TOWER FERMENTATION OF WHEY PERMEATE
AND
SUCROSE-ENRICHED WHEY PERMEATE TO ETHANOL

A thesis presented in partial fulfilment
of the requirements for
the Degree of Doctor of Philosophy
in Biotechnology
at Massey University

CHATURONG BOONTANJAI

1983

ABSTRACT

Tower fermentation of sulphuric acid whey permeate using *Kluyveromyces marxianus* Y42 has been investigated. The tower fermenter used was 0.025 m in diameter and 2.69 m high. The straight section of the tower was 2.37 m. The total tower volume was 2.9 litres and the separator section made up 1.6 litres of the total volume. The operating temperature was 30°C. The optimum medium feed rate was observed at a superficial liquid velocity of 0.24 mm/s. It was found that a tower height of only 0.82 m was required, excluding the separator section, and the corresponding residence time was 1 hour. An exit ethanol concentration of 16 g/l was produced at a productivity of 16 g/lh from 45 g/l lactose in the whey permeate feed (94% utilization). This was an ethanol yield of 71% on lactose utilized. If the separator section were considered, the ethanol productivity was 5 g/lh and the exit ethanol concentration was 19 g/l, while the overall retention time was 3.7 hours. The cell concentration inside the tower varied between 10 and 100 g/l dried weight (54 and 350 g/l wet weight) being greatest at the bottom of the tower.

K. marxianus was found to be inhibited by a high level of ethanol in the growth medium and unable to ferment completely a high concentration of lactose when tested in 10 litre-scale-batch fermentation. Further tests in the presence of sucrose and lactose found that this yeast exhibited diauxic behaviour by utilizing sucrose before lactose. This behaviour generally resulted in incomplete lactose utilization in the tower. In the screening for a flocculent lactose-fermenting yeast, the yeast strain *K. marxianus* was found to be the only flocculent yeast, but it was only moderately flocculent. Further investigation found that it had good flocculence when grown in media which support good growth, and poor flocculence when grown in acidic media and in media which do not support good growth. A subculture of this yeast strain showed moderate flocculence when grown in whey permeate.

Tower fermentation of whey permeate enriched with molasses by mixed culture of *Saccharomyces cerevisiae* CFCC39 and *K. marxianus* Y42 was found to be difficult. The difficulty arose because of incomplete lactose utilization even at a very low feed rate (up to 0.14 mm/s) and incompatible flocculation properties of the two yeast species employed. Blockage of the separator and gas slug formation were caused by the very flocculent yeast mass of *S. cerevisiae* CFCC39. This caused

K. marxianus to be slowly washed out of the tower fermenter. Sucrose was completely utilized at the bottom of the tower fermenter, while lactose utilization was slow and incomplete. The incomplete lactose utilization has been attributed to the diauxic behaviour of *K. marxianus*, ethanol inhibition and molasses inhibition (probably due to its reaction with whey permeate during autoclaving).

Results of tower fermentation of cane molasses have also been given for characterization of the tower fermenter used.

Experiments to isolate an ethanol tolerant *K. marxianus* using a serial subculture in a medium containing increasing ethanol concentrations were performed. The isolate obtained could tolerate up to 50 g/l ethanol. It could ferment lactose in whey permeate to produce ethanol at a faster rate than the parent strain and other lactose-fermenting yeast tested. The isolate was found to be stable. It was not used in the tower fermenter as it was non-flocculent.

An attempt was made to isolate a sucrose-negative *K. marxianus*. This was only partially successful. The mutant did not grow on sucrose agar but reverted to the wild type when grown in liquid medium containing both sucrose and lactose.

An experiment to isolate a diauxie-negative *K. marxianus* strain using D-glucosamine as a glucose analogue was also described. This was unsuccessful because *K. marxianus* was able to grow on lactose in presence of the analogue.

TO MY KIWI AND THAI PARENTS

ACKNOWLEDGEMENT

During the course of his attempt to complete this study, the author owed gratitude to many people and wishes to thank the following :

Professor R.L. Earle and Dr. Mary D. Earle for the kindness and help given to him and to the development of Food Technology and Biotechnology studies in Thailand and in particular at Khonkaen University.

The DSIR for providing financial support during part of this study.

His supervisors Drs. Vidar Friis Larsen and John D. Brooks for their guidance and advice .

Dr. Ian S. Maddox for his able guidance, advice and forever readiness to help and answer the many questions from the author on general industrial microbiology.

Dr. Graham J. Manderson for his forever readiness to give help and advice on yeast morphology and behaviour, and photomicroscopy.

Dr. Noel W. Dunn of the School of Biological Science, University of New South Wales, Sydney, Australia, for his guidance, advice and planning of the experiments on the culture improvement of *Kluyveromyces marxianus*.

Assoc. Professor Anthony M.MacQuillan of the Dept. of Microbiology, University of Maryland, College Park, Maryland, USA, for providing two lactose fermenting yeast cultures in which one of them was flocculent. Thus, enabling the completion of this study, and for his advice on *Kluyveromyces* species mutation.

Dr. Marion Ewen of the Dairy Research Institute (DRI), Palmerston North, for her various help and advice , particularly for providing references on whey technology, giving some first hand knowledge on tower fermentation and reading the manuscript.

Dr. Roy J. Thornton of the Dept. of Microbiology, Massey University, for giving consultation and advice on yeast mutation.

The staff of DRI, Palmerston North. The Whey product Section : Messrs. Peter Hobman, Mike O'Connell and John Bligh for their assistance in providing the whey permeate . The DRI library staff for their able assistance in particular Lorraine Tremain.

The technical and secretarial staff of the Faculty of Technology : John Alger, Paul Shaw, Derek Couling, Mike Stevens, Robyn Calder, Mark Lubbers, Astrid Ndzinge, Melvin Smith, Terry Gracie, Margaret Bewley,

Raewyn Cheer, Beverly Hawthorn and Carol Clouston for their able assistance.

The staff of Massey University Library in particular the Serials and Interloan Departments.

Claire Mudford for her assistance during the experiments on tower fermentation, and thesis preparation.

The boiler house staff for their cooperation in providing the steam outside office hours.

Bruce Walker for his assistance in the transportation of whey permeate.

His fellow students : Tom Clark, Sheelagh Wilkinson, Moazzem Hozzain, Tipavana Ngarmsak, Pisanu Vichiensanth, Tony Retter, Richard Gapes, John Mawson, Warren Hollaway, Wong Tze Sen and Pamela Palfreyman. Many thanks for their helpful and encouraging words, advice and sharing sadness during bad times.

Many thanks to the many friends in the Faculty of Technology who have provided a very friendly atmosphere to work in.

Brian Wilkinson, Dick Poll, Mr. & Mrs Meredith and their respective families for their kindness and encouraging words and for offering the warmth of their homes.

Mrs. Bunnak Wickham and (Aunty) Bertha Zurcher for finding and letting a comfortable accommodation.

Sarah Sant for proof reading part of the final manuscript.

The Computer Centre staff for their cooperation and assistance in the preparation of the manuscript.

Vivienne Mair and Joane Charles for typing part of the manuscript.

Mr. A. Eustace, Sunanta Juntakul and Priscilla Burton for their kind assistance during photocopying.

Christine Samaniego and Linda Poll for their assistance in the preparation of the manuscript for binding.

Finally, his Kiwi parents (Mr. & Mrs. Moxon) and their family for all the love, kindness, warmth, care, encouragement and help that they have given which could not be expressed in a few words. Without their assistance the completion of this study would have been extremely difficult.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

PREFIX

Δ	change in concentration, g/l or %

SUBSCRIPTS

a	average
E	effective
i	condition at a particular tower height or section
l	lactose
o	overall
r	residence time
s	sucrose or superficial
t	total sugar
u	substrate utilization

NOTATIONS

A	aluminium sulphate, $Al_2(SO_4)_3$
AFEB	attached-film-expanded-bed fermenter
B	broth
B	95% confidence interval uncertainty
Ca	calcium sulphate, $CaSO_4$
CB	yeast cleaning buffer($CaSO_4$wash)
CP	*Candida pseudotropicalis*
CC39	*Saccharomyces cerevisiae* CFCC39
CSTR	continuous stirred tank reactor
D	dilution rate
DGA	D-glucosamine
DW	cell dried weight, g/l DW
E	ethanol concentration, g/l
E'	volumetric rate of ethanol production, g/lh
EF	extremely flocculent
F	membrane filtration (0.45 μm)
FM	flocculating medium (acetate buffer)
G	glucose
H,H_E	tower height, effective tower height, mm or m
H*	average tower height
KL	*Kluyveromyces lactis*
KM	*Kluyveromyces marxianus*

KMY42 *K.marxianus* Y42

LVE limiting volumetric efficiency

M malt extract broth

M* malt extract broth (Oxoid)

Ma maltose

MBN modified Burn's number

MBN* non-standard modified Burn's number

Me malt extract powder medium

MF moderately flocculent

Mo molasses

Ms malt extract syrup (Maltexo)

P whey permeate

P percentage uncertainty

Pe peptone

P4.6 whey permeate with no pH adjustment

q specific rate of substrate utilization, g/gh

Q volumetric flow rate, ml

r (Linear regression) correlation coefficient

R rough

S substrate concentration, g/l

S' volumetric rate of substrate utilization, g/lh

SC *Saccharomyces cerevisiae*

SC146 *S.cerevisiae* FT146 (AWRI 350)

SG_e exit specific gravity

SM spent malt extract broth

S_u substrate utilization, %

T_r residence time, h

T^*_r average residence time, h

T_{ro} overall residence time, h

TS subcultured from the tower fermenter

V_E effective tower volume, ml

V_i volume of a section of the tower fermenter, ml

V_s superficial liquid velocity, mm/s

VF very flocculent

WF weakly flocculent

X total cell number or cell weight, cell/ml or g/l

X_a average cell concentration, g/l

X_{KM} *K.marxianus* cell number or weight, cell/ml or g/l

Y	yield cofficient, yield of ethanol on substrate utilized, % yeast extract
10	100 g/l whey permeate solution
44,46	ratio of lactose to sucrose of 40:40 g/l and 40:60 g/l
5	pH 5.0

GREEK NOTATIONS

μ	specific growth rate, g/gh
ν	specific rate of ethanol production, g/gh
ϕ	diameter

CHAPTER 1

WHEY, ITS UTILIZATION AND DISPOSAL

1.1 INTRODUCTION

The rapid increase in oil price since 1973 and 1979 has greatly stimulated the search for alternative energy sources and in particular renewable ones. Ethanol produced from biomass has received considerable attention as an alternative liquid fuel and Brazil has a very extensive ethanol fuel programme based on sugar cane and cassava.

In New Zealand, there is no large indigenous supply of easily fermentable sugars for ethanol production. However, fodder beet, sugar beet, and whey have considerable potential for development as fermentation feed stock and have received considerable interest (Tichener 1980).

Whey is a waste product of the manufacture of cheese and casein and contains between 40-50 g/l lactose, depending upon the process. Disposal of whey safely to the environment is a constant problem in the dairy industry.

1.2 TYPES OF WHEY

Whey is defined as the fluid obtained by separating the coagulum from milk, cream or skim milk. It is produced in the manufacture of cheese, rennet casein or acid casein (fig.1.1), and each process gives a characteristic whey. Sweet whey is derived from the manufacture of cheese or rennet casein and has a pH value greater than 5.5. Acid whey is derived from the manufacture of cottage cheese, lactic casein or mineral acid casein. It has pH value less than 5 (Short 1978b).

1.3 WHEY COMPOSITION

The composition of whey varies considerably according to the product or process from which it derives. Whey usually contains half the initial total solid content of the influent milk and averages about 6% total solids, of which the major constituents and their compositions are given in table 1.1.

Whey protein is approximately 20% of the original protein content of milk , and there are a number of protein fractions, both soluble and insoluble, which can be recovered by ultrafiltration or heat precipitation. Approximately 90% of the total protein fraction comprises α-lactalbumin and β-lactoglobulin. Whey proteins are nutritionally superior to most other proteins in human and animal nutrition, principally due to their

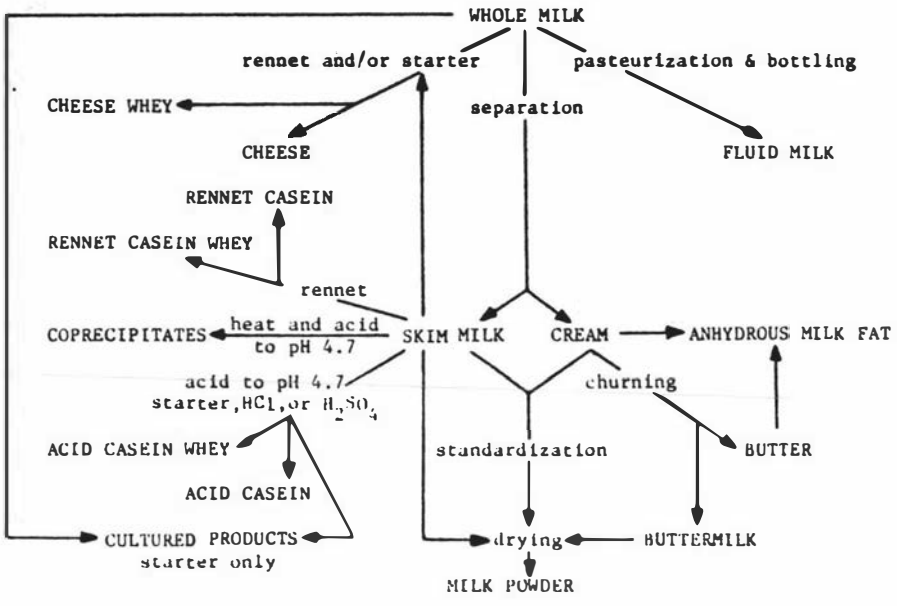


Fig.1.1 Milk utilization. Major products are butter, cheese, skim milkpowder, acid & rennet casein. Minor products are anhydrous milkfat, coprecipitates, whole milk powder, butter milk powder, cultured products and frozen cream. Waste products are cheese whey, acid & rennet casein wheys. (Webb & Whittier 1970)

Table 1.1 *Typical composition of wheys produced in New Zealand (g/kg).* (Short & Doughty 1977)

	Cheese Whey	Casein Wheys	
		Lactic	Sulphuric
Total Solids	67	64	59
Lactose	50	44	47
Protein	5.7	5.7	5.3
Non Protein Nitrogen	0.5	0.5	0.3
Fat	0.3	0.2	0.4
Ash	5.3	5.8	6.7

Table 1.2 *Composition of deproteinated wheys of various sources.* (Short 1978b)

	Cheddar cheese	Cottage cheese	Lactic casein	Lactic casein	Pretreated lactic casein	Sulphuric casein
Total solids g kg	57.0	58.0	56.8	57.3	43.7	56.4
Total nitrogen g kg	0.26	0.33	0.64	0.51	0.22	0.37
Non protein nitrogen g kg	0.24	0.30	0.46	0.46	0.17	0.32
Ash g kg	5.0	5.6	5.7	7.3	4.2	7.9
Lactose g kg	49.0	43.0	44.8	41.0	37.4	46.0
Lactic acid lactate g kg	1.4	4.4	3.4	5.7	0.2	–
Ash TS %	8.8	9.7	10.0	12.7	9.7	14.0
Lactose TS %	86.0	74.0	79.0	71.0	86.0	82.0
pH	5.5	5.0	5.0	5.0	5.5	5.0

favorable amino acid composition and in particular to the high lysine content (Smith 1979 ; Ewen 1980).

Deproteinated whey is basically a dilute solution of lactose with mineral salts,lactic or mineral acid, soluble nitrogenous compounds and vitamins. typical compositions of deproteinated sweet and acid wheys are given in table 1.2.

The major mineral constituents of whey are potassium, calcium, sodium, magnesium, chloride, and phosphate. Calcium and phosphate are retained to a certain degree in cheese but the other minerals are present in virtually the same quantities as in whole milk (Ewen 1980).

1.4 WHEY PRODUCTION

For each tonne of cheese manufactured,7.6 tonnes of whey are produced and for each tonne of casein 25 tonnes of whey are produced. During the 1977/78 dairying season some 70,000 tonnes of cheese whey and 1,300,000 tonnes of casein whey were produced in New Zealand (Marshall 1978). In 1974, over 14 million tonnes of whey were produced in the USA and it was estimated that only a little over one half was utilized; the rest was disposed of as waste (Bernstein et al 1977). In 1973, the world whey production was estimated at 72 million tonnes (Coton 1976). Table 1.3 gives estimated quantities of whey production in the USA, Canada, Australia, and New Zealand for the 1975-82 period showing continuous increase in whey production.

Whey production in New Zealand is seasonal as shown in fig.1.2. It follows the seasonal milk flow pattern which builds up from June/July to a peak in October/November and then decreases to a level by April/May.

1.5 WHEY UTILIZATION AND DISPOSAL

In order to utilize its valuable contents and reduce its pollution strength, whey can be further processed. Fig.1.3 gives a summary of various possible alternatives. The total N.Z. production of sweet whey is almost completely utilized, except for a number of small isolated factories, either for the production of lactose by crystallization or for incorporation into special baby food (Marshall 1978). Acid whey can be processed to remove whey protein by ultrafiltration or heat treatment (Short 1978b). The annual production of whey powder, which can be used for human or animal consumption in baked food, ice cream, processed meat, and cheese, is given in table 1.4.

Table 1.3 Estimated quantities of fluid whey and whey solids produced in some countries.(Zall et al 1979 ; Hobman 1982)

	1000 of tonnes														
	USA[1]			CANADA			AUSTRALIA[2]				NEW ZEALAND				
	1975	1976	1977	1975	1976	1977	1975	1976	1977	1978	1975	1976	1977	1978	1982
Sweet whey															
Fluid whey	11490	13570	13720	1086	1130	1210	749	856	788	858	770	901	785	671	912
Whey solids	747	882	892	71	73	79	49	56	51	56	50	59	51	44	100
Acid whey															
Fluid whey	1910	1940	1920	143	153	158	338	388	420	465	765	1213	1282	1343	1480
Whey solids	123	126	125	93	99	103	22	25	27	30	50	79	83	87	108
Total															
Fluid whey	13400	15500	15640	1229	1283	1368	1087	1244	1208	1323	1530	2114	2067	2014	2392
Whey solids	870	1008	1017	80	83	89	71	81	79	86	100	138	134	131	208

1. USA figures for sweet whey were estimated from cheese production assuming 9 kg whey/1 kg cheese and that averaged total solids content of whey was 65%. For acid whey it was assumed that there were 6 kg whey/1 kg Cottage cheese.
2. Australia and New Zealand figures were estimated from cheese production assuming 7.6 kg whey/ 1 kg cheese, and casein production assuming 25 kg whey/ 1 kg casein, and averaged total solids of whey was 6.5%.

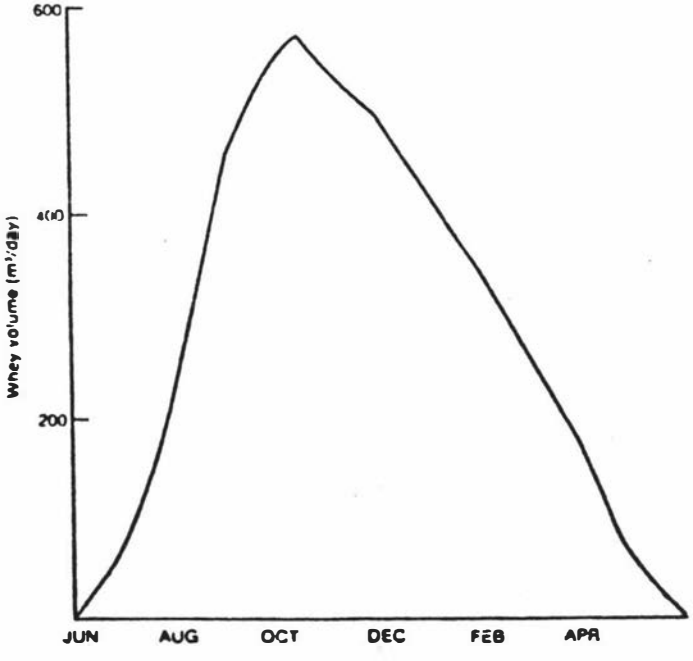


Fig.1.2 Typical annual whey production of a N.Z. dairy factory.(Marshall 1981)

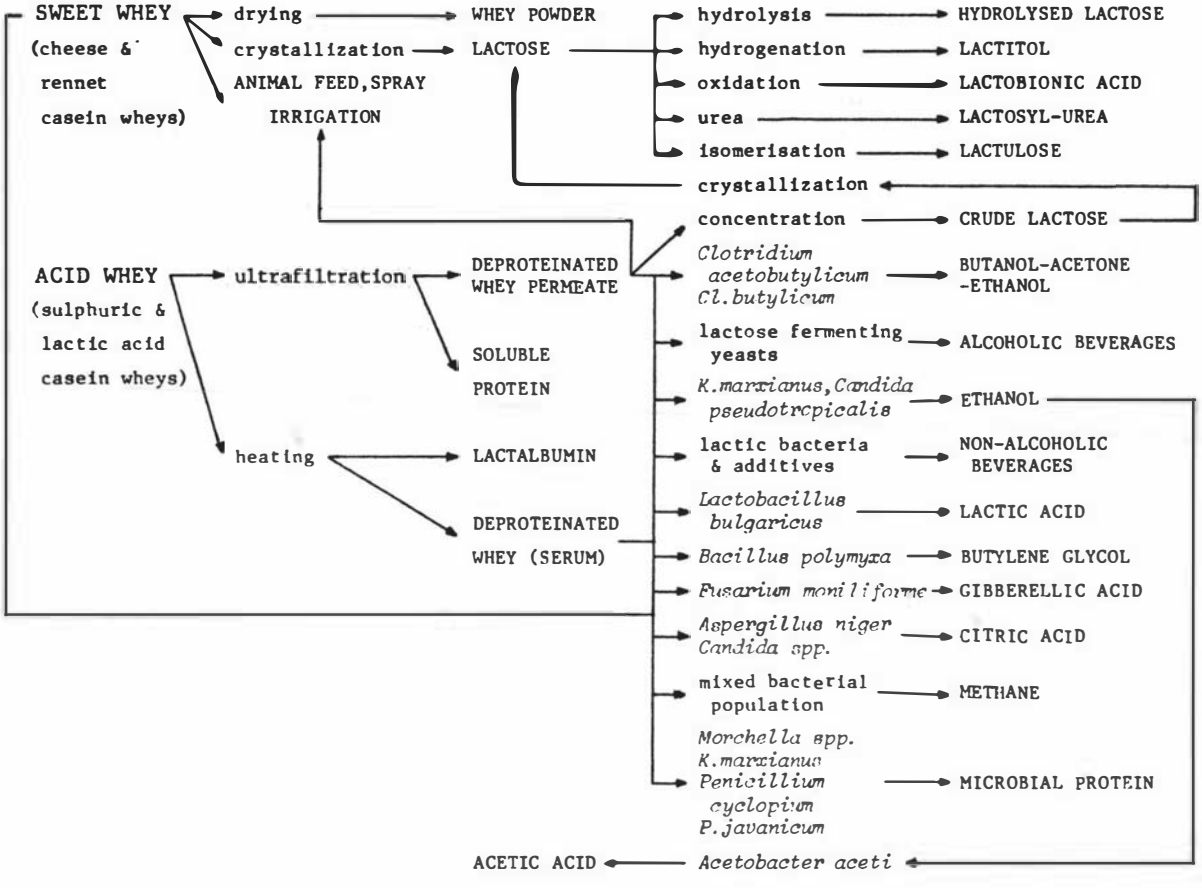


Fig.1.3 Summary of processes for whey utilization and disposal.

TABLE 1.4
Whey powder production

	1966	1970	1971	1972	1973	Whey drying as % of total whey supply 1973
	('000 tons)					
USA, total	214	282	308	346	338	40
of which for food	110	133	145	171	178	—
Canada	19	20	24	25	24	36
EEC	93	210	274	325	389	30
of which France	26	80	115	148	170	39
Netherlands	28	51	65	71	99	53
Germany, FR	15	43	48	56	66	26
United Kingdom	11	13	14	15	15	15
Belgium	6	7	10	9	9	55
Others	7	16	22	26	30	6
Austria	1	7	8	9	11	35
Finland	6	12	14	17	17	65
Total 13 countries	333	531	628	722	779	34

1 ton = 1.016 tonne (Coton 1976)

1.5.1 Whey disposal

Unutilized whey has generally been discarded either by spray irrigation or discharged into natural waterways or municipal sewers. Very few factories have effluent treatment plants (Bissett & Riddle 1976; Oborn 1968) because of theplant capital cost required to treat the high Biological oxygen demand (BOD) of whey.

Spray irrigation is a common disposal method used in N.Z. About 20% of the effluent from all butter, cheese and milk powder factories and 50% of the effluent from all casein factories are disposed in this manner. Factories are normally situated in rural areas and disposal of effluent to sewage treatment plants is difficult. The area required for spray irrigation is determined by the total waste volume, the application rate, and the return cycle. The return cycle is set by the infiltration rate recovery, which in turn is dependent upon the BOD application rate (Parkin & Marshall 1976).

Feeding whey to animals is another traditional disposal method. In the UK , 1973, 40% of the whey produced was still used as pig feed (Coton 1976).

1.5.2 Protein production

One of the oldest means of recovering protein from acid casein whey is the lactalbumin process. The whey is heated to 98^{o}C for 20 min and the protein precipitate is recovered and dried. The product called "lactalbumin" is tan in colour and has a gritty texture which limits its application in food stuffs (Robinson et al 1976).

Ultrafiltration of whey proteins is a new technology which is gaining wide popularity, as the recovered proteins have high solubility, whippability, heat gelation, and stability in acid solution. They are used in the flour and sugar confectionery, soft drink and baby foods industries (Coton 1976)

1.5.3 Lactose production

Lactose is almost exclusively produced from whey and in particular sweet whey. It is a major component in the infants or invalids formulae, being added to cow's milk to approximate the lactose content of human milk. When added to food, it contributes to body and viscosity of the food without excessive sweetness; absorbs and stabilizes food and beverage flavours and aromas; promotes and strengthens agglomerate structure. Refined grade lactose is used in the pharmaceutical industry as a

fermentation substrate for antibiotic production, a filler and inert carrier for capsules and tablets. It has also been used in production of β-galactosidase. Lactose can react with urea to form lactosylurea which is used as a nitrogen supplement in ruminant feeds (Widell 1979)

Deproteinated whey can be concentrated by heating at 40-44^{o}C to produce crude lactose through crystallization, ion exchange or electrodialysis. Crude lactose finds application in formulated dairy foods (Webb & Whittier 1970).

1.5.4 Fermentation of whey

Whey can be fermented, using different microorganisms, to obtain many different products.

(a) Beverage production Non-alcoholic and alcoholic beverages have been producuced from whey. In the production of non-alcoholic beverages, deproteinated whey is used as a base for a number of carbonated acid beverages fermented by lactic bacteria (Short 1978b).

Alcoholic beverages may be produced by a fermentation in which whey is utilized as the medium for ethanol production or a more readily fermentable sugar such as glucose may be added to the liquor (Short 1978b).

(b) Lactic acid production Lactic acid is a syrupy liquid used in food and beverage manufacture, and is produced by *Lactobacillus bulgaricus*. The lactic acid can be recovered from the fermented liquor by the addition of calcium lactate, calcium carbonate, and calcium hydroxide. After further treatment sulphuric acid is added to yield lactic acid and calcium sulphate crystals. The lactic acid obtained is cleaned using activated carbon (Campbell 1953).

(c) Citric acid production Citric acid is commonly used as a food acidulant because of its ease of assimilation, palatability and low toxicity. Most of the world's supply of citric acid is produced by fermentation of molasses or other carbohydrate sources using selected strains of *Aspergillus niger* or *Candida sp.*. Since the optimum environment for citric acid production is, for most microorganisms, around pH 2.5-3.5, acid whey should be a suitable medium. However, there is little published work on production of citric acid from whey. Generally 15-20% sugar concentration is required for fermentation and if this falls below 5% microbial cells only are produced (Hossain 1983).

(d) Acetic acid fermentation Acetic acid is produced by oxidation of ethanol using *Acetobacter aceti*. Ethanol can be produced from whey

and after the yeast has been separated from the broth, a suitable starter culture of *Acetobacter* may then be added. Vinegar bacteria grow both in the liquid and on exposed surfaces to form a gelatinous film. Large scale fermentation vessels are usually packed columns, which provide the large surface area required for rapid oxygen transfer. The production of vinegar fron whey has already been commercialized in France, USA, and Switzerland (Short 1978b).

(e) Microbial protein production Lactose in whey can be converted to microbial protein by fermentation. The protein produced may be processed by cooking, extruding, texturising etc. into food grade products similar to textured vegetable protein or may be hydrolyzed to form the base for stocks, gravies and sauces or used as animal feed stuff for high quality meat producing animals such as cattle, pigs and chicken (Bernstein et al 1977)

Yeasts of the *Brettanomyces, Candida* & *Kluyveromyces spp.* can grow on lactose under aerobic conditions (Meyrath & Bayer 1979; Wasserman et al 1960). A yield of around 1.5 kg yeast mass per kg of whey solid has been reported (Bernstein & Everson 1974). A plant in France is reported to produce *Penicillium cyclopium* from whey ultrafiltrate (Kosaric & Miyata 1981).

(f) Butanol production Butanol is used as cosurfactant in tertiary oil recovery; solvent for paints, varnish and cellulose esters and its derivatives are used in the pharmaceutical and perfume industries. The bacteria *Clostridium butylicum* and *Cl. acetobutylicum* are used for its production by fermentaion. Whey is an ideal substrate for fermentation to produce butanol because of its low sugar content, as butanol levels greater than 12 g/l inhibit the fermentation. The proportion of butanol, acetone and ethanol produced varies according to the strain used but normally the ratio is 6:5:2 respectively (Gapes 1982).

(g) Production of other fermentation products A process for the production of butylene glycol, a raw material for butadiene production, from whey has been described using *Bacillus polymyxa* (Speckman & Collin 1982). *Fusarium miniforme* has been used to produce gibberellic acid from whey (Maddox & Richert 1977). Many digester designs have been described for anaerobic treatment of whey to produce methane (Boening & Larsen 1982; Archer,Larsen & McFarlane 1983).

(h) Ethanol production Whey is currently being used as the feed stock for potable and industrial ethanol production in many parts of the world, using *Kluyveromyces marxianus*. Presently, there is a plant

producing potable ethanol from whey permeate by batch fermentation at Carbery, Southern Ireland (Sandbach 1981). Microbial protein and ethanol are produced from whey at a Milbrew Inc. plant in Juneau, Wisconsin. This process could operate in a batch, semi-continuous or continuous mode (Anon 1977). A continuous pilot plant has been successfully tested in Denmark (Reesen 1978) whereas in New Zealand the New Zealand Co-operative Dairy Co. commissioned its first full scale whey to ethanol fermentation plant in September, 1980 at Reporoa. This plant utilises lactalbumin serum and a batch fermentation process. A second plant at Tirau, which uses a continuous stirred tank fermentation process and lactalbumin serum, was commissioned in September 1982.

The total annual production will supply all of the 4.5 to 5.0 million litres market requirements in New Zealand with a surplus of 1.6 to 2.1 million litres (Howell 1981). There are two more batch fermentation plants producing ethanol from whey at Edgecumbe (Mawson 1983) and Temuka (Gooding 1982). It is worth mentioning here that if all lactose in acid whey produced in New Zealand were fermented to ethanol it would replace only 1% of all New Zealand motor spirit consumption (Marshall 1978). Its impact will, however, be large in a region where the dairy industry is concentrated such as in the Waikato and Taranaki. If all the ethanol produced was used only within the regions, it could replace a large portion of the petrol used within the regions.

Ethanol is used as a chemical intermediate in the synthesis of a large variety of compounds by simple chemical reactions, such as reaction with carboxylic acids to fats, waxes; dehydration to produce olefins and ethers; sulphonation and ethoxylation to form surfactants for use as detergents; and other synthesis yielding compounds for use as plasticizers, emulsifiers, lubricants, emollient and foaming agents. It may be dehydrated to ethylene, an important synthetic chemical starting point. It is second in importance only to water as a solvent for drugs, lacquers, perfumes, cosmetics, detergents and plastics. Recent applications are in aerosol and mouth-wash products, motor and rocket fuels(Anon 1974).

1.6 SUMMARY

When all the fermentation processes described here for the utilization of whey are considered, only beverage, lactic acid and ethanol productions have been carried out on a commercial scale, while the other processes are still being investigated. The production of ethanol from whey provides a very attractive alternative through its simple fermentation process and great demand for ethanol. The seasonal nature of whey production and the

cost of ethanol from whey are of considerable importance when considering the alternatives to be used for the treatment of whey. Greater utilization of whey will help to reduce the need to dump it into natural waterways and reduce environmental pollution. The ethanol that can be produced will provide another product for export from New Zealand.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION TO ETHANOL FERMENTATION

Ethanol fermentation is one of man's oldest technologies. The Sumerians and Babylonians were known to produce beer before 6000 BC. Much later, about 4000 BC, the Egyptians discovered the use of yeast for baking and ancient Egyptians relief drawing dating from 2400 BC described the methods of baking and brewing. Kui, a Chinese rice beer, has been traced back to 2300 BC (Demain & Solomon 1981). Pasteur, in 1851, began a research project which led him to the conclusion that "alcoholic fermentation is an act correlated with the life and organization of yeast cells". Subsequently he came to another conclusion that alcoholic fermentation was the result of life without oxygen. Later many other researchers have contributed to the knowledge of the fermentation processes and established that the yeast is responsible for the alcoholic fermentation. Today, fermentation technology is very important to man in the production of wine; beer; bread; cheese; organic chemicals such as ethanol,citric acid, and antibiotics; animal feed and waste treatment (Nord & Weiss 1958 ; Harrison & Graham 1970).

Before the advent of the petrochemical industry, all industrial ethanol was produced by fermentation of carbohydrate substrates. However, ethanol production from agricultural sources declined from the 1930's as petrochemical-based ethanol was considerably cheaper to produce. The demand for fermentation-derived ethanol in the food industry has however been steady whereas the demand for ethanol as a fuel varied considerably. In Europe, immediately after the first World War, when the supply of petroleum was uncertain and its price was relatively high, there was intensive research into "alcohol fuels". For a number of inter-war years from 1922 to 1935 an ethanol-petrol blend was the sole motor spirit in France (Titchener1980). In the Mackay district of Queensland, ethanol-petrol blend was in continuous use from 1929 to 1956 (Titchener1980). Racing car and motor cycle engines have made use of alcoholic fuels for many years. During and after World War II circumstances revived interest in alcohol as a motor fuel in a number of Third World countries such as India.

In the 1970's, the OPEC nations increased the price of oil dramatically and this led to renewed interest in ethanol as an alternative liquid fuel. There was also a general conclusion that petrol supplies will dwindle in the coming decades and must in the near future be replaced by an alternative fuel. This has led many countries including New Zealand to search for alternative fuel sources, and Brazil mounted an ambitious ethanol programme based on sugar cane and other starchy root crops (Tichener 1980; Pimentel 1980). Thus, ethanol may once again be used as a substitute for petroleum-based fuels.

2.1.1 General biochemistry

The main substrates for ethanol fermentation are simple carbohydrates. These are the hexoses : glucose, fructose, galactose, and mannose ; disaccharides : sucrose, maltose, lactose ; and the trisaccharide : maltotriose. More complex carbohydrates, such as starches and celluloses, must normally be hydrolysed by acids or enzymes to simple sugars before fermentation can proceed (Kosaric et al 1980).

The conversion by yeasts or bacteria of sugars to ethanol proceeds through a series of enzymatic reactions that were elucidated in the 1930's and which later become known as the Embden-Meyerhof-Parnas (EMP) glycolytic pathway (fig. 2.1). Under aerobic conditions, glycolysis provides pyruvate for entry into the tricarboxylic acid cycle and thence by oxidative phosphorylation ATP, carbon dioxide and water are produced. This gives the maximum quantity of energy per unit of sugar utilized. Under anaerobic conditions or high sugar concentration yeasts convert the pyruvate to ethanol and carbon dioxide. Less energy is produced per unit sugar utilized than under aerobic conditions, since ethanol has remaining electron pairs available for oxidation.

The stoichiometric relationship for glucose oxidation to ethanol may be used as a basis from which actual yields may be evaluated.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

glucose ethanol

Thus, theoretically the yield is 51.1 % (W/W) ethanol and 48.9 % (W/W) CO_2 from glucose. However, in practice some sugar is converted to cell mass and other minor products such as glycerol and succinate, giving a practical ethanol yield of approximately 90 % of the above theoretical maximum. These minor compounds are produced only in trace quantities but are of great importance for the aroma and taste of alcoholic beverages (Harrison & Graham 1970 ; Nord & Weiss 1958).

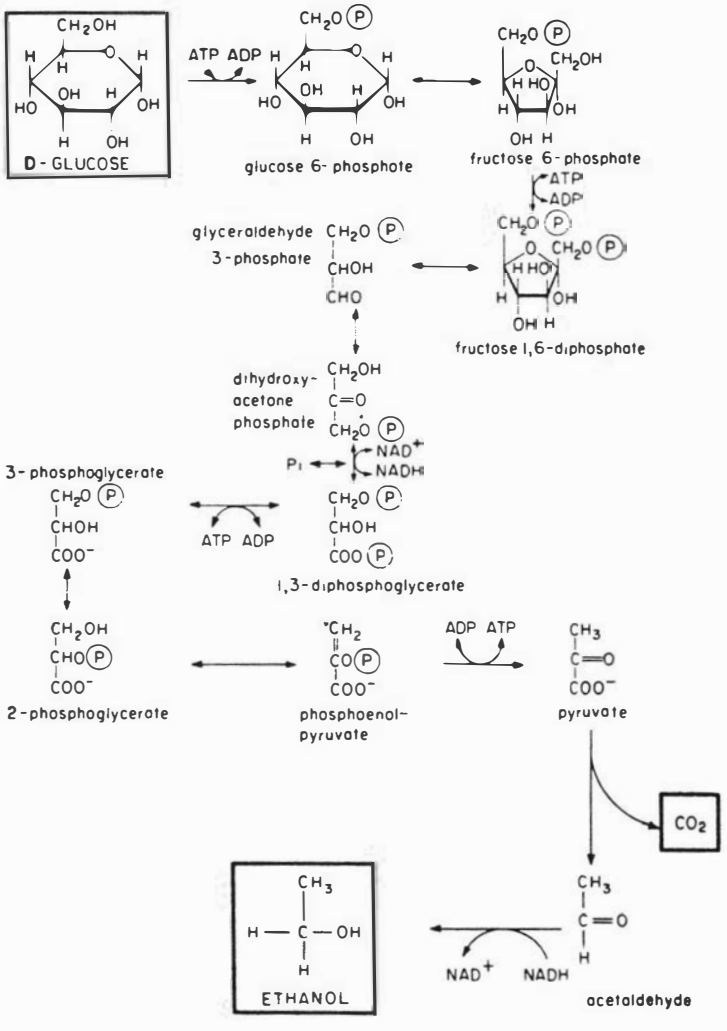


Fig. 2.1 Embden-Meyerhof-Parnas pathway (Rose 1977).

2.1.2 General microbiology

There are a large number of yeasts that can ferment carbohydrates to ethanol. The yeasts from the genus *Saccharomyces* are the most widely used and studied for potable and industrial ethanol production. One limitation of the yeasts from this genus is that they cannot ferment such sugars as lactose, arabinose, cellobiose, rhamnose, sorbose and xylose (Lodder 1970 ; Harrison 1971). *K.marxianus*, on the other hand, can utilize lactose. It has been used for studies of the production of microbial protein and ethanol from lactose(Meyrath & Bayer 1979). It has also recently been shown that some strains of *K.marxianus* can ferment xylose to ethanol under aerobic conditions (Margaritis & Bajpai 1982).

Yeasts of the genus *Schwanniomyces* have been investigated for use in the production of microbial protein or ethanol from starch without the

need of an enzyme hydrolysis step (Wilson et al 1982), whereas *Candida tropicalis* and *Pachysolen tannophilis* have been used to convert xylose to ethanol (Wong, Manderson, & Larsen 1982).

Recently, there has been considerable interest in the use of *Zymomonas mobilis* for ethanol fermentation because of its high specific rates of sugar uptake and ethanol production plus improved yields. However, it can not utilize starch or cellulose directly (Rogers et al 1980).

A saprophytic filamentous fungus of the genus *Monilia* has been investigated for use in the direct conversion of cellulose to ethanol (Gong et al 1981).

2.2 ETHANOL FERMENTATION FROM WHEY

2.2.1 Microorganisms

Early studies on lactose fermentation found *Candida pseudotropicalis* (*Torula cremolis*) to be the best of four yeast species owing to its rapid fermentation and high ethanol yield. It was able to utilize 46 g/l lactose in whey in 22 h at 30-34^{o}C (Browne 1941).

In a similar study *C.pseudotropicalis* required 55 h to utilize 50 g/l lactose at 30-34^{o}C (Rogosa et al 1947). Although it appeared to be considerably slower in this work than in the previously reported work (Browne 1941) it was selected as the most efficient organism out of the 11 yeasts strains tested. Among the other yeasts tested were another strain of *C.pseudotropicalis*, *K.lactis* (*S.lactis*) and *K.marxianus* (*S.fragilis*).

When the growth medium was concentrated whey,containing 200 g/l lactose, *C.pseudotropicalis* (ATCC 8619) was also selected out of nine yeast strains (Izaguirre & Castillo 1982). It produced 99 g/l ethanol in 192 h, at 30^{o}C. The long fermentation time was a result of the completely anaerobic fermentation condition used in this study.

K.marxianus (*K.fragilis*) was also found to be a rapid lactose fermenter. *K.marxianus* CBS 397 fermented 150 g/l lactose in whey, without any additive, in 72 h at 28^{o}C (Laham-Guillaume et al 1979). It was better than 8 other yeasts tested including one *C.pseudotropicalis* and three other *K.marxianus* strains. A different *K.marxianus* strain (CBS 5795) fermented a similar concentration of lactose in whey in 36 h yielding 92% ethanol (Burgess & Kelly 1979). Two *K.marxianus* strains (Y18 & Y42) were found to be the greatest ethanol producers from 100 g/l lactose medium in comparison with 26 other lactose fermenting yeast strains (Yoo 1974).

They produced up to 6.3% v/v (50 g/l ethanol) in ten days, under static conditions and 28°C.

Further information on lactose fermenting yeasts and their synonyms can be found in Lodder (1970) or Barnett et al (1979).

2.2.2 Metabolism of lactose to ethanol by yeast

The metabolism of lactose is essentially the same as for glucose (fig.2.1) with the exception of the lactose hydrolysis and transport mechanism. The lactose is transported across the yeast cell membrane by an inducible specific enzyme system. This is followed by hydrolysis inside the yeast cell by an intracellular enzyme, β-galactosidase, with the production of glucose and galactose. Glucose is converted to ethanol via the EMP pathway. Galactose, on the other hand, is converted to D-glucose-6-phosphate through three intermediary reaction steps before it is converted to ethanol via the EMP pathway (Yoo 1974).

2.2.3 Yeast environmental consideration

(a) Substrate utilization Early investigation reported fermentation time of 22 h to ferment 46 g/l of lactose in whey, by *C.pseudotropicalis*, at 30°-34°C. The resulting lactose utilization rate was 2.1 g/lh (Browne 1941). This utilization was considerably higher than reported for a different strain of *C.pseudotropicalis* which required 55 h to ferment 50 g/l lactose in whey at the same temperature. This gave a lactose utilzation rate of 0.9 g/lh (Rogosa et al 1947). These workers did not provide information on residual lactose concentration or indicate whether agitation was used.

K.marxianus (CBS 5795) has been reported to ferment 50 g/l lactose in whey in 17 h in aerated shake flasks. The reported lactose utilization was 100% and this gave a rate of utilization of 2.9 g/lh. *C.pseudotropicalis* (NCYC 744) fermented similar medium, under the same conditions, in 12 h which gave utilization rate of 4.2 g/lh again with reported 100% lactose utilization (Burgess & Kelly 1979).

Fermentation time of 12 h has been reported for different strains of *K.marxianus* & *C.pseudotropicalis* on whey ultrafiltrate containing 50 g/l lactose under similar conditions as above (Moulin et al 1980). No data were given on the residual lactose but this gave an average utilization rate of 4.2 g/lh. These reported rates were very similar to the commercial scale fermentation time of 16 h using whey containing 41 g/l lactose. The utilization rate was 2.8 g/lh (Howell & Tichbon 1980).

When the fermentation condition was completely anaerobic, *K.marxianus* (NCYC 151) & *C.pseudotropicalis* (ATCC 8619) required 72 h to ferment 99% of 51 g/l lactose in whey, at pH 4.5 & 30°C giving a rate of lactose utilization of 0.7 g/lh (Izaguirre & Castillo 1982). This was between 4 to 6 times slower than the rate indicated previously when agitation and some air were available.

Concentrated whey Fermentations of concentrated whey containing between 100 to 200 g/l have been reported (table 2.1) using both *K.marxianus* and *C.pseudotropicalis*.

Table 2.1 Fermentation of concentrated whey.

yeast strains	S_{1o} g/l	S_{1u} %	S'_1 g/lh	time h	T °C	pH	reference
(a) partially aerobic							
KM CBS 5795	100	>95*	3.1	32	28	4.6	1
CP NCYC 744	100	>95	4.2	24	28	4.6	1
KM CBS 5795	150	>95	3.9	38	28	4.6	1
CP NCYC 744	150	>95	5.0	30	28	4.6	1
CP IP 513	200	>95	3.2	63	28	4.6	2
(b) anaerobic							
CP ATCC 8619	100	>95	0.7	92	30	4.5	3
KM NCYC 151	150	88	0.7	192	30	4.5	3
CP ATCC 8619	150	93	0.7	192	30	4.5	3
KM NCYC 151	200	60	0.6	192	30	4.5	3
CP ATCC 8619	200	84	0.9	192	30	4.5	3
KM NCYC 151	250	46	0.6	216	30	4.5	3
CP ATCC 8619	250	48	0.6	192	30	4.5	3

KM - *K.marxianus* ; CP - *C. pseudotropicalis*

S_{1o} - initial lactose concentration ; S_{1u} - lactose utilization

S'_1 - rate of lactose utilization ; time - fermentation time

T - fermentation temperature ; pH - initial pH of whey

* - >95 indicates complete lactose utilization

1. Burgess & Kelly 1979

2. Moulin et al 1980

3. Izaguirre & Castillo 1982

Complete lactose utilization was reported for fermentation of concentrated whey containing as high as 200 g/l lactose under partially aerobic fermentation (Burgess & Kelly 1979 ; Moulin et al 1980). Fermentation of concentrated whey containing 250 & 300 g/l, under the same fermentaton conditions, was reported to be incomplete but no data were given on residual lactose and fermentation time (Moulin et al 1980).

K.marxianus (NRRL Y 1109) utilized only 70% of lactose in concentrated whey. This was reported to be a result of ethanol inhibition (Burgess & Kelly 1979).

Fermentation of concentrated whey under completely anaerobic fermentation condition required long fermentation time (table 2.1) (Izaguirre & Castillo 1982). There was incomplete lactose utilization, when the lactose content was greater than 150 g/l, and even after a prolonged fermentation time as long as 200 h.

At all concentrations of lactose, *C.pseudotropicalis* utilized lactose at a slightly faster rate than *K.marxianus*.

In summary, complete lactose utilization occurred between 50 and 200 g/l lactose depending on the organisms. Lactose utilization decreased as lactose concentration increased. Increased lactose utilization and higher lactose utilization rate occurred when there was agitation and partially aerobic fermentation conditions. In all cases, the extent of utilization of lactose and rate of utilization were also found to be dependent on the strain of yeast.

(b) Ethanol production Two early independent investigators reported that in the fermentation of whey, ethanol yield based on lactose utilized was 80 & 91% when the lactose concentrations were 46 & 50 g/l, respectively (Browne 1941 ; Rogosa et al 1947). These workers did not provide data on ethanol concentrations.

Later investigators reported ethanol production from 50 g/l lactose to be from 21 to 26 g/l (table 2.2) because different fermentation conditions were used. The corresponding ethanol productivity varied from 0.3 to 2.0 g/lh. The lower productivity value of 0.3 was obtained under anaerobic fermentation conditions, while the productivity of 2.0 g/lh was obtained under partially aerobic conditions. The yield of ethanol on lactose used varied from 79 to 97% with no clear trends between different yeast strains and fermentation conditions. Some investigators used a term called " ethanol yield efficiency" which was calculated as the ratio of the amount of ethanol produced to the amount of total substrate available. The results showed decreasing yield efficiency as lactose concentration

Table 2.2 Ethanol concentration, productivity and yield in whey fermentation.

	lactose, g/l						ref.
	50	100	150	200	250	300	
(a) *K.marxianus* CBS 5795, 25°C, pH 4.6, partially aerobic.							1
S_{lu}, g/l	50	100	150				
E, g/l	28	45	74				
Time, h	17	32	38				
E', g/lh	1.4	1.4	1.9				
$Y_{e/ts}$, %	87	84	92				
(b) *K.marxianus* CBS 397, 28°C, pH na, partially aerobic.							2
S_{lu}, g/l	na	na	na	na	na	na	
E, g/l	25	47	69	90	90	79	
Time, g/l	12	na	na	na	na	na	
E', g/l	2.0	na	na	na	na	na	
$Y_{e/ts}$, %	90	90	87	86	68	50	
(c) *K.marxianus* NCYC 151, 30°C, ph 4.5, anaerobic.							3
S_{lu}, g/l	50	na	132	110	106		
E, g/l	22	na	63	29	28		
Time, h	72	na	192	96	144		
E', g/lh	0.3	na	0.3	0.3	0.2		
Y , %	82	na	89	50	48		
(d) *C. pseudotropicalis* NCYC 744, 28°C, pH 4.6, partially aerobic.							1
S_{lu}, g/l	50	100	150				
E, g/l	21	44	70				
Time, h	12	24	30				
E', g/lh	1.8	1.9	2.3				
$Y_{e/ts}$, %	79	83	86				
(e) *C.pseudotropicalis* IP 513, 28°C, pH na, partially aerobic.							2
S_{lu}, g/l	na	na	na	200	na	na	
E, g/l	25	46	70	90	69	87	
Time, h	12	na	na	63	na	na	
E', g/lh	2.0	na	na	1.4	na	na	
$Y_{e/ts}$, %	90	87	89	86	68	55	
(f) *C.pseudotropicalis* ATCC 8619, 30°C, pH 4.5, anaerobic.							3
S_{lu}, g/l	50	89	140	178	114		
E, g/l	26	51	75	98	32		
Time, h	72	120	192	216	216		
E', g/lh	0.4	0.4	0.4	0.5	0.1		
Y, %	97	100	100	100	52		

E - ethanol concentration ; E' - rate of ethanol production
Y - ethanol yield on substrate utilized
$Y_{e/ts}$ - ethanol yield on total substrate available

1. Burgess & Kelly 1979 ; 2. Moulin et al 1980 ;
3. Izaguirre & Castillo 1982.

increased. However, the report did not provide all fermentation times or residual lactose concentrations (Moulin et al 1980 ; Burgess & Kelly 1979).

At higher lactose concentrations of 100 g/l,the ethanol concentration varied from 44 to 51 g/l, productivity from 0.4 to 1.9 g/lh, and yield from 83 to 100%. Again the lower productivity was from anaerobic fermentation. The high value of ethanol yield (100%) was obtained for *C.pseudotropicalis* (ATCC 8619) under anaerobic conditions. Some reservations must be placed on these 100% yields because other workers found yields of between 80 to 91% only for the same yeast species but different strains (Izaguirre & Castillo 1982). Similar productivity and yield were found at lactose concentrations of 150 & 200 g/l except in the case of *K.marxianus* NCYC 151, which produced only 29 g/l ethanol from 200 g/l lactose.

When the lactose concentrations were 250 & 300 g/l, there were poor ethanol production and the maximum ethanol concentration was 90 g/l under partially aerobic fermentation of 200 g/l lactose (Moulin et al 1980). The ethanol productivity was only 0.1 to 0.2 g/lh for the anaerobic condition. There were no data available for partially aerobic conditions and the yield was very low (between 48 to 52%) for the anaerobic condition.

Thus, the maximum rate of ethanol production reported was 2 g/lh. The ethanol concentrations and yields were virtually independent of lactose concentration up to 200 g/l. At higher lactose concentrations, there was poorer ethanol production and much lower yield. Ethanol yield was found generally to be between 79 to 92%. Different yeast strains showed a different capability to produce ethanol.

It was found that prolonged fermentation could reduce the ethanol yield because both yeast species changed from lactose to ethanol metabolism (Burgess & Kelly 1979). *K.marxianus*, however, grew poorly on ethanol as a carbon source (Sarfacon et al 1972).

(c) Ethanol inhibition Ethanol inhibition was found to reduce lactose consumption and ethanol yield efficiency (Yoo & Mattick 1969). It had been found that 35 g/l ethanol could reduce the activity of β-galactosidase in *K.marxianus* by 77% (Wendorf et al 1970a). Adaptation to high ethanol concentration had been reported to improve ethanol tolerance to up to 79 g/l for some strains of *K.marxianus* (Gawel & Kosikowski 1978). Different strains of yeasts had been found to have different ethanol tolerance. Ethanol added to a final concentration of

55 g/l could kill more than 90% of the initial cell population of *K.marxianus* in 48 h. Ethanol was added at the beginning of the fermentation (Yoo 1974).

(d) Aeration and agitation It has been found that the fermentation rate of lactose-fermenting yeasts were very slow in the complete absence of aeration and agitation (Burgess & Kelly 1979 ; Yoo 1974). One *K.marxianus* strain (Y18) was able to ferment 150 g/l lactose to completion in 10 days if it were aerated during the initial 24 h. Static culture could not utilize lactose completely after 20 days (Yoo 1974). *K.marxianus* (CBS 5795) was found to ferment the same lactose concentration in 38 h under constant agitation and partial aerobic conditions. (Burgess & Kelly 1979).

(e) Nutrient requirements Most reported investigations on fermentations of whey to ethanol did not involve nutrient addition to the whey (O'leary et al 1977 a & b ; Moulin et al 1980 ; Izaguirre & Castillo 1982). Burgess & Kelly (1979), however, added yeast extract and urea to their whey permeate at a rate of 1 and 0.5 g/l, respectively. Owing to the different fermentation conditions used by various workers, it was not possible to compare the resultant fermentation rates.

In the production of microbial protein from whey using *K.marxianus* NRRL Y1109, the addition of $(NH_4)_2SO_4$, K_2HPO_4, and yeast extract at rates of 5,5,1 g/l, respectively, was required to improve the cell yield (Wasserman et al 1958). NaCl and $CaCl_2$ at a level of 15 g/l or higher were found to cause noticeable inhibition of fermentation by *K.marxianus* (Gawel & Kosikowski 1978).

(f) Temperature It was reported that between 30 and 42°C, a strain of *C.pseudotropicalis* was able to ferment lactose most rapidly at 37°C but the optimal temperature was found to be 33-34°C (Rogosa et al 1947). The rate of lactose fermentation of another strain (NCYC 744) was found to increase with temperature within the range of 25 to 35°C. The time required to utilize completely 150 g/l lactose was reduced from 22 to 20 h as the temperature was increased from 30 to 35°C (Burgess & Kelly 1979).

It was reported that when the fermentation temperature was between 22 to 32°C there was greater cell population at lower temperatures. As the temperature increased, the time required to reach the maximum cell level decreased. The rate of ethanol production was more rapid at higher temperatures. The optimal temperature for ethanol production from lactose was 28°C (Yoo 1974).

(g) pH It was found that a pH between pH 4 to pH 6 had no effect on the fermentation of lactose to ethanol by either *C.pseudotropicalis* or *K.marxianus*. The optimal fermentation pH was reported to be between pH 4.5 to pH 5.0 (Rogosa et al 1947 ; Yoo 1974 ; Burgess & Kelly 1979). Viable yeast cells were destroyed faster at lower pH (Yoo 1974).

In summary, there were considerable variations in the time reported for whey fermentation by lactose-fermenting yeasts. The time required was affected by the initial substrate level, fermentation temperature, inoculum size, aeration, ethanol produced, and the yeast strain used. The fermentation time was reduced when a fast lactose-fermenting yeast strain, increased temperature, inoculum size and sufficient aeration was used.

2.3 ETHANOL FERMENTATION PROCESSES.

Ethanol fermentation can be carried out in batch or continuous reactors, with batch fermentation being the most widely used process for producing industrial ethanol. Three quarters of the estimated total world industrial ethanol production of 3,200 million litres is produced using the batch fermentation process (Maiorella et al 1981). The process is simple and reliable, but slow. The overall productivity for a batch process is typically between 1.8 to 2.5 g/lh for fermentation of molasses containing 100 g/l sucrose , with 28-32 h fermentation at $30^{o}C$, while for whey, rates of up to 2.0 g/lh are reported.

There are many ethanol fermentation processes in operation and under development which will improve the process over that of batch fermentation. Simple criteria for comparing various processes have been described as low operating and capital costs. Low operating cost is made possible through a continuous process with simple operation, low energy input and near complete sugar utilization. Low capital cost is the result of high productivity through small reactor volume and a mechanically simple reactor (Maiorella et al 1981).

2.3.1 Alternative processes to batch ethanol fermentation

(a) Continuous stirred tank fermentation (CSTR) Continuous fermenters offer many advantages over batch fermenters. These include reduction in operating and maintenance costs, consistency of products and reduction in processing time for the same holding capacity. The process is however less flexible than the batch process and it is essential that the contamination by foreign organism be

avoided, as this can affect the function of the yeast in the system (Hospodka 1966). The theory of continuous fermentation is well reviewed (Hough & Wase 1966 ; Fredrickson & Tsuchiya 1977 ; Levenspiel 1980).

It has been reported that several plants in the USSR use CSTR processes for the production of industrial ethanol (Hospodka 1966). The total productivity of a CSTR system is limited, however, by the low cell density (10-12 g/lDW) achieved in the fermentation (typically 5-10% of the influent sugar concentration) and the cell maximum specific growth rate. The overall productivity was reported to be about 6 g/lh from 100 g/l sugar input which was 3 times that of batch productivity (Maiorella et al 1981). By adding a continuous cell recycle, cell densities as high as 83 g/l DW could be maintained with ethanol productivity of 30-40 g/lh from 100 g/l sugar feel (Maiorella et al 1981). The CSTR systems,however, required constant energy input to provide agitation, to transfer liquor from one vessel to the next and for centrifugation (Maiorella et al 1981).

(b) Vacuum fermentation This process was developed to overcome ethanol inhibition in order that a high sugar concentration could be used. By drawing a vacuum of 73 kPa (55 mmHg), at 35^{o}C, ethanol was continuously boiled off from the liquid and withdrawn from the fermenter. In continuous mode, 333 g/l glucose feed was fermented to give total ethanol productivities of up to 82 & 40 g/lh with and without cell recycling, respectively. The productivities obtained here were up to 20 to 40 times that of batch productivity (Cysewski & Wilke 1977). There were some drawbacks to this process. It was reported that the energy consumed in providing the vacuum led to a total requirement 5% greater than for the conventional process. There was also a constant pure oxygen requirement to meet the yeast oxygen maintenance requirements under vacuum. This added 0.5 cents(US) to the cost of every litre of ethanol produced. The vacuum pumps required must operate at unusually low pressures and are extremely large and they would be difficult to control. There was also the extra capital required to provide the vacuum pumps. Vacuum operation could increase the likelihood of fermenter contamination and shutdown. Doubts were expressed as to whether the outstanding productivity of the vacuum fermenter could compensate for these potential difficulties (Maiorella et al 1981).

A process called "flash fermentation" modified the simple vacuum fermentation process to overcome some of its operating difficulties. The fermentation was carried out in an atmospheric pressure fermenter so that the yeast oxygen requirement could be cheaply met with sparged

air. Ethanol was removed by rapidly recycling the beer, via a flow regulating valve, to a small auxillary flash vessel where it boiled off under vacuum. Ethanol was recovered as the flash vessel overhead product and the ethanol depleted beer was pumped back to the fermenter. The contamination problem was greatly reduced as only the small flash vessel was under vacuum. The energy requirement was slightly lower than the vacuum fermenter but the flash fermentation plant was more complicated than the vacuum fermenter because it required an added vessel and associated beer cycling pumps. Again, there were doubts as to whether the high productivities possible would offset the operation and control difficulties and the likely high vacuum pump costs (Maiorella et al 1981).

(c) Rapid batch fermentation Rapid batch fermentation is a process that used a large cell inoculum to achieve short fermentation times. A honey solution containing 250 g/l total sugar was fermented to 100 g/l ethanol in 3 h, at 30°C. The inoculum was 10^9 cells/ml or 50 g/l DW and this gave a productivity of 33 g/lh. The drawback with this fermentation was loss of viability with only 2% viable yeast at the end of the fermentation. Continuous aeration to maintain the dissolved oxygen level at 13% of saturation and reduction of the temperature to 15°C increased cell viability to 94%. The fermentation time was however increased to 6 h, while ethanol productivity reduced to 17 g/lh (Nagodawithana et al 1974 ; Nagodawithana & Steinkraus 1976). At this low temperature, energy would be required to provide refrigerated cooling to remove the heat of the fermentation. As this process was a batch process, the productivity would be reduced when the down time was taken into account. The time and fermentation capacity required to prepare such a large inoculum would also be considerable.

(d) Fermentation by immobilized cells Cells can be immobilized by entrapment in a gel matrix such as alginate gel. This process employs the entrapped cells in a packed-bed column fermenter. There is no requirement for agitation or yeast recovery equipment. The medium is fed from the bottom of the column. An investigation using immobilized *Zymomonas mobilis* to ferment 150 g/l glucose at 30°C, reported an ethanol productivity of 53 g/lh, ethanol concentration of 63 g/l and a residence of 1.2 h. There was 30% reduction in the cell activity after 33 days of continuous operation (Grote et al 1980). Immobilized *S.cerevisiae* were employed to ferment molasses solution containing 197 g/l reducing sugars, with a reported ethanol productivity of 25 g/lh, ethanol concentration of 71 g/l , residence time of 2.9 h, and cell half life of 1800 h

(Ghose & Bandyopadhyay 1980).

Continuous fermentation of whey using immobilized yeasts has been reported. One investigation described stable continuous fermentation operation for one month but the results showed a steady reduction of the ethanol concentration with time (Linko et al 1981). When the inlet lactose levels in demineralized whey were 50, 100, and 150 g/l, the ethanol levels were initially 25, 45 and 48 g/l, but after one month, these levels decreased to 21, 30 and 33 g/l, respectively. Thus, ethanol productivities were reduced from 6.4, 5.4 and 3.2 to 5.4, 3.6 and 2.2 g/lh, respectively. The residence time were 3.9, 8.3 and 15 h, respectively. The initial pH and temperature were 4.5 and 25^{o}C, respectively.

K.marxianus (NRRL Y 1109) immobilized in acrylamide gel was used in a different study to ferment whey containing 51 g/l lactose (Dillon 1980). The optimum dilution rate reported was .15 h^{-1} which corresponded to a residence time of 6.8 h. The productivity obtained was 3.1 g/lh which resulted in 21 g/l ethanol at the exit. There was 93% lactose utilization and 79% yield of ethanol on lactose utilized. The yeast cells were found to lose less than 10% of the initial fermentative activity in 10 days and had a half life of 50 days.

Finally, this process may give considerably higher ethanol productivity than batch fermentation but the cells have a short stable life. This means frequent replacement of immobilized cells in order to maintain continuous fermentation operation. It had been estimated that gel cost contributed24% of the operating cost of a fermentation-distillation plant producing ethanol from whey as a substrate, using entrapped *K.marxianus* with half life of 50 days (Dillon 1980).

A modification to the fixed bed reactor system used in the immobilized cell fermenter is the attached-film-expanded-bed fermenter (AFEB) in which cellulose acetate was used as the yeast supporting medium.When fermenting a 100 g/l lactose feed, the residual lactose increased gradually from 22 to 83 g/l, while ethanol decreased from 35 to 7.6 g/l as the dilution rate increased from .07 to 0.9 h^{-1}. The ethanol productivity was 2.4 g/lh at dilution rate of .07 h^{-1} whereas it was 6.9 g/lh at D of 0.9 h^{-1}. The corresponding values of sugar utilization were 78 & 17%, respectively (Chen & Zall 1982).

(e) Tower fermentation The continuous tower fermentation process employs a single tubular reactor with a conical bottom and an expanded top section to facilitate yeast settling. The fermentor is filled with flocculent yeast and the cell concentration of 200 to 400 g/l WW is maintained in the tower. Typically, an ethanol productivity of 10 g/lh

and concentration of 39 g/l are obtained at a residence time of 3.8 h from a feed beet molasses solution containing 100 g/l reducing sugar. The advantages of tower fermentation lie in the simplicity of construction and mode of operation. No agitation is required to keep the yeast cells in suspension. Yeast separation and recycling are carried out inside the tower (Hough et al 1976 ; Coote 1974). Thus, there is considerably less energy and capital requirements compared with fermentation systems described previously. Tower fermenters have been operated successfully on a commercial scale to produce different products such as beer and vinegar (Greenshields & Smith 1974).

2.3.2 Industrial and pilot plant processes used for the production of ethanol from whey

At present, there are 5 plants producing ethanol from whey by batch fermentation. These plants are located at Reporoa, N.Z. (Howell 1981) ; Edgecumbe, N.Z. (Mawson 1983) ; Temuka, N.Z. (Gooding 1982) ; Carbery, Ireland (Sandbach 1981) ; and Juneau, Wisconsin, USA (Anon 1977). The process at Reporoa which is similar to the process at Carbery produces 18 g/l ethanol from 41 g/l lactose in 16 h. This gives an ethanol yield of 90% and productivityof 1.1 g/lh. There were no technical data available for other plants.

A commercial continuous fermentation process employing three fermenter tanks in series (CSTR) is used at Tirau, N.Z. The plant processes a maximum of 1500 m^3 of deproteinated whey per day to produce 32,000 litres of 96% ethanol (Howell 1981).

A pilot plant scale investigation was carried out in Denmark. The process employed two fermenters in series to carry out continuous fermentation. The minimum residence time indicated was 12 h (Reesen 1978).

Thus, among all the ethanol fermentation processes considered in these last two sections (sect.2.3.1 & 2.3.2), with the exception of the batch process, only the CSTR and the tower fermentation processes are in commercial use. The tower fermentation process fits all the simple criteria of low operating and capital cost required to improve the economy of the process. Its high productivity is surpassed only by the energy intensive vacuum fermentation process. It has better productivity than the CSTR processes and is considerably less energy and capital intensive.

2.4 ETHANOL PRODUCTION BY TOWER FERMENTATION

2.4.1 History of tower fermentation

The concept of tower fermentation was described as early as 1945 (Alzola 1945). The process described employed a column divided into six parts. The feed medium was pumped in from the bottom of the column and the carbon dioxide produced from the fermentation provided agitation. A similar design was subsequently used for continuous fermentation but with the feed medium gravity fed from the top of the column (Owen 1948). A process which employed a series of tanks stacked one on top of another to form a tower was patented in the same year (Victorero 1948). The feed liquor entered from the bottom of the lowest tank, passing through the upper tanks and out at the top of the tank.

A continuous fermentation process whereby a single vertical tubular fermenter was employed, was patented in 1960 (Watson & Shore 1960) for APV Co Ltd. The tower fermenter was filled with a high concentration of microoganisms and had an expanded top section which permitted the microorganisms to settle and return to the tower's lower section. This process was intended for beer production but other applications have been described (Smith & Greenshields 1974).

Other types of tubular column fermenters have been described by various workers, such as the gradient-tube continuous fermenter (Portno 1967), multistage tower fermenter (Paca & Gregr 1979), tower fermenter with two fluid feed nozzles (Goto et al 1981), column fermenter packed with immobilized yeast (Linko & Linko 1981). A summary of tower fermenters is given in table 2.3. The following is concerned mainly with the APV tower fermenter type.

2.4.2 The characteristics and operation of the tower fermenter

The tower fermenter behaves as a heterogeneous system, with a progression from substrate solution to fully fermented liquor within the single vessel. The APV tower fermenter is made up of a vertical cylindrical tower with a conical bottom (fig.2.2). The medium enters at the bottom and passes up through a dense suspension of yeast cells. At the top, the vessel expands into a large settling zone in which yeast is separated from the effluent liquor and returns to the main body of the tower, while the clear alcoholic liquor overflows from the top of the tower. Fermentation proceeds as the liquor rises while the yeast cells settle back and are retained. Thus, the superficial medium velocity must not exceed the settling velocity of the cell flocs. The APV tower fermenter

Table 2.3 Industrial ethanol tower fermentation studies or processes.

reference	fermenter sizes & other details	media
Alzola 1945	6-sections column ; mash enter from the bottom & agitated by CO_2 produced (patent).	na
Victorero 1948	tanks stack one on top of another ; mash enter from bottom & exit at top (patent).	na
Owen 1948	73mmϕ x760mm high ; six-sections(108mm long)-glass tower ; molasses feed from top to bottom.	molasses
Watson & Shore 1960	APV tower, single stage tower using flocculent yeast ; 1.1mϕ x 10 m high & 1.8mϕ x 12.5m high (6.5 & 7.2m straight section height respectively ; commercial sizes) ; 150mmϕ x 7m (pilot size).	various
Coote 1974	Pyrex, 25.5mmϕ x 1.75m high(887ml) + 80mmϕ x .54 m high(2.714 l) + expanded spherical section 60mmϕ x 60mm liquid depth(57ml) ; total height 2.35m ; varying feed rate ; sugar concentrations 100-250 g/l ; used flocculent *S.cerevisiae* ; 20-28°C.	lager beer beet molasses
Henderson Smith 1982	Perspex, 100mmϕ x four 1.5 l sections ; 6 l total volume ; 3 perforated plates ; feed rate varied ; sugar concentrations 100-150 g/l ; used flocculent *S.cerevisiae* ; 35°C.	fodder beet & sugar beet extracts
Prince & Barford 1982	Pyrex, 75mmϕ straight section ; 150mmϕ yeast settling section ; overall H/ϕ 22:1 ; 10.1 l total volume ; flocculent *S.cerevisiae*, *S.diastaticus*, & *Z.mobilis* with flocculent aid ; feed rate varied ; sugar concentrations 100-250 g/l ; 30°C.	cane juice whey cane stillage

ϕ - internal diameter ; H - height

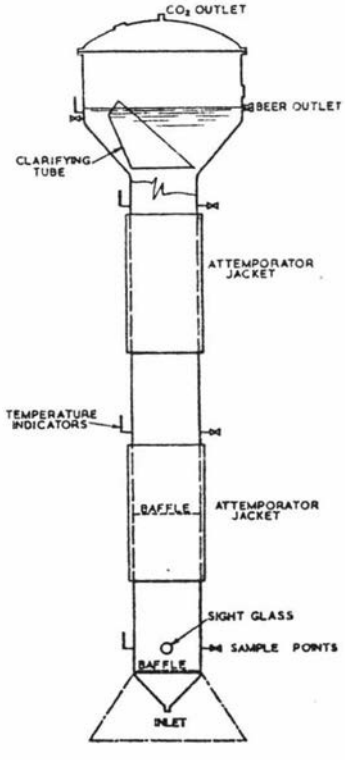


Fig.2.2 Schematic diagram of the APV tower fermenter (Greenshields & Smith 1971).

used for commercial beer production has an overall aspect ratio (straight section to internal diameter) between 7:1 to 10:1 and tower diameters from 0.9 to 2.0 m (Royston 1966). Baffles are incorporated within the tubular section of the fermenter to reduce gas channelling and prevent back mixing of the yeast and the beer (Klopper et al 1965).

At relatively low liquid flow rates, the tower fermenter can be considered as a fixed bed, catalytic reactor and at relatively high flow rates as a fluidized bed catalyst reactor. Stoke's law has been used to described the characteristics of yeast flocs inside tower fermenters. However, it was found that this could not adequately describe the characteristics of the yeast flocs (Greenshields & Smith 1971).
A summary of the equation for estimating the hindered settling velocity of particles in a liquid has been described by Boening & Larsen (1983) but these require exact knowledge of particle size, density and sphericity as well as the voidage of the fermenter if reasonable results are to be obtained.

(a) Organisms for tower fermentation A flocculent strain of organism is essential in tower fermentation to prevent wash out, as the density of the yeast cells differs only slightly from that of the liquid. Flocculent yeast that have been used in tower fermentation are from the genus *Saccharomyces*. One study (Coote 1974) carried out extensive tests on flocculent *Saccharomyces* strains for tower fermentation of beer and molasses. More than 20 strains of flocculent yeasts were listed. *S.cerevisiae* NCYC 1257 was used in the study on tower fermentation of cane juice (Prince & Barford 1982). *S.cerevisiae* CFCC 39 was used in the tower fermentation of beet juices (Henderson & Smith 1982). A flocculent strain of *Z.mobilis* has also been tested for use in ethanol production from cane juice using a tower fermenter (Prince & Barford 1982). Further discussion on yeast behaviour in tower fermentation is given in sect.2.5.

(b) The effect of tower height In tower fermentation of beer there is a gradual decrease in the wort specific gravity with increasing height from the tower inlet (fig.2.3). The wort specific gravity decreases sharply from 1.036 to 1.010 for most flowrates over the first half of the tower, whereas the decrease is lower (1.010 to 1.005) over the second half of the tower. As the superficial liquid velocity increases there is an increase in the specific gravity at various tower heights. The initial rapid fall of wort gravity is due to fermentation of glucose, fructose, sucrose, and some maltose, whilst the slower fall over the middle and top of the tower is due to fermentation of the remainder of the maltose

and maltotriose in the wort. It is also found that, at a particular superficial liquid velocity, higher wort gravity results in higher final specific gravity of the effluent beer.

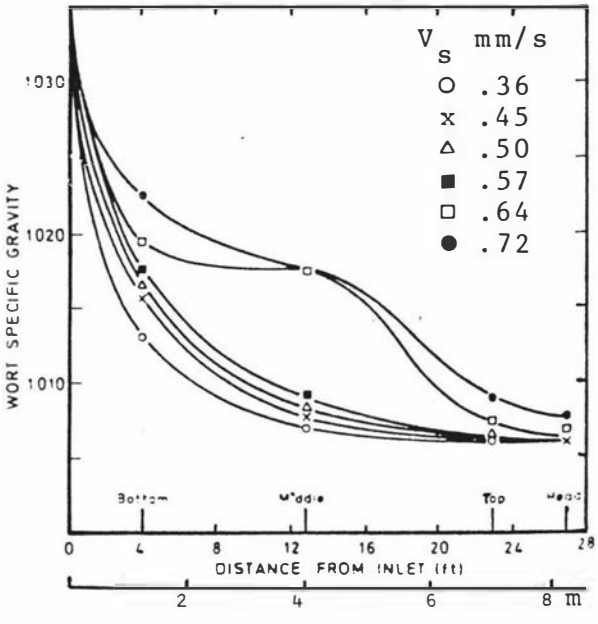


Fig.2.3 Progressive reduction of wort gravity in a tower fermenter at various flow rates using physically limited yeast. (Greenshields & Smith 1971)

(c) Residence times in tower fermentation There were considerable differences between the residence times for the gas, liquid, and solid phases in a tower fermenter (Smith & Greenshields 1973). The rise velocity of the gas bubbles was reported to be dependent on the floc size and concentration and medium throughput. In a full scale (0.9 m diameter) tower, reported rise velocity was 0.1 m/s and the residence time was 80 s.

The average residence time for the yeast in tower fermentation of beer has been estimated to be approximately 100 h (Smith & Greenshields 1973). At present there is no published information on the effect of the medium specific gravity, feed rate, aeration and foaming on the residence time of yeast in tower fermenters.

In beer fermentation , liquid residence times between 4-6 h have been reported for wort with an initial specific gravity of 1.035 (58 g/l reducing sugars), with 81% sugar utilization, at 21^{o}C. The sugars present in the beer wort were found to have different fermentation times inside the tower. No free sucrose was detected after 25 min, whereas glucose and fructose were both utilized in 40 min. Some maltose was utilized during this period but 2 h was required for complete utilization of maltose. Maltotriose required 3.75 h to be completely utilized with

virtually no utilization in the first 1.5 h. Thus, the necessary 4-6 h residence time in beer tower fermentation was due to the time required to ferment maltotriose. This still compares favorably with 48 to 72 h required for batch fermentation (Ault et al 1969).

This difference in times is reflected when using a plot of wort gravity vs apparent fermentation time (fig.2.4). It shows that the wort gravity is reduced from 1.035 to 1.010 in 1 h but further reduction from 1.010 to 1.005 required 19 h (Klopper et al 1965).

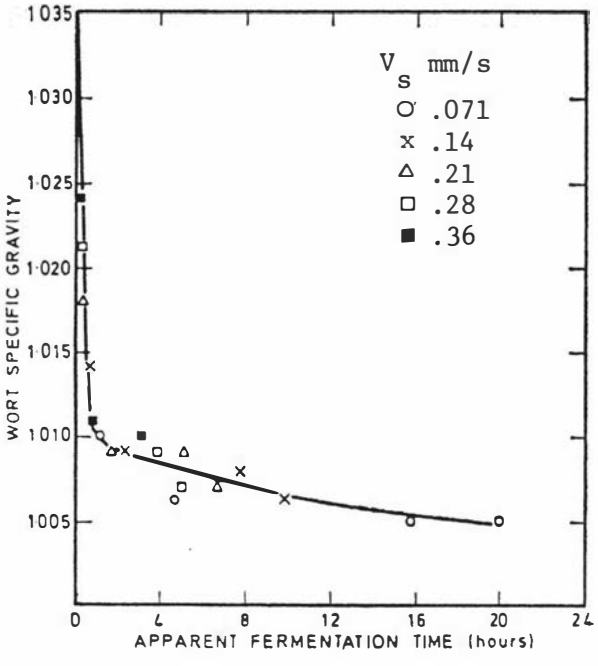


Fig.2.4 The relationship between feed liquor specific gravity and apparent fermentation time in tower fermentation of beer using physically limited yeast. (Klopper et al 1965)

In the fermentation of beet molasses (Coote 1974), containing 100 g/l reducing sugars, the limiting volumetric efficiency (the maximum ratio, between the volume of beer produced in 24 h and the void volume of tower fermenter, at which there was 80% sugar utilization using feed liquor of specific gravity 1.050) was found to be 4.7 or a dilution rate of 1.1 h^{-1}. This was equivalent to an estimated T_r of 0.9 h. The exit ethanol concentration was 39 g/l.

(d) The effect of feed liquor specific gravity on limiting volumetric efficiency It was found that, for a particular original feed liquor gravity, there was a limiting volumetric efficiency at which wash out occured. This increased rapidly as the original gravity was decreased from 1.090 to 1.030 (fig.2.5). When the original gravity was 1.030, the volumetric efficiency could be increased without wash out (Greenshields & Smith 1971). This made the tower fermenter ideal for use in the fermentation of dilute feed medium if an appropriate flocculent organism were available.

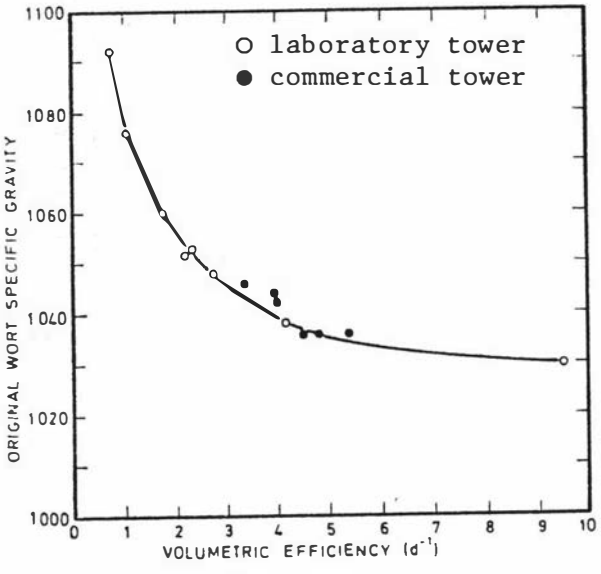


Fig.2.5 The effect of original feed liquor specific gravity on the limiting volumetric efficiency for a physically limited yeast. (Greenshields & Smith 1971)

(e) Aeration Aeration was found to be important in the operation of the tower fermenter (Ault et al 1969). It allowed a greater degree of yeast growth and improved yeast viability. During anaerobic fermentation, aeration of the feed medium to 70-75% saturation, was found to be sufficient to maintain yeast viability. During the cell build up period, the tower was aerated directly. The superficial air velocity was limited to 10 mm/s to avoid foaming and slug flow. The typical air to liquid volumetric flow ratio used was 50:1.

2.4.3 Applications of tower fermentation

The APV tower fermenter has been used successfully in ale brewing. The tower fermenter has also been reported to be used commercially for the production of vinegar (Greenshields & Smith 1974). The production of microbial protein and waste treatment using the tower fermenter have been investigated (Morris et al 1973 ; Ewen 1980 ; Burgess 1977).

At present there is no commercial application of the tower fermenter for industrial ethanol production although there have been a few laboratory scale investigations.

2.4.4 Industrial ethanol tower fermentations

(a) Laboratory scale investigations It was indicated previously that there is no commercial scale use of the APV type tower fermenter for industrial ethanol production. There were three reported laboratory scale investigations. The details of the tower sizes used are given in table 2.3 (Coote 1974 ; Henderson & Smith 1982 ; Prince & Barford 1982).

(b) Medium used and the effect of sugar concentration Industrial ethanol production by tower fermenter reported to date has used only sucrose based substrates such as beet molasses (Coote 1974), cane juice (Prince & Barford 1982), fodder and sugar beet extracts (Henderson & Smith 1982). The sugar concentrations used vary from 100 to 200 g/l.

In the tower fermentation of beet molasses (Coote 1974), it was found that, at a particular reducing sugar level, there was an optimum feed rate or volumetric efficiency at which 80% sugar utilization occured and above which the utilization decreased and cell wash out occurred. The limiting volumetric efficiency decreased with an increase in the reducing sugar concentration of the feed medium (table 2.4). The ethanol productivity also decreased with an increase in the sugar concentration. The reduction in the productivity was due to LVE as percentage sugar utilization and percentage ethanol yield remained constant. The reduction in the LVE was a result of the reduction in the yeast settling velocity due to increase in liquid specific gravity with increase in sugar concentration.

Table 2.4 The effect of sugar concentration on the limiting volumetric efficiency (LVE) in the tower fermentation of molasses (Coote 1974)

reducing sugars, g/l	100	150	175	200
LVE, day^{-1}	4.7	2.5	2.0	1.1
V_s, mm/s	.53	.29	.23	.13
D , h^{-1}	1.1	.59	.47	.26
*ethanol productivity, g/lh	10	8.0	8.2	4.9

* The productivity was calculated based on the overall residence time inside the tower including the residence time inside the separator.

In the tower fermentation of cane juice (Prince & Barford 1982) it was found that the dilution rate required to achieve a given sugar utilization and productivity was at an optimum when the sugar concentration was 123 g/l (fig.2.6). As the sugar concentration was increased further, it was necessary to reduce the dilution rate and hence the productivity in order to maintain the same level of sugar utilization. For 95% sugar utilization, the optimum dilution rate and ethanol productivity were .27 h^{-1} & 16 g/lh, respectively.

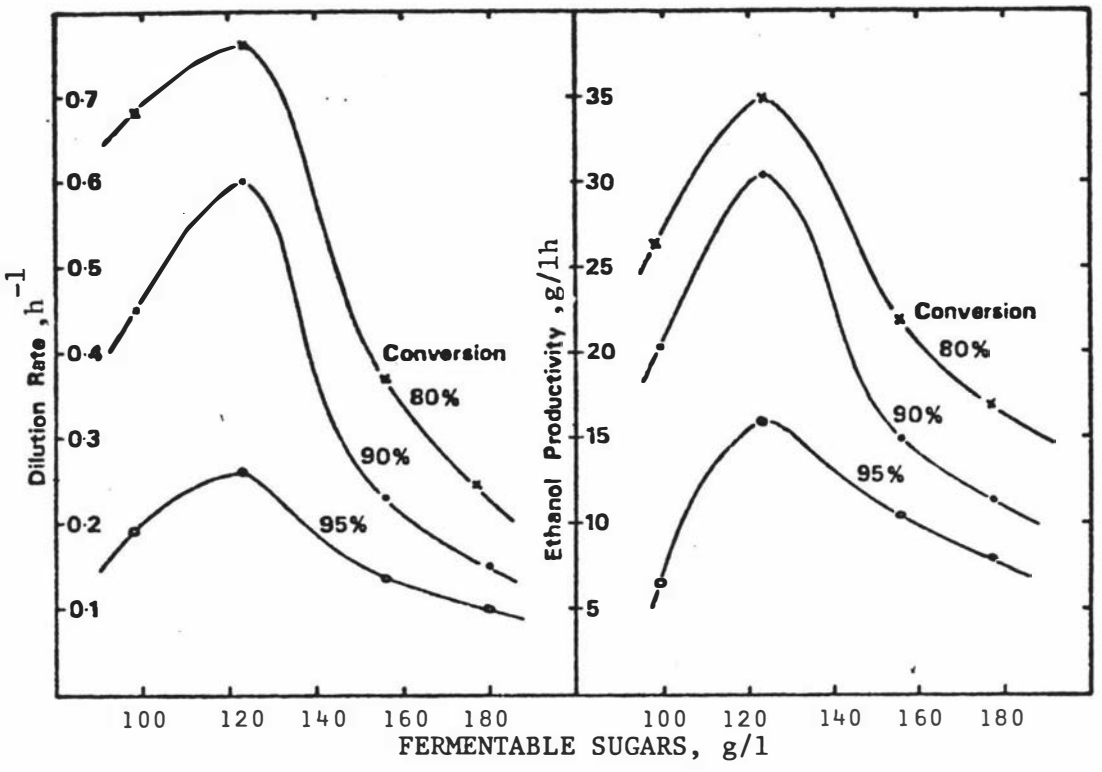


Fig.2.6 The effect of fermentable sugars concentration on the dilution rate and ethanol productivity of tower fermentation of cane juice (Prince Barford 1982).

In the tower fermentation of beet juices containing 100 g/l sucrose (Henderson & Smith 1982), it was found that there was 91 & 77% sugar utilization at dilutions rates of .20 & .33 h^{-1}, respectively. The ethanol concentrations and the productivities were 49,40 g/l and 9.8, 13 g/lh, at D of .20 & .33 h^{-1}, respectively. When the sucrose level was increased to 150 g/l, it was found that there was no sugar utilization after the ethanol concentration reached 60 g/l due to ethanol inhibition. This corresponded to 70% sugar utilization.

In summary, the dilution rate decreased as the feed sugar concentration increased. The ethanol productivity decreased with an increase in the sugar concentration.

(c) Fermentation temperature The operating temperature of tower fermenter differed from one group of investigators to another. In the tower fermentation study of beet molasses the temperature used varied from 20 to 28^{o}C. It was found that this temperature increase resulted in 6% increase in substrate utilization rate from 193 to 204 g/lh. There was 4.2% increase in the ethanol production rate but 1.5% reduction in ethanol yield efficiency. The concentration of the fermentable sugar used was

150 g/l and the volumetric efficiency was 7.2 (V_s= .82 mm/s) (Coote 1974).

A temperature of 30°C was used during tower fermentation of cane juice (Prince & Barford 1982). In the tower fermentation of sugar beet extract, a higher temperature of 35°C was used (Henderson & Smith 1982).

(d) Operating pH No data on the effect of pH of operation has been reported in the literature. A pH of 4.5 was used during a tower fermentation of beet molasses (Coote 1974). This was the same pH used in a different investigation to ferment sugar beet solution (Henderson & Smith 1982). A lower pH of 4.0 was used in a tower fermentation of cane juice (Prince & Barford 1982).

(e) Conclusions Finally, it is evident that there have been very few investigations on industrial ethanol tower fermentation. The three groups of workers reviewed here used different approaches to present their data and so it was difficult to compare their results. None of the three papers referenced indicated directly the straight section of the tower . Coote (1974) used the parameter "volumetric efficiency". This is a very difficult parameter to use in comparison with other workers because this parameter is not very often quoted by other workers. In order to estimate the superficial liquid velocity and dilution rate, Coote's values were multiplied by 208.3 to give a medium feed rate in ml/h. This conversion factor was obtained from the author's data which indicate that a volumetric efficiency of 4.67 was equivalent to 972 ml/h. The ratio of these figures gave 208.3 ml/h per one unit of volumetric efficiency, assuming that the relationship was constant for all feed rates. The feed rate values in ml/h were then converted to superficial liquid velocity and dilution rate using the quoted information on the tower diameter (25 mm) and tower height (1.75 m).

The results reported by the three groups of investigators showed that tower fermenters gave high ethanol productivities ranging from 5 to 16 g/lh. The residence time varied from 0.9 to 3.9 h for 80% sugar utilization upward. These values were affected by the concentration of the feed sugar and temperature.

2.5 FLOCCULATION OF YEASTS

2.5.1 Flocculent yeast classification

Yeast for ethanol fermentation in tower fermentation can be classified into three groups, based on their flocculent properties (Greenshields & Smith 1971).

(a) Non-flocculent These yeasts do not attain a concentration above 30 g/l centrifuged wet weight (WW) and are rapidly washed out from the tower.

(b) Flocculent-physically limited These yeasts attain a concentration in the tower of 200-300 g/l WW and form fine flocs up to 2 mm in diameter. At any particular specific gravity of the wort, there is a critical superficial liquid velocity which, if exceeded, causes a complete wash out of this type of yeast. At superficial liquid velocities up to this critical wash out value the normal fermentation of the wort is achieved.

(c) Flocculent-fermentation limited These yeasts attain high cell concentrations (250-400 g/l WW) and form heavy "sticky" flocs up to 13 mm in diameter. They are retained in the tower at all wort gravities and up to relatively high liquid velocities. At a certain critical superficial liquid velocity (V_s), the complete fermentation of the wort can no longer be achieved with this class of yeasts due to insufficient liquid residence time. If the superficial liquid velocity is furthur increased complete wash out occurs.

2.5.2 Quantitative measurement of yeast flocculation

Several methods have been used by various workers for the measurement of yeast flocculation, of which some have undergone subsequent modification.

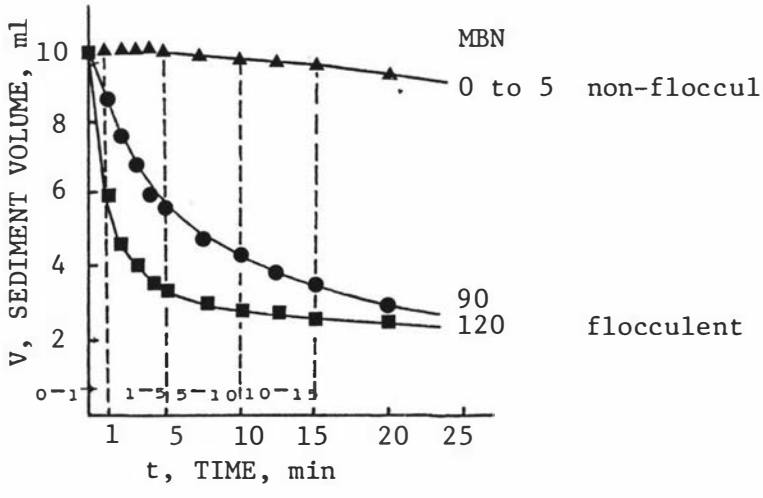
(a) Burn's method as modified by Helm et al (1953) Burn's method is a relatively simple method of flocculation measurement. One gram WW of yeast cells was carefully weighed into 10 ml calcium sulphate solution (0.51 g/l) in a graduated tapering 15 ml centrifuged tube. After mixing, the tube was allowed to stand. The volume of yeast sediment after 10 min became the Burn's number. It was, however, found that the Burn's number could not be related to the performance of flocculent yeasts in the tower fermentation. Different strains of flocculent yeasts gave different limiting volumetric efficiency in the tower fermenter whereas the Burn's number could not distinguish between them (Greenshields et al 1971).

(b) Burn's method as modified by Stewart (1975) This was another simple method very similar to the one described previously (sect.2.5.2a) to determine quickly yeast flocculence. Yeast cells cleaned in deionized water were suspended in 10 ml of deionized water containing 80 mg/l

calcium chloride at pH 4. The degree of flocculence was determined after 10 min of mixing by visual examination using a subjective scale (0-5) ranging from extremely flocculent (5) to non-flocculent (0).

This method has the same limitation as the previous method but any amount of cells can be used. Thus, it can be used to quickly distinguish between flocculent and non-flocculent yeasts before the more tedious modified Burn's number is determined.

(c) Sharp's method (Greenshields et al 1972) A further modification to the method described in sect.2.5.2(a) gave flocculence as modified Burn's number (MBN). This value was obtained by plotting the yeast sedimented volume against time at 1 min interval for 15 min on 1/10 inch graph paper (fig.2.7).



$$MBN = \{(\frac{V_0-V_1}{t_1-t_0}) + (\frac{V_1-V_5}{t_5-t_1}) + (\frac{V_5-V_{10}}{t_{10}-t_5}) + (\frac{V_{10}-V_{15}}{t_{15}-t_{10}})\} \times \frac{1}{4}$$

V_i is the sediment volume (ml) at time t_i (min).

Fig.2.7 Determination of modified Burn's number (MBN) (Greenshields et al 1972).

The slopes of the straight lines between 0-1,1-5,5-10,and 10-15 min were summated and divided by 0.4 to give the MBN value. The factor of 0.4 was necessary because 2 ml of yeast was plotted as 1 inch while 5 min was plotted as 1 inch ie. a slope of 1 was equivalent to 0.4 ml/min. The method is more tedious and time consuming than the previous two methods. It was, however, found that the MBN value can be related proportionately to the performance of yeasts in the tower fermentation. This was because the method takes into account the yeast settled volume during the first five min where very rapid settling of flocculent yeast

occurs. The previous two methods did not take this into account. Very flocculent yeast gave MBN values as high as 160. For tower fermentation, the yeast should have MBN greater than 70 (Coote 1974).

(d) Spectrophotometric method A spectrophotometric method measured the absorbance of 0.1 g of washed yeast suspended in 4 ml acetate buffer in a 10 mm spectrophotometer cuvette (Greenshields et al 1972). The yeast flocs were loosened and dispersed by gentle agitation with a small spatula followed by slow inversion of the cuvette 10 times. The cuvette was then inserted rapidly into an automatic recording spectrophotometer. Flocculent yeasts showed rapid decrease in absorbance (at 670 nm) from 1.8 to almost zero in less than 40 s, whereas non-flocculent yeasts showed little decrease in absorbance with time. The absorbance normally remained at 1.9 after a time period of 2.5 min. This method is still in the development stage.

2.5.3 Factors influencing yeast flocculence

The tower fermentation system can operate only with a highly flocculent yeast (Royston 1966). There are two determining factors that influence the flocculence of yeast, one being the genotype and the other external factors which refer to the growth medium and other environmental factors such as temperature, pH, chemicals and the presence of other organisms (Atkinson & Daoud 1976).

Microbial flocs and flocculation in fermentation processes is well reviewed (Atkinson & Daoud 1976) while the flocculent behaviour of the *Saccharomyces* spp is extensively studied particularly in brewer's yeasts (Stewart 1975). The flocculent behaviour of yeast described here applies mainly to *Sacchamyces* spp unless stated otherwise.

2.5.3.1 Inherited flocculent properties in yeast

(a) Flocculent genes It is known that there are four genes regulating the flocculation in yeast. They have been labelled as *FLO 1*, *FLO 2*, *flo 3* and *FLO 4*. All are dominant genes except "*flo 3*" (Stewart & Russel 1977), and only one of these genes need to be present for flocculation to occur. Spontaneous gene mutation or mitotic segregation rates from flocculent to non-flocculent are high and much higher than those rates in the reverse direction (Lewis et al 1976). It is known that spontaneous yeast mutation can occur and is considered to be of importance in continuous fermentation (Thorne 1968, 1970).

(b) The yeast cell wall The inheritable character is expressed in the yeast cell wall structure, which shows differences in various yeast

cultures, grown under different conditions (Jayatissa & Rose 1976). The yeast cell wall is thought to consist essentially of two layers, the outer mannan-phosphate protein layer which is connected to the inner structure glucan layer. In general the relative composition of each component is 40% glucans, 40% mannans, 10% proteins and the remaining proportion are hexosamine, lipids and inorganic materials (Stewart 1975 ; Lyons & Hough 1971).

(c) Comparison of the cell walls of flocculent and non-flocculent yeasts There was evidence of a higher level of phosphorus in flocculent walls than in non-flocculent walls (Lyons & Hough 1970, 1971). 70% of the phosphorus was incorporated as phosphodiester which bonds mannan and protein of the yeast cell wall together. Walls from flocculent yeasts were found to bind on average twice as much calcium as did walls from non-flocculent yeasts. Removal of the phosphorylated manno-protein decreased the capacity of the cell walls to bind calcium ions and rendered the wall non-flocculent. It was postulated that the phosphate of the glycoprotein complexes with calcium ions to form bridges between adjacent cells. In non-flocculent walls the level of the phosphate is presumably not sufficiently high to permit formation of stable salt bridges (Lyons & Hough 1970, 1971).

Subsequent studies on yeast mannan structure of a number of *S.cerevisiae* mutants which have altered mannan structures found a mutant which had a lower phosphate content than its parent strain but exhibited a much higher degree of flocculence (Ballou et al 1973). These workers also detected no difference between the phosphate level of flocculent and non-flocculent cell walls (Cawley & Ballou 1972). It was found that there was a much higher content of total carbohydrate in the flocculent yeast cell walls than in non-flocculent walls. The increased carbohydrate content was found to be due to an elevated level of mannan while the glucan level appeared to be very similar (Stewart 1975 ; Beavan et al 1979).

Hence, there were disagreements on the different phosphorus levels in flocculent and non-flocculent yeast cell walls. Later investigation found higher total carbohydrate content in the walls of flocculent than in non-flocculent yeast.

(d) Chemical effects on the disulphide bridge Chemical modification with reagents, known to act on disulphide bridges, carboxyl and/or phosphate groups, phenolic groups, amino groups and imidazole groups, was found to destroy the ability of yeast cells to flocculate. This is

a strong indication that these functional groups of amino acid residues of the protein are essential for the floc forming ablility of brewer's yeast cells (Nishihara et al 1977).

(e) The fimbria of yeast cell wall Some flocculating yeast strains possess minute fimbria (0.5 μm) on their cell wall. These fimbria can be easily removed when treated with α-amylase and treated cells lose the ability to flocculate. It was suggested that the fimbriae may be the surface mannan-protein complex known to be involved in flocculation (Day et al 1975).

(f) The cell wall ionic charge The nature and density of ionic charges on the surface of cell envelope and interference from adsorbed material from the medium were thought to have an effect on flocculation (Jayatissa & Rose 1976) but electrophoretic mobility studies on strains of top and bottom fermenting yeasts, at pH 3-7, have shown that the mobilities were independent of the flocculation characteristics of yeasts. This indicates that flocculence and surface charge of the yeast cells are not directly interrelated (Beaven et al 1979).

2.5.3.2 Environmental effects on yeast flocculation

Environmental conditions exert a subsidary influence either at the cell surface or indirectly on the cell metabolism. These include the pH, temperature, and medium composition (Rainbow 1966).

(a) Flocculation aids Calcium, magnesium and manganese ions were found to induce flocculation. Maximum flocculation of yeast occurred at a Ca^{2+} concentration of 0.2 mM (22 mg/l). Above this concentration there was no further increase in flocculation (Mill 1946b). The optimum concentration for magnesium and manganese were found to be 10 mg/l but the flocculation induced was of reduced intensity when compared with calcium. A ten times increase in their concentration did not increase the flocculation intensity. Low concentrations (1-10 mg/l) of either sodium or potassium ions were found to induce flocculation of yeast strains displaying intense flocculation with calcium ions but high concentrations of either ions (50-100 mg/l) were found to antagonise floc formation (Mill 1964b ; Stewart & Goring 1976).

Certain organic substances such as furfural, certain polysaccharides (treberin), ethanol, and colloidal wort components (such as humic acid, metanoidins, and phlobaphene) are known to cause flocculation (Rainbow 1966). However, their optimum concentrations for maximum flocculation were not reported.

It is worthwhile noting here that typical whey permeate was found to contain calcium, potassium and sodium in concentrations of 1.17, 1.45 & 0.6 g/kg, respectively (Anon 1981). It is evident from the data just described that whey permeate contain a greater concentration of calcium than that required for maximum flocculation. The concentrations of potassium and sodium, however, are high in the range which reduces flocculation. Hence, whey contains a flocculating agent as well as deflocculating agents.

(b) Mechanism of ionic induced flocculation It was suggested that divalent ions act by bridging cells through negative charges on the cell surface, whereas monovalent ions induce flocculation via a "counter ions" effect where the repellent forces of the negative charges on the cell surface are neutralized, thus allowing some floc formation due to hydrogen bonding or other types of non-ionic bonding between cells. The antagonism of sodium or potassium ions may be due to neutralization of all available cell surface charges by the monovalent ions and thus prevent cell to cell hydrogen bonding (Stewart & Goring 1976).

(c) Deflocculating agents Certain anions which can complex with calcium such as EDTA, potassium salts of phosphate, fluoride, bicarbonate, oxalate, citrate, diglycollate and nitrilotriacetate can affect flocculation. The lowest inhibiting concentration of each salt was found to be in proportion to its complexing power. EDTA (at 10 mM) was effective but the inhibitory effect of EDTA was reversible after washing (Taylor & Orton 1973 ; Stewart 1975). A similar effect was found for *Schizosaccharomyces pombe* (Calleja 1970). Some unspecified proteins have been reported to prevent flocculation (Rainbow 1966).

There was evidence that some sugars fermentable by brewers' yeast, such as sucrose and maltose, could prevent flocculation but the results were inconclusive (Mill 1964b).

(d) Growth media The composition of the growth and flocculating medium exert considerable influence on yeast flocculation. Some ale yeast strains have been reported to be able to flocculate when cultured in a defined medium of glucose, ammonium salts, vitamins and ions (Stewart et al 1975) while some required the presence of nitrogen containing inducer in the growth medium. A peptide has been identified as the inducer of flocculation in wort (Stewart et al 1975). It was found to contain a high level of acidic amino acid residues. Most flocculent lager strains examined were able to flocculate after growing in a defined medium without the peptide inducer. A strain of brewers' yeast grown in medium containing ammonium phosphate as a nitrogen source was

found to flocculate more weakly than cells grown in medium containing urea or ammonium sulphate and failed to flocculate when grown in medium deficient in magnesium. The minimum concentration required was found to be 20 μM (Nishihara et al 1976a).

Thus, the growth media effect yeast flocculence by providing nutrients necessary for flocculation.

(e) Temperature Yeast cell flocs have been reported to be stable between 20-50°C and at higher temperature rapid deflocculation occurred. If the temperature were not high enough to kill the yeast cells, the flocs reappeared upon cooling (Mill 1964a ; Calleja 1970).

(f) pH Flocculent yeast cells grown in medium of initial pH less than 3 were found to flocculate poorly (Nishihara et al 1976a). Another study observed that flocculation was low at pH 2 but increased with increasing pH and reached a maximum between pH 4.5 to 5.5 (Mill 1964b).

(g) Coflocculation Some strains of brewers' yeast which are non-flocculating, were found to flocculate readily in presence of an appropriate partner ie. another strain (Eddy 1958). This type of flocculence has been termed "coflocculation" (Stewart 1975).

(h) Agitation Agitation of the growing medium exerts a constant action on microbial aggregates (Atkinson & Daoud 1976). It has been indicated that in the practical operating range of tower fermentation when superficial liquid velocities are greater than .14 mm/s. The yeast flocs appeared to be affected by an increase in the liquid flow rate but the yeast bed expanded with this increase which eventually lead to wash out when a high enough velocity was reached (Greenshields & Smith 1971).

2.6 MIXED CULTURE AND MIXED SUBSTRATE FERMENTATION

The concept of growing a mixed culture on a mixed substrate is important in dairy fermentation, alcoholic fermentation and biological waste treatment processes. Mutual existence of species is termed symbiosis. There are a variety of possible interactions and several of these can take place simultaneously (Table 2.5) (Bungay & Bungay 1968). Studies on mixed culture have been reviewed by many workers (Bungay & Bungay 1968 ; Bungay & Krieg 1966 ; Veldkamp & Jannasch 1972 ; Jannasch & Mateles 1974). A number of kinetic models have also been proposed by many workers (Chiu et al 1972 ; Yoon et al 1977 ; Fredricson & Tsuchiya 1977 ; Yoshida et al 1979).

There have been very few investigations on the concept of improving ethanol productivity by utilising two yeast species with different abilities.

Table 2.5 Common terms for microbial interactions

interaction	definition
neutralism	lack of interaction
competition	a race for nutrients and space
commensalism	one member benefits while the other is unaffected
mutualism	each member benefits from the other
predation	one member feeds on another
parasitism	one member steals from another
amensalism	one member adversely changes the environment for the other
inhibition	excretion of a factor harmful to the other
synergism	combination synthesizes by cooperative metabolism

One study on the application of binary mixtures of yeasts in continuous beer fermentation found that in certain pairs of yeasts, one of the strains becomes dominant within a few days, indicating competition for substrate, although in other mixtures the original proportions were maintained indicating mutualism. The efficiency of a mixture was usually less than the average value for its individual components (Rudin & Hough 1959). Improved productivity resulted when the two yeasts were chosen because of their different inhibition properties at high sugar and high ethanol concentration respectively (Jones & Greenfield 1981).

When there is more than one sugar source available, the sugar which is the easiest to utilize will be metabolized first. This growth pattern has been termed "diauxic behaviour" (Monod 1947). Three terms are now used : catabolite repression describes the inhibition of specific enzyme synthesis by the preferred substrate ; catabolite inhibition describes the inactivation of specific enzymes by the preferred medium ; and catabolite inactivation involves inactivation of already existing enzymes including their degradation (Holzer 1976). D-glucose has been shown to exert catabolite inhibition on D-xylulose, D-xylose and D-xytitol metabolism in some yeasts (Hsiao et al 1982).

Growth of a mixed culture on mixed substrate in which one substrate is utilized at higher efficiency can lead to steady state accompanied by an incomplete utilization of the other substrates present in the medium. However, if different growth limiting substrates are used by different organisms, coexistence will occur (Chain & Mateles 1968 ; Yoon & Blanch 1977).

When two organisms are competing for the same growth-limiting substrate and no other interactions between these organisms occur, their

behaviour can be predicted from the known relationships between substrate concentrations and growth rates.

If one organism has a greater growth rate and lower K_s (substrate saturation constant) than another, then for any substrate concentration, it will dominate and the other organism will be selectively excluded (fig.2.8a). If the saturation curves of both organisms cross (fig.2.8b). The dilution rate will determine the dominating organism.

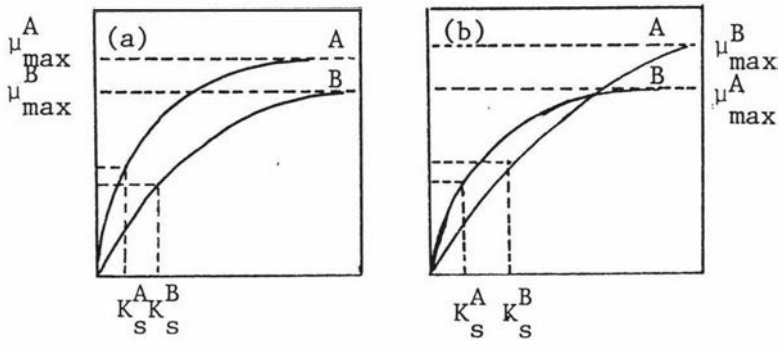


Fig.2.8 μ-S relationship of two organisms A and B. (a) $K^A_s < K^B_s$ and $\mu^A_{max} > \mu^B_{max}$; (b) $K^A_s < K^B_s$ and $\mu^A_{max} < \mu^B_{max}$ (Veldkamp & Jannasch 1972).

There is one substrate concentration for which the corresponding growth rates of both organisms are equal. When this substrate concentration is maintained in the chemostat, the concentrations of both organisms will also be maintained constant. Such behaviour has been observed experimentally (Veldkamp & Jannasch 1972 ; Chain & Mateles 1968).

2.7 CONCLUSIONS

The review has shown that *K.marxianus* and *C.pseudotropicalis* are rapid lactose fermenting yeasts. *K.marxianus* is, however, more suitable for industrial fermentation of whey to ethanol because, in contrast with *C.pseudotropicalis*, it has not been reported to be pathogenic. It is also more widely used. Its optimum operating pH is between 4.5 to 5.0, and optimum temperature of 28 to 30°C.

Tower fermentation is a simple and relatively cheap continuous fermentation process with high productivity in comparison with the batch fermentation and other alternative ethanol fermentation processes. Its productivity is affected greatly by the flocculating ability of the yeast used. This is because the yeast flocculence limits the medium and thus the medium feed rate. It is used in a number of industrial applications but has not yet been used for industrial ethanol production. Thus, it is a process that could be used to ferment whey rapidly to produce ethanol, as an alternative to the low productivity batch

fermentation, if a flocculent lactose fermenting yeast were available. This would enable the dairy industry to cope with the ever increasing volume of whey.

Yeast flocculence is a genetically inherited behaviour which is influenced by various environmental factors.

Yeasts exhibit diauxic behaviour when more than one sugar source is available, the sugars are utilized sequentially.

Fermentation studies using mixed culture of yeasts found no interaction between the yeasts used. These topics have been included in the review because it was intended to investigate the possibility of using *K.marxianus* and *S.cerevisiae* together to ferment whey permeate enriched with sucrose to increase productivity by using 100 g/l total sugar and to improve the economic viability of the plant by removing the seasonal variability in the feedstock supply.

CHAPTER 3
MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

Inorganic and organic chemicals (analytical reagent grade) were obtained from BDH Chemicals (NZ) Ltd (Palmerston North, NZ) with the exception of D-glucosamine which was obtained from Sigma Chemical Co St Louis, Missouri, USA).

3.1.2 Gases

Carbon dioxide and hydrogen for the gas chromatograph were obtained from New Zealand Industrial Gas Ltd (Palmerston North,NZ).

3.1.3 Media

(a) Whey permeate Sulphuric acid whey permeate was supplied by the Dairy Research Institute (DRI), Palmerston North, in 40 litre containers. It was stored at -20^{o}C until required, when it was thawed and autoclaved prior to use. The lactose content of the whey permeate varied from 39 to 50 g/l and the pH was 4.6. There was one batch which had a pH of 4.2 but this was adjusted to 4.6 by addition of calcium hydroxide before autoclaving. Whey permeate was used without any additional nutrient. Table 3.1 gives typical composition of the whey permeate used.

Table 3.1 Typical composition of sulphuric whey permeate (Anon 1981).

lactose	TS	ash	Ca	Cl	K	Na	PO_4	SO_4	
42.6	56.9	7.8	1.17	.09	1.45	0.6	1.92	1.51	g/kg

(b) Molasses Molasses was supplied by the Chelsea Sugar Refinery Ltd (Auckland, NZ) in cylindrical steel drums containing 292 kg of molasses. The sucrose content varied from 500 to 600 g/kg of molasses.

(c) Tower fermentation start up media During inoculum preparation and start up of tower fermentation of whey permeate, the growth medium used was whey permeate enriched with malt extract syrup (20 g/l) (Maltexo, Wilson Malt Extract Ltd, Dunedin), Marmite (2 g/l) (Sanitarium Health Food Co Ltd ,Auckland) and .51 g/l of each of calcium chloride, ammonium sulphate and diammonium hydrogenphosphate ($(NH_4)_2HPO_4$).

In the tower fermentation of whey permeate enriched with molasses, there were further additions to the above medium. The additions were molasses (120 g molasses/l of total medium volume) and urea (1 g/l).

The start up molasses medium for the tower fermentation of molasses contained 100 g/l sucrose. Urea and diammonium hydrogenphosphate were added using concentrations as indicated above.

(d) Tower fermentation media In the tower fermentation of whey permeate no additive was used. In the tower fermentation of whey permeate enriched with molasses, the medium used contained the ratio of lactose to sucrose of 40:60 g/l. Urea (1 g/l) and diammonium hydrogenphosphate (0.5 g/l) were also added. Molasses (100 g/l sucrose) was used as the fermentation medium in the tower fermentation of molasses. Urea and diammonium hydrogenphosphate were added using the same concentration as given above.

(e) Culture preservation media YM agar (Difco Laboratory, Detroit, Michigan, USA, supplied by Fort Richard Ltd, Wellington, NZ) was used for culture preservation at $4^{o}C$. Nutrient broth (Difco), containing 30% V/V glycerol, was used to maintain the stock culture at $-20^{o}C$.

(f) Flocculation test media The media used are listed in sect 6.2 for ease of cross reference during results presentation because of the large number of media and variations used.

(g) Basic nutrient base For carbohydrate utilization tests.

Ammonium chloride	NH_4Cl	1 g/l
Dipotassium hydrogenphosphate	K_2HPO_4	0.5
Nitrogen base (Difco)		2
(Agar (Difco)		15-30)
pH 5		

If broth medium were required only the appropriate sugar was added.

If agar plates were required, agar was added. Normal agar plates contained 15 g/l agar, replicating plates contained 30 g/l agar.

(h) Whey broth and agar This was used extensively in mutation and culture improvement experiments.

Yeast extract (Difco)	3 g/l
Ammonium chloride	1
Dipotassium hydrogenphosphate	0.5
(Agar	15-30)
(95% ethanol (.7897 gm/ml) was added as needed.)	
pH 5	

Whey permeate was used as the make up liquid. High concentration of agar was required because the agar would not set at the normal concentration of 15 g/l. Ethanol was added aseptically as needed after autoclaving.

(i) Lactose agar Lactose was added to the nutrient base (sect 3.1.3 g) using concentrations of 1.7 and 40 g/l for mutation and *K.marxianus* plate counting, respectively. Lower lactose concentration (1.7 g/l) was used during mutation in order to avoid interference from lactose. This concentration was the minimum required for growth.

(j) Sucrose broth and agar Sucrose was added to the basic nutrient medium described in sect 3.1.3(g) at concentrations of 1.7 g/l when making mutation sucrose agar plates and 20 to 60 g/l for fermentations tests.

(k) Total cell plate count agar YM agar (Difco) was used for total cell plate counts.

(l) pH adjustment of media The pH of all media was adjusted using 0.5 M sulphuric acid or 1 M sodium hydroxide before autoclaving. For tower fermentation of whey permeate and flocculation tests, calcium hydroxide was used, since sodium ions could interfere with the yeast cell flocculation.

3.1.4 Organisms

Twelve lactose fermenting yeasts and three *Saccharomyces cerevisiae* strains were used (table 3.2). They were obtained as slant or freeze dried cultures.

Table 3.2 Yeast cultures used.

no	species	strains	sources	forms
1	*Candida pseudotropicalis*	CBS 2234	BT	S
2	*Kluyveromyces lactis*	NCYC 416	DRI	FD
3	" "	NCYC 469	BT	S
4	*K.marxianus*	ATCC 10022	DRI	FD
5	"	CBS 397	BT	S
6	"	NCYC 100	DRI	FD
7	"	NCYC 587	BT	S
8	"	NRRL Y1109	DRI	FD
9	"	UCD-FST 71-58	BT	S
10	"	X DRI	BT	S
11	"	Y 18 (NRRL Y-610)	UM	D
12	"	Y 42	UM	D

no	species	strains	sources	forms
13	*S.cerevisiae*	AWRI 350 (FT146)	AWRI	S
14	"	CFCC 39	UA	S
15	"	Y16	BT	S

AWRI - Australian Wine Research Institute, Adelaide, Australia.
BT - Department of Biotechnology, Massey University, Palmerston North, New Zealand.
DRI - Dairy Research Institute, Palmerston North, New Zealand.
UA - Department of Biological Science, University of Aston in Birmingham, U.K.
UM - Department of Microbiology, University of Maryland, College Park, Maryland, U.S.A. 20742.
D - Dried on sterilized filter paper.
FD - Freeze dried culture.
S - Agar slope culture.

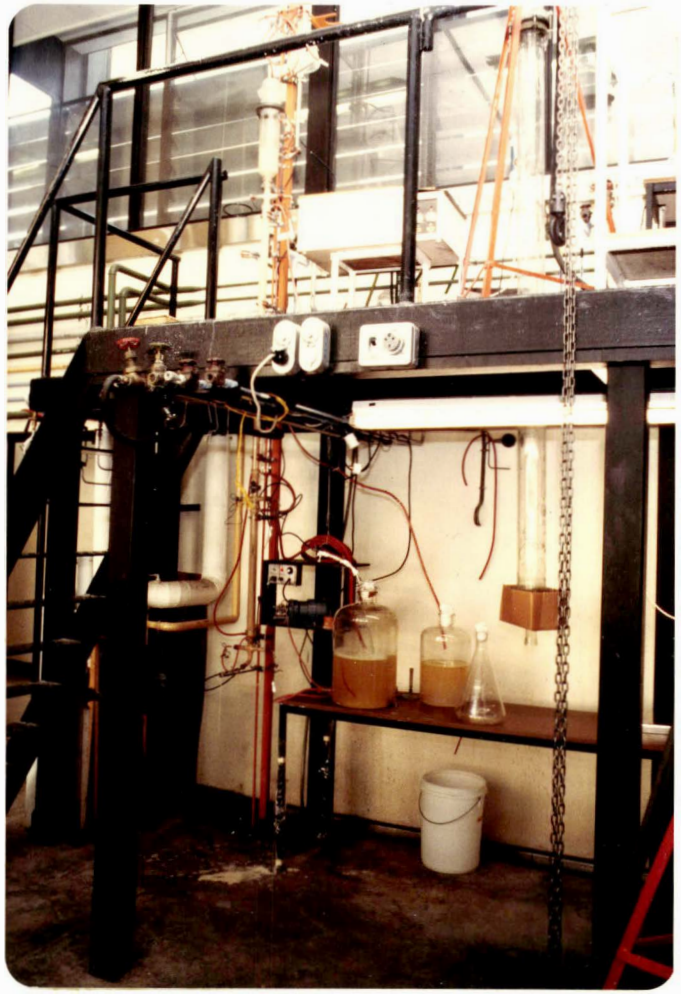
Yeast no.12 is a maltose-utilizing hybrid of yeast no.11 and *S.dobzhanskii* Y 1976 (Wickerham and Burton 1956).

3.2 EQUIPMENT

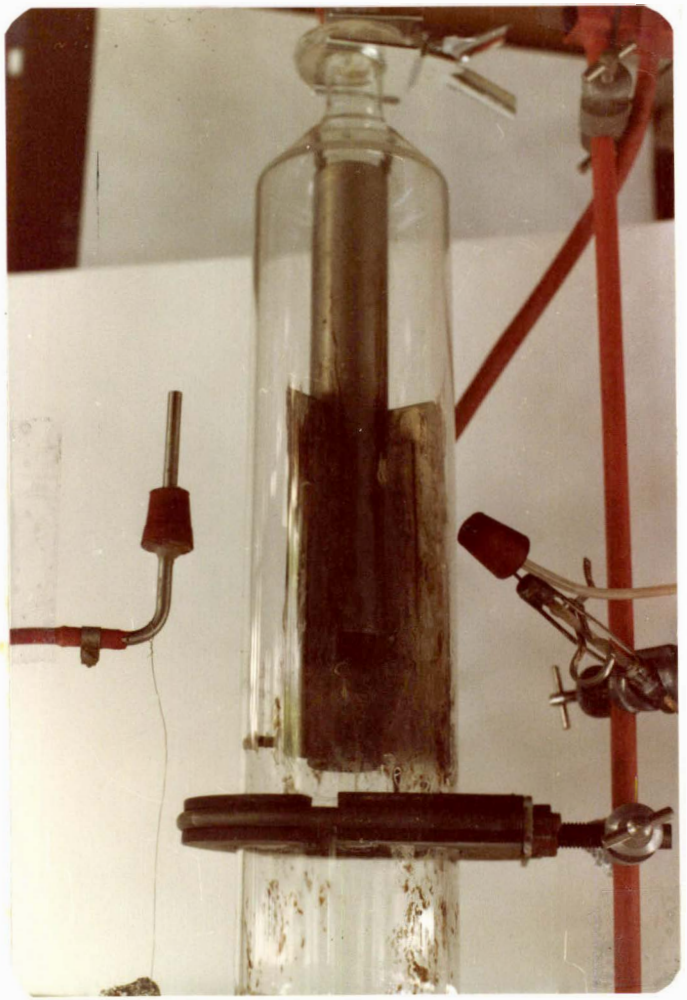
3.2.1 Tower Fermenter

The tower fermenter (figure 3.1 and 3.2) used in this study was constructed from 3 jacketed Pyrex glass pipes (Jobling Lab. Div., Stratfordshire, England) with an enlarged section situated at the top to act as yeast/liquid/gas separator. The internal diameter of the lower and separator sections were 25 and 100 mm respectively. The total length of the fermentation section was 2.37 m and overall height was 2.7 m giving a fermentation and total volume of 1.29 and 2.92 l respectively. The overall view of the tower fermenter in situ is given in figure 3.1(a), while figure 3.1(b) shows from left to right the medium and air inlets the separator section, and a sampling tube.

The column was fitted with 4 sampling points at regular intervals along the column as given in figure 3.2 samples were drawn from the vertical centre line of the tower at each sample point. In addition samples were drawn at the inlet (sample point 0) and exit (sample point 5). A bottom inoculation port was fitted to the tower at the same level as sample port no.1. The bottom of the column was fitted with a rubber



(a)



(b)

Fig.3.1 (a) Tower fermenter set up for continuous fermentation of whey permeate suowing aerated feed medium (large vessel) and effluent collector (small vessel). (b) Separator section (centre) ; medium feed and air inlets (left) ; and a sampling port tube (right).

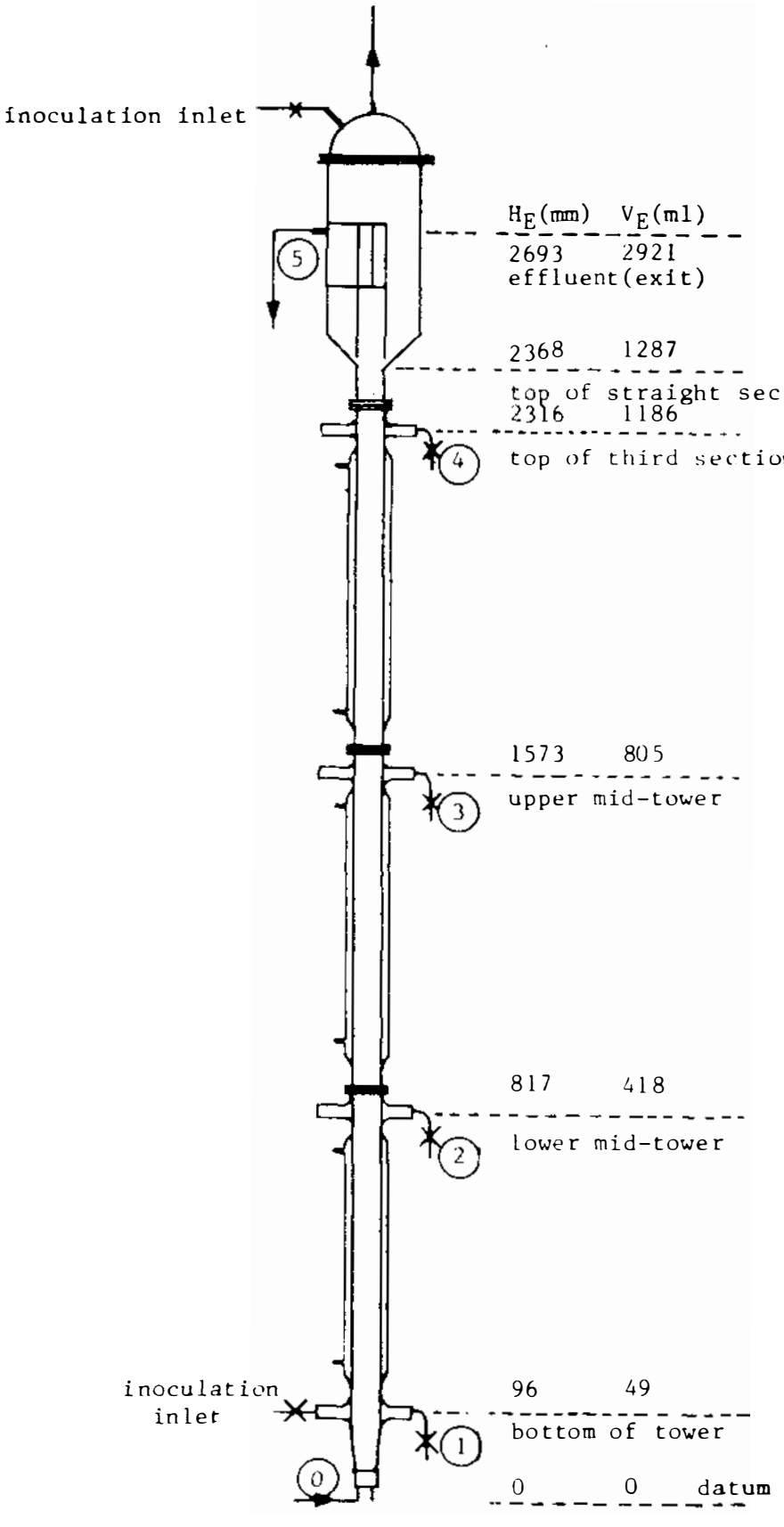


Fig .3.2 Schematic diagram of the tower fermenter.

Internal diameter 25.5 mm, cross sectional area 5.12 cm^2,
H_E - effective height,mm; V_E - effective volume,ml

stopper fitted with medium and air inlet pipes (figure 3.1(b)).

(a) The separator The separator (figure 3.3) consisted of a Pyrex glass housing (supplier as in section 3.1.1) and a concentric stainless steel (grade 18:8) draught tube (N.Z. Steel and Tube Ltd , Auckland). The Pyrex housing consisted of a truncated inverted cone 25 mm high, a straight pipe section 400 mm high and a hemisphere of 100 mm radius. The top and bottom radii of the inverted cone were 100 mm and 25 mm respectively while the radius of the pipe section was 100 mm (figure 3.1(a)).

A liquid effluent port was positioned 250 mm from the bottom of the straight section while gas exit and inoculum ports were positioned at the top of the hemisphere.

The draught tube (figure 3.3 (b) and (c)) was 38 mm in diameter and 300 mm long. The draught tube was supported on three stainless steel pins 15 mm x 5 mm (the bottom end of the pins were tapered to follow the contour of the glass tube), thus the bottom of the draught tube was 15 mm above the top of the fermenter straight section and the top of the draught tube 15 mm above the liquid effluent port.

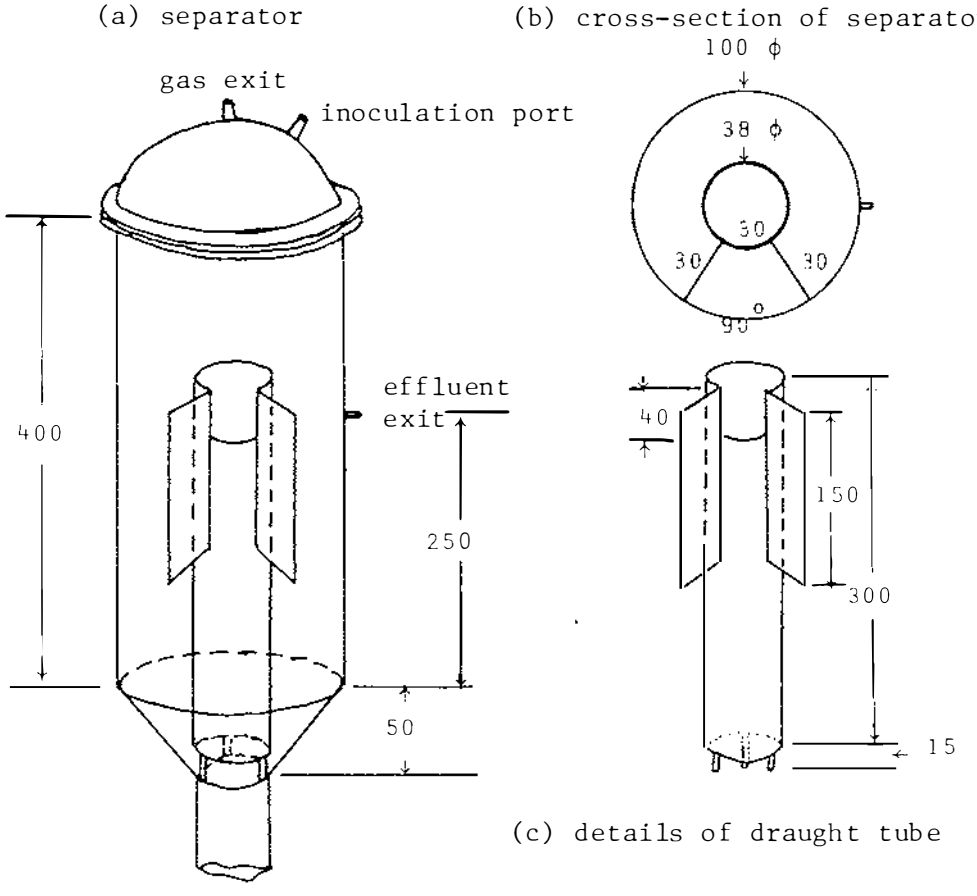
Two rectangular baffles, 90^{o} apart were positioned on the outside of the draught tube. The baffles were 150 mm in height and of sufficient width to reach the internal wall of the Pyrex glass housing.

In between the baffles a 30 mm section of the draught tube was cut to a depth of 40 mm forming a weir for the liquid effluent. The weir was positioned diametrically opposite to the liquid effluent port.

The flow pattern in the separator was therefore as follows: The liquid effluent from the fermenter flowed up the draught tube and over the weir into the zone between the baffles. At this point it was forced to flow down under the baffles and up into a quiescent zone where its superficial velocity decreased. The cross-sectional area of the quiescence zone was 50 cm^{2}. This decrease in superficial liquid velocity allowed the yeast flocs to settle under gravity and the clear liquid effluent flowed out of the tower via the liquid effluent port.

The effective working volume of the separator was 1.6 l.

(b) Temperature Control The temperature of the fermenter was controlled by circulating water $30^{o}C$ from a hot water bath (Compenstat water circulator, Gallenkamp, London, England, supplied by Smith-Biolab



All dimensions are in mm. ϕ = internal diameter

Fig.3.3 Schematic diagram of the separator and the draught tube.

Auckland, New Zealand) through the 3 water jackets (figure 3.4). The jacketed sections were connected in series, with the water entering the bottom of the lowest section and returning to the water bath via the top exit of the uppermost jacketed sections.

The temperatures at the four sample points on the tower were monitored using copper-constantan thermocouples connected to a 12 input - 3 channel - chart recorder (Versaprint, Honeywell, USA).

(c) Tower and medium aeration The tower was aerated from the bottom via a stainless steel capillary tube to keep air bubble size very small (figure 3.2(a) and 3.4) at a rate of 120 ml/min., 50 kPa during the cell build up period. The medium was aerated for 1 h before being pumped into the tower.

(d) Air supply and filter The air supply was provided by the University central service. The supply to the tower was fitted with a vapour - oil filter and pressure regulator (Norgren, type F04, supplied by Kidd Garrett, Auckland). The air supply for medium reservoir and tower aeration was sterilized by passage through an air filter constructed of stainless steel tube 25 mm ID x 350 mm packed with fibreglass. A similar filter was fitted to the gas exit line of the tower. The air flow rate was monitored using a 0-1000 ml/min variable area flowmeter (GAP, Basingstoke, UK supplied by Homersham Ltd., Christchurch, New Zealand).

(e) Medium pump The medium was fed continuously into the bottom inlet of the tower (figure 3.2(a) and 3.4) using a Masterflex Peristaltic pump, model no. WA IR 051 (Cole-Parmer, Chicago, USA supplied by Smith-Biolab, Auckland, New Zealand) fitted with pump head no.7013 for feed rates up to 350 ml/hr and no.7014 for feed rates up to 1000 ml/hr. Silicone tubing was used throughout. Calibration curves for delivery rate to the tower at various motor speeds are given in Appendix E

3.2.2 10 l Batch fermenter

The fermenter vessel was a 15 l New Brunswick Scientific Co Ltd, Model CMF 14, glass vessel (Watson Victor Ltd, Wellington, New Zealand). The fermenter support and control unit was constructed by the Department of Biotechnology workshop, Massey University. The unit has facilities for aeration, control of temperature, agitation speed, and foaming. The fermenter vessel was 15 l total volume with a working volume of 10 l.

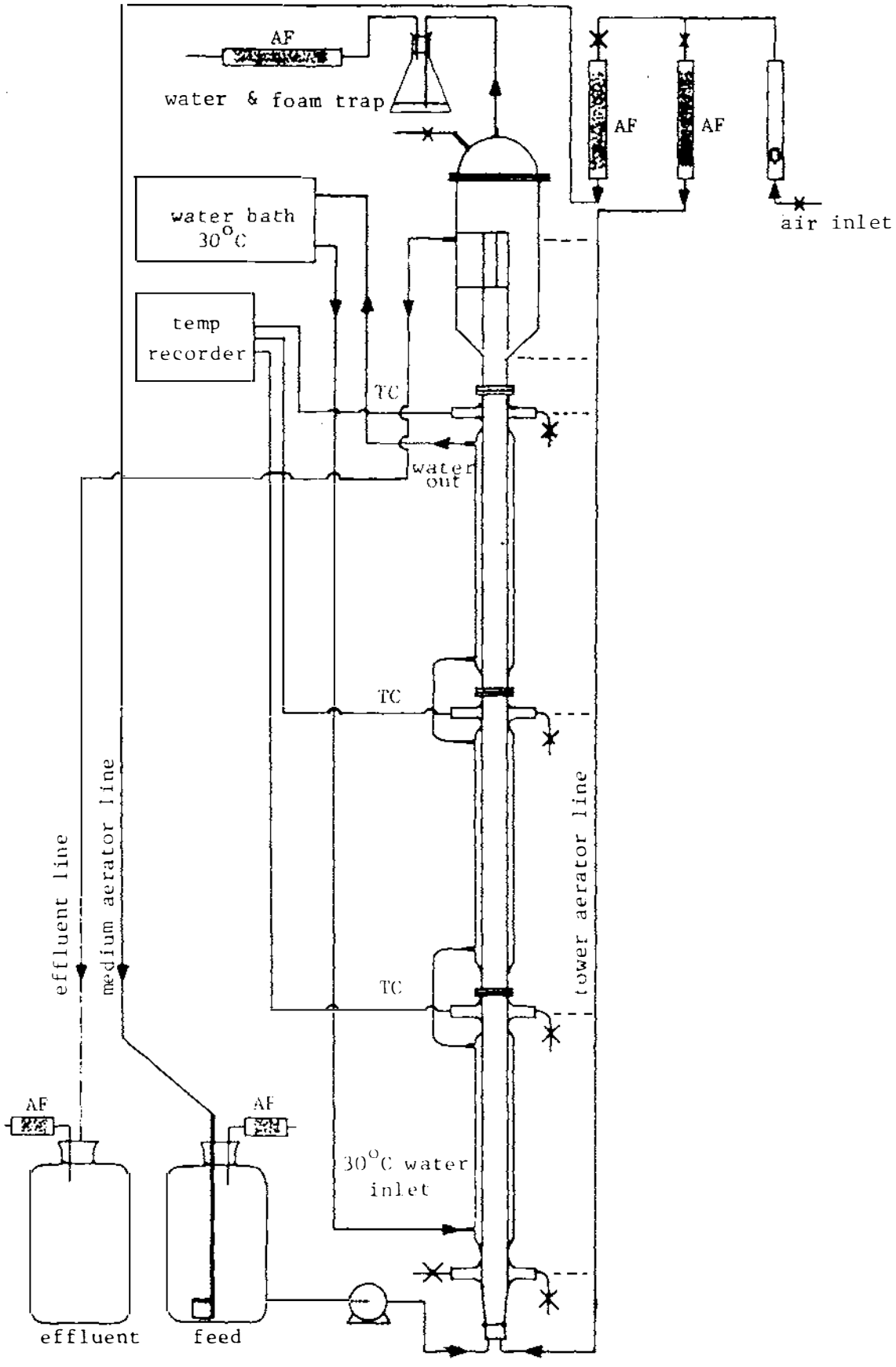


Fig.3.4 Water heating and air filtration systems for the tower fermenter. AF - air filter ; TC - thermocouple

Fig.3.5 Batch fermenter (10 litre working volume).

Fig.3.6 Replication equipment. Wooden supporting base and replicating cloth.

The medium temperature was maintained at 30°C by recirculation of hot water. The agitation speed was 250 rpm (figure 3.5).

3.2.3 UV lamp

The UV lamp used for mutation experiments was a CAMAG universal - UV - lamp (29200) operating at a wavelength of 254 nm, at 300 mm above the yeast cell suspension.

3.2.4 Replica plating

Replica plating was carried out using a simple round wooden base having a diameter slightly smaller than presterilized plastic agar plate. The transfer cloth (figure 3.6) was held in place by an adjustable Jubilee clip steel band which can be removed easily from the base. The transfer cloths were stacked in groups of 50 with paper towels as separators and autoclaved at 100 kPa, 120°C, 15 min before being dried overnight at 70°C.

3.2.5 Glassware

Standard laboratory glassware obtained from commercial sources was used throughout this study. All glassware was routinely washed in hot water containing "pyroneg" (Diversey-Wallace Ltd , Auckland, New Zealand), rinsed with distilled water and air dried at ca. 50°C.

3.3 STERILIZATION

3.3.1 Medium and glassware sterilization

All media were sterilized at 100 kPa for 15 min. The media in large volume containers of 4 to 20 l were sterilized under the same conditions for 25 min.

Glassware was sterilized at 160°C for 2 h.

3.3.2 Tower fermenter

The tower was thoroughly cleaned with water containing Pyroneg and sterilized by free steaming for a total of 24 h. The steaming was carried out for 12 h, stopped for 12 h, and then steaming was continued for 12 h. After the second steaming the tower fittings were immediately installed. All tower fittings were autoclaved at 100 kPa for 15 min before installation.

During the commissioning run using molasses medium, purity tests using nutrient agar were carried out to test the effectiveness of

the sterilization. No contamination was detected.

3.4 ANALYTICAL METHODS

3.4.1 Lactose

Lactose was analyzed using a YSI industrial sugar analyzer, model 27 (Yellow Spring Ins. Co , Ohio, USA). The sugar analyzer was fitted with a lactose membrane containing galactose oxidase, immobilized by glutaraldehyde and allowed direct measurement of lactose concentration.

3.4.2 Sucrose

The same sugar analyzer was used as in section 3.4.1 but the analyzer was fitted with a glucose membrane which contained the enzyme glucose oxidase immobilized by glutaraldehyde Sucrose was first hydrolysed to glucose and fructose using yeast invertase concentrate (BDH) which was added at a rate of 250 µl/g sucrose in the samples. The sample solution was then incubated at 30°C for 1 h before injection into the analyzer. The glucose reading obtained was multiplied by 1.9 (the ratio of molecular weight of sucrose to glucose) to give the sucrose concentration.

3.4.3 Ethanol

Ethanol concentrations in the samples were determined using a Varian Aerograph gas chromatograph, model 600 D (California, USA). The column was packed with Porapak Q (Applied Science Lab Inc., State College, Pennsylvania, USA) and operated at 170°C. Isopropanol solution was added to the samples as internal standard. The external standards used contained equal concentration of ethanol and isopropanol and covered the concentration range from 10 to 50 g/l in steps of 10 g/l. An additional standard of 5 g/l were used for low ethanol concentration. The injection volume used was 1 µl in all cases and all ethanol concentrations were expressed in g/l.

3.4.4 Cell concentration

(a) Cell dried weight and wet weight (DW and WW) Samples of 10 ml volume were centrifuged and the supernatant fluid was decanted. The wet yeast cell pellets were weighed to give the centrifuged cell wet weight (WW) (g/l).

The cells were then dried at 105°C for 24 h to determine the cell dried weight (DW) (g/l).

(b) Plate count The total live cell count was obtained from YM agar incubated aerobically at 25°C while lactose agar (section 3.1.3 i) was used for the *K.marxianus* count. Peptone water (5 g/l peptone) (Difco) was used as dilution medium.

For flocculent yeasts, the dilution medium contained NaCl(9 g/l) and EDTA (4 g/l). NaCl provided an osmotic pressure balance and EDTA induced deflocculation. During dilution, each dilution was vigorously agitated before the next dilution step was made. Serial dilution was continued to give approximately 30-300 colonies when 1 ml aliquots were plated. Duplicate plates were made for each dilution. The agar plates were incubated at 25°C for 3 days before counting.

(c) Haemacytometer cell count The total cell number was obtained by using a haemacytometer (Assistent, Germany). Samples to be counted that came from very cloudy media were usually diluted 5-10 times in order that cell count could be made.

3.4.5 Measurement of yeast flocculence

Two methods were used for the measurement of yeast flocculence. The first method was adapted from Helm et al (1953) and Stewart (1975). This method used visual observation to give a flocculation scale. The second method was an adaptation of that described by Greenshields et al (1972) using yeast settling volumes in relation to time to give a numerical flocculation value.

(a) Flocculation scale method

1. Yeast cells were prepared as described in section 3.9. One gram of centrifuged cells was resuspended in 9 ml of the testing medium and transferred to a 15 ml capacity graduated tapered centrifuged tube, giving a total volume of 10 ml. The tube was allowed to reach thermal equilibrium with ambient, then gently shaken for 5 minutes to ensure all flocs were evenly broken up.
2. The yeast settled volume was recorded at 1,2,5,10,15 and 60 minutes.
3. The flocculent scale was then determined using the following subjective scale (Stewart 1975).
4. Each flocculation test was carried out in duplicates from step 1 to 3.

5	extremely flocculent	EF
4	very flocculent	VF
3	moderately flocculent	MF
2	weakly flocculent	WF
1	rough flocculent	R
0	non-flocculent	NF

This method is very subjective. The procedure used here differed from that of Helm et al (1953) in that the subjective scale was used instead of using the yeast settled volume after 10 min. The method also differed from that of Stewart (1975) in that 1 gm wet yeast was used in a total test medium volume of 10 ml. The cleaning and test media used were also different. The flocculation scale used was the same.

(b) Sharp's modified Burn's number (MBN) Yeast flocculation was also measured by the modified Burns number(Greenshields et al 1972).The yeast volumes recorded (as in section 3.4.5(a)) after 0,1,5,10 and 15 min. were used to calculate MBN or MBN* for the yeast tested depending on the growth and the test medium used.

If the yeasts were grown, prepared and tested following the standard method described by Greenshields et al (1972), the value obtained was designated as "MBN". However if the yeasts were not grown and tested in the standard media, the value obtained was designated as "MBN*".

3.4.6 pH

The pH of the medium was measured using a Triac DPH-1 pH meter fitted with a combination electrode (E.L. Kay Ltd, Auckland, New Zealand).

3.4.7 Specific gravity

Medium specific gravity was measured using a Zeal hydrometer (G.H. Zeal Ltd, London, England, supplied by Smith-Biolab Ltd, Auckland, New Zealand) at $20^{o}C$.

3.5 CULTURE PRESERVATION AND MAINTENANCE

The yeast cultures were maintained on YM agar slopes at $4^{o}C$. Two slopes of the culture were maintained. One slope was used for experiments while the other slope was kept as stock culture. Cultures were subcultured onto new slopes at six month intervals. Another set

of stock cultures were maintained at -20°C in nutrient broth containing 30% v/v glycerol.

3.6 INOCULUM PREPARATION

Inocula for experiments were prepared using the following steps. The number of steps required increased with the volume of inoculum required. The incubation period for each step was 24 h and the volume transferred was 5% v/v of the final total volume of the next step.

1. Stock culture
   ↓ loop
2. 10 ml medium in 25 ml bottle.
   ↓ 5 ml
3. 95 ml medium in 250 ml shake flask (100 ml)
   ↓ 50 ml
4. 450 ml medium in 2 l shake flask (500 ml)
   ↓ 500 ml
5. 9.5 l medium in 15 l batch fermenter (10 l)
   (concentrated before inoculation)
6. 2 x 450 ml medium in 2 l shake flasks (1 l)
   (used inoculum prepared in step 3)
7. 12 x 450 ml medium in 2 l shake flasks (6 l)
   (used inoculum prepared in step 6)
   (concentrated before inoculation)
8. 30 l medium in 45 l batch fermenter (30 l)
   (used inoculum prepared in step 6)
   (concentrated before inoculation)

3.7 FERMENTATION CONDITIONS

All fermentations were carried out at 30°C. All shake flask fermentations were agitated at 150 rpm. For normal fermentation the inoculum volume was 5% v/v of the final total medium volume. For some mutation and culture improvement shake flask fermentations an inoculum volume of 10% v/v of the final total medium volume was used.

Ten litre batch fermentation was agitated at 250 rpm and the inoculum was prepared as in steps 1 to 4 in section 3.6.

Agar plates were incubated at 25°C for 3 days. The lower incubation temperature was used to reduce the drying of the agar.

3.8 TOWER FERMENTATION

3.8.1 Start up

(a) Initial start up

1. The inoculum was grown on the start up medium (whey permeate with Maltexo) using steps 1 to 5, 6 & 8 in section 3.6.

2. After inoculation into the tower, the flocs were allowed to settle and the spent liquid was decanted off at sample point 2 (.82 m). The tower was then filled with fresh medium, and an aerated batch fermentation took place for 4 h after which the yeasts were allowed to settle for a period of 1 h.(Aerobic fermentation of whey was reported to require 3 to 4 h for complete utilization of 40 g/l lactose on whey using an initial cell concentration of 2×10^9 cells/ml.)(Wasserman et al 1958).

3. The procedure of decanting of supernatant liquid, refilling the tower, and batch fermentation was repeated 3 times. In this way large cell concentrations were obtained in the tower.

4. After the last batching, the supernatant liquid was decanted. A continuous slow feed of 80 ml/h was commenced with aeration at 120 ml/min for 10 days in order to increase the cell concentration further before sampling could begin.

(b) Subsequent start up After one month of operation the experiment was terminated because of whey shortage. A slightly different inoculation procedure was employed in the subsequent start up for ease of operation and to reduce contamination risk.

The inoculum was prepared using steps 1 to 4, 6 & 7 in section 3.6. The inoculum was aseptically concentrated and inoculated into the tower. Then the start up steps 2 to 4 described in section 3.8.1 (a) were followed.

The tower was considered ready for continuous operation when the settled yeast volume occupied 2/3 of the tower straight section.

In the tower fermentation of whey permeate enriched with molasses, the inocula of the two yeast cultures were prepared separately using similar steps but the growth medium for *S.cerevisiae* CFCC 39 was molasses medium (100 g/l sucrose) while *K.marxianus* Y 42 was grown in the whey permeate with Maltexo start up medium. They were then inoculated into the tower together. The procedure described in section 3.8.1 (a) from steps 2 to 4 was followed using whey permeate enriched with molasses and Maltexo as the start up medium.

Tower fermentation of molasses was started using the "subsequent start up" procedure and molasses solution (100 g/l sucrose) as the start up medium.

3.8.2 Sampling procedure

Daily sampling of pH and cell centrifuged wet weight for evaluation of the onset of steady state was performed at sampling point 2 (.82 m) and the exit (sampling point 5) together with the specific gravity and flow rate of the effluent.

Once steady state was established as seen by two subsequent analyses agreement to within 5%, complete sets of samples were collected from all sampling points including the exit (figure 3.1). Approximately 15 ml samples were taken at sample points 0 to 4. The effluent was collected prior to sampling for approximately 1 h duration. A 10 ml sample from each sampling point was centrifuged immediately after collection. The supernatant liquids were analyzed for sugar & ethanol. The centrifuged cell wet weight (WW) and the cell dried weight (DW) were determined. The remaining samples were used for pH measurement. It was possible to monitor the SG of the effluent only, as the tower fermenter volume was too small to permit the removal of large volumes at each of the sample points. An example of the data sheet is given in Appendix E.

Further sets of samples were collected after minimum periods of 2 residence times. There were generally five sets of samples at each feed rate.

In the tower fermentation of whey permeate enriched with molasses, a similar procedure as above was followed with some additions. After samples were obtained from various sampling points, 1 ml of each sample was aseptically transferred to sterilized 9 ml EDTA-saline solution for use in the determination of the cell numbers by plate count section 3.4.4 b).

The remainder of each sample was processed using similar procedure as for pure culture fermentation. The parameters measured were pH ; glucose, sucrose, lactose and ethanol concentrations ; total cell count and *K.marxianus* cell count.

In the tower fermentation of molasses similar sampling procedure as for the tower fermentation of whey permeate was used.

For the last two tower fermentations only two complete sets of samples were taken for each feed rate. However, daily sampling at sample point 2 and the exit was continued to ensure steady state. This was necessary because of the difficulties in carrying out extensive sampling of mixed culture and mixed substrate fermentations (see chapter 5), and the limited amount of molasses available.

3.8.3 Continuous operation

When the start up operation was completed, the air was turned off and the medium was fed to the tower at the slowest feed rate. After 2-3 days at this feed rate, daily sampling began, once steady state was established. Once the required sets of samples were collected, the medium feed rate was increased to the next feed rate and the same procedure was repeated. This was carried out until the wash out flow rate was reached.

3.9 FLOCCULATION TESTS

Flocculation tests involved two major steps:

1. Yeast cell preparation was carried out by growing the yeast to be tested in a medium of interest and observing flocculating behaviour during fermentation.
2. Measurement of yeast flocculence to determine flocculent scale.

The yeast cells were grown as follows:

1. The yeast to be studied was grown in the medium of interest, with or without added nutrients. The initial pH, final pH and formulation of the medium were recorded. Observations during fermentation were also recorded.
2. The yeast cells were harvested after 48 h (total collected 1g centrifuged wet weight) for use in flocculation measurements. If the yeast were tested for flocculence in its growth medium, no cleaning was necessary. If the yeast were tested in a different medium, that medium was used to clean the yeast twice. Most yeast types were tested for flocculence in the flocculation testing medium (section 6.2.8 medium 36.FM). The cleaning procedure involved twice washing in calcium sulphate (.51 g/l) and centrifuging at 2500 rpm for 5 min.
3. The cleaned yeast cells were then tested in an appropriate medium to determine the flocculation scale or modified Burns number as described in section 3.4.5.

3.10 CULTURE IMPROVEMENT

3.10.1 Isolation of ethanol tolerant *K.marxianus* using ethanol gradient agar

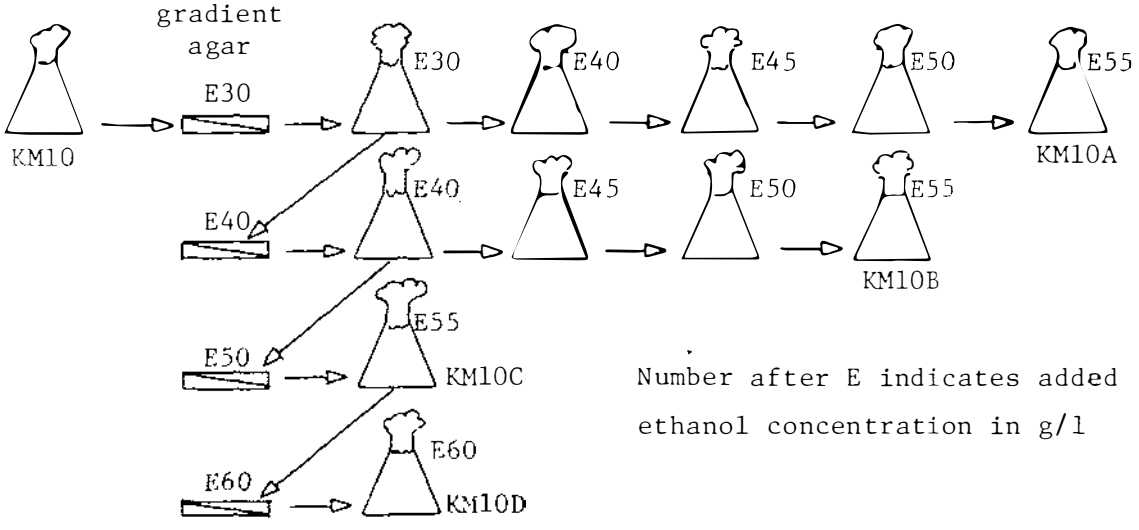


Fig.3.7 Subculturing steps used in the isolation of ethanol tolerating *K.marxianus*.

The subculturing sequence used in this experiment is summarised in figure 3.7. In pouring the gradient alcohol concentration agar, normal whey agar was poured first on a slope. When the agar solidified, the agar plates were placed on a level surface and whey agar with added ethanol was poured over the sloping agar.

1. A 48 h culture of *K.marxianus* UCD FST 7158, grown in whey permeate (100 g/l lactose) was used to inoculate 30 g/l ethanol gradient agar plates using .1 ml inoculum and spread on the agar surface.

2. After incubation, single colonies growing at the end of the agar plate with the highest ethanol concentration wereused to inoculate a 30 g/l ethanol whey broth (100 ml) and fermented for 48 hours.
3. After 48 h, this culture was used to inoculate 40 g/l ethanol gradient agar and whey broth. These were incubated as before.
4. The steps that followed are given in figure 3.7.
5. Once the sequence described above was complete, the cultures in 55 and 60 g/l ethanol whey broth were compared on their ability to ferment 100 g/l lactose whey permeate. These cultures were called KM 10A, KM10B, KM10C and KM10D. Each culture was used to inoculate a separate flask containing 100 ml whey permeate (100 g/l lactose) and incubated. Samples of 5 ml were collected at 0,24, and 48 h and pH, cell number, lactose and ethanol were measured.
6. The culture found to be the fastest lactose fermenter was put through a further series of subculturing in whey broth containing 35 and 55 g/l ethanol to ensure that the ethanol tolerance was stable. The culture was subcultured using a series of 100 ml whey broths with 35 and 55 g/l ethanol added. The inoculum size used was 10% v/v in order to compensate for the cell loss due to ethanol.

3.10.2 An attempt to isolate sucrose negative *K.marxianus* strains

The culture was irradiated with UV light to induce mutation. The procedure involved the establishment of a yeast-kill-curve by UV irradiation in order to determine the optimum irradiation time, followed by a replica plating experiment to isolate the desired yeast.

(a) Determination of optimum irradiation time

1. A 24 hour culture of alcohol tolerant *K.marxianus* (KM10D10) (section 3.10.1) was prepared in 10 ml whey permeate broth in a 250 ml shake flask.
2. The yeast suspension was washed by centrifuging and resuspending the cells in 10 ml of 9 g/l saline solution. This step was repeated once more.
3. The suspension was shaken and placed in a sterilized petri dish and irradiated with 254 nm UV light, at a distance of 300 mm above the dish for the following times: 1,1.5,2,3,4,5,7, and 10 min.
4. For each irradiation time, the number of viable yeast cells was estimated by taking 0.1 ml of suspension and preparing a dilution

series using peptone water and plating appropriate dilutions on YM agar. Two plates were poured for each concentration. The plating dilutions used are given in table 3.3. It should be noted here that steps (3) and (4) should be carried out in darkness to prevent repair by visible light repair mechanisms.

Table 3.3 Plating dilutions used to determine optimum UV irradiation time

UV time(s)	0	1	1.5	2	3	4	5	7	10
Dilutions	-8	-7	-7	-7	-7	-6	-6	-6	-5
(tens)	-9	-8	-8	-8	-8	-7	-7	-7	-6

5. The viable cell count obtained was plottedvs irradiation times. The optimum time was that which gave 99.99% kill (figure 3.8).

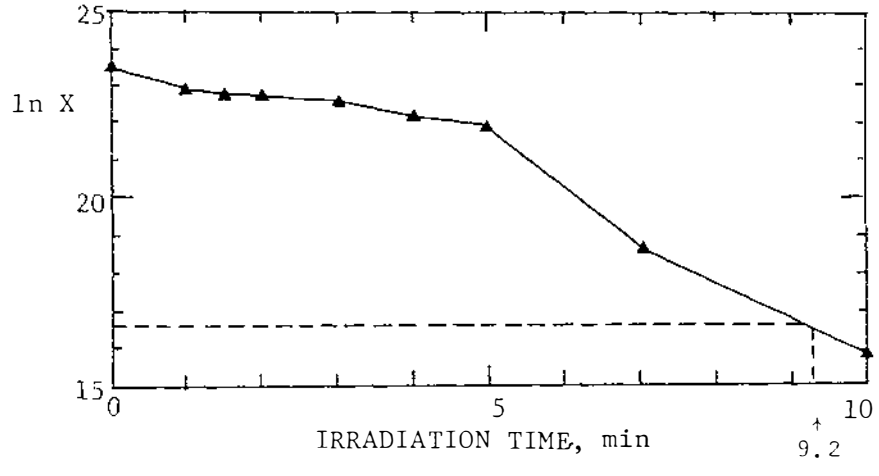


Fig.3.8 Survival of cells irradiated with UV light. KM10D10. (246 nm, distance 300 mm, in 9 g/l saline solution)

(b) Replica plating and isolation

1. Steps 1 to 3 of section 3.10.3(a)were followed except that the irradiation time used was 9 min 15 s.
2. The irradiated cells were centrifuged and resuspended in 10 ml whey broth and allowed to express for 6 hours at 30°C, 150 rpm.
3. Then the number of viable cells were determined by plating onto whey agar and incubated at 25°C for 3 days in order to determine the dilution which gave a count of approximately 100 colonies/ plate.

4. The dilution which contained 10^3 cells/ml (i.e. 100 cells/plate) was used to plate 300 plates of whey permeate agar which were incubated at 25°C for 3 days.
5. The plates were used for replication onto sucrose agar plates (see figure 3.5). After incubation, the two plates, original and replicated plates, were compared and missing colonies in sucrose agar plates were noted.
6. The colonies which were found to be missing on sucrose agar plates were streaked from the original whey permeate agar plates onto a pair of sucrose and whey permeate agar plates to check for growth. Those colonies which showed growth in both plates were rejected, those only on whey were retained.
7. Those possible sucrose negative isolates were streaked and plated onto a series of pairs of sucrose and whey agar plates to check for stability, i.e. no growth in sucrose agar during subculturing before a fermentation check was carried out initially in whey permeate broth to build up population and then in a mixed substrate medium of lactose and sucrose (40:40 and 20:20 g/l).
8. For the fermentation test, a number of single colonies were used to inoculate whey permeate (100 ml in 250 ml shake flask), one colony per flask, and fermentation was carried out for 48 hours at 30°C, 150 rpm. The cell population, lactose, pH, and ethanol were measured. These cultures were then used to inoculate lactose and sucrose media, correspondingly numbered and fermentation was carried out.

3.11 CALCULATION METHODS

3.11.1 Tower fermentation

Volumetric flowrate (Q) (ml/h)

Q = effluent volume (ml) collected over time t (h).

Residence time (T_{ri}) (h) at a sample point

T_{ri} = tower volume at this sample point (ml) ÷ Q (ml/h).

Residence time (T_r) (h) for the total tower straight section was calculated using the straight section volume of 1287 ml.

T_r = 1287 ÷ Q (ml/h)

Dilution rate (D) (h^{-1})

D = 1 ÷ T_r (h)

Mean residence time (T_r^*) (h) for a particular tower section

$$T_r^* = T_{ri} + \{(T_{ri+1} - T_{ri}) \div 2\}$$

where T_{ri} and T_{ri+1} are residence times at the bottom and the top of the section respectively.

Superficial liquid velocity (V_s) (mm/s)

$$V_s = Q \div (\text{tower cross sectional area})$$
$$= \{Q \text{ (ml/h) x } 10\} \div \{(5.1195 \text{ (cm}^2\text{) x } 3600 \text{ (s/h)}\}$$
$$= Q \times 5.4257 \times 10^{-5}$$

The mean volumetric rate of substrate utilization (S') within a particular tower section was calculated from the amount of substrate utilized within the section during the time period that medium was in the section.

$$S' = (S_i - S_{i+1}) \div (T_{ri+1} - T_{ri}) \qquad \text{g/lh}$$

where S_i and S_{i+1} are substrate concentrations at the bottom and the top sample points of the section respectively (g/l).

T_{ri} and T_{ri+1} are the residence times at the bottom and the top sample points of the section respectively (h).

The mean specific rate of substrate utilization (q) within a particular tower section

$$q = S' \div [(X_i + X_{i+1}) \div 2] \qquad \text{g/lh}$$

where X_i and X_{i+1} are cell concentrations at the bottom and the top sampling points of the sections respectively.

The cell concentration in the bottom section of the tower (up to 0.096 m) was assumed to be the same throughout. This assumption was made because there was no sampling at zero tower height for the cell concentration.

The mean volumetric rate of ethanol production (E') within a particular tower section was calculated from the amount of ethanol produced within the section during the time period that the medium was in the section.

$$E' = (E_{i+1} - E_i) \div (T_{ri+1} - T_{ri}) \qquad \text{g/lh}$$

where E_i and E_{i+1} are ethanol concentrations at the bottom and the top sample points of the section respectively (g/l).

The mean specific rate of ethanol production (ν) within a particular tower section was calculated from the mean volumetric rate of ethanol production (E') within the section divided by the mean cell concentration in the section.

$$\nu = E' \div [(X_i - X_{i+1}) \div 2] \quad g/gh$$

where X_i and X_{i+1} are cell concentrations at the bottom and the top sample points of the section respectively (g/l).

Ethanol productivity at a particular tower height is

$$= E_i \div T_{ri} \quad g/lh$$

where E_i is ethanol concentration at that height (g/l).

The yield of ethanol (Y) on substrate utilized based on theoretical yield at a particular tower height,

$$Y = (E_i \times 100) \div [(S_o - S_i) \times 0.538] \quad \%$$

where S_o is the feed substrate concentration (g/l) and

S_i is the substrate concentration at this height (g/l).

The mean specific growth rate (μ). for the tower excluding the separator,

$$\mu = (1/X) \times (dX/dt) = X_e \div (X_a T_r) \quad g/gh$$

where X_e is the cell concentration leaving the tower and

X_a is the mean cell concentration for the tower from the bottom of the tower up to 2.32 m height. T_r is the residence time at this height. Growth inside the separator was negligible.

3.11.2 Batch fermentation

The sugar utilization rate and ethanol production rate used were averaged over the fermentation time period.

$$S'_t = (S_i - S_{i+1}) \div (t_{i+1} - t_i) \quad g/lh$$

$$E'_t = (E_{i+1} - E_i) \div (t_{i+1} - t_i) \quad g/lh$$

$$q_t = S'_t \div X_{at} \quad g/gh$$

$$\nu_t = E'_t \div X_{at} \quad g/gh$$

$$Y = [(E_{i+1} - E_i) \times 100] \div [(S_i - S_{i+1}) \times 0.538] \quad \%$$

$$t = t_i + [(t_{i+1} - t_i) \div 2] \quad h$$

$$X_{at} = (X_i + X_{i+1}) \div 2 \quad g/lDW$$

where S is the substrate concentration (g/l),

S' is the mean volumetric rate of substrate utilization (g/lh),

E' is the mean volumetric rate of ethanol production (g/lh),

q is the mean specific rate of substrate utilization (g/gh),

ν is the mean specific rate of ethanol production (g/gh),

Y is the ethanol yield based on theoretical yield (%),

t is the fermentation time (h),

X_a is the mean cell concentration (g/l DW).

Percentage substrate utilization,

S_u = (feed sugar concentration - residual sugar concentration) x 100 ÷ feed sugar concentration.

CHAPTER 4

TOWER FERMENTATION OF WHEY PERMEATE

In this study, *K.marxianus* Y42 was used in the tower fermenter to ferment whey permeate continuously. The continuous fermentation operation was carried out at different superficial liquid velocities (V_s) until cell wash out was observed. Such fermentation parameters as lactose, ethanol and cell concentrations, and the medium pH were monitored at different locations on the tower including the exit. These and other parameters were considered with respect to the height in the tower, the residence time and the superficial liquid velocity in order to study the performance of the tower fermenter when used to ferment whey permeate and to determine optimum operating conditions for this substrate and yeast culture.

The averaged data for each superficial liquid velocity have been given in Appendix B.3. The following conversion data can be used to convert the superficial velocity (V_s) to other related parameters as required.

Dilution rate (D) based on the total tower height of 2.37 m.

$D = 1.44\ V_s$ (mm/s) $\quad h^{-1}$

Volumetric flow rate (Q)

$Q = 1880\ V_s$ (mm/s) $\quad$ ml/h

Five medium feed rates were used :

V_s mm/s	D h^{-1}	Q ml/h
0.044	0.063	80
0.080	0.12	150
0.17	0.25	310
0.24	0.34	440
0.30	0.43	550

4.1 THE RELATIONSHIP BETWEEN TOWER HEIGHT AND VARIOUS FERMENTATION PARAMETERS

4.1.1 Lactose concentration and utilization

The lactose concentration of the fermentation broth declined with an increase in the height in the tower (fig. 4.1).

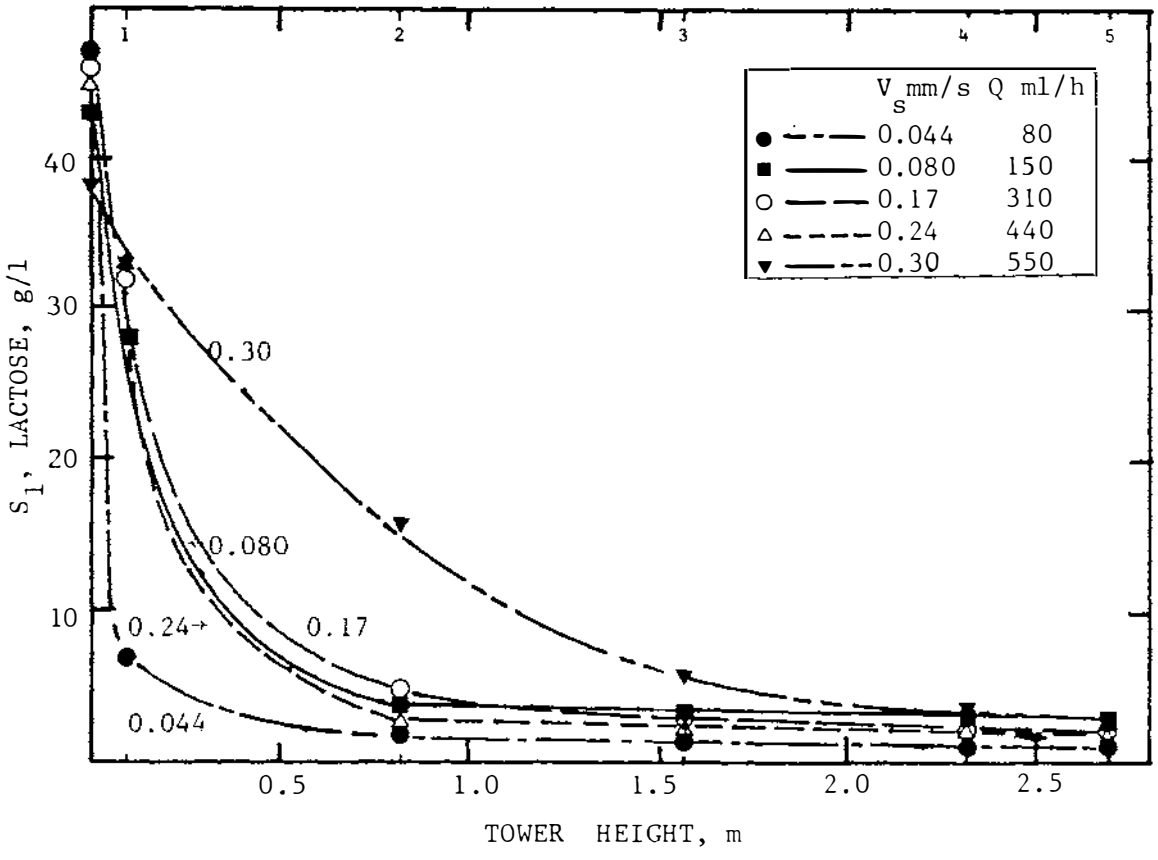


Fig.4.1 Lactose concentration vs tower height at various superficial liquid velocities.

There was a very rapid decrease in lactose concentration, from 40 to 8 g/l over the first 0.096 m of the tower to sample point 1 for a low superficial liquid velocity (V_s) (0.044 mm/s). The lactose concentration then decreased slowly to 2 g/l, at 0.82 m height (sample point 2), and reduced further to 1 g/l, at 1.57 m. It remained at this concentration over the remainder of the tower height. This corresponded to 85 and 96% lactose utilization at heights of 0.096 and 0.82 m, respectively (table 4.1).

The rate of reduction in lactose concentration with height was lower at velocities between 0.080 and 0.24 mm/s. The concentration was reduced from 40 g/l at the inlet to 32 g/l at 0.096 m and to 4 g/l at 0.82 m when the velocity was 0.080 mm/s. Further increase in height had only a marginal effect on further reduction of lactose concentration which was 1.5 g/l at the exit (2.69 m). The corresponding lactose utilization at 0.096 and 0.82 m was 35 and 91%, respectively. Similar profiles with only minor variations in lactose concentration at 0.096 and 0.82 m were observed for velocities of 0.17 and 0.24 mm/s (table 4.1).

Table 4.1 Lactose utilization at various tower heights and superficial liquid velocities.

location/height		S_{lu}(%), at various V_s (mm/s)				
	m	0.044	0.080	0.170	0.24	0.30
1	0.096	85	35	32	27	14
2	0.82	96	91	91	90	57
3	1.57	98	92	93	94	84
4	2.32	97	92	94	95	90
5	2.69	97	93	95	96	92

When the velocity was increased to 0.30 mm/s the reduction in concentration with height was very much slower. The concentration remained high at 34 g/l, at 0.096 m, and 17 g/l at 0.82 m. At this velocity 2.32 m of height were required to reduce the concentration to 4 g/l. The corresponding lactose utilization at 0.096, 0.82 and 2.32 m was 14, 57 and 90%, respectively.

It was evident that at a constant velocity there was a decrease in the concentration of lactose with the increasing height. The height at which lactose was reduced to a particular concentration increased when the velocity was increased. At a height of 0.82 m, lactose was reduced to less than 4 g/l for superficial velocities below 0.24 mm/s corresponding to a lactose utilization of 90 to 94%. This compared favorably with the batch fermentation utilization of 93% (Appendix B.2).

The results presented here were averages of five samples with at least two residence times (T_r) between each sampling. This represented more than 10 residence times for each velocity used. The exception to this was at the velocity of 0.30 mm/s where only 2 sets of consecutive samplings were used. There was a slow reduction in the cell concentration inside the tower (sect. 4.6) and an increase in the exit effluent specific gravity (SG_e) (fig. 4.26a). This was an indication of cell wash out. This superficial velocity thus represents an approximation of the critical velocity above which the tower cannot be operated. Clearly this set of readings cannot be regarded as steady state data.

Similar trends of decreasing sugar concentration with increasing tower height have been observed for tower fermentation of beer (Greenshields & Smith 1971) where the specific gravity reduced rapidly from 1.035, at the inlet, to 1.010 as the height increased to 2 m, when the velocity was 0.36 mm/s. Further increase in height to 7 m caused a slow decrease in specific gravity to 1.006.

The trend observed in the present work of rapid reduction in lactose concentration with height at the low velocity of 0.044 mm/s was very similar to that observed in the tower fermentation of fodder beet extract (Henderson & Smith 1982). Sucrose concentration showed a rapid reduction from 100 to 8 g/l as the height increased from 0 to 0.095 m at a velocity of 0.042 mm/s ($D = 0.2\ h^{-1}$). Further increase in height to 0.48 m caused a slow decrease in sucrose concentration to 3 g/l.

4.1.2 Volumetric rate of lactose utilization (S_1')

The rate of lactose utilization (S_1') described here was calculated as the mean for each of the 5 tower sections (fig. 3.2) and plotted against the height at the mid-point of each section.

The mean volumetric rate decreased rapidly with increasing height in the tower (fig. 4.2).

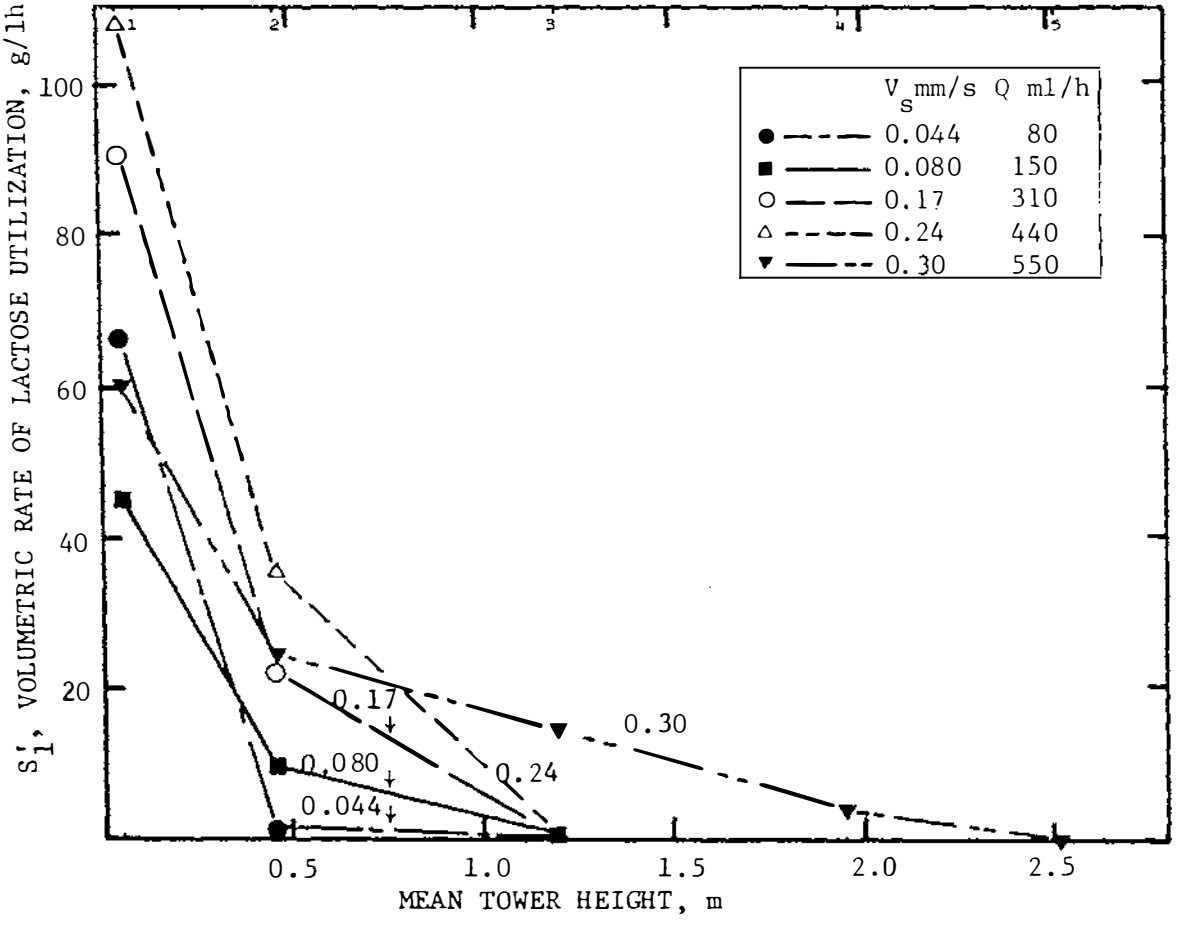


Fig. 4.2 Volumetric rate of lactose utilization vs mean tower height at various superficial liquid velocities.

It was greatest in the first tower section below 0.096 m and reduced to near zero as the mean height increased to 1.20 m for all superficial liquid velocities except the highest (0.30 mm/s). At the lowest superficial velocity (0.044 mm/s), there was a rapid reduction in the rate from 66 g/lh at 0.048 m to 1 g/lh at 0.46 m. It then decreased to a rate below 1 g/lh over the remaining height.

At 0.080 mm/s, the rate reduced from 45 to 10 g/lh as the mean height increased from 0.048 to 0.46 m. It then reduced further to less than 1 g/lh at 1.20 m. Similarly for velocities of 0.17 and 0.24 mm/s, the rate reduced to less than 1 g/lh above 1.20 m.

At the critical velocity (0.30 mm/s) the mean rate over the initial section of the tower (up to 0.46 m) was low compared to those at lower velocities, suggesting a lower yeast concentration was present. Utilization occurring in the higher section of the tower (from 1.20 to 1.95 m) was greater than at velocities less than 0.30 mm/s because of the greater concentration of yeast and lactose in these sections.

The result showed that the greatest mean rate of lactose utilization occurred at the lower section of the tower up to 0.82 m and for all velocities up to 0.24 mm/s. There was little lactose utilization at heights greater than this. At a velocity of 0.30 mm/s fermentation was observed over the full height of the tower including the separator.

The trend observed here was similar to the trend observed for the tower fermentation of fodder beet extract (Henderson & Smith 1982). At a velocity of 0.042 mm/s ($D = 0.2\ h^{-1}$), the volumetric rate of sucrose utilization decreased rapidly from 72 to 3 g/lh as the height increased from 0.095 to 0.29 m. A further reduction to 1 g/lh occurred between 0.38 m and the exit at 0.76 m.

4.1.3 Specific rate of lactose utilization (q_1)

The mean specific rate of lactose utilization (q_1) decreased rapidly to a low value as the mean tower height increased (fig. 4.3). The specific rate decreased from 0.68 to 0.02 g/gh as the mean height increased from 0.048 to 0.46 m at the lowest velocity (0.044 mm/s). Beyond this mean height the specific rate was less than 0.02 g/gh. This was because lactose was 96% utilized when the height of 0.82 m was reached so there was very little lactose utilization after this height.

At the higher velocities of 0.080, 0.17 and 0.24 mm/s, the specific

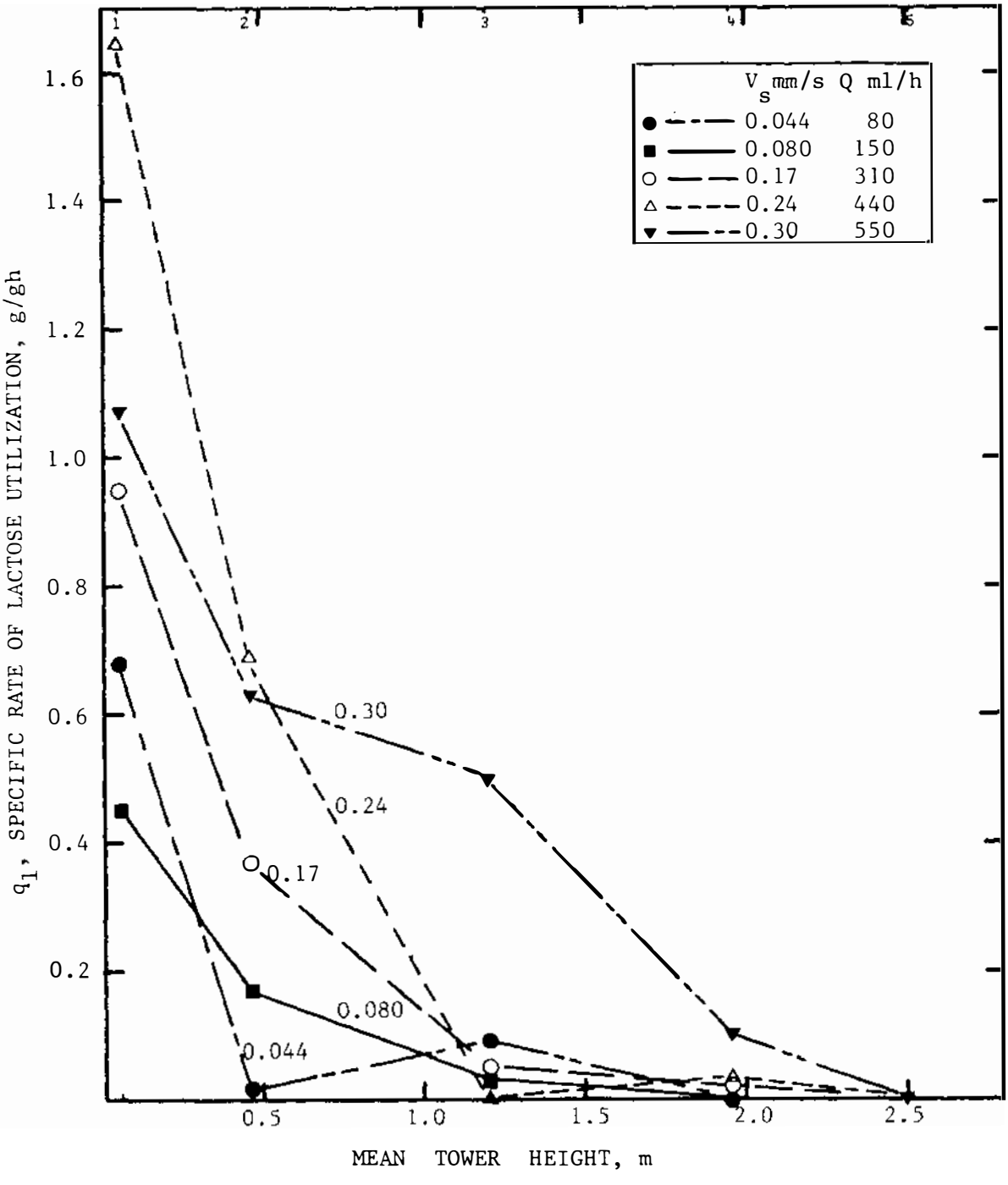


Fig.4.3 Specific rate of lactose utilization vs mean tower height at various superficial liquid velocities.

rate decreased from between 0.45 and 1.64 g/gh to less than 0.1 g/gh as the mean height increased from 0.046 to 1.20 m, at this height lactose was 95% utilized (table 4.1). Beyond this height, the specific rate was lower than 0.1 g/gh since there was little lactose left to be utilized.

At the highest velocity of 0.30 mm/s, the specific rate decreased

less rapidly than at lower velocities. The specific rate fell from 1.07 to 0.10 g/gh as the mean height increased from 0.048 to 1.95 m. This slow decrease occurred because there was lactose utilization throughout the tower as a result of the decreased residence time. The cell concentration at the bottom of the tower was reduced while there was an increase in lactose concentration at greater heights as the velocity increased. Thus, lactose utilization occurred throughout the tower.

In the separator, the specific rate was zero for all velocities.

The results showed that the specific rate of lactose utilization decreased as the height increased and the height at which the specific rate was zero depended on the residence time and the cell concentration. It was possible that ethanol cencentration would have an effect on the specific rate, since ethanol concentration increased with height (fig. 4.4), thus, ethanol inhibition could increase with height. However, the maximum ethanol concentration reached was 23 g/l which was considerably lower than a reported inhibiting concentration of 30 g/l (Wendorf et al 1970a).

The trends observed were similar to those obtained in the tower fermentation of molasses (fig.C.3). However, the specific rate of sugar utilization was greater in the molasses and at a similar velocity, fermentation was completed at a mean height of 1.20 m. The specific rate was greater due to the higher feed sucrose concentration used (100 g/l) and the greater rate at which sucrose could be utilized.

4.1.4 Ethanol concentration (E)

Ethanol concentration increased with increasing height for a given superficial velocity (fig.4.4). The ethanol concentration rose rapidly with height at the lowest velocity (0.044 mm/s). The concentration increased from 0 to 20 g/l at 0.096 m and further increase in the height had minor effect on the concentration, which was 22 g/l at 2.32 m.

There was a slower rate of increase in ethanol concentration with height at greater velocities up to 0.24 mm/s. When the velocity was 0.080 mm/s, the concentration increased to 5.8 g/l at 0.096 m, to 17 g/l at 0.82 m, and to 18 g/l at 1.57 m. Further increase in height had no significant effect on the concentration which remained at 18 g/l until the exit was reached. Similar profiles with minor variations in concentration were observed for velocities of 0.17 and 0.24 mm/s.

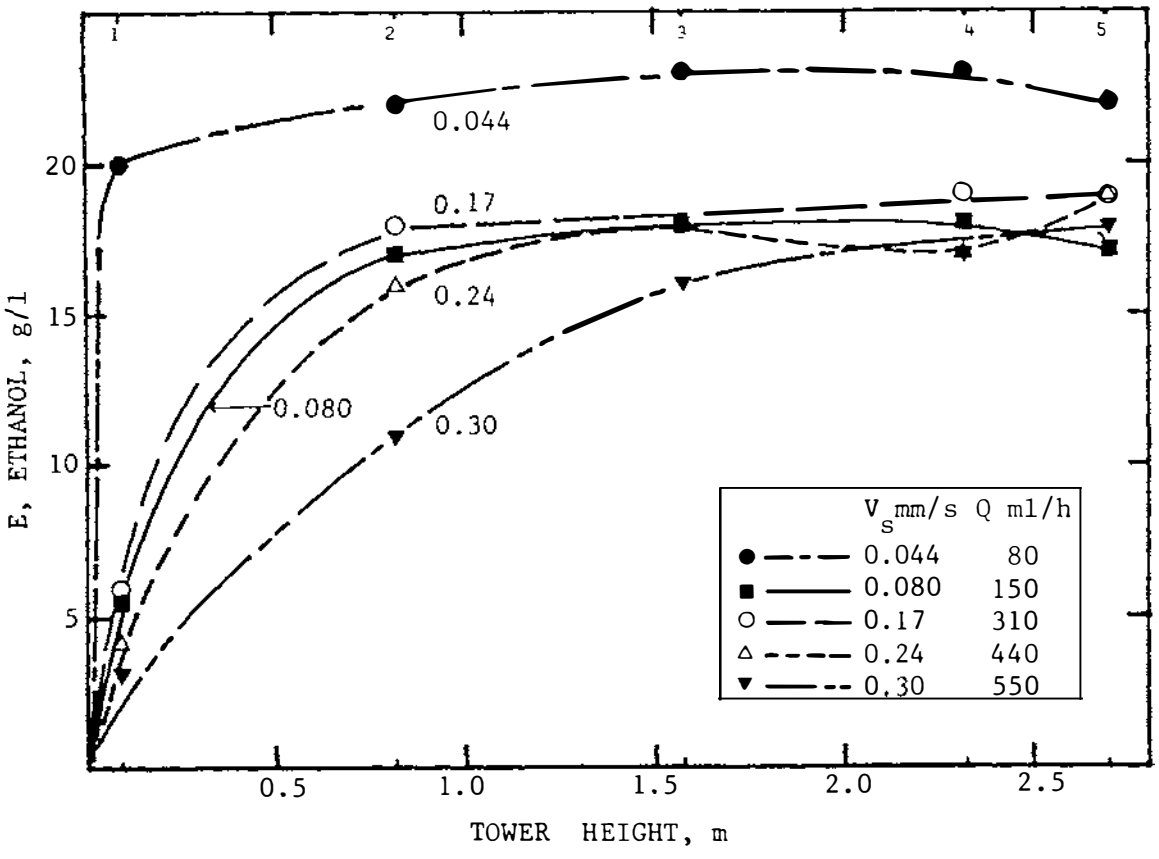


Fig.4.4 Ethanol concentration vs tower height at various superficial liquid velocities.

There was a slower increase in the concentration with height at the greatest velocity (0.30 mm/s). A small amount of ethanol was produced in the initial 0.096 m of the tower and at 0.82 m less than 2/3 of the final ethanol concentration was produced (11 g/1). At this velocity the full fermenter height including the separator (2.69 m) was required to achieve an ethanol concentration of 18 g/1.

The results showed that for all velocities up to 0.24 mm/s, most of ethanol was produced within the first 0.82 m of height. This was a reflection of the trend obtained for the lactose reduction because for these velocities lactose was more than 90% utilized within this height. At the greatest velocity (0.30 mm/s), ethanol concentration increased continuously as the height increased because lactose was being utilized continuously over the entire tower height.

The trend observed here, at the lowest velocity (0.044 mm/s) was similar to the increase in ethanol concentration with height observed in the tower fermentation of sugar beet extract (Henderson & Smith 1982). They noted that the concentration increased rapidly to 48 g/1

as the height increased to 0.095 m when the velocity was 0.042 mm/s. From 0.095 m to the exit, the concentration increased by only 3 g/l to 51 g/l.

4.1.5 Ethanol yield (Y)

For all superficial velocities but one (0.24 mm/s), the ethanol yield coefficient did not vary greatly between 0.82 and 2.32 m (fig.4.5).

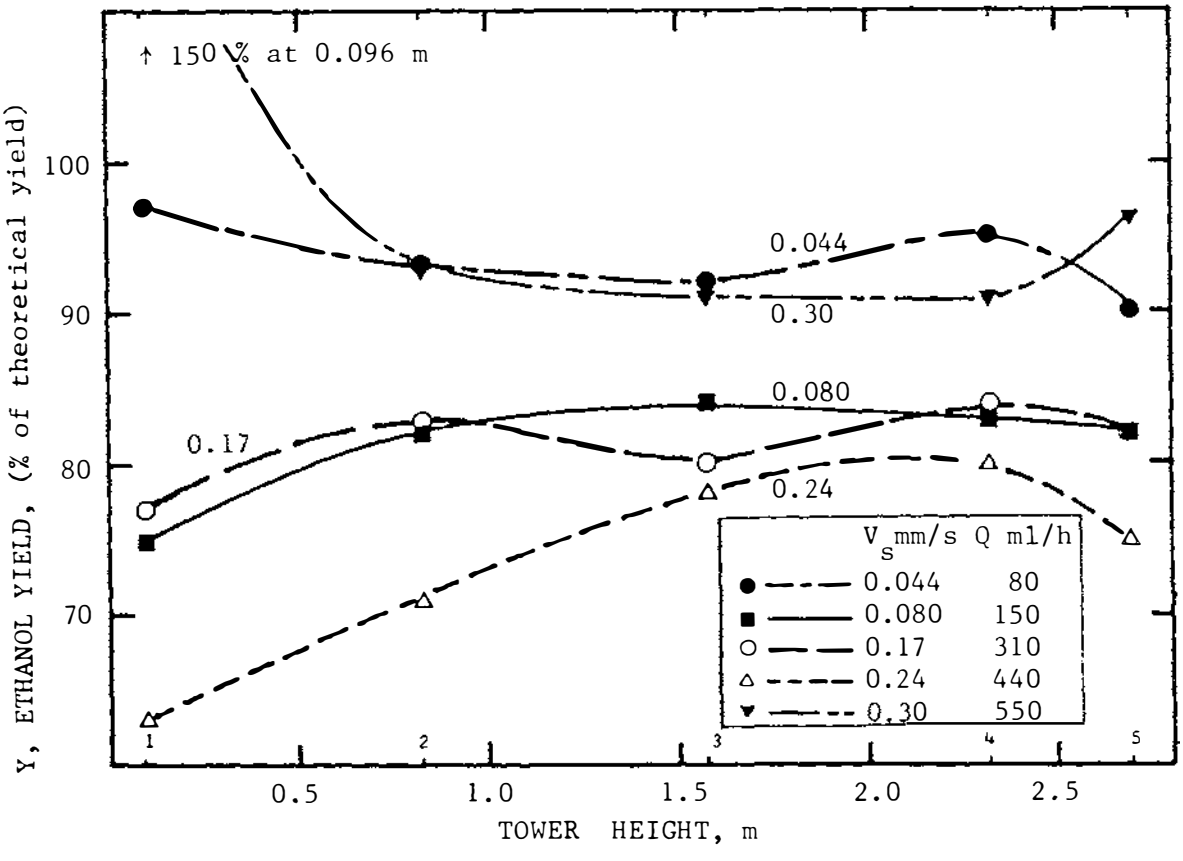


Fig.4.5 Ethanol yield vs tower height at various superficial liquid velocities.

The yield was between 82 and 93%. At velocity of 0.24 mm/s, the yield increased from 71 to 80% as the height increased from 0.82 to 2.32 m.

The yield at 0.096 m varied considerably between 63 to 150% for all velocities. The probable explanation for this behaviour is that, because of the high sugar and low ethanol concentrations at this point, small fluctuation in the measured sugar and ethanol concentrations resulted in considerable variation in the calculated yield value. The

uncertainty in the yield value at this height was estimated to be as high as 490% (sect. E.3).

When the effluent left the tower (2.69 m), the yield was generally lower than inside the tower (between 1 and 5%) for all velocities except 0.30 mm/s. This small reduction could have been caused by gas stripping of ethanol when carbon dioxide left the tower and consumption of ethanol by *K.marxianus* (Sarfacon et al 1972). However, this was not studied further.

The trend observed between 0.82 and 2.32 m was similar to the trend observed in the tower fermentation of fodder beet extract (Henderson & Smith 1982). They observed that the yield was invariant with height for all velocities used. The yield was between 94 and 98%.

Thus, for all velocities, there was little variation in the yield of ethanol within analytical uncertainty (12% from sect. E.3) within the heights of 0.82 to 2.32 m at a particular velocity. At 0.096 m, there was considerable variation in the yield due to analytical error. The yield of ethanol in the effluent was slightly lower than that in the tower.

4.1.6 Volumetric rate of ethanol production (E')

The volumetric rate of ethanol production (E') (fig.4.6) was plotted in a similar way to that described for the volumetric rate of lactose utilization (sect. 4.1.2).

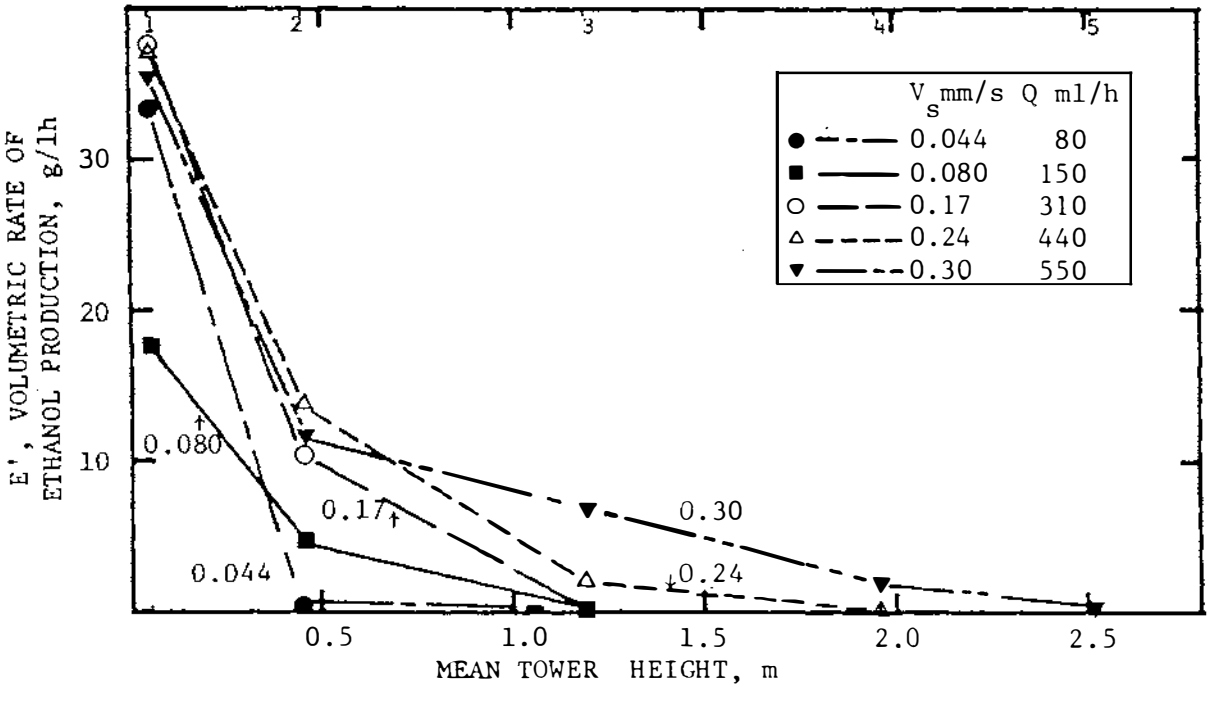


Fig.4.6 Volumetric rate of ethanol production vs mean tower height at various superficial liquid velocities.

At a constant velocity, the volumetric rate of ethanol production decreased with increasing mean height in a similar way to the trend obtained for the volumetric rate of lactose utilization (fig.4.2).

At the lowest velocity (0.044 mm/s), the volumetric rate decreased rapidly from 33 g/lh to less than 1 g/lh as the mean height increased from 0.048 to 0.46 m and remained below 1 g/lh over the remaining sections of the tower. The profile was similar for velocity of 0.17 mm/s.

At velocities of 0.24 and 0.30 mm/s initial rates remained high, but ethanol production occurred over more of the total tower height.

For all velocities ethanol production ceased inside the separator (2.51 m).

In all cases, the volumetric rate of ethanol production was high within the first 0.82 m because of the high lactose and cell concentrations present. At greater heights,the volumetric rate was very low because there was only a small amount of lactose left to be utilized.

Thus, the volumetric rate of ethanol production at a particular tower height followed the observed trend for the volumetric rate of lactose utilization. For all velocities under 0.30 mm/s, the height greater than 0.82 m was not essential.

The trend observed here was similar to that observed in tower fermentation of fodder beet extract (Henderson & Smith 1982). They found that the volumetric rate of ethanol production decreased rapidly from 38 to 2 g/lh over the first 0.38 m of the tower height when the velocity was 0.042 mm/s ($D = 0.2\ h^{-1}$). It then reduced further to a low value (0.6 g/lh) and showed no significant change between heights of 0.38 and 0.76 m. The effluent left the tower at 0.76 m.

4.1.7 Specific rate of ethanol production (ν)

At a constant velocity, the specific rate of ethanol production (ν) showed a general trend of decrease from high value (between 0.2 and 0.7 g/gh) at a mean height of 0.048 m to zero with increasing height in the tower (fig.4.7). The mean height at which the specific rate reduced to zero was affected by the velocity being,further up the tower at a greater velocity.

The specific rate decreased with height because there was a decrease in lactose concentration as the height increased but the cell concentration, at a particular velocity, between 0.82 and 2.32 m, showed

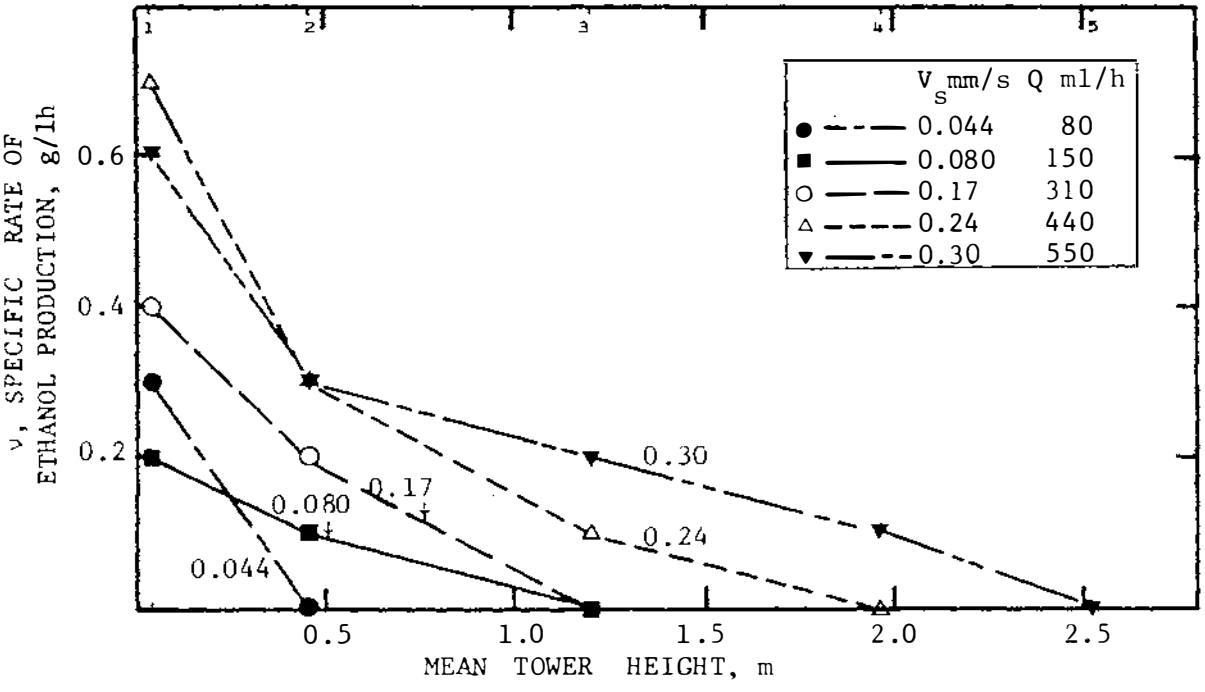


Fig.4.7 Specific rate of ethanol production vs mean tower height at various superficial liquid velocities.

little change as the height increased due to cell recycling (fig.4.9). Thus, the amount of ethanol produced by an almost constant cell concentration, decreased with height. Hence, the specific rate of ethanol production decreased with height.

The trend of decrease was similar to the trend observed in the tower fermentation of molasses (sect. C.1.2) at similar velocity but the rate observed in the molasses fermentation was higher (between 2.8 and 7.0 g/gh, at a mean height of 0.048 m).

There was a trend of decreasing specific rate of ethanol production with an increase in the ethanol concentration (fig.4.8). However, the evidence available was insufficient to conclude that this reduction was due to ethanol concentration alone because there was low lactose concentration when ethanol concentration was high (fig.4.1 compared with fig.4.4) while the cell concentration was kept high and changed little between 0.82 and 2.32 m (fig.4.9).

Thus, the reduction of the specific rate with an increase in ethanol concentration was probably due to a combined effect of the availability of lactose, ethanol concentration and the high biomass concentration.

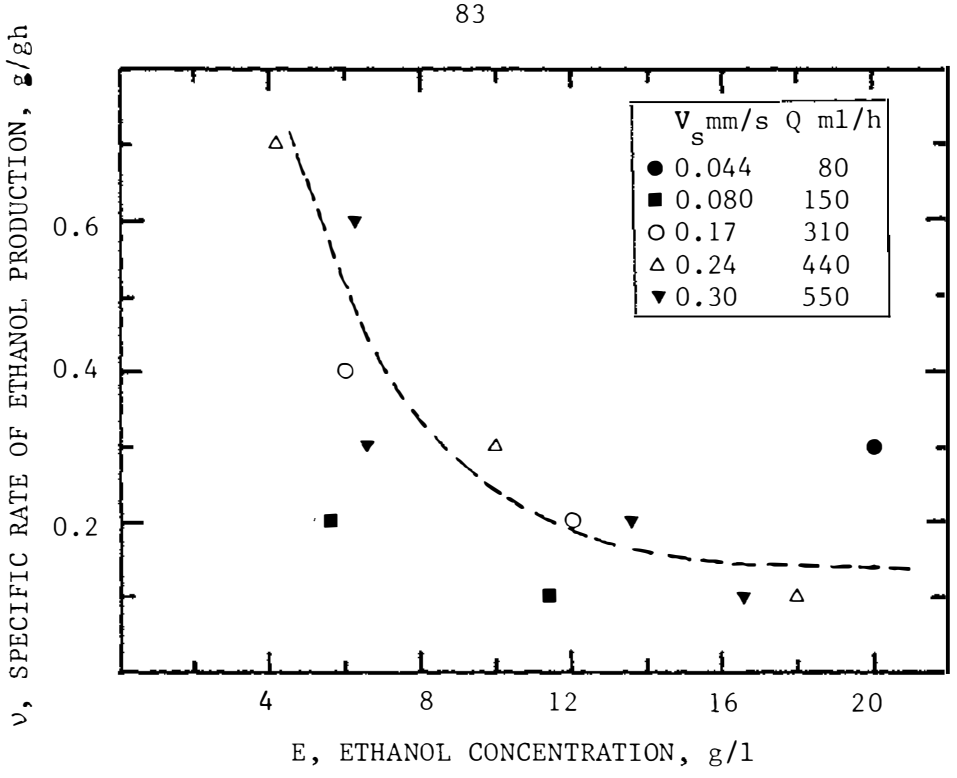


Fig. 4.8 Specific rate of ethanol production vs ethanol concentration at various superficial liquid velocities.

4.1.8 Cell concentration

For all values of superficial liquid velocities (V_s), the cell concentration (fig.4.9) was highest at height of 0.096 m with values in the range of 56 to 100 g/l dried weight (DW) and 220 to 350 g/l centrifuged wet weight (WW). The exact values depended on the velocity and will be discussed in sect. 4.3.4.

As the height in the tower increased from 0.096 to 0.82 m, the cell concentration decreased rapidly. Then as height in the tower increased further to 2.32 m, the concentration decreased at a slower rate. The exceptions to this trend were for the velocities of 0.24 and 0.30 mm/s at which the cell mass was more evenly distributed throughout the tower because of fluidization of the flocs. In all cases, the concentration of cells leaving the fermenter from the separator was less than 0.5 g/l DW (18 g/l WW).

The results showed that the cell concentrations inside the tower between 0.82 to 2.32 m were much lower than at height of 0.096 m. Highest concentrations corresponded with the greatest fermentative activity (up to 0.82 m). An increase in the superficial velocity resulted in the gas carbon dioxide being produced at a higher rate because of the increase in the rate of lactose utilization with velocity (sect. 4.3.3).

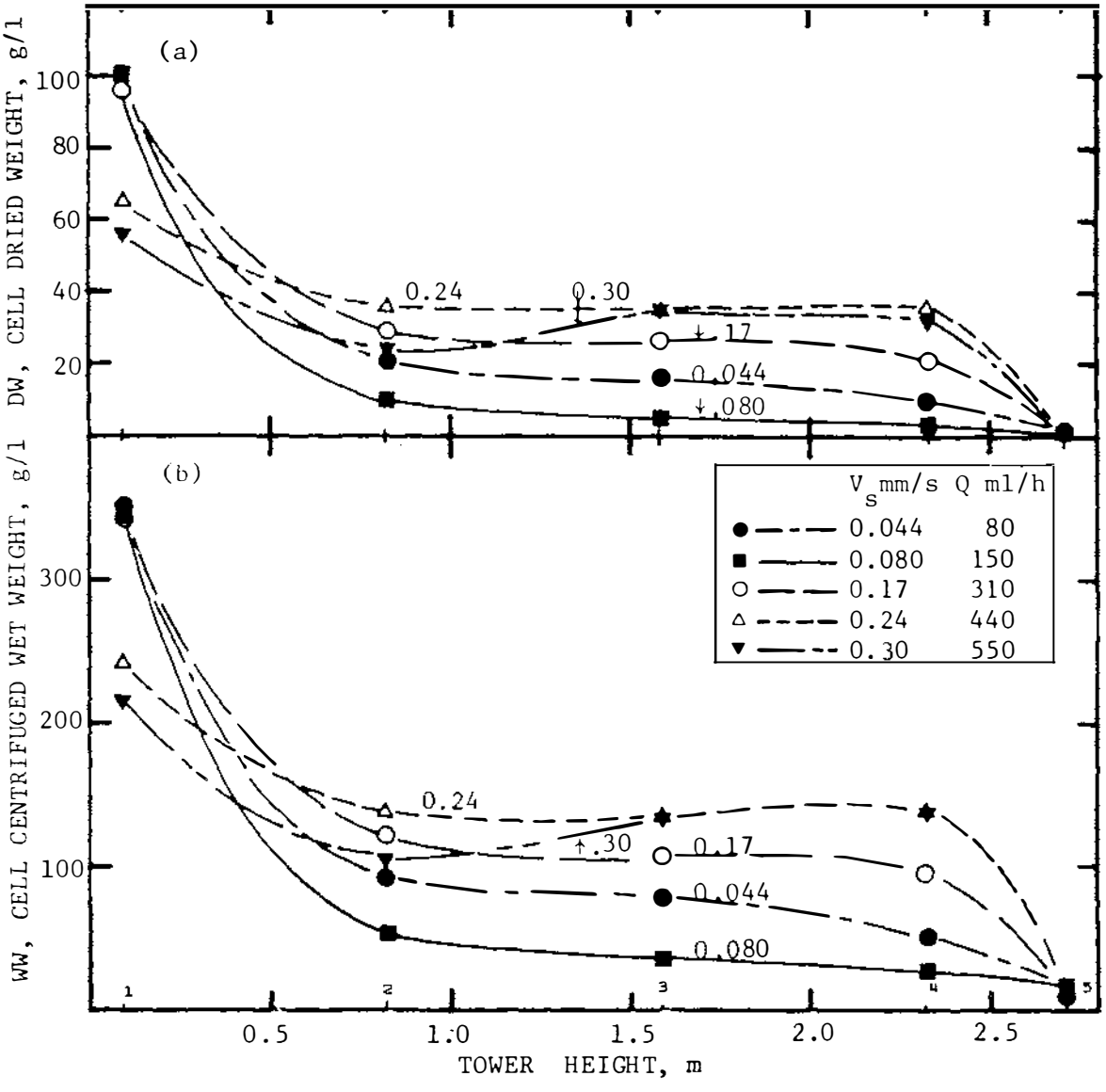


Fig.4.9 Cell concentration vs tower height at various superficial liquid velocities.

Thus, the upflow movement of this gas and the fermenting liquor combined to lift part of the cell population to a greater height. At the highest velocity (0.30 mm/s), this combined uplifting action caused the deviation in the profile from the general trend of decreasing cell concentration with height between 0.82 and 2.32 m. There was a corresponding increase in the fermentative activity within these greater heights.

In general, the cell concentration reached inside the tower (between 0.82 and 2.32 m) was lower than 40 g/l DW (150 g/l WW) because the yeast used was only moderately flocculent in whey permeate.

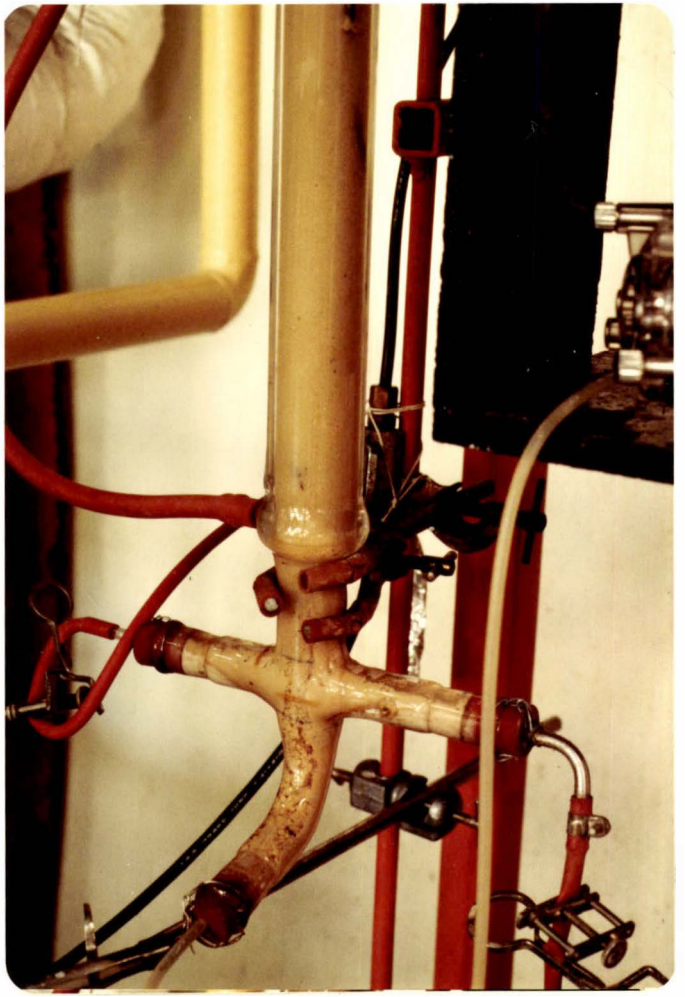
This was lower than the cell concentration reached in other tower fermentations using *S.cerevisiae*.

An average value of 57 g/l DW (250 g/l WW) was reported for beer fermentation inside a tower fermenter (Greenshields & Smith 1971). Prince & Barford (1982) operated their tower fermentation of cane juice using cell concentrations of 54 to 83 g/l DW (220 to 230 g/l WW). Henderson & Smith (1982) operated their tower fermentation of beet extracts using cell concentrations of 18 to 80 g/l DW (90 to 320 g/l WW).

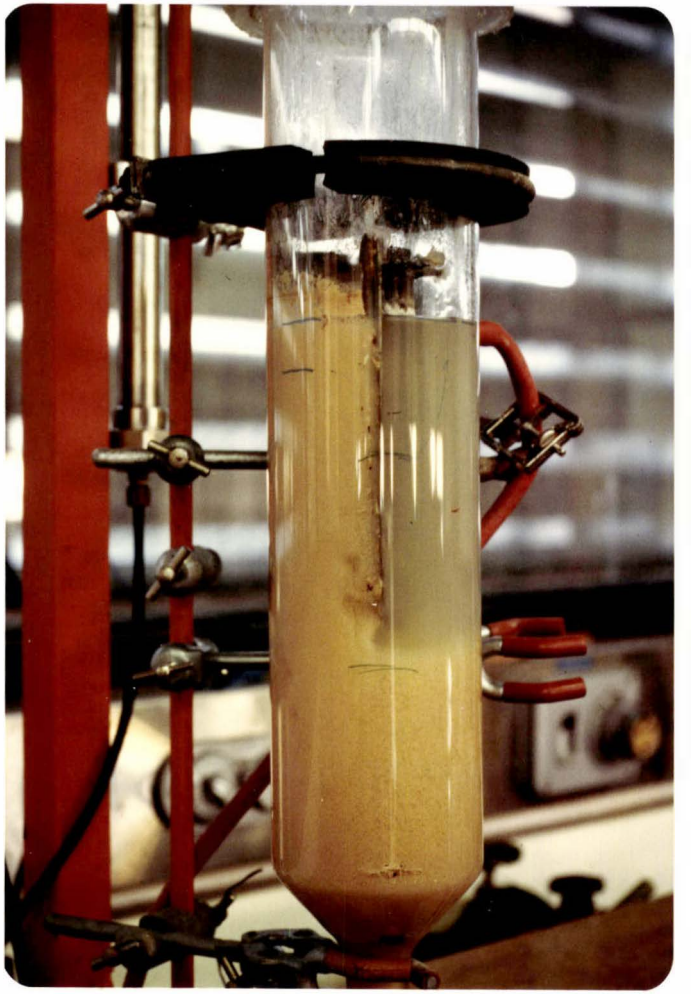
On the other hand, Chen & Zall (1982) operated their AFEB fermenter to ferment whey using a non-flocculent lactose-fermenting yeast at a concentration of 4 to 22 g/l DW. The very flocculent yeast used by the first two groups of workers permitted a much greater medium throughput than in this study. The cell concentration reached in the present study of the tower fermentation of whey permeate was approximately half that reached in the tower fermentation using more flocculent *S.cerevisiae* strains. Thus, the fermentation capability of the culture as a whole was limited by the yeast strain used.

The typical appearance of *K.marxianus* Y42 flocs in the tower at the velocity of 0.17 mm/s is shown in fig. 4.10. In the inlet zone and sample point 1 (0.096 m), the cell concentration was very high (100 g/l DW) (fig.4.10 a). The flocs were large (1 to 3 mm in diameter). Some large flocs were broken up by streams of whey permeate entering the tower. From sample point 1 upward (above 0.096 m), there was considerable carbon dioxide production. The upward movement of this gas and the medium provided lifting action to suspend and disperse the yeast flocs throughout the tower.

Inside the separator (fig.4.10 b), the beer and yeast flocs entered the baffled section of the separator from the top of the separator draught tube (upper left hand side). Carbon dioxide separated from the beer and left the tower via the top exit port (not shown). The beer and the yeast flocs flowed down past the bottom of the baffled section and then up toward the effluent exit (on the right hand side). The yeast flocs that were sufficiently dense to resist the upward motion of the fluids returned to the tower straight section. There was a very clear separation between the effluent and the floc suspension. It was observed that this zone of suspended yeast flocs acted as a filter that trapped free yeast cells to form larger flocs. When this zone of suspended yeast flocs was not present (at the low velocities of 0.044 and 0.080



(a)



(b)

Fig.4.10 Tower fermenter during whey permeate fermentation at superficial liquid velocity of 0.17 mm/s (310 ml/h). (a) Tower bottom showing large yeast flocs ; feed and air inlet (lower centre) ; sampling port 1 (mid-right) , (b) Separator.

mm/s), there was a slightly greater cell loss with the effluent.

4.1.9 Medium pH

The pH of the fermentation broth decreased with height over the initial 0.82 m (fig.4.11). The influent pH of 4.7 to 5.4 was reduced to between pH 4.4 and 4.8 over the initial 0.096 m, and then decreased slowly to between pH 4.2 and 4.6 as the height increased to 0.82 m. Further height increase beyond this to the exit (2.69 m), resulted in no significant change in pH. It remained between pH 4.2 and 4.6.

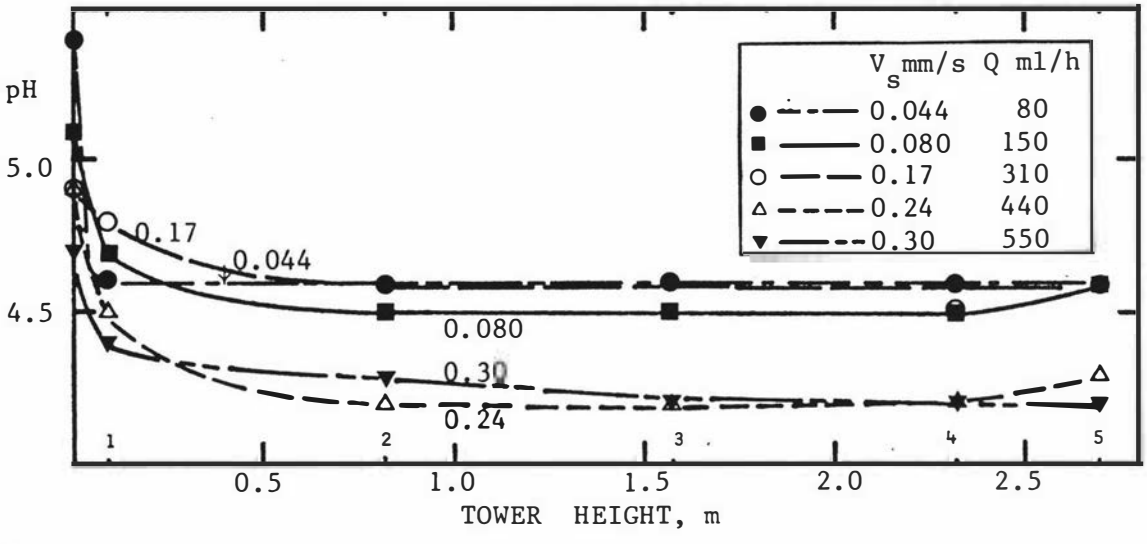


Fig.4.11 Medium pH vs tower height at various superficial liquid velocities.

The high initial pH of greater than pH 5 was due to the aeration of the medium. Aerated medium was found to have pH between 5.0 to 5.4 even though the pH before autoclaving was 4.6. Once the medium was in the inlet zone at the bottom of the tower, there was considerable fermentative activity. Ethanol and carbon dioxide were produced. This caused the initial pH reduction. The slower reduction, between 0.096 and 0.82 m, was a result of a much less intense fermentative activity. Above 0.82 m, the pH remained constant because there was virtually no fermentation taking place.

The lower effluent pH of 4.2, when the velocities were 0.24 and 0.30 mm/s, was caused by a new season whey permeate having a lower initial pH ranging from pH 4.2 to 4.4. Even though the pH was corrected to pH 4.6 before autoclaving, the operating pH still decreased to between 4.2 and 4.4 inside the tower.

The trend observed was similar to that observed in the tower fermentation of molasses (sect. C.1.5) but the operating pH was higher at pH 5.0.

During the tower fermentation of whey permeate, a sudden decrease in the pH to below pH 4.0 in association with a reduction of ethanol concentration inside the tower was found to be an indication of bacterial contamination (sect. 4.6).

4.2 THE EFFECT OF THE RESIDENCE TIME ON VARIOUS FERMENTATION PARAMETERS

The liquid residence times(T_r) used in this section were calculated using the tower height at the respective sample points. It could be referred to as " apparent fermentation time ". The mean residence time (T_r^*) was, however, calculated in a different manner from the true residence time (T_r). It was calculated using the mean height of each tower section (sect.3.11.1 and Appendix B.3). The mean residence time was plotted versus the mean rates in each tower section.

4.2.1 Lactose concentration (S_1)

Lactose concentration decreased rapidly from about 40 g/l to about 6 g/l as the residence time increased to 1 hour and then showed a small reduction to between 2 and 4 g/l as the residence time increased further to 15 hours (fig.4.12).

The rapid reduction of lactose concentration during the first hour in the tower fermenter was a result of the high cell concentration within the lower tower section (up to 0.82 m). Lactose was utilized without any delay normally required to build up the cell concentration in batch fermentation.

The fermentation time observed here was very much less than the 16 hours required to complete the batch fermentation (Appendix B.2). It was also very much shorter than the 12 hours reported for continuous fermentation of whey containing 58 g/l lactose using two continuous stirred-tank fermenters in series (Reesen & Strube 1978). Further comparison could be made with the longer residence time of 3.9 hours reported for 85% utilization of sugar in continuous fermentation of 50 g/l lactose in whey using an immobilized yeast system (Linko et al 1981).

The residence time of 1 hour for 90% lactose utilization observed in the present work was only slightly longer than a residence time of

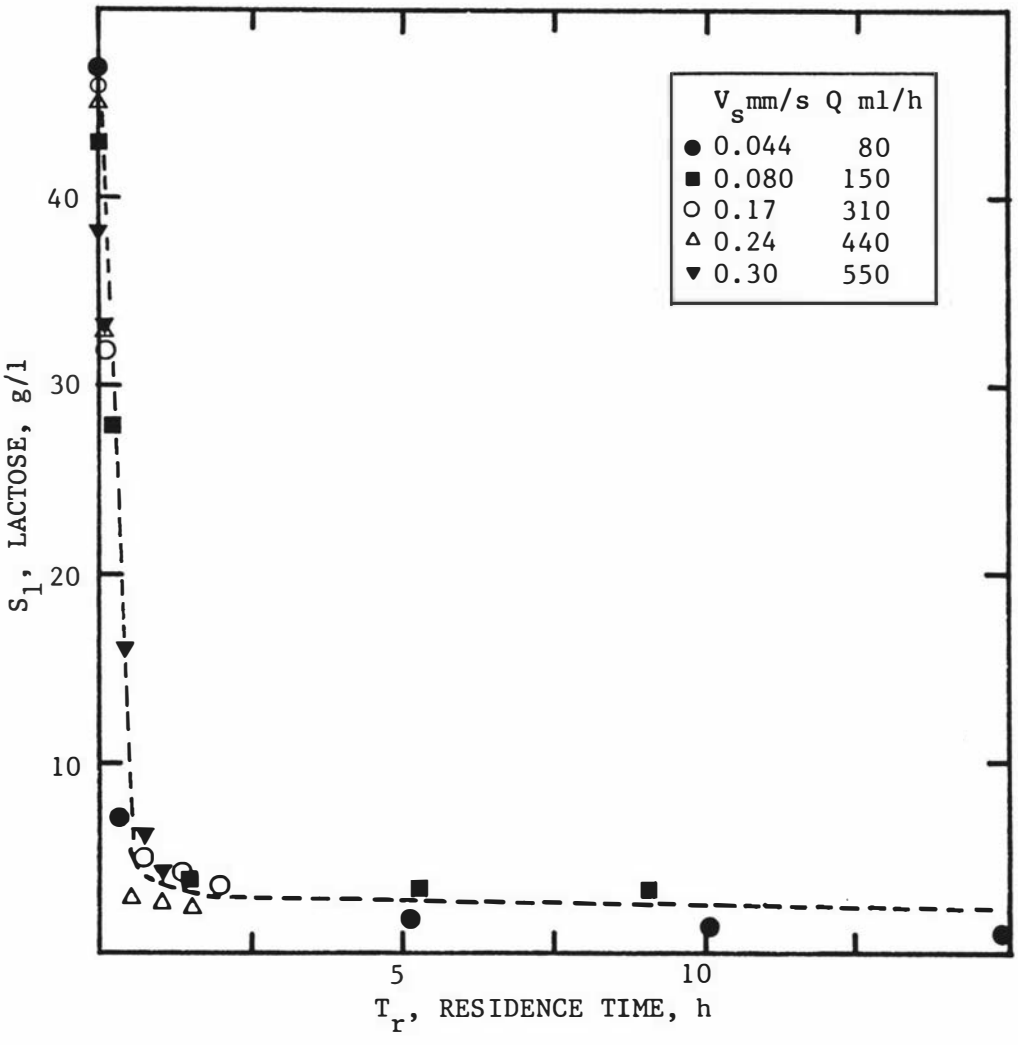


Fig.4.12 Lactose concentration vs residence time at various superficial liquid velocity.

of 0.7 hour observed during tower fermentation of cane molasses in which 96% utilization of 100 g/l sucrose occurred (fig.C.7a).

The trend observed here was similar to that reported for tower fermentation of beer (Greenshield & Smith 1971). However, the beer wort contained many different sugars. Thus, as the residence time increased, there was a slower reduction in the sugar concentration following the initial rapid reduction. One hour was required to reduce the fermenting liquor specific gravity from 1.035 to 1.010, but further reduction from 1.010 to 1.005 required 19 hours.

4.2.2 Ethanol concentration (E)

Ethanol concentration increased with increasing residence time (fig.4.13). The concentration increased rapidly from 0 to greater than 16 g/l as the residence time increased from 0 to 1 hour. Further increase

in the residence time resulted in a small increase in the concentration to greater than 17 g/l. The final ethanol concentrations reached in these experiments were different for various superficial liquid velocities because of the variation in the feed lactose concentration (Appendix B.3).

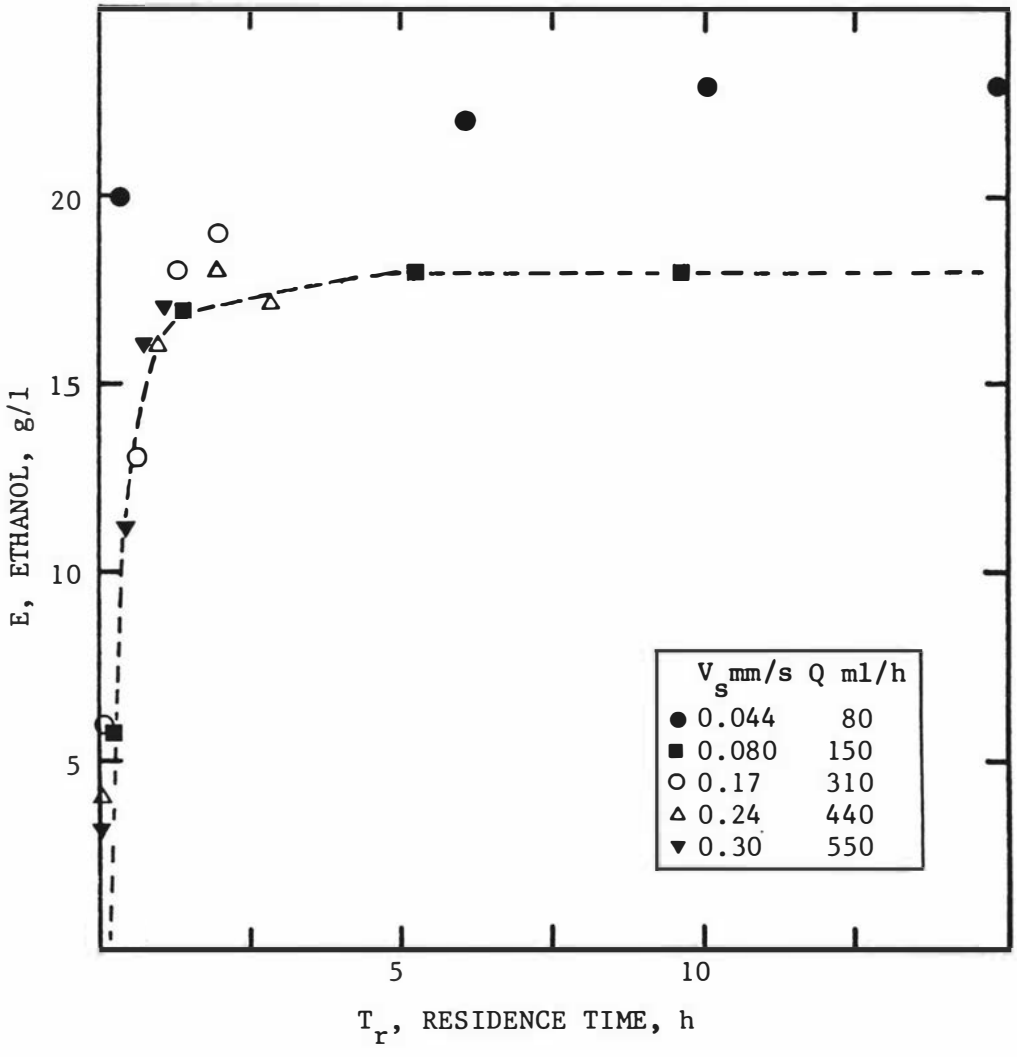


Fig.4.13 Ethanol concentration vs residence time at various superficial liquid velocities.

It was shown previously that up to 90% lactose utilization occurred in 1 hour (fig.4.12). Thus, as would be expected, most ethanol was produced during this time.

The trend observed here was different from that observed during the initial period of batch fermentation (fig.7.1). During the early stage of batch fermentation, there was a short delay before ethanol was produced. This period was then followed by a moderately rapid increase in the ethanol concentration to the maximum concentration. A fermentation time of 16 hours was required to produce 18 g/l ethanol compared with the

1 hour required to produce 16 g/l ethanol in tower fermentation.

It is evident that in the tower fermentation of whey permeate, the majority of the lactose was utilized with the production of ethanol during the first hour in the fermenter. There was no delay in the production of ethanol as observed in batch fermentation.

4.2.3 The rates of lactose utilization and ethanol production

The volumetric rates of lactose utilization (S_1') and ethanol production (E') reduced from high values (108 and 37 g/lh) to 1 g/lh as the mean residence time increased to 2 hours and were less than 1 g/lh when the mean residence time was greater than 2 hours (fig.4.14 and 4.15). Similar trends were observed for the specific rate of lactose utilization q_1 (fig.4.16) and the specific rate of ethanol production (ν) (fig.4.17). For all velocities, the two specific rates (q_1 and ν) were less than 0.03 g/gh at mean residence times greater than 2 hours.

The results showed that, for all velocities, there was no significant lactose consumption after 2 hours in the tower and as a result, there was also no ethanol production after 2 hours. This rapid fermentation was made possible by the high yeast cell concentration achieved by biomass feedback from the separator. Thus, an increase in the residence time of the fermenting medium inside this tower beyond two hours would result in no further utilization of the lactose or further production of ethanol. The high biomass concentration was greater than the concentration that could be supported in a stirred-tank fermenter with no feedback utilizing the same feedstock.

A similar trend was observed in the tower fermentation of molasses (sect.C.2.2). The mean residence time at which the two rates of lactose utilization (S_1' and q_1) were almost zero was 1.5 hours,while for the two rates of ethanol production (E' and ν), the time was 1.2 hours. This was shorter than the time obtained in the tower fermentation of whey permeate, even though the optimum velocity reached in the tower fermentation of molasses was higher (0.33 mm/s) and the feed sucrose was also higher (100 g/l).

In the tower fermentation of fodder beet extract (Henderson & Smith 1982), the mean residence time required for the volumetric rate of sucrose utilization to reduce from 100 g/lh to less than 3 g/lh was 2 hours,and a shorter time of 1.5 hours was required for the volumetric rate of ethanol production to reduce from 50 g/lh to less than 3 g/lh. This was similar

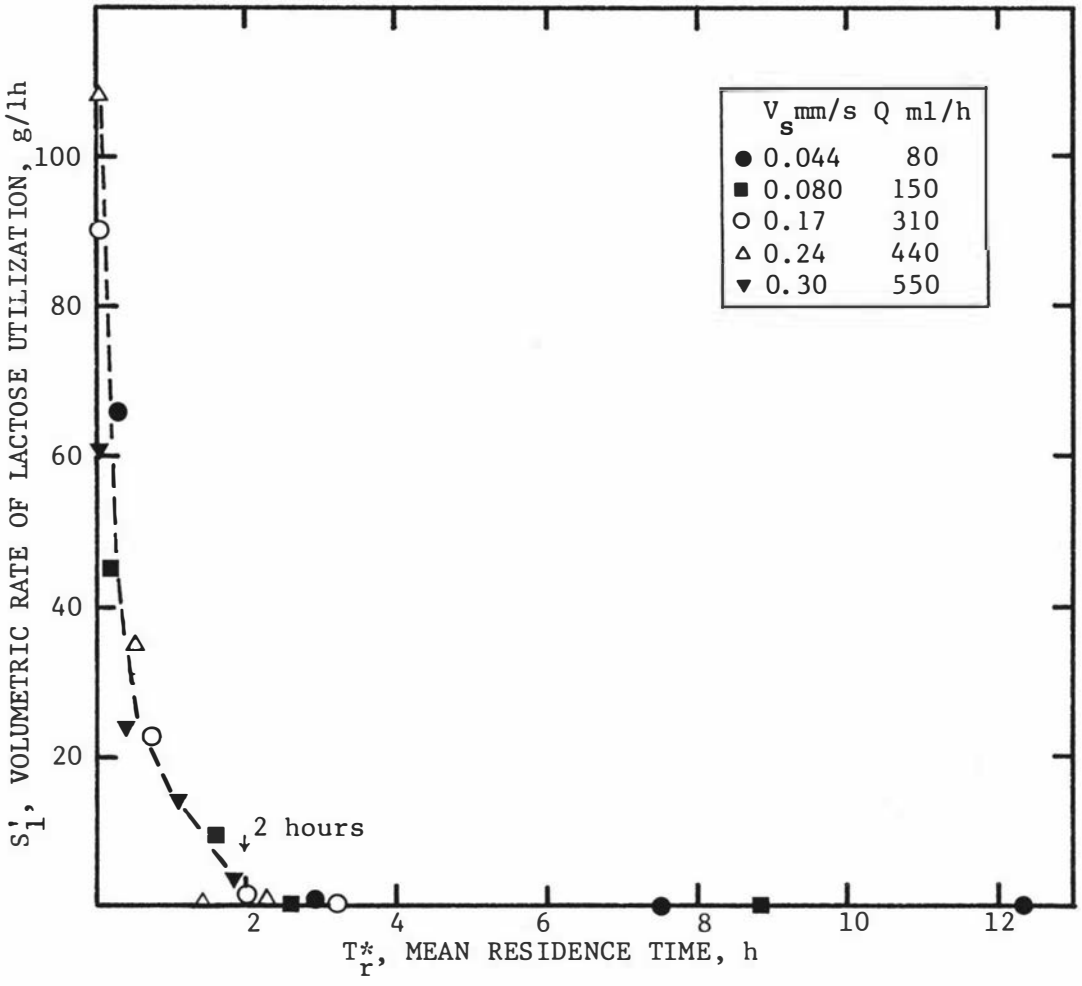


Fig.4.14 Volumetric rate of lactose utilization vs mean residence time at various superficial liquid velocities.

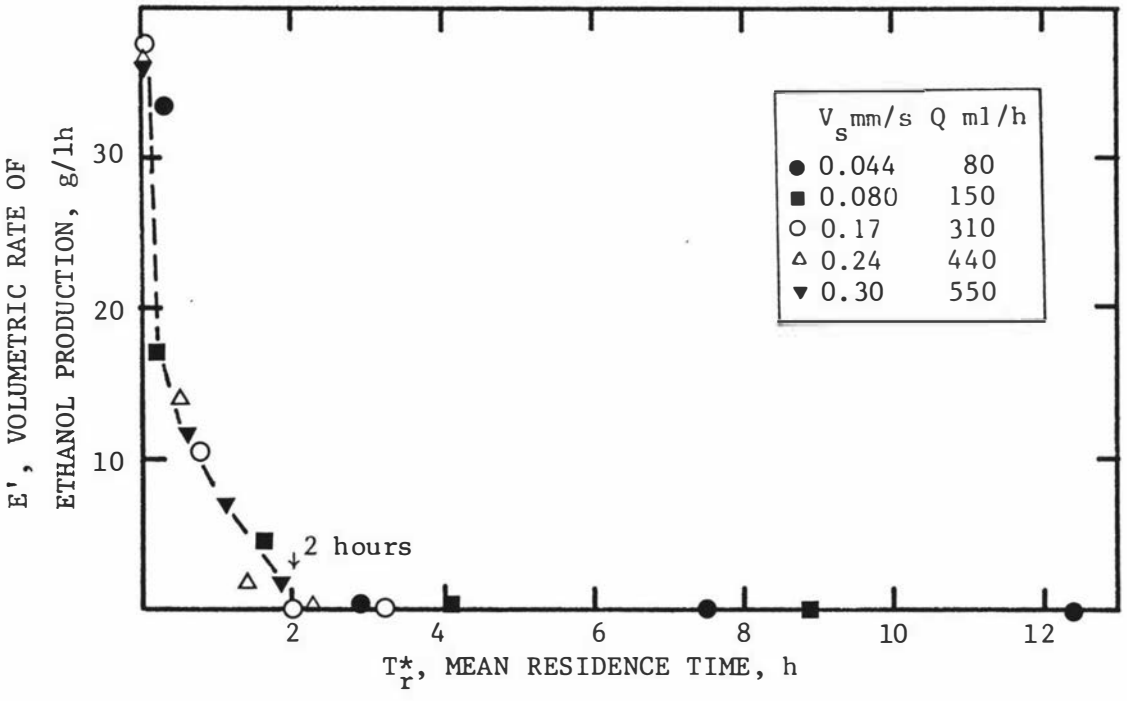


Fig.4.15 Volumetric rate of ethanol production vs mean residence time at various superficial liquid velocities.

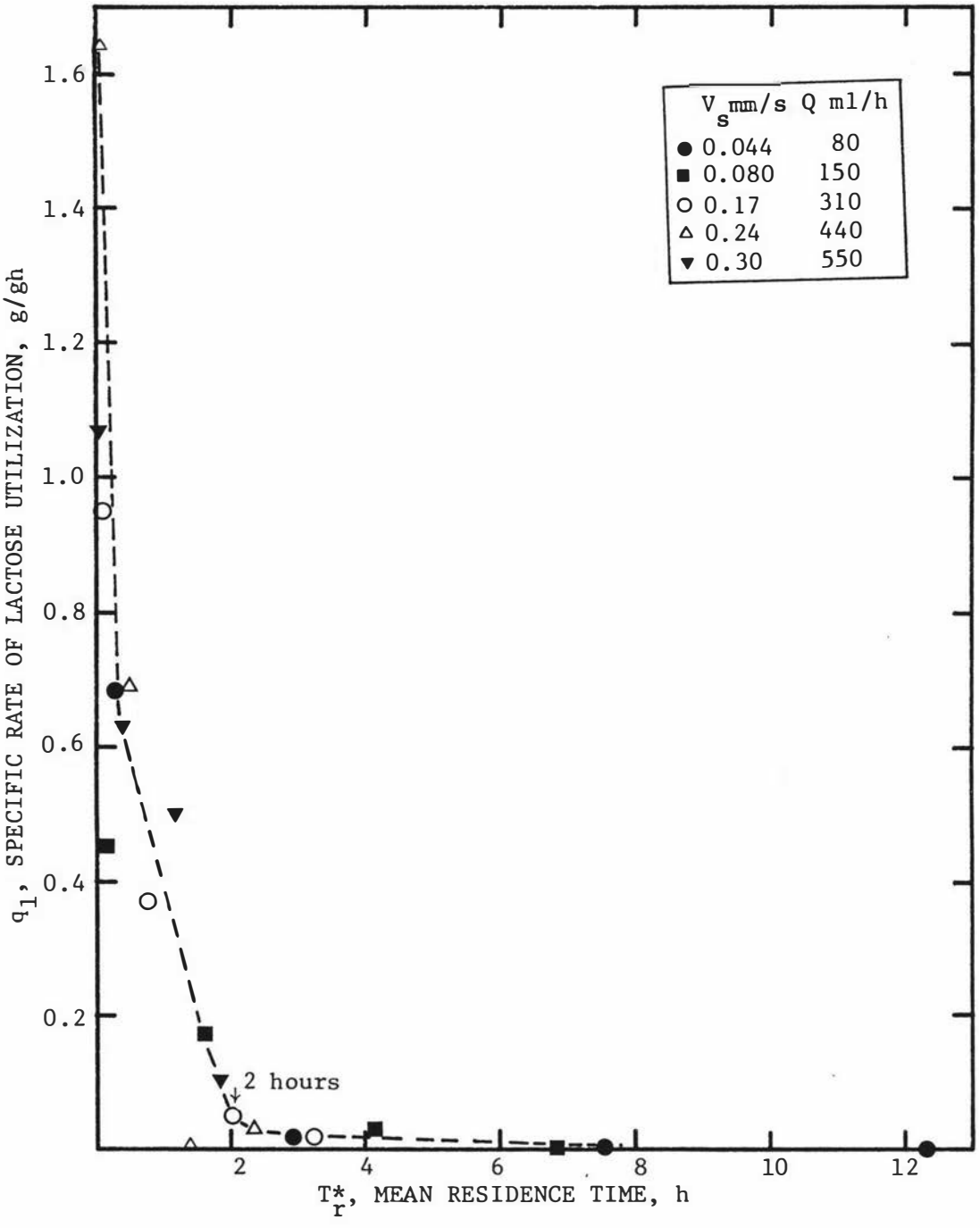


Fig.4.16 Specific rate of lactose utilization vs mean residence time at various superficial liquid velocities.

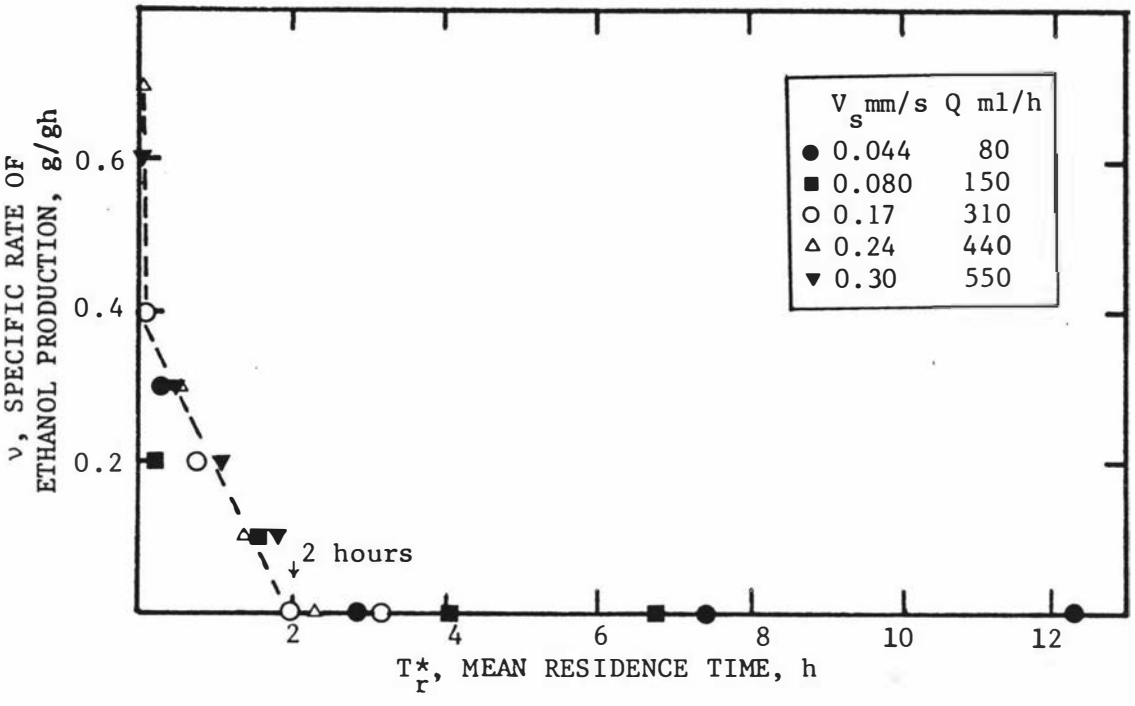


Fig.4.17 Specific rate of ethanol production vs mean residence time at various superficial liquid velocities.

to the time of 2 hours observed in the present work for tower fermentation of whey permeate. Thus, in a tower fermenter a sucrose based substrate would be used up at a faster rate than lactose based substrate such as whey and so ethanol production would be completed in a shorter time. The fermentation section of such a tower could therefore be shortened.

4.3 THE EFFECT OF SUPERFICIAL LIQUID VELOCITY ON VARIOUS FERMENTATION PARAMETERS

4.3.1 Lactose concentration (S_1)

The effect of the superficial liquid velocity (V_s) on the lactose concentration varied with the height in the tower (fig.4.18).

At the bottom of the tower (0.096 m, curve 1), the concentration increased rapidly from 7 to 28 g/l as velocity increased to 0.30 mm/s, but did not reach the feed lactose concentration. At heights from 0.82 to 2.32 m including the separator (2.69 m) (curves 2 to 5), there was a constant low level of lactose at approximately 5 g/l at all velocities up to 0.24 mm/s. When a velocity of 0.30 mm/s was reached, there was an increase in lactose concentration.

The increase in lactose concentration at 0.096 m (curve 1) was

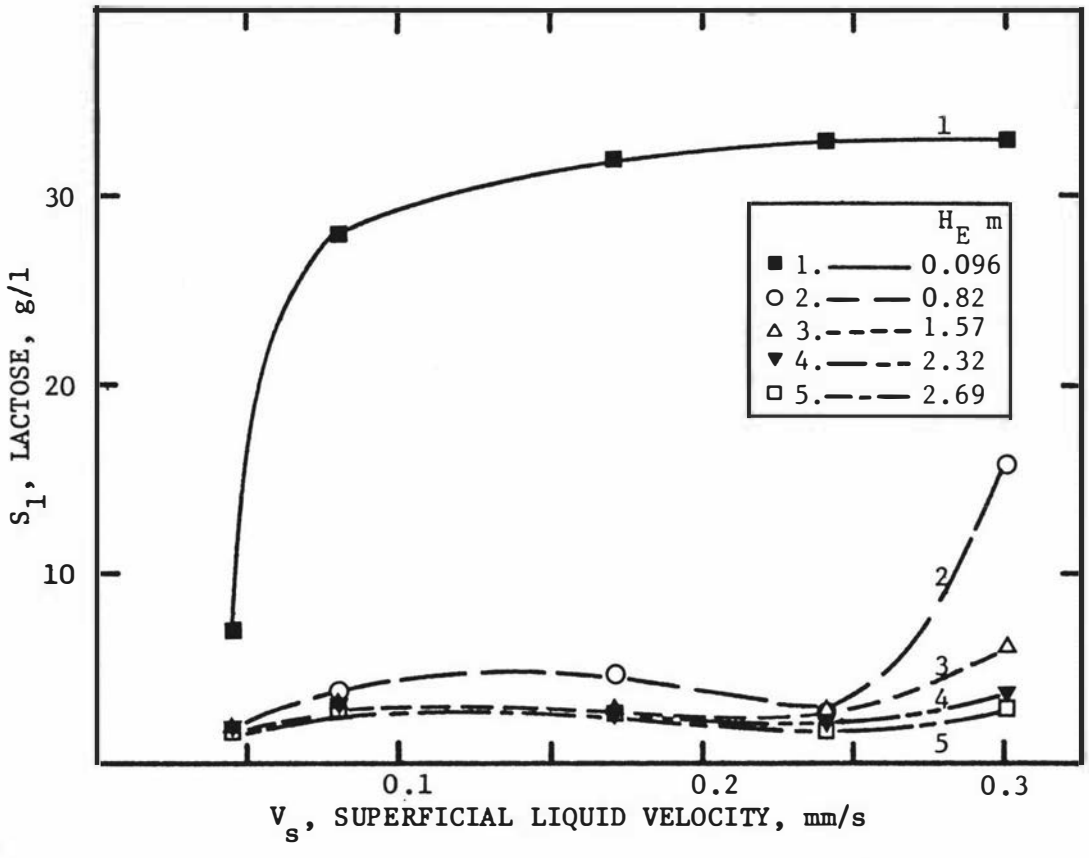


Fig.4.18 Lactose concentration vs superficial liquid velocity at various tower heights.

because this sampling location was very close to the inlet. As the velocity increased there was an increase in the feed liquor velocity and carbon dioxide production. These two factors loosened and lifted up the yeast flocs at the bottom of the tower. There was a constant lactose concentration between heights of 0.82 and the exit (2.69 m) at velocity up to 0.24 mm/s because lactose was reduced to 5 g/l in the first 0.82 m of the column.

The variations in lactose concentration at heights greater than 0.096 m at velocities above 0.24 mm/s were due to the shifting of the yeast bed up the column (fig.4.25) and the reduction of residence time in each tower section. Above this velocity (0.24 mm/s) cell wash out occurred.

The results showed that for all velocities up to 0.24 mm/s, 88% of available lactose was utilized in the first 0.82 m, and at the highest velocity (0.30 mm/s) wash out occurred.

A similar trend was observed in the tower fermentation of molasses (fig.C.10a). The sucrose concentration increased rapidly with velocity

increase at a height of 0.096 m. At heights between 0.82 and 2.32 m, the concentration was generally lower than 4 g/l and increased when the velocity was greater than 0.33 mm/s. At velocities greater than this, there was cell wash out and incomplete sucrose utilization. Thus, the wash out velocity during growth on molasses was greater than for whey permeate even though the feed molasses had a greater density than that of whey permeate, and that the yeast strain (*S. cerevisiae* FT146), used in the molasses fermentation, was only moderately flocculent.

A similar trend, was observed in the tower fermentation of fodder beet extract (Henderson & Smith 1982). The sucrose concentration at a constant height increased with the velocity. Henderson & Smith (1982) showed an increase in the sucrose concentration even at a velocity of 0.07 mm/s (their maximum velocity) which was much lower than the velocity of 0.24 mm/s reached in the present study. This could be due to the different tower fermenter internal construction (their tower was baffled) since they used a more flocculent yeast strain and similar range of cell concentration (20 to 80 g/l DW).

Prince & Barford (1982) observed that the exit sucrose concentration in tower fermentation of cane juice was initially constant as the velocity increased and then, after a certain velocity was reached, the concentration of sucrose increased with an increase in the velocity. This limiting velocity decreased as the feed sucrose concentration increased.

It is evident that, in tower fermentation, the substrate concentration at the bottom of the tower increases with velocity while the exit substrate is not affected by velocity increase until the wash out velocity is reached, at which point the substrate concentration increases with velocity.

4.3.2 Ethanol concentration (E)

The ethanol concentration showed a general trend of decreasing concentration with an increase in the velocity (fig.4.19). However, there were exceptions to this trend.

Ethanol concentration at height of 0.096 m (curve 1) decreased rapidly from 20 to 6 g/l with a small increase in the velocity to 0.080 mm/s and then decreased slowly to 3 g/l with further increase to 0.30 mm/s. At greater heights between 0.82 and 2.32 m (curves 2 to 4), ethanol concentration was relatively constant (between 17 and 18 g/l) until a velocity of 0.24 mm/s was reached at which there was a decrease in the ethanol concentration with velocity. The high ethanol concentration (between 20 to 23 g/l) at the lowest velocity of 0.044 mm/s was due to

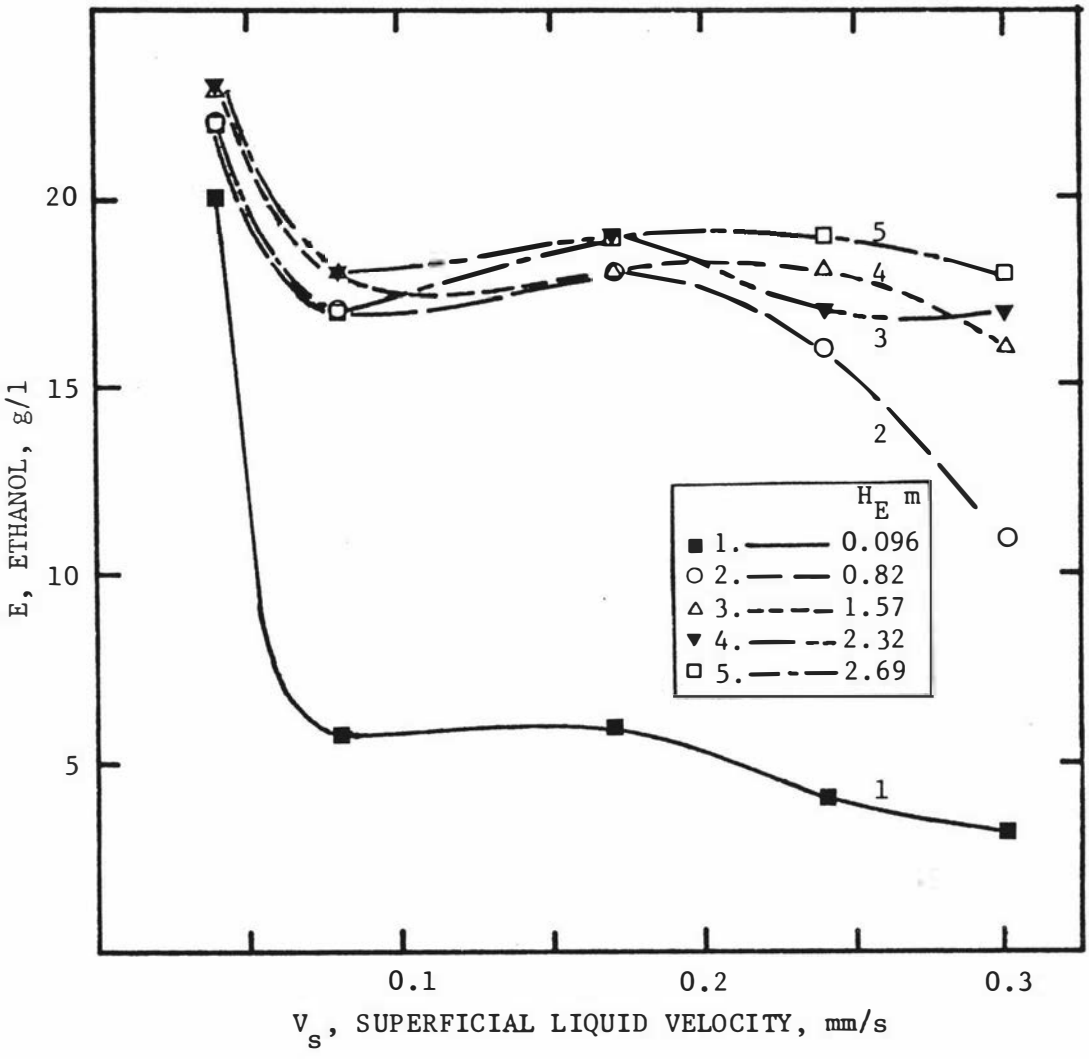


Fig.4.19 Ethanol concentration vs superficial liquid velocity at various tower heights.

the greater feed lactose concentration (47 g/l, Appendix B.3) than for other velocities. At the exit (curve 5), the ethanol concentration was affected by the fluctuation of the feed lactose concentration for each velocity.

The results showed that as the velocity increased, the major ethanol production occurred at heights greater than 0.096 m. For velocities up to 0.24 mm/s, most ethanol was produced within the first 0.82 m.

The trend observed was similar to that obtained in the tower fermentation of cane molasses (fig.C.10b). Prince & Barford (1982) showed that at velocity greater than 0.28 mm/s ($D = 0.50\ h^{-1}$), the exit ethanol concentration decreased with velocity when the feed sucrose concentration was 100 g/l. Coote (1974) showed that at a velocity

greater than 0.17 mm/s there was a reduction in ethanol concentration with velocity when the feed sucrose concentration in the beet molasses was 100 g/l.

The trend was different in the tower fermentation of fodder beet extract (Henderson & Smith 1982), the ethanol concentration decreased as the velocity increased at all heights in the tower including the exit. This difference was probably because their tower was much shorter (0.76 m maximum compared with 2.69 m in the present study). Thus, their tower corresponded with the bottom of the present apparatus.

Thus, in tower fermentation, ethanol concentration at the bottom of the tower decreased with an increase in the velocity, but at the tower exit the concentration decreased with the velocity only when the fermenter was operated at wash out velocity.

4.3.3 Rates of lactose utilization and ethanol production

The volumetric (S_1') and specific (q_1) rates of lactose utilization (fig.4.20 and 4.21), and the volumetric (E') and specific (υ) rates of ethanol production (fig.4.22 and 4.23), all showed similar changes with velocity.

At a mean height of 0.048 m (curve 1), all the rates were high and increased with velocity to 0.24 mm/s and then decreased as the velocity increased further to 0.30 mm/s. The exception to this trend occurred at a velocity of 0.080 mm/s at which there was a reduction in the rates. This reduction was probably a result of lower feed lactose concentration (43 g/l) than for the lower velocity Of 0.044 mm/s (47 g/l). Measured rates for two sample sets obtained at this mean heights and velocity, were low thus reducing the mean value obtained. The reduction of the rates at 0.30 mm/s and 0.048 m, was due to the reduction in the cell concentration and the residence time caused by the increase in the velocity. This caused a reduction in the overall lactose utilization and a reduction of the rates compared with those at the lower velocity of 0.24 mm/s.

A similar trend was observed at a mean height of 0.46 m (curve 2). However, because lactose and cell concentrations were lower than in the first section of the tower, these rates were lower.

At mean heights of 1.20 and 1.95 m (curves 3 and 4), there was no change in the rates until a velocity of 0.24 mm/s was reached, at which point the rates increased with further velocity increase. The increase in the rates at velocities greater than 0.24 mm/s was a result of the incomplete utilization of lactose in the lower sections. An increase

in the cell concentration in these sections was observed (fig.4.24) and this was consistent with the greater availability of lactose as the velocity increased.

In the separator (curve 5), the rates were effectively zero for all velocities up to 0.30 mm/s. At the wash out velocity (0.30 mm/s), there was some lactose utilization in the separator (0.2 g/1h) and so some ethanol production occurred (0.3 g/1h). Thus, at most operating velocities the separator was not involved in lactose utilization,and its sole function was separation of the yeast from the effluent broth and return of the yeast cells to the active column.

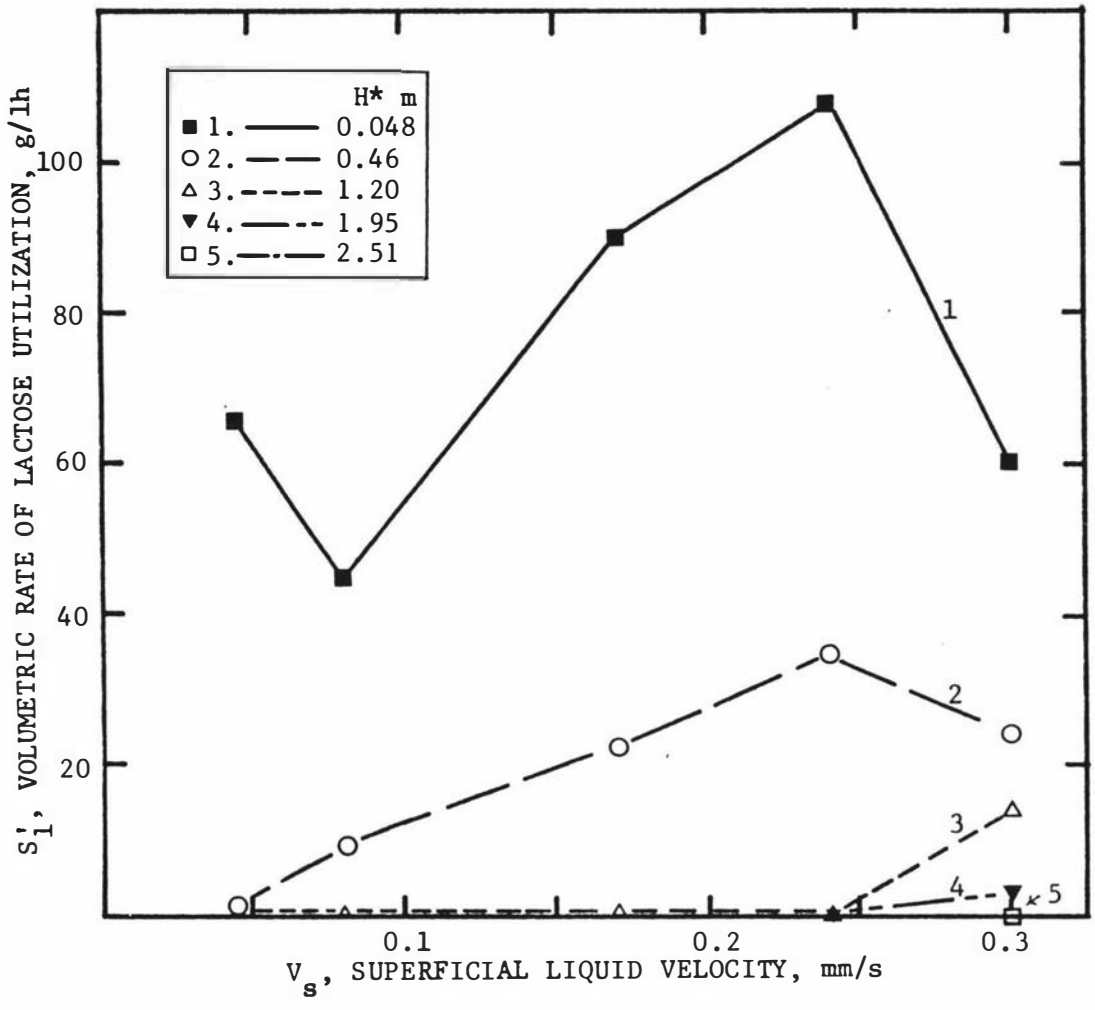


Fig.4.20 Volumetric rates of lactose utilization vs superficial liquid velocity at various mean tower heights.

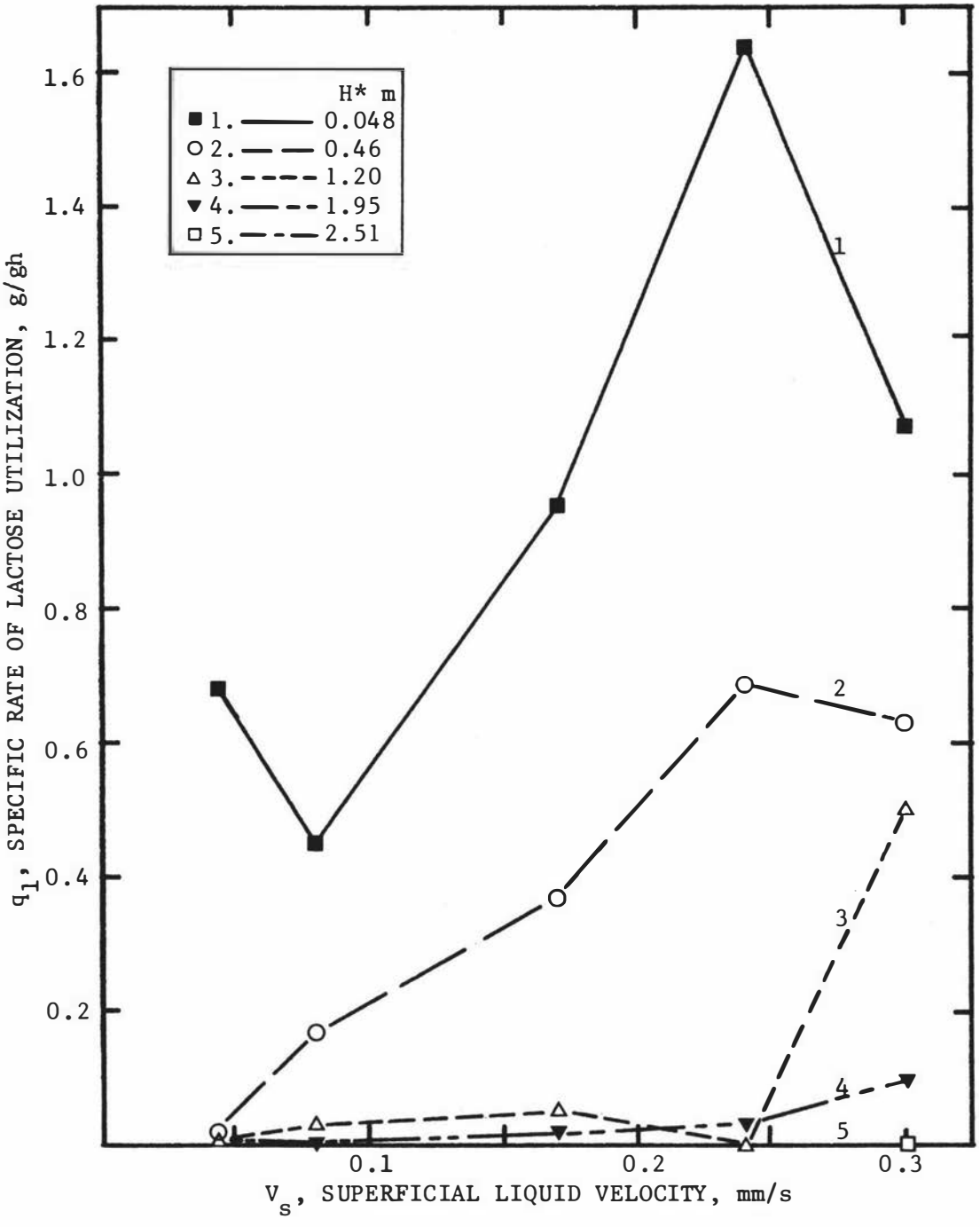


Fig.4.21 Specific rate of lactose utilization vs superficial liquid velocity at various mean tower heights.

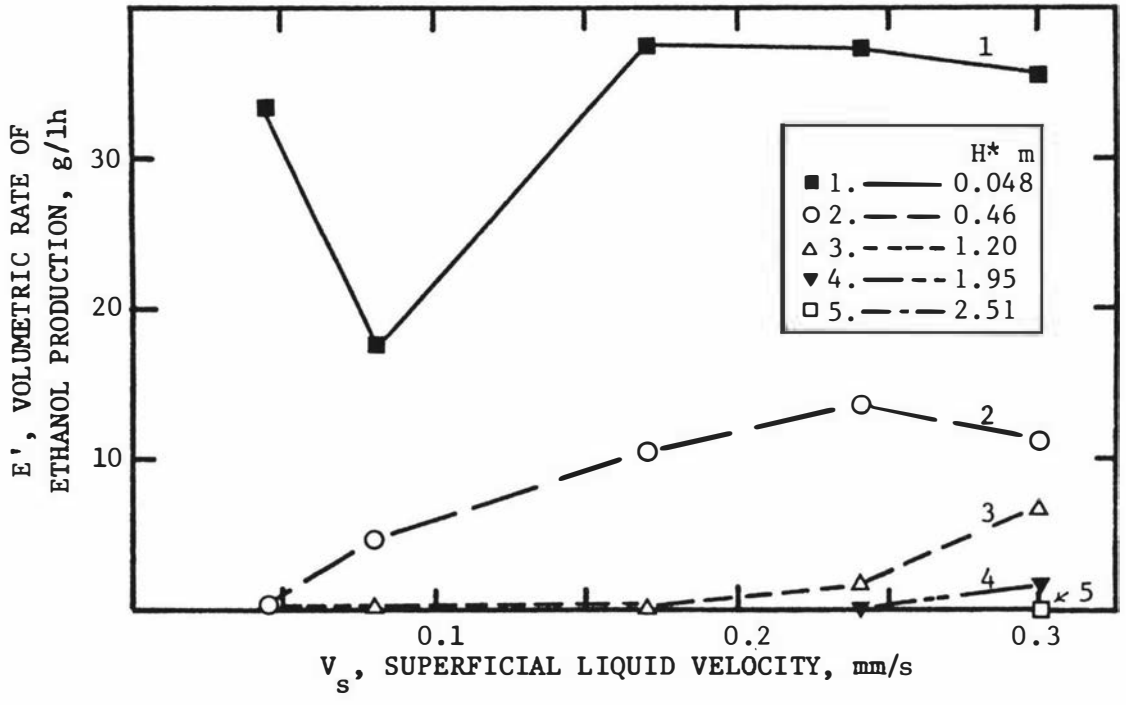


Fig.4.22 Volumetric rate of ethanol production vs superficial liquid velocity at various mean tower heights.

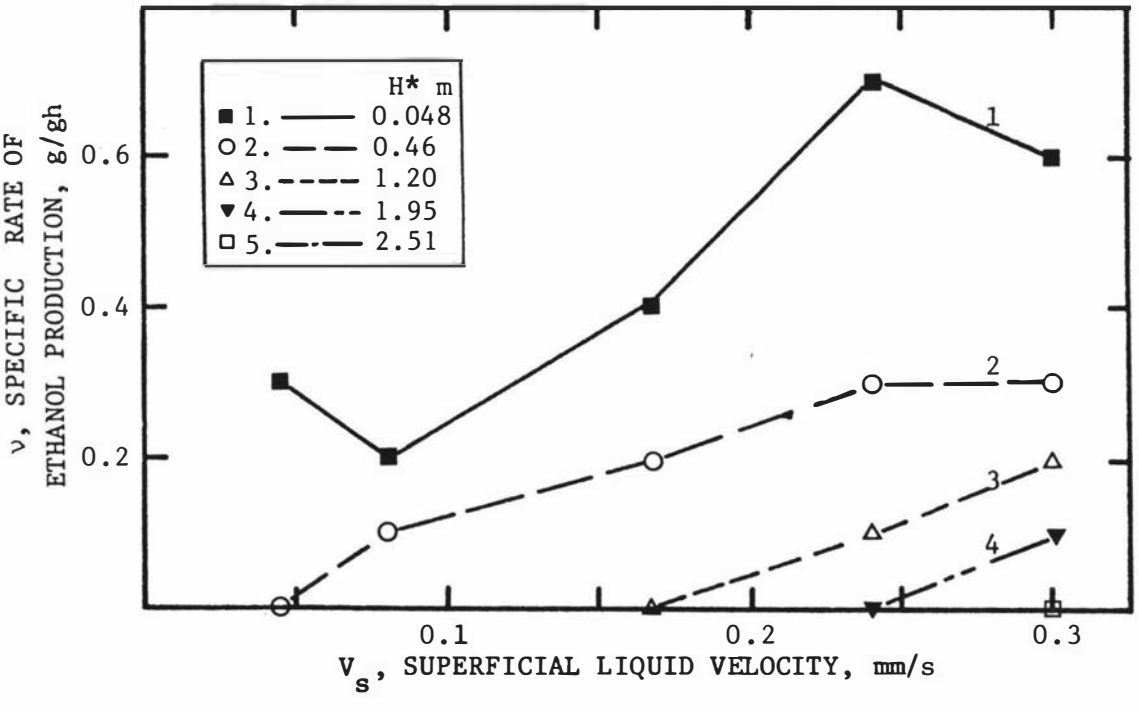


Fig.4.23 Specific rate of ethanol production vs superficial liquid velocity at various tower heights.

The results showed that as the velocity was increased, more of the tower was involved with lactose fermentation and less of the total ethanol was produced in the bottom of the tower. The increase in the specific rates with velocity implies that the culture at mean heights of 0.48 to 1.95 m was limited by the carbon feed rate. The results also showed that for all velocities up to 0.24 mm/s, there was negligible lactose utilization and ethanol production for mean heights greater than 0.48 m because there was little lactose remaining. Thus, tower sections higher than 0.82 m were redundant.

The trend observed here was similar to that found in the tower fermentation of molasses (C.3.3). However, the optimum velocity (0.33 mm/s) was greater than in the tower fermentation of whey permeate in which a lower concentration (40 g/l lactose) was used. Thus, the overall productivity on sucrose was superior. The specific rates observed in the whey fermentation were as low as 1/5 of those observed in the tower fermentation of molasses (fig.C.11 and C.12) at similar velocities. This showed that the utilization of lactose and ethanol production from lactose by *K.marxianus* occurred at a much slower rate than the utilization of sucrose and ethanol production from sucrose by *S.cerevisiae*.

A slightly different trend was observed in the tower fermentation of fodder beet extract (Henderson & Smith 1982). The maximum volumetric rates of sucrose utilization and ethanol production were observed at the lower part of the tower (below 0.095 m), and increased with velocity. However, at heights greater than this the two volumetric rates were not affected by velocity and over the small range of velocities studied (0.04 to 0.07 mm/s), the cell flocs were still concentrated at the bottom of the tower. The bed was not lifted up by the low velocity used. Thus, most sucrose was utilized with the production of ethanol in the lower tower section. Henderson & Smith (1982) indicated that their fermentation was limited by the medium used.

Further comparison could be made between the specific rates of ethanol production (ν) obtained in batch and continuous fermentation (Appendix B.2). The highest specific rate during batch fermentation was found to be 1.4 g/gh. This was twice the maximum observed at a velocity of 0.24 mm/s in the tower fermentation of whey permeate. However, these two observations were not strictly comparable, since in the continuous tower fermentation the cell concentration was considerably higher than in the batch fermentation because of cell recycle (fig.4.23). Thus, in

the batch fermentation, the specific rate of ethanol production was limited by the cell physiology but in the continuous tower fermentation, the specific rate was limited by the feed rate, which in turn was limited by the floc settling velocity.

It was not possible to compare the specific rates observed here with the results of other tower fermentation investigations because insufficient data on the cell concentration were provided. However, it was reported that for a continuous stirred-tank (CSTR) fermentation of glucose medium (9.4 g/l) by *S.cerevisiae*, the specific rate of ethanol production (ν) increased from 0.23 to 1.83 g/gh with an increase in the dilution rate from 0.072 to 0.42 h^{-1} (Bazua & Wilke 1977). The biomass concentration, in contrast, decreased from 1.5 to 0.69 g/l DW with the same increase in the dilution rate at constant glucose feed concentration.

Thus, in the tower fermentation of whey permeate, the specific rate of ethanol production increased with an increase in the superficial liquid velocity at heights up to 0.82 m and velocity below wash out (up to 0.24 mm/s). The calculated specific rate, however, was lower than for batch and continuous stirred-tank fermentations due to the high biomass concentration employed to provide rapid conversion of lactose to ethanol.

Finally, the conclusions to be drawn from these measurements are that at heights up to 0.82 m, there was an increase in the various rates of lactose utilization and ethanol production with an increase in the velocity to 0.24 mm/s. At heights greater than this there was no change in the rates and the rates were generally zero. Therefore, these sections of the tower were redundant for this operating range as shown previously (sect.4.1.1). The specific rates were lower than the specific rates observed for fermentation systems using lower biomass concentration.

4.3.4 Cell concentration

The effect of the superficial liquid velocity on the cell concentration was closely related to height (fig.4.24). At the bottom of the tower (0.096 m, curve 1), the concentration was relatively unaffected by an increase in the velocity from 0.044 to 0.17 mm/s. The average concentration was 98 g/l DW (344 g/l WW). However, as the velocity increased further to 0.30 mm/s, it showed a rapid reduction to 56 g/l DW (220 g/l WW). This was an indication that the cell plug at the bottom of the tower was slowly being lifted by the liquid to greater heights.

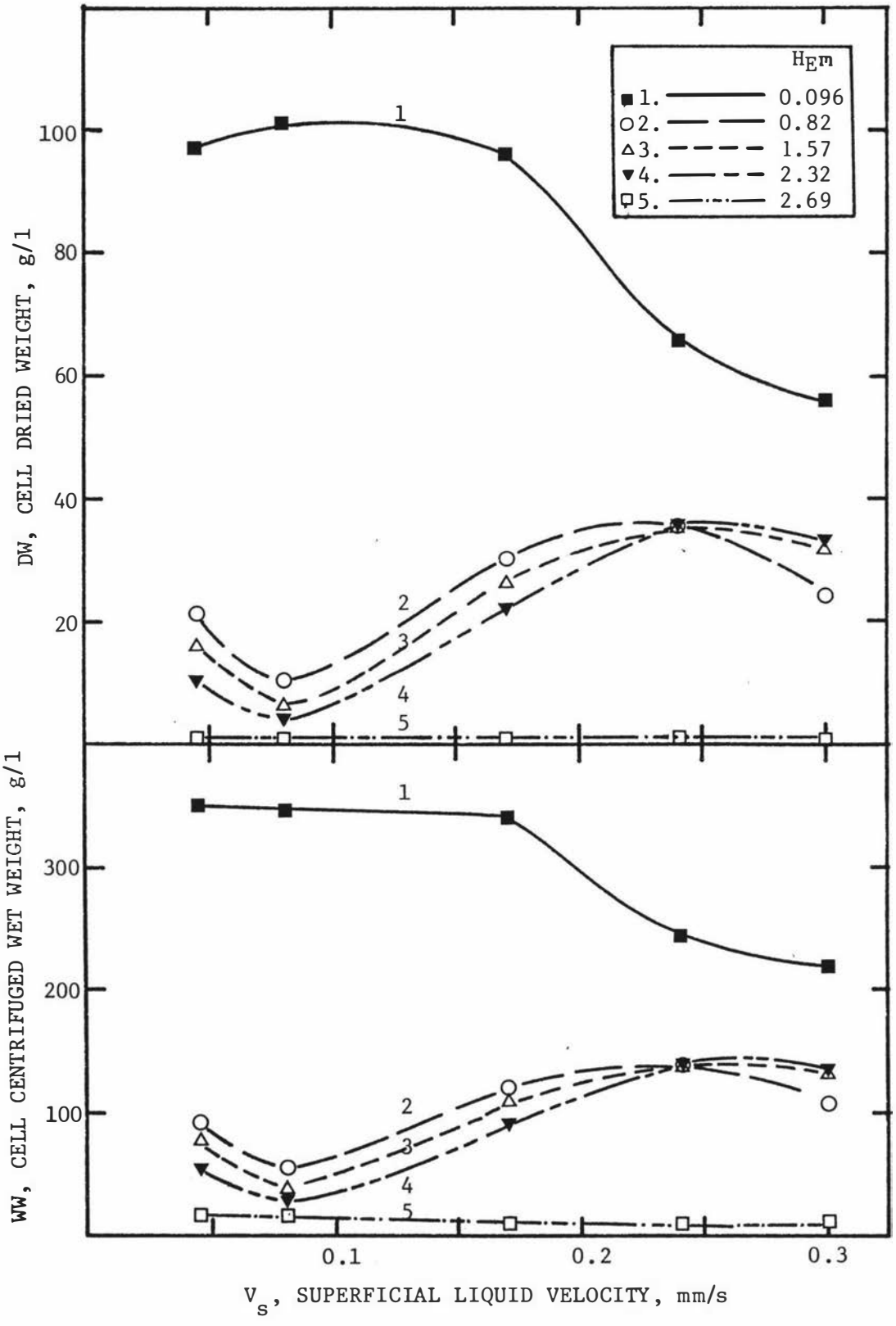


Fig.4.24 Cell concentration vs superficial liquid velocity at various tower heights.

There would be less cell compaction also. Thus, the cell concentration at this location decreased as the velocity increased.

At a greater height of 0.82 m, the trend was different. The concentration decreased from 21 to 10 g/l DW (92 to 55 g/l WW) as the velocity increased from 0.044 to 0.080 mm/s and then increased to a peak of 36 g/l DW (140 g/l DW) as the velocity increased further to 0.24 mm/s. There was further decrease in the concentration to 24 g/l DW (106 g/l WW) with a further velocity increase to 0.30 mm/s. A similar trend was observed at heights of 1.57 and 2.32 m (curves 3 and 4). As the velocity increased, the concentration of cells in the upper parts of the tower increased, indicating fluidization and washout of the biomass. At 0.24 mm/s, the cell concentration was uniform between 0.82 and 2.32 m. At 0.30 mm/s, washout was apparent.

The reduction of the cell concentration as the velocity increased from 0.044 to 0.080 mm/s, may be due to different cell compaction. At the lowest velocity, there could be a greater cell compaction between 0.82 m and 2.32 m, because the liquid velocity was not high enough. Thus, the cell concentration was greater than at the higher velocity (0.080 mm/s). The increase in the cell concentration at velocities between 0.17 and 0.30 mm/s for heights between 0.82 and 2.32 m, corresponded with the reduction in the cell concentration at the bottom of the tower (0.096 m).

Finally, at the exit of the tower after the effluent left the separator, the concentration was practically constant at between 0.2 and 0.5 g/l DW (10 and 18 g/l WW) as the velocity increased from 0.04 to 0.08 mm/s. This in effect showed that the exit cell concentration was unaffected by the velocity even at the washout velocity. The results indicated that the separator was functioning very effectively for the yeast KM Y42. If the velocity were greater than 0.30 mm/s, an increase in the exit cell concentration would be expected.

A different trend was obtained in the tower fermentation of cane juice (Prince & Barford 1982), the concentration (exact sampling height was not indicated) increased from 67 to 83 g/l DW with an increase in the velocity from 0.14 to 0.54 mm/s ($D = 0.25$ to $0.95\ h^{-1}$) when the feed sugar concentration was 100 g/l. This difference was probably due to carbon limitation at lower velocities. However, since the sampling location was not indicated, it is difficult to make further speculation on the cause of this difference.

In summary, in the tower fermentation of whey permeate, the cell concentration at the bottom of the tower (0.096 m) was not affected by

the increase in the velocity until a velocity of 0.17 mm/s was reached, after which the concentration decreased with an increase in the velocity. At greater heights between 0.82 and 2.32 m, the concentration increased, with an increase in the velocity, to a peak at 0.24 mm/s and then decreased with further velocity increase and washout occurred.

4.3.5 Specific growth rate (μ_{KM})

Estimates of specific growth rate are useful for purposes of comparison of different processes.

The mean specific growth rate was calculated using the exit cell concentration based on the average cell concentration in the tower up to 2.32 m and the residence time at this height.

$$\mu = X_e \div (X_a T_r) \qquad g/gh$$

where

X_e = the exit cell concentration, g/l DW.

X_a = the mean cell concentration between 0 and 2.32 m.

T_r = the residence time at 2.32 m.

The specific growth rate increased from 3.9×10^{-4} to 6.3×10^{-3} g/gh as the velocity increased from 0.044 to 0.33 mm/s (table 4.2).

Table 4.2 Mean specific growth rate at various superficial liquid velocities.

V_s, mm/s	0.044	0.080	0.17	0.24	0.30
μ_{KM}, g/gh	3.88×10^{-4}	8.96×10^{-4}	1.21×10^{-3}	3.21×10^{-3}	6.25×10^{-3}
X_a, g/l DW	32.4	25.7	40.4	41.6	34.3

X_a = the estimated average cell concentration in the tower calculated from concentrations at various heights.

The increase in specific growth rate with velocity was due to the reduction in the residence time as the velocity increased to 0.17 mm/s since the exit cell concentration was constant at 0.2 g/l DW. At greater velocities than this, there was an increase in the exit cell concentration with velocity (0.4 and 0.5 g/l DW). This increase would also contribute to the increase in the specific rate with the velocity since there was little change in the average cell concentration throughout this velocity increase (sect.4.3.4).

The results showed that the overall specific growth rate was low because of the high cell concentration resulting from biomass feedback. The values obtained here were lower than in the tower fermentation of

molasses (4.4×10^{-3} to 0.059 g/gh from Appendix B.5) but showed a similar trend of increase with velocity. This was because the exit cell concentration was higher in the molasses fermentation.

Thus, there was a low mean specific growth rate in the tower fermentation of whey permeate as a result of the high yeast cell concentration used, and the employment of the yeast separator as an integral part of the fermenter to recycle the yeast cell which resulted in a low exit cell concentration. In the tower fermentation of whey permeate the dilution rate was not equal to the specific growth rate.

4.4 TOWER FERMENTER PERFORMANCE

It was shown that in the tower fermentation of whey permeate, there was 90% lactose utilization and greater than 16 g/l of ethanol was produced over the first 0.82 m of the height in the tower for all velocities up to 0.24 mm/s (sect.4.1.1 and 4.1.4). This was strongly reflected by high rates of lactose utilization and ethanol production within the first 0.82 m of the tower (sect.4.1.2, 4.1.3, 4.1.6, and 4.1.7). The rates were negligible at greater heights.

It was also shown that for all velocities used, there was greater than 90% lactose utilization in 1 hour (sect.4.2.1). This was also the time during which more than 16 g/l of ethanol was produced (sect.4.2.2). There were high rates of lactose utilization and ethanol production during the first 2 hours in the tower and the rates were negligible at residence times greater than this (sect.4.2.3). This reflected the rapid rates of lactose utilization and ethanol production in this tower fermenter.

The resulting yield of ethanol based on lactose utilized was betweeen 70 and 96% at heights between 0.82 and the exit for all velocities (sect.4.15).

The cell concentration which gave this rapid lactose utilization and ethanol production was between 66 and 100 g/l DW (244 and 348 g/l WW) at a height of 0.096 m for all velocities up to 0.24 mm/s (sect.4.1.8).

4.4.1 Optimum superficial liquid velocity (V_s)

The optimum velocity for the tower fermentation of whey permeate was 0.24 mm/s. This was lower than the optimum velocity reached by other tower fermentation investigations with one exception (table 4.3).

Table 4.3 Comparison of optimum superficial liquid velocities from various tower fermentation studies.

medium	S_{lo} g/l	V_s mm/s	D h^{-1}	references
1.Whey permeate	40	0.24	1.0	This study
2.Cane molasses	100	0.33	1.4	Appendix C, sect.C.4
3.Beet molasses	100	0.53	1.1	Coote 1974
4.Cane juice	100	0.34	0.60	Prince & Barford 1982
5.Fodder beet extract	100	0.05	0.24	Henderson & Smith 1982
6.Beer wort	58	0.57	-	Ault et al 1969

S_{lo} - feed sugar concentration

3. Coot's value was calculated from limiting volumetric efficiency of 4.7.

In this study, the optimum velocity was lower than that observed by most other workers because the yeasts used by the other workers were more flocculent. It was expected that if a more flocculent *K.marxianus* were available the optimum velocity could be as high as the velocity obtained in the other investigations since whey permeate is less dense than the more concentrated sugar solutions used by the other workers. It was shown that the maximum operating superficial liquid velocity for a particular flocculent yeast strain decreased as the medium concentration increased (Prince & Barford 1982 ; Coote 1974). Thus, the lower density whey could permit operation at a higher velocity if the yeast were more flocculent. Of course, the specific rate of ethanol production would remain lower.

It is worth noting that the use of the parameter "superficial liquid velocity" (V_s) to define tower fermenter performance is more suitable than use of "dilution rate" (D). In table 4.3, the values of D for the first three tower fermentation studies were high and were 1 h^{-1} or greater. This was because D was calculated based on the tower height up to the bottom of the separator only. The superficial liquid velocity, on the other hand, is not affected by the change in the tower height or the overall volume. It is related directly to the fermenting medium input and output rate. Thus, it is a more realistic parameter for use to describe the performance of a tower fermenter.

4.4.2 Residence time and tower height

The minimum tower height of 0.82 m with respect to the velocity of 0.24 mm/s giving a residence time of 1 hour (excluding the time in the separator) was comparable to other tower fermentation values (table 4.4).

Table 4.4 Comparison of the effective tower heights and residence time of various tower fermentation studies.

medium	H_E m	ϕ m	H_E/ϕ	T_r h	V_s mm/s	references
1.Whey permeate	0.82	0.025	32	1.0	0.24	This study
2.Cane molasses	0.82	0.025	32	0.7	0.33	Appendix C, sect.C.4
3.Beet molasses	1.75	0.025	69	0.9	0.53	Coote 1974
4.Cane juice	1.65	0.075	22	1.7	0.34	Prince & Barford 1982
5.Fodder beet extract	0.38	0.10	3.8	2.1	0.05	Henderson & Smith 1982
6.Beer wort	6.96	1.8	3.9	3.3	0.57	Ault et al 1969

H_E - Effective tower height excluding the separator.

ϕ - Tower diameter.

H_E/ϕ - Aspect ratio.

Coote (1974) did not optimize his tower height but optimized the velocity (limiting volumetric efficiency). The height quoted here was his tower straight section up to the bottom of the separator. Prince & Barford (1982) also did not optimize their tower height but optimized the velocity at various medium feed concentrations. They also optimized their medium feed concentration. However, insufficient information on the tower dimensions were given. The height quoted here was based on the overall aspect ratio of 22:1. The height indicated for tower fermentation of fodder beet extract (Henderson & Smith 1982) was optimized from the author's data which showed very little fermentative activity at height greater than 0.38 m. This gave a corresponding residence time of 2.1 hours. A similar approach was used for the tower fermenter used for beer fermentation (Ault et al 1969). The height obtained was 6.96 m and the residence time was 3.3 hours.

Table 4.4 showed that the residence time for sucrose based substrates was short between 0.7 and 2.1 hours. The high value of 2.1 hours was from a tower fermenter which had a slightly different internal construction from the other fermenters (Henderson & Smith 1982). The beer wort which contained mixed sugar has a longer residence time of 3.3 hours because there was a sequential sugar utilization (Ault et al 1969).

It was also evident here that the effective tower height was a function of the residence time and the superficial liquid velocity. The residence time was determined by the feed medium concentration and the yeast flocculence. The superficial velocity, on the other hand, was limited by the yeast settling velocity and the yeast fermentation rate. When these two parameters were determined, the corresponding height could then be determined.

The tower diameter and superficial velocity (medium throughput) are related. An increase in the tower diameter at a constant superficial velocity and tower height would increase the medium throughtput and reduce the aspect ratio. The residence time, however, would remain constant. This was the reason for the high aspect ratios of the laboratory scale tower fermenters (22 to 69) when compared with the aspect ratio of the commercial scale APV tower fermenter of 3.9:1.

4.4.3 Sugar utilization

The proportion of feed lactose utilized reached in this study was greater than 90%. This was similar to the level reached in other tower fermentation investigations which were 80% in beer fermentation (Ault et al 1969), 80% on molasses fermentation (Coote 1974), 90 to 95% in cane juice fermentation (Prince & Barford 1982), and 90 to 96% in fodder beet extract fermentation (Henderson & Smith 1982).

4.4.4 Yield of ethanol

The yield of ethanol at the exit, which varied between 75 and 96%, was comparable with the yields reported by other workers. In the tower fermentation of beet molasses (Coote 1974), the exit yields reported were between 74 and 84%. In the tower fermentation of fodder beet extract, the yield was between 91 and 97% (Henderson & Smith 1982).

In summary, the fermenter used here gave low residence time and high productivity which are expected from a tower fermenter. The performance shown was comparable to that observed by other tower fermentation investigations. The performance was limited only by the moderately flocculent nature of *K.marxianus*.

4.5 OPTIMUM CONDITIONS FOR THE TOWER FERMENTATION OF WHEY PERMEATE

It was shown in the previous section (sect.4.4) that the maximum velocity for stable continuous tower fermentation of whey permeate was 0.24 mm/s. The exit conditions at this velocity are summarized

in table 4.5.

Table 4.5 Exit conditions at velocity of 0.24 mm/s (from Appendix B.3).

T_r h	pH	S_l g/l	S_{lu} %	E g/l	Y %
6.7	4.3	2	97	19	75

Inlet lactose concentration = 45 g/l

Tower straight section T_r = 3 hours

The productivity (E') at 2.32 m was 6.2 g/lh while the overall productivity (E'_o) was 2.8 g/lh if the residence time in the separator of 2.7 hours were considered.

However, it was shown that at this velocity there was very little lactose utilization (fig.4.18) and ethanol production (fig.4.22) at heights greater than 0.82 m. Thus, the tower sections above this height were redundant. At 0.82 m, the residence time was 1 hour and the overall residence time including the separator became 3.7 hours. This gave a true ethanol productivity of 16 g/lh at 0.82 m and an overall ethanol productivity of 5.1 g/lh (calculated from the exit ethanol concentration of 19 g/l from 95% lactose utilization, from fig.4.11 for T_r= 3.7 hours).

4.5.1 Comparison with other tower fermentation investigations

It was considered that for comparison of the productivity of tower fermenters only the active fermenter section should be considered ie. the separator section should be excluded from the calculation. The overall productivity values (E') strictly apply only to those tower fermenters with the same dimensions. However, previous investigations (Coote 1974 ; Henderson & Smith 1982 ; Prince & Barford 1982) did not provide sufficient data to permit comparison on this basis. Therefore, the productivity (E') excluding the separator section was calculated from their data by assuming that the exit ethanol concentration was the same as the concentration entering the bottom of the separator. This calculation does not take account of the redundant tower sections prior to the separator (see above).

The productivity (E') observed in the present investigation using whey permeate was lower than those calculated from the available data for tower fermentation of cane molasses (table 4.6, row 2) and beet molasses (table 4.6, row 3) using similar size tower fermenters. This

Table 4.6 Comparison of optimum tower fermentation conditions.

	S_o g/l	S_u %	E g/l	Y %	E' g/lh	E'_o g/lh	V_s mm/s	D h^{-1}	T_r h	T_{ro} h
1. Whey permeate	45	95	19	83	16	5.1	0.24	1.0	1.0	3.7
2. Cane molasses	100	96	50	92	67	14	0.33	1.4	0.7	3.4
3. Beet molasses	100	90	37	77	41	9.8	0.53	1.1	0.9	3.8
4. Cane juice	123	95	56	89	17	16	0.15	0.26	3.8	4.3
5. Fodder beet extract	100	93	49	97	12	11	0.05	0.24	4.2	-

S_o - Feed sugar concentration.
S_u - Percentage sugar utilization.
E' - Productivity excluding the separator.
E'_o - Overall productivity including the separator.
T_r - Residence time in the tower excluding the separator.
T_{ro} - Overall hold up time including the separator.

1. This study
2. Appendix C, sect.C.4.
3. Coote 1974 . The limiting volumetric efficiency used was 4.7.
4. Prince & Barford 1982 .
5. Henderson & Smith 1982.

Table 4.7 Comparison of tower fermentation of whey permeate with batch fermentations of whey.

organisms	S_{lo} g/l	S_{lu} %	E g/l	Y %	E' g/lh	T h	references
1.KM Y42	45	95	19	83	16	3.7	This study.
2.KM Y42	40	90	18	94	1.1	16	Appendix B.2
3.KM CBS5795	50	100	23	87	0.96	24	Burgess & Kelly 1979
4.KM"Irish Yeast"	41	94	19	90	1.2	16	Howell & Tichbon 1981
5.CP NCYC744	50	100	21	79	2.1	10	Burgess & Kelly 1979

KM - *K.marxianus*
CP - *C.pseudotropicalis*
T - Fermentation time
1. - The value of T indicates the overall residence time in the tower fermenter.

was due to three factors. Firstly, these two media (cane and beet molasses) were based on sucrose, which could be utilized by yeasts more rapidly than lactose. Secondly, the yeasts used were more flocculent than *K. marxianus* and could remain in the tower fermenters at a higher superficial liquid velocity. Thirdly, the sugar concentrations used were greater than in the present study. Thus, more ethanol was produced over a similar residence time.

The true productivity of 16 g/lh was, however, comparable to those obtained for the tower fermentation of cane juice (table 4.6, row 4) and fodder beet extract (table 4.6, row 5).

4.5.2 Comparison with batch fermentation of whey

Further comparison can be made with batch fermentation of whey (table 4.7). In the 10 litre-batch fermentation of whey permeate by *K. marxianus* Y42, 16 hours was required to ferment 40 g/l lactose (90% utilization) to produce 18 g/l ethanol (table 4.7, row 2). The ethanol productivity was 1.1 g/lh which was approximately 1/15 that of the tower fermentation of whey permeate.

The results of the tower fermentation of whey permeate were also better than the batch fermentation reported by other workers who used a different strain of *K. marxianus* (Burgess & Kelly 1979) (table 4.7, row 3). The productivity was more than 17 times the value of 0.96 g/lh reported by these workers.

Comparison with the industrial batch fermentation at the NZ Dairy Companies plant at Reporoa showed over 13 times the productivity of this plant (1.2 g/lh, table 4.7, row 3) (Howell & Tichbon 1981). The plant had a high yield of 19 g/l ethanol from 41 g/l lactose and employed cell recycling which increased the cell concentration at inoculation time. Thus, the resulting fermentation time was shorter than would have been without cell recycling, therefore improving the productivity. If the down time were considered, the comparison would be further in favour of the tower fermentation of whey permeate.

A final comparison can be made with a batch fermentation by *C. pseudotropicalis* (table 4.7, row 5) (Burgess & Kelly 1979). This yeast strain gave a productivity of 2.1 g/lh (1/8 the productivity of the tower fermentation of whey permeate).

Thus, tower fermentation of whey permeate generally gave a greater productivity than batch fermentation.

In summary, the optimum values obtained for various parameters were comparable to other tower fermentation investigations. If a more flocculent *K.marxianus* were available, the operating velocity could be higher. Thus, the residence time could be reduced and this would result in increased productivity. Hence, further investigations are warranted to improve the flocculence of *K.marxianus*.

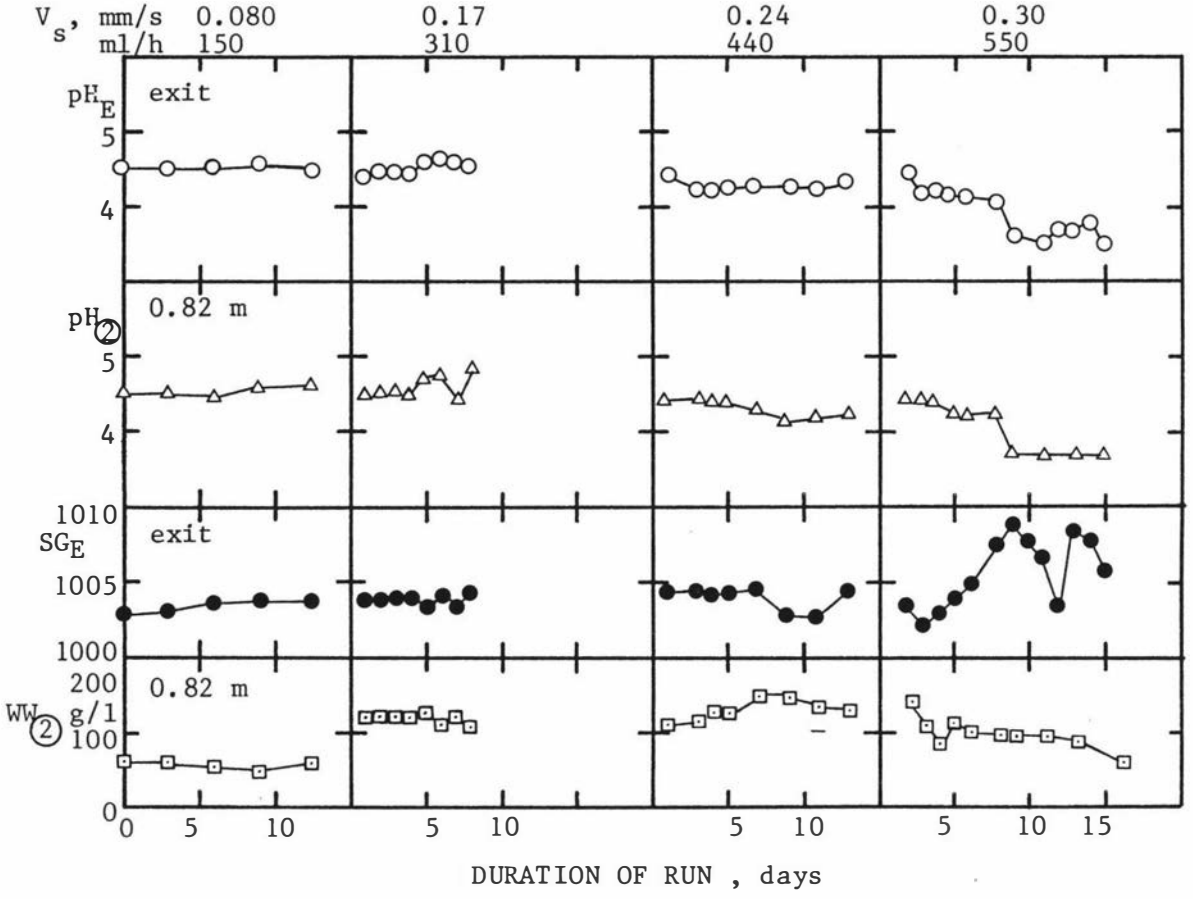
4.6 CONTINUOUS OPERATION AND DIFFICULTIES

4.6.1 Organism

K.marxianus Y42 was found to be the only flocculating lactose fermenting yeast (Chapter 6). It was reported to be a rapid lactose fermenter (Yoo 1974). Batch fermentation tests (Appendix B.2) on whey permeate found it to be a moderately rapid lactose fermenter in this medium when compared with the ethanol tolerating *K.marxianus* isolated in this study (KM10D10, sect.7.3). The flocs formed by this yeast during tower fermentation were very small and were not spherical. As a result of this limitation, the washout flow rate was reached when the velocity was 0.30 mm/s. At this velocity, the cell cencentration decreased slowly inside the tower straight section and lactose concentration increased inside the tower. Coote (1974) reported a limiting feed rate of molasses solution containing 100 g/l reducing sugar at a velocity of 0.53 mm/s for a tower fermenter of similar dimensions using a very flocculent *S.cerevisiae* strain. This was more than double the optimum velocity reached in this study, even though the feed medium contained more than two and a half times the fermentable sugar concentration found in whey permeate. During start up, 14 days were required to build the yeast cell concentration up to 50 g/l DW. In the tower fermentation of beer (Klopper et al 1965) up to 70 g/l DW was built up during a similar period. The lower cell concentration reached in the present study was a combined effect of the lower feed sugar concentration used (40 g/l lactose) and a less flocculent yeast. This resulted in greater cell loss due to aeration during the cell build up period.

4.6.2 Continuous operation monitoring curves

The monitoring curves of pH and cell concentration at 0.82 m (sampling point 2), and the exit pH and specific gravity at various velocities are given in fig.4.25 (a). The curves show the steady state obtained at velocities between 0.080 and 0.24 mm/s. However, at a velocity of 0.30 mm/s, the cell concentration decreased and the specific



(a)

(b)

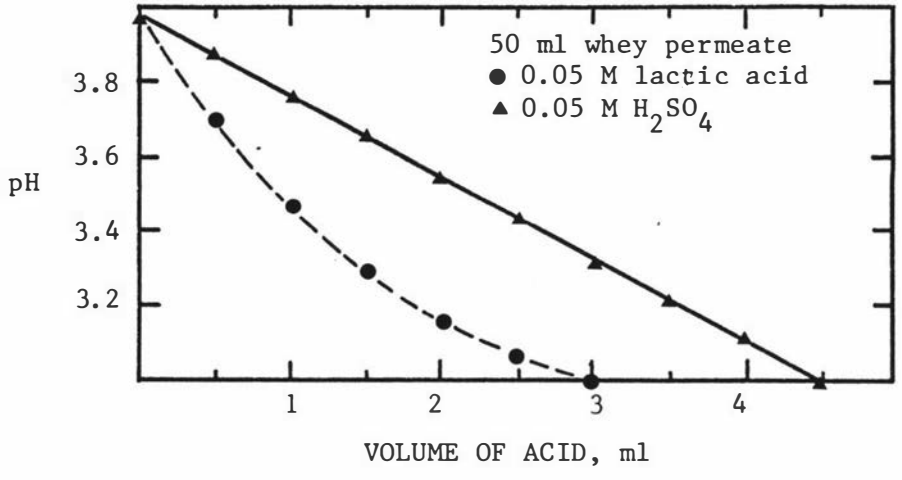


Fig.4.25 (a) Tower operation-monitoring curves for steady state determination at different medium feed rates, (b) Titration curves of a new batch of whey permeate with 0.05 M H_2SO_4 and 0.05 M lactic acid in order to show the small amount of acid required to reduce the pH to below pH 4.0.

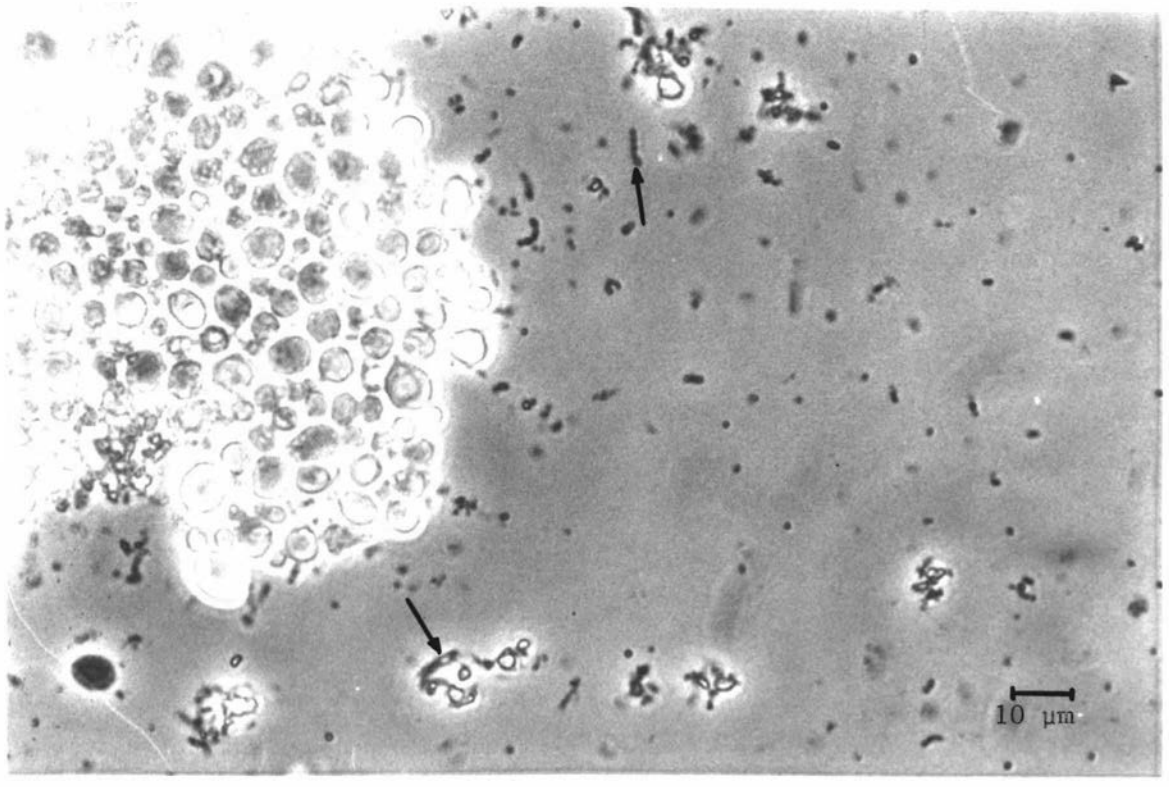
gravity increased as the continuous operation time increased. This was an indication of nonsteady state operation or washout. There was also a reduction in the pH as continuous operation time increased at this velocity. This was a result of the poor buffering capacity of the new season whey permeate that was used.

4.6.3 Contamination

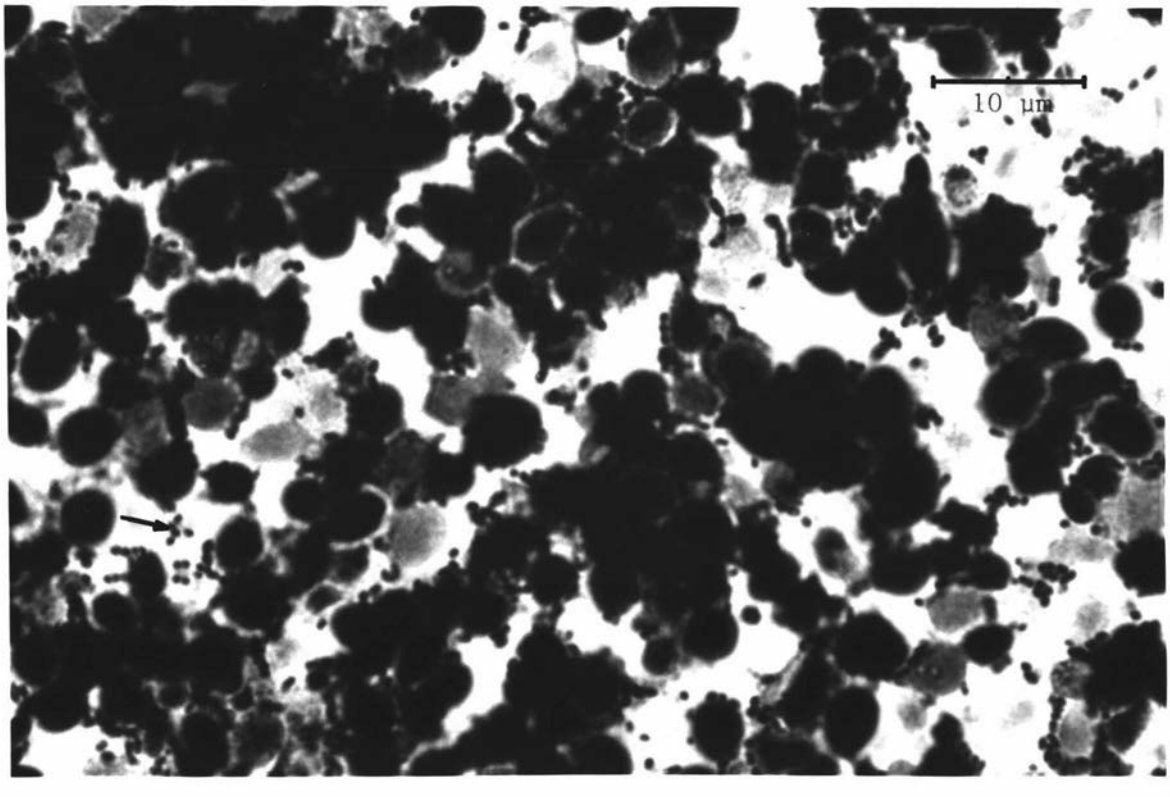
The whey permeate was prone to contamination by gram-positive-short -rod bacteria and wild yeast. This was reduced by frequent change of feed medium holding vessel and the feed lines, the medium was aerated for only 1 hour, and greater care was taken during aseptic sampling. Contamination inside the tower was difficult to control, once it had occurred. This caused a sudden drop of the pH to below pH 4 and the lactose uptake was reduced resulting in a slight increase in the exit residual lactose concentration. A titration showed that little lactic acid was required to cause such a change in the pH (fig.4.25 b). It is also worth noting here that *K.marxianus* can utilize lactic acid in whey (de Sanchez & Castillo 1980), but this does not result in the formation of ethanol.

The contaminating bacterial cells inside the tower were trapped within and between the yeast flocs (fig.4.26). Thus, they were difficult to remove. In fig.4.26 (a), spore formers could be seen in the clear medium. They were probably *Bacillus spp.*. Coote (1974) did not encounter any contamination problem during a six-months continuous tower fermentation of molasses and considered that contamination would be selectively washed out by the feed medium flow. In this study, the addition of pennicillin and streptomycin to the feed medium did not bring immediate decontamination but helped to contain the infection. This run was, however, terminated and the fermentation restarted.

In continuous beer production, bacterial infections have been reported to be a more serious problem than spontaneous mutation. These were overcome by careful attention to the maintenance of a vigorous yeast population and an overall improvement in the hygiene standards (Brightwell 1978). In the tower fermentation of beer, lactic acid bacterial infection was found to be almost impossible to remove and in one instance, a concentration of 3×10^6 cells/ml developed in 3 days (Ault et al 1965 ; Hough et al 1976). Bacterial infection of stored wort prior to continuous fermentation has also been reported (Hough & Button 1972). This was probably the result of incomplete sterilization and poor hygeine.



(a) Wet mount (800X) (Arrows indicate examples of contamination .)



(b) Gram stain (2000X)

Fig. 4.26 Bacterial contamination (smaller dark cells) among *K. marxianus* Y42 cells. Sample was taken from the tower.

4.6.4 Feed lactose concentration

There was also difficulty in maintaining a constant feed of lactose in whey permeate to the tower. The lactose content varied from batch to batch. The variation was between 30 and 47 g/l lactose in the same batch (See sudden drop of the exit SG during the run at 0.33 mm/s in fig.4.25 (a)). Because of this, considerable care was required during medium preparation. A quick check of the specific gravity was performed to ensure that the whey permeate had specific gravity close to 1.020.

4.7 CONCLUSIONS

K.marxianus Y42 was used successfully to carry out continuous fermentation of whey permeate containing 40 g/l lactose in a tower fermenter.

The optimum operating conditions were as follow :

Minimum tower height (excluding the separator)	= 0.82	m
Superficial liquid velocity	= 0.24	mm/s
Residence time in the tower straight section	= 1	hour
Overall residence time	= 3.7	hours
Lactose utilization	= 95	%
Ethanol produced	= 19	g/l
Overall ethanol productivity	= 5.1	g/lh
Cell concentration (at 0.096 m)	= 60 to 100	g/l DW
	= 240 to 350	g/l WW
(above 0.096 m to 2.32 m)	= 36	g/l DW (140 g/l WW)

The maximum operating superficial velocity was limited by the moderately flocculent nature of *K.marxianus* Y42. Thus, there was a need to improve the flocculence of this yeast strain.

Finally, continuous tower fermentation of whey permeate was prone to contamination.

Using this work as a basis, further investigations were carried out using *K.marxianus* Y42 in conjunction with a flocculent *S.cerevisiae* to ferment whey permeate enriched with molasses to increase the sugar concentration to 100 g/l in the same tower fermenter (Chapter 5).

4.8 SUMMARY

1. Continuous tower fermentation of whey permeate required only 0.82 m of the lower sections of the tower fermenter to utilize more than 90% of the feed lactose,and produce greater than 16 g/l of ethanol, for all superficial liquid velocities up to 0.24 mm/s. This occurred in a short residence time of only 1 hour.

2. The volumetric and specific rates of lactose utilization and ethanol production for all velocities, decreased rapidly to a negligible value (less than 2 g/lh and 0.05 g/gh) as the height increased to 0.82 m. There was little fermentative activity at greater heights and when the mean residence time was greater than 2 hours.

3. The effect of the velocity on the cell concentration was closely associated with the height in the tower. The cell concentration decreased with an increase in height. The trend altered when the washout superficial velocity was reached.

The cell concentration at the bottom of the tower decreased as the superficial liquid velocity increased while generally the concentration inside the tower increased as the superficial velocity increased and then decreased as the velocity increased further.

4. The tower operating pH was between 4.2 and 5.0 and reduced to less than pH 4.0 when there was bacterial infection.

5. Bacterial contamination was a problem to continuous fermentation operation and careful attention to the maintenance of a high hygiene standard was essential.

CHAPTER 5

TOWER FERMENTATION OF WHEY PERMEATE ENRICHED WITH MOLASSES

It was established previously that the moderately flocculent strain of *K. marxianus* Y42 could be employed in the tower fermentation of whey permeate (sect.4.7). It was also found from flocculation tests that mixed culture of *K. marxianus* Y42 and *S. cerevisiae* CFCC39 growing on whey permeate enriched with molasses formed large and very flocculent flocs (sect.6.2 f). Thus, tower fermentation studies were carried out using whey permeate enriched with molasses (using a lactose to sucrose ratio of 40:60 g/l) and a mixed culture of the two yeast strains indicated above. The *S. cerevisiae* (CCFC39) strain used here was different from the *S. cerevisiae* (FT146) strain used during the tower commissioning using molasses as the feed medium (Appendix C) because the latter yeast was the only flocculent yeast available in the Department of Biotechnology, Massey University at commissioning. Various fermentation parameters were considered with respect to the tower height at different superficial liquid velocities of the medium in order to determine the optimum operating conditions similar to those obtained for the tower fermentation of whey permeate (sect.4.7).

These parameters were not considered with respect to the residence time because, at constant superficial velocity, residence time is linearly related to tower height.

The data obtained from this tower fermentation study are given in Appendix B.4.

5.1 THE RELATIONSHIP BETWEEN TOWER HEIGHT AND VARIOUS FERMENTATION PARAMETERS

5.1.1 Sugar concentrations and utilization

(a) Sucrose (S_s) Sucrose concentration of the fermentation broth declined with increase in the tower height (fig.5.1). The concentration decreased very rapidly for the lowest superficial liquid velocity (0.087 mm/s), from 60 to 13 g/l over the first 0.096 m of the tower height and then decreased slowly to 0.5 g/l at 0.82 m which gave a sucrose utilization of 78 and 99%, respectively (table 5.1). No further reduction in the concentration occurred as the height increased

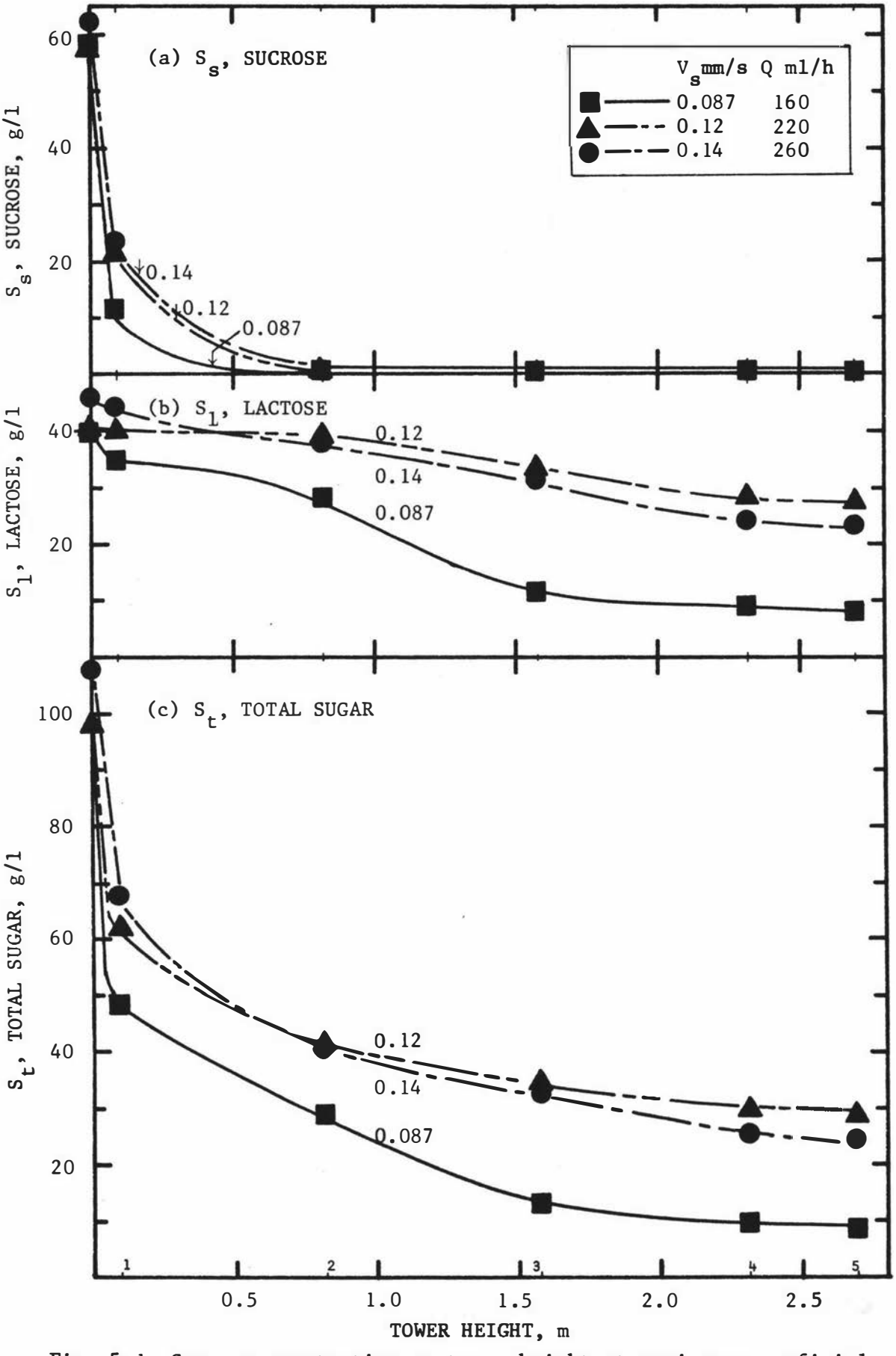


Fig. 5.1 Sugar concentration vs tower height at various superficial liquid velocities. (a) Sucrose , (b) Lactose , (c) Total sugar.

further to 2.32 m. The decrease of sucrose concentration with height was slower for the two greater velocities (0.12 and 0.14 mm/s) over the initial 0.096 m, where the concentrations were 22 and 23 g/l at the respective velocities.

Table 5.1 Percentage sugar utilization at various tower heights and superficial liquid velocities

V_s, mm/s		0.087			0.12			0.14		
location,m		S_{su}	S_{lu}	S_{tu}	S_{su}	S_{lu}	S_{tu}	S_{su}	S_{lu}	S_{tu}
1	0.096	78	12	51	62	1	37	62	4	37
2	0.82	99	29	71	96	3	58	97	16	62
3	1.57	99	68	87	98	17	64	98	31	70
4	2.32	99	77	90	98	30	70	98	47	76
5	2.69	99	80	91	98	33	71	98	49	77

S_{su} - Sucrose utilization, %
S_{lu} - Lactose utilization, %
S_{tu} - Total sugar utilization, %

However, at 0.82 m, the sucrose concentration was reduced to a similar value as for the lowest velocity and showed no further reduction with increasing height. This difference in sucrose concentration at 0.096 m was attributed to an increase in the liquid velocity, with the resulting decrease in the **residence** time at a particular tower height, as well as the reduction in the total cell weight (fig.5.8).

A similar rapid reduction of the sugar concentration was obtained in the tower fermentations of whey permeate (fig.4.1) and molasses (fig.C.1 a) at the same low superficial velocities. In all cases, the sugars were completely utilized in the first 0.82 m height of the tower.

(b) Lactose (S_l) Although there were minor variations from one superficial velocity to another, there was generally only a small decrease in lactose concentration up to a height of 0.82 m, after which a moderate decrease occurred until a height of 2.32 m was reached (fig.5.1 b).

At the lowest velocity, there was a moderate lactose utilization

over the first 0.82 m, with a reduction in concentration from 40 to 34 g/l. A significant reduction occurred over the next 0.75 m with lactose concentration being 11 g/l at 1.57 m. A near linear decrease occurred over the remaining height of the tower.

The lactose concentration profiles with heights were nearly identical for the two higher velocities (0.12 and 0.14 mm/s) with one exception. For both velocities, there were small decreases in concentration over the first 0.82 m height where the concentration was 39 and 40 g/l, respectively.

The small reduction in lactose concentration below 0.82 m, was attributed to the diauxic growth behaviour of *K. marxianus* Y42. The presence of sucrose in the medium up to 0.82 m would inhibit lactose utilization through glucose repression of the enzyme β-galactosidase. The small amount of lactose utilized could possibly be attributed to a diffusion effect : visual observation of the tower indicated that for all three velocities the tower did not behave as a fluidized bed but as a packed bed and channelling of the liquid medium was observed (fig.5.9). Sucrose could have been rapidly utilized by the yeast cells close to the liquid channels and as the medium diffused into the inner layers of the packed bed, only lactose remained in the medium. The reduction within the initial tower sections was slightly greater at the lowest velocity because there was a greater cell concentration (table 5.2) and the fermentation time was longer as a result of the lower velocity. Sucrose was completely utilized below 0.82 m at this low velocity. Thus, lactose utilization could occur below 0.82 m at the lowest velocity. This would explain the greater reduction in lactose concentration at 0.087 mm/s than at higher velocities.

Comparison of the estimated cell weight of *K. marxianus* (table 5.2) within the tower up to 0.82 m with those in the tower fermentation of whey permeate (without enrichment) at similar velocities, showed that the cell dried weight (57 g/l) at 0.087 mm/s was more than double the cell weight (25 g/l) at a similar velocity (0.080 mm/s) in the whey permeate fermentation. For the two higher velocities, the cell concentration was comparable with those in the whey permeate fermentation, but this did not result in a comparable lactose utilization, since the fermentation of lactose in the mixed sugar fermentation was dependent on the absence of sucrose and probably sugar diffusion to the inside of the yeast flocs.

Table 5.2 Comparison of lactose utilization with that of the tower fermentation of whey permeate with respect to the cell dried weight *K. marxianus* within tower sections above and below 0.82 m.

	Whey permeate + molasses			Whey permeate	
V_s, mm/s	0.087	0.12	0.14	0.080	0.17
1. Tower heights between 0 and 0.82 m					
X_{KM}, g DW	57	24	35	25	28
S_{lu}, %	29	3	16	91	90
2. Tower heights between 0.82 and 2.32 m					
X_{KM}, g DW	77	59	47		
S_{lu}, %	48	27	31		

X_{KM} - Estimated *K. marxianus* cell dried weight from table 5.4.
S_{lu} - Percentage of lactose utilized from table 5.1.

The slow reduction in lactose concentration at heights greater than 0.82 m and the incomplete lactose consumption at the exit for all velocities were probably a result of ethanol inhibition of the cell growth. It has been reported that an ethanol concentration greater than 30 g/l could reduce the activity of the enzyme β-galactosidase obtained from *K. marxianus* (NRRL Y1109) by 70% (Wendorf et al 1971 b) and similar inhibition was observed for another strain of *K. marxianus* (UCD FST 7158) in batch culture (sect.7.3). As the ethanol concentration in the tower was greater than 25 g/l for all velocities (fig.5.3) it was likely that ethanol inhibition took place.

This was supported by the estimated concentration of *K. marxianus* present within these heights ; the estimated total *K. marxianus* cell weight was between 2 - 3 times the concentration that was required to achieve 90% lactose utilization in the first 0.82 m in the tower fermentation of whey permeate at similar velocities (table 5.2).

These observations should be interpreted with caution, since the cell weights of *K. marxianus* estimated in mixed culture were subject to considerable uncertainty (12 - 460%) see sect.E.6.4.

As a result of the diauxic behaviour of the yeast and probably ethanol inhibition, the profiles observed were different from those in

the tower fermentation of whey permeate in which lactose concentration was reduced to 4 g/l within the first 0.82 m and showed little change with further height increase.

(c) Total sugar (S_t) The total sugar curve is the sum of the sucrose and lactose curves (fig.5.1 c). Thus, there was an incomplete sugar utilization due to incomplete lactose utilization.

The trend observed here was similar to that observed for tower fermentation of beer wort which contained mixed sugars. Such sugars as sucrose, glucose and fructose were all utilized in the lower regions (0 to 1.4 m) of the tower while maltose and maltotriose required a longer time to be utilized. Thus, they remained in the tower up to a greater height (between 1.4 and 8.4 m) in a similar manner to lactose (Ault et al 1969).

In summary, in the mixed sugar fermentation of lactose and sucrose, sucrose was utilized within the first 0.82 m of the tower, while lactose uptake was repressed due to the diauxic behaviour of *K. marxianus* and a small amount of lactose was utilized. At heights greater than 0.82 m, there was only slow uptake of lactose, due probably to ethanol inhibition.

5.1.2 Volumetric rate of sugar utilization (S')

All volumetric and specific rates given here were calculated as mean values between subsequent sampling points, and the mean heights are the midpoint between these sampling points (table B.4).

(a) Sucrose (S_s') The volumetric rate of sucrose utilization decreased rapidly from a high value between 148 and 208 g/lh to less than 1 g/lh as the mean height increased from 0.048 to 1.20 m for all velocities (fig.5.2 a). It was less than 1 g/lh over the remaining mean heights.

This showed that sucrose utilization occurred rapidly up to 0.46 m as a result of high cell and sucrose concentrations, and both yeast species consumed sucrose.

The trend of change in the rate as the mean height increased was similar to those observed in the tower fermentation of whey permeate (fig.4.2) and molasses (fig.C.2 a) using a similar velocity. The rate reduced to a negligible value as the height increased to 1.20 m.

(b) Lactose (S_l') The rate was low for all velocities studied and showed some fluctuations with heights (fig.5.2 b). The fluctuations

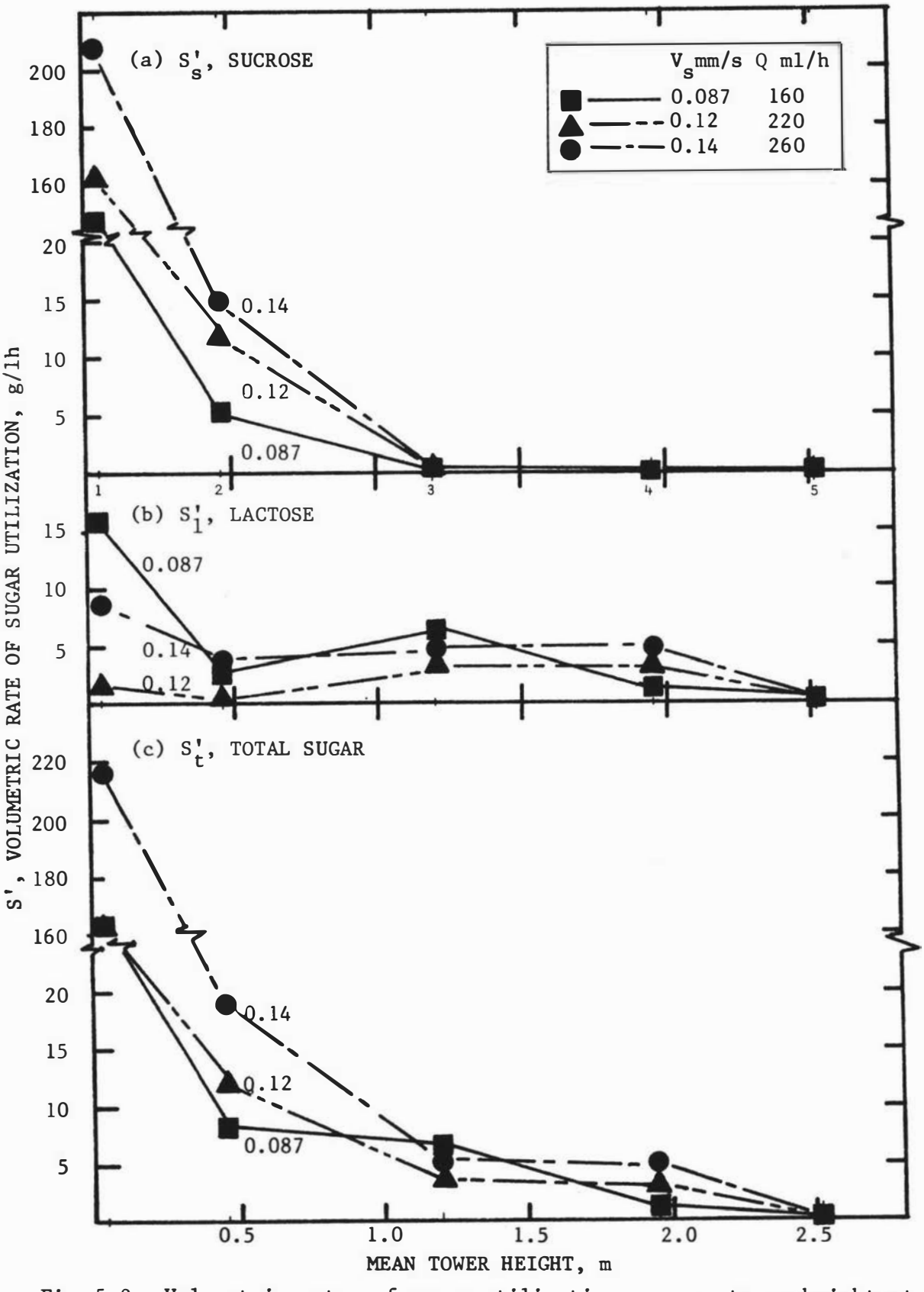


Fig. 5.2 Volumetric rates of sugar utilization vs mean tower height at various superficial liquid velocities. (a) Sucrose, (b) Lactose, (c) Total sugar.

were due to diauxic behaviour and hence the presence of sucrose in the lower region, and possibly to ethanol inhibition of β -galactosidase in the upper regions. Some redistribution of the biomass as velocity increase may also have had some effect upon the rates of utilization.

The trends obtained were different from those obtained in the tower fermentation of whey permeate, where the initial rate was much higher and fell to less than 1 g/lh above 0.46 m when sugar was almost exhausted (fig.4.2).

(c) Total sugar (S_t') The volumetric rate of total sugar utilization at a mean height of 0.048 m was between 164 and 217 g/lh. Sugar utilization occurred throughout the tower, but the rates were low at mean heights above 0.46 m.

The greatest rate achieved (217 g/lh) was lower than that obtained for molasses fermentation (350 g/lh at 0.17 mm/s) using the same concentration of sugar (100 g/l) in the feed.

5.1.3 Specific rates of sugar utilization (q)

(a) Sucrose (q_s) The specific rate of sucrose utilization was calculated using the total cell weight since both yeast species utilize sucrose. The specific rate decreased with an increase in the mean tower height (fig.5.3 a).

At the lowest velocity (0.087 mm/s), the specific rate decreased rapidly from 0.43 to 0.03 g/gh as the mean height increased from 0.048 to 0.46 m and then decreased to zero as the mean height increased further to 1.20 m. It remained at this level over the remaining tower sections. Similar profiles with higher specific rates were observed at the two higher velocities (0.12 and 0.14 mm/s).

The results showed that the yeast cells were very active in utilizing sucrose. At the mean height of 1.20 m upward , there was no sucrose utilization since all sucrose was used up below 0.82 m (sect.5.1.1 a). The trend observed was similar to those in the tower fermentation of molasses (sect.C.1.2 b) at similar velocities.

(b) Lactose (q_l) The specific rate of lactose utilization was low for all velocities studied and showed some fluctuations with height (fig.5.3 b).

The irregular trend observed was considered to be due to the different concentrations of *K. marxianus* at various heights in the tower (table 5.4). At all velocities, there was the effect of ethanol

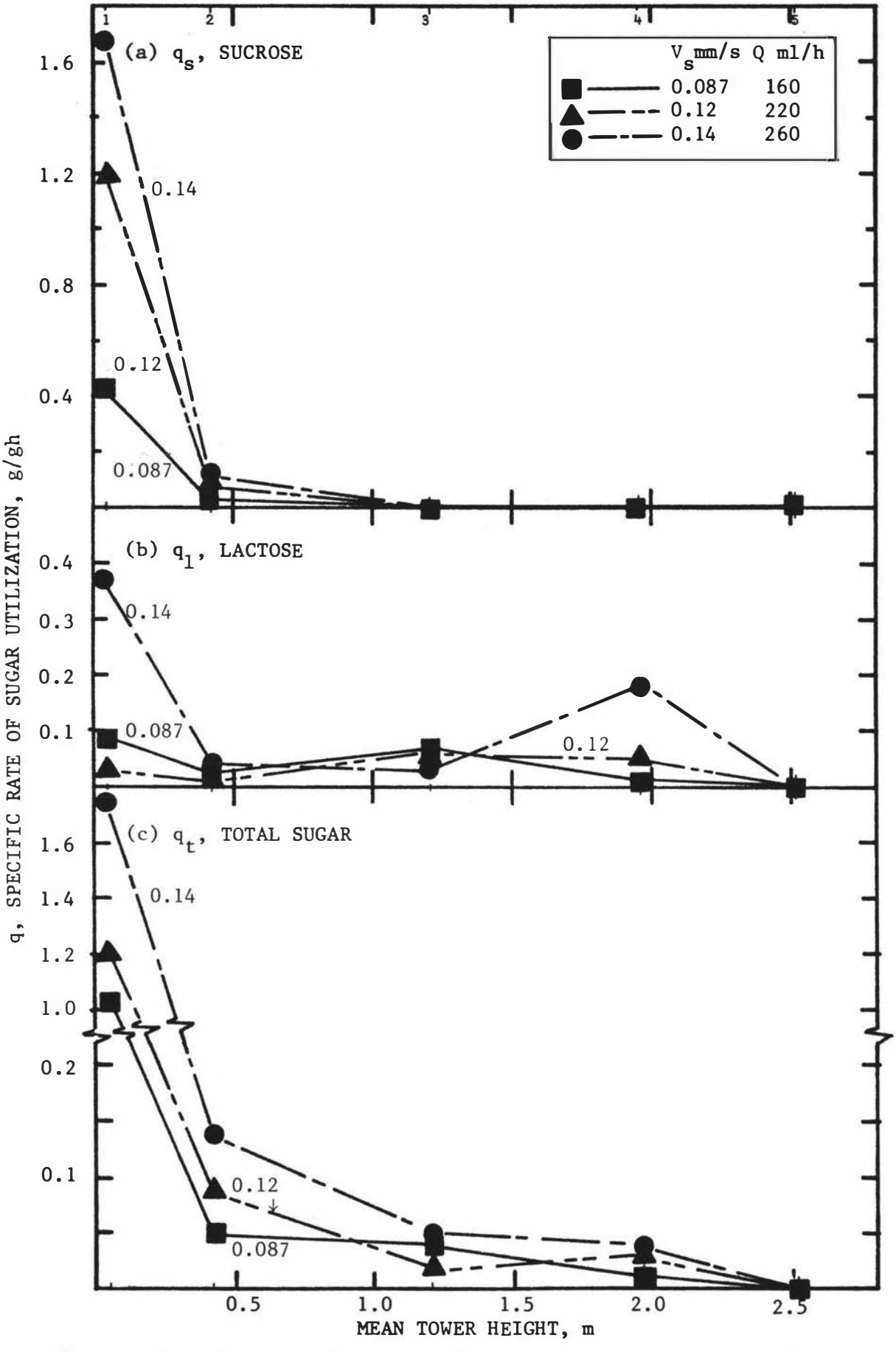


Fig. 5.3 Specific rate of sugar utilization vs mean tower height at various superficial liquid velocities.

concentration on the specific rate of lactose utilization between 0.82 and 2.32 m. Within these regions, the ethanol concentration was high (between 26 and 43 g/l) (fig.5.4), and would contribute to lowering the specific rate of lactose utilization (Wendorf et al 1971 a).

Thus, *K. marxianus* cells were active throughout the tower. However, differing mean cell concentrations at each mean height led to different specific rates throughout the tower.

The results obtained here were different to the trend observed in the tower fermentation of whey permeate, which showed a reduction of the specific rate as the height increased (sect.4.1.3). Here the rates generally decreased with height, but there was significant lactose utilization throughout the entire length of the tower.

(c) Total sugar (q_t) The resulting mean specific rate of total sugar utilization (fig.5.3 c) showed rapid decrease from between 1.03 and 1.75 g/gh to between 0.05 and 0.14 g/gh as the mean height increased from 0.048 to 0.46 m. The specific rate was between 0.01 and 0.05 g/gh over the remaining mean height increase to 1.95 m and was zero in the separator.

These trends were similar to those of the volumetric rate of total sugar utilization described previously (sect.5.1.2 c). However, the high cell concentration used in this mixed culture fermentation gave a very low specific rate between 0.46 and 1.95 m and only *K. marxianus* was involved in the fermentation. Thus, the specific rates observed in this fermentation were lower than those observed in the tower fermentation of molasses (as high as 10.8 g/gh) (sect.C.1.2 b).

5.1.4 Ethanol concentration (E)

Ethanol concentration (fig.5.4) increased rapidly as the height increased to 0.82 m and as the height increased further to 2.32 m, the concentration increased slowly.

The concentration increased to 36 g/l over the first 0.82 m of the tower height at the lowest velocity (0.087 mm/s). It then increased slowly to 43 g/l as the height increased further to 2.32 m. The concentration was 41 g/l as the effluent liquor left the tower at the exit. A similar profile was observed for the two higher velocities (0.12 and 0.14 mm/s) but the concentration was lower at all heights. The effluent ethanol concentrations reached were 33 and 34 g/l, respectively.

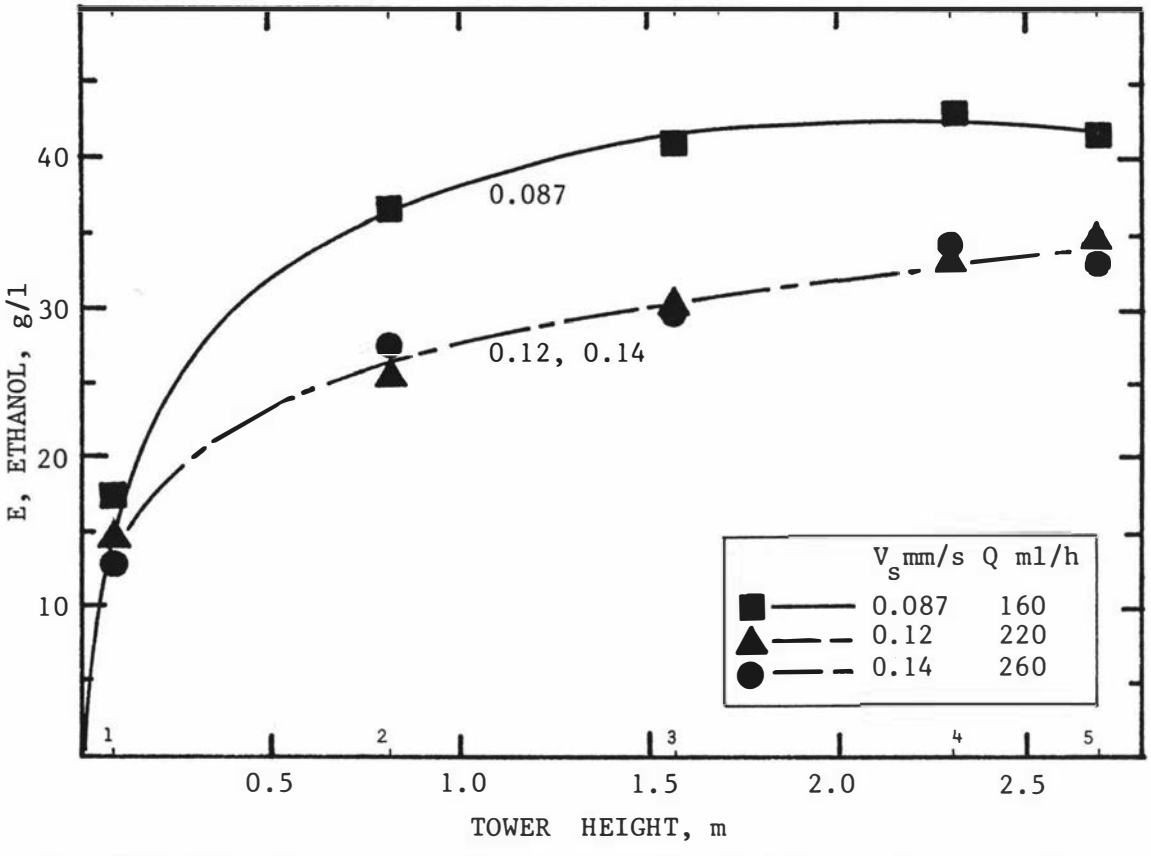


Fig. 5.4 Ethanol concentration vs tower height at various superficial liquid velocities.

The initial increase in the concentration with increase in height up to 0.82 m was mainly a result of sucrose utilization. As the tower height increase further to the tower exit there was a slow utilization of lactose, resulting in the slow increase in the ethanol concentration. The smaller increases in the ethanol concentration with height at the two higher velocities was a result of incomplete lactose utilization. This was discussed previously (sect.5.1.1).

The trend observed was similar to those observed at similar velocities in the tower fermentation of whey permeate (fig.4.4) and molasses (fig.C.1 b), in that there was a rapid initial increase in ethanol concentration. There was a difference at heights between 0.82 and 2.32 m in which there was a slow increase in the ethanol concentration in the mixed sugar fermentation, but this did not occur in the two pure sugar fermentations, since most ethanol was produced in the lower regions of the tower.

5.1.5 Ethanol yield (Y)

Ethanol yield increased with increasing height in the tower (fig.5.5). The yield was lower at greater superficial liquid velocities and ranged between 74 and 97%. In the lower 0.096 m of the tower, the yield at all velocities was low.

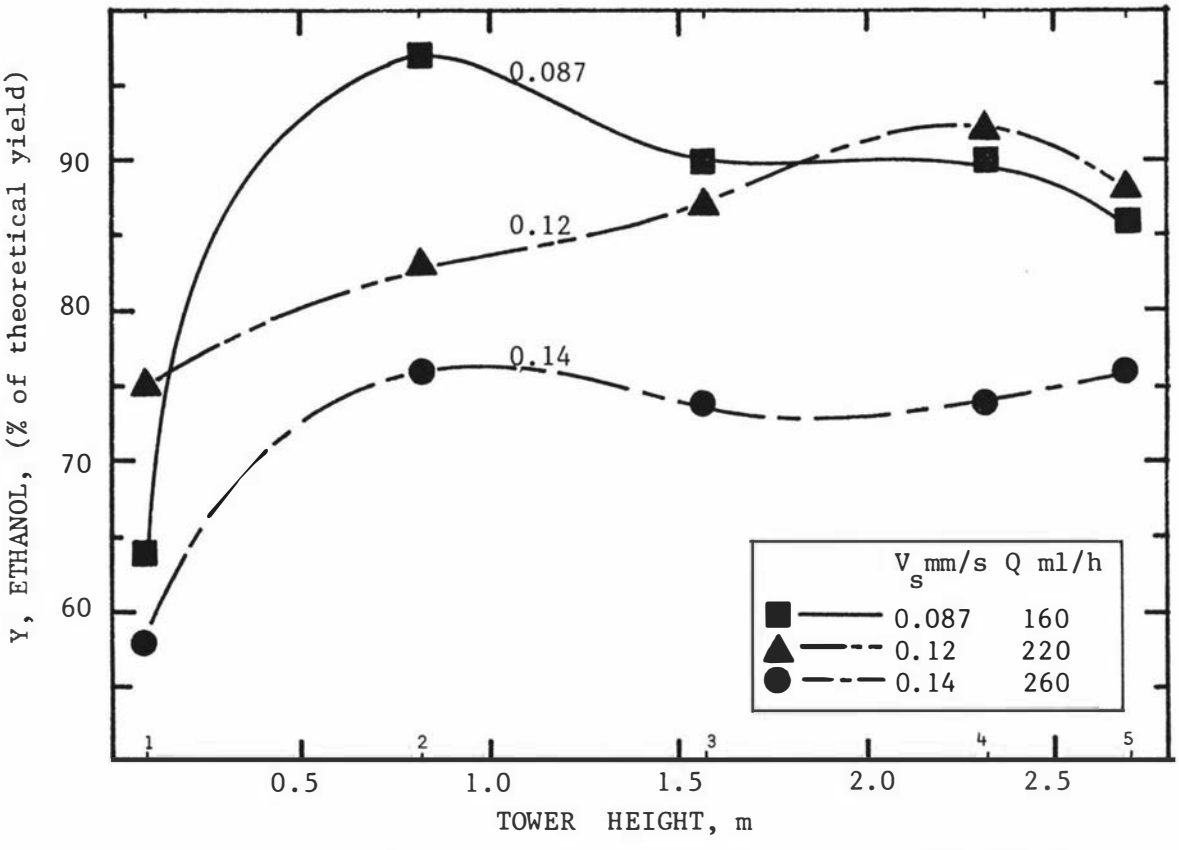


Fig. 5.5 Ethanol yield vs tower height at various superficial liquid velocities.

The results observed were similar to those in the tower fermentation of whey permeate (fig.4.5) in that the yield was lower at 0.096 m and then increased, at 0.82 m, to a level where it showed little change within analytical error as the height increased to 2.32 m and at the exit. It was explained previously that the lower yield at 0.096 m was affected by analytical uncertainty more than at the other heights, because of high sugar concentration and low ethanol concentration (sect.4.1.5).

5.1.6 Volumetric rate of ethanol production (E')

The volumetric rate of ethanol production (E') (fig.5.6) followed a similar trend to that observed for the volumetric rate of total sugar utilization (fig.5.2 c). The rate decreased rapidly from 57 to 2 g/lh

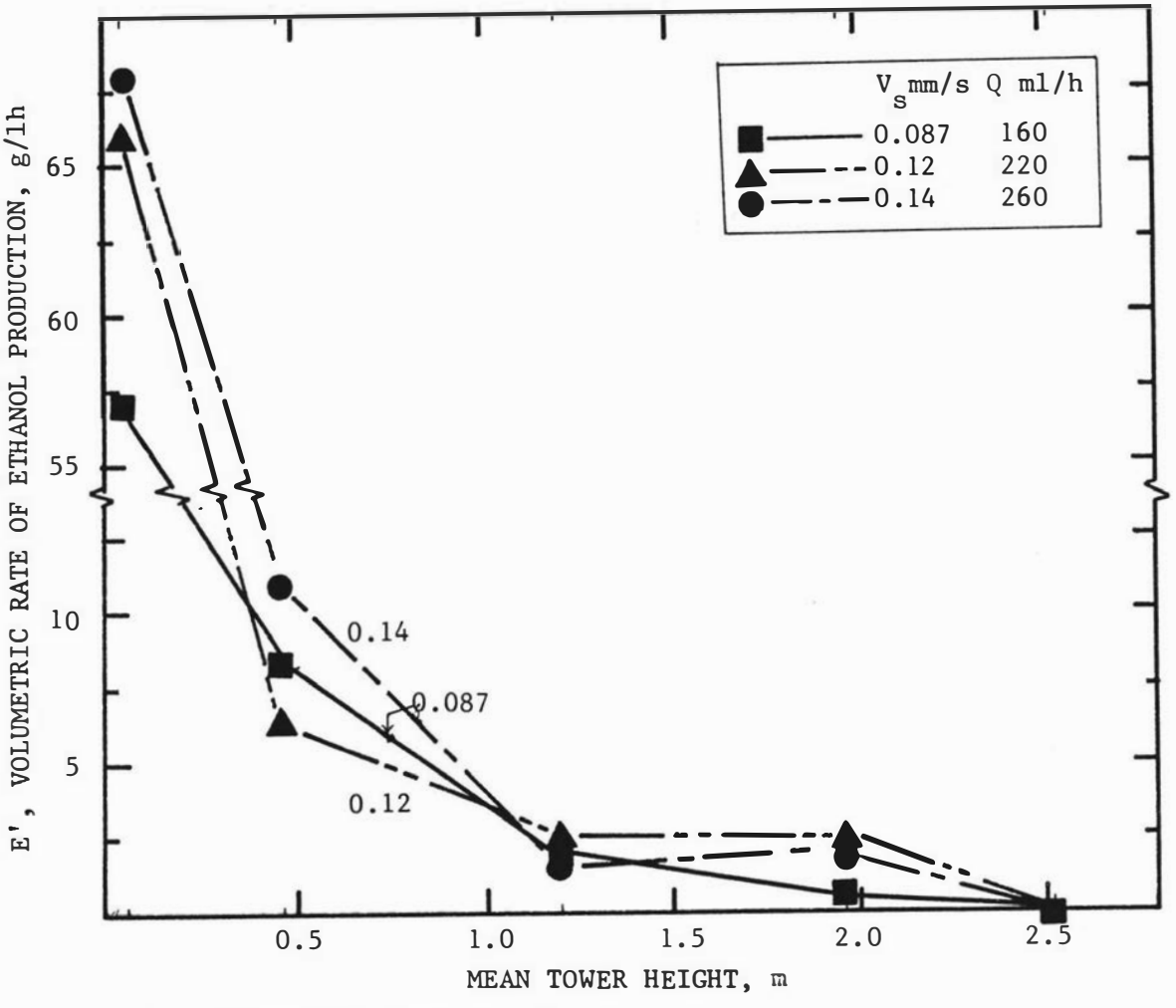


Fig. 5.6 Volumetric rate of ethanol production vs mean tower height at various superficial liquid velocities.

as the mean height increased from 0.048 to 0.46 m at the lowest velocity (0.087 mm/s). It then decreased slowly to 0.7 g/lh as the mean height reached 1.95 m. A similar trend was observed for the two higher velocities. Inside the separator(2.51 m), the rate was zero for all velocities.

This showed that there was ethanol production throughout the tower, though the volumetric rate of ethanol production was highest in the lower section of the tower (less than 0.82 m) due to high sucrose utilization. Most of the ethanol was produced within this section. The rate was lower in the upper section of the tower (greater than 0.82 m) because of the low rate of lactose utilization. The initial trend (up to 0.82 m) was similar to the trend observed for similar velocities in the tower fermentation of whey permeate (fig.4.6) and molasses (fig.C.2 b) in which there was a rapid reduction in the rate

to less than 1 g/lh within the same tower sections.

5.1.7 Specific rate of ethanol production (ν)

The specific rate of ethanol production decreased to zero as the mean height increased (table 5.3).

Table 5.3 Specific rate of ethanol production (ν) (g/gh) at various mean tower height and superficial liquid velocities.

V_s mm/s	ν (g/gh) at various mean height (m)				
	0.048	0.46	1.20	1.95	2.51
0.087	0.4	0.1	0	0	0
0.12	0.5	0	0	0	0
0.14	0.5	0.1	0	0	0

At the lowest velocity, the specific rate decreased from 0.4 to 0.1 g/gh as the mean height increased from 0.048 to 0.46 m and then decreased to zero as the mean height increased further to 1.20 m and remained at this level up to the exit. Similar trends were observed at the two higher velocities. The rate was zero inside the separator (2.51 m) for all velocities.

This showed that there was a high specific rate of ethanol production at the bottom of the tower for all velocities studied. It was shown previously that ethanol was produced throughout the tower (sect.5.1.5), however , the specific rate was effectively zero at mean heights greater than 0.46 m because the amount of ethanol produced within these heights (between 1 and 5 g/l) was very small compared to the total cell weight (between 58 and 185 g/l DW). It should be noted that the specific rate was calculated based on the total cell weight, but at heights greater than 0.82 m only lactose was being utilized by *K. marxianus* since sucrose was all used up (sect.5.3.1 a). Thus *S. cerevisiae* cells were not involved in ethanol production but were included in the calculation of the specific rate.

5.1.8 Cell concentration

(a) *K. marxianus* concentration (X_{KM}) The number of *K. marxianus* cells in the tower between 0.096 to 2.32 m was high (between $3.5x10^8$ and $2.4x10^9$ cells/ml) and was very low (between $7.2x10^5$ and $1.1x10^7$ cells/ml) in the effluent liquor (fig.5.7 a). Similar profiles were obtained at all velocities studied. At the lowest velocity, the cell number decreased from $2.4x10^9$ to $6.5x10^8$ cells/ml as the height increased from 0.096 to 2.32 m. This resulted in a high log mean cell number of $1.2x10^9$ cells/ml and gave an overall lactose utilization of 80%. The corresponding cell dried weight at this velocity was estimated from fig.B.1 relating plate count to dried weight, and was found to be higher than the cell concentration (at 0.080 mm/s) measured in the tower fermentation of whey permeate (table 5.4). The estimated cell dried weights (table 5.4) showed some discrepancies when compared with the cell number (table B.7). It was, however, the only method available to determine the *K. marxianus* cell dried weight from the cell plate count number and these estimated weights were essential for comparison with the cell dried weight obtained in the tower fermentation of whey permeate.

Considering the next velocity of 0.12 mm/s, the cell number changed little as the tower height increased from 0.096 to 2.32 m and the log mean cell number within these heights was $7.6x10^8$ cells/ml.

At the highest velocity (0.14 mm/s), the cell number was relatively constant throughout the tower with the exception of the cell number at 0.82 m ($2.3x10^9$ cells/ml). The log mean cell number was $5.3x10^8$ cells/ml.

In the separator, the yeast cells were mainly *K. marxianus* numbering between $7.2x10^5$ and $1.1x10^7$ cells/ml. There was between $2.2x10^5$ and $2.6x10^6$ cells/ml of *S. cerevisiae*. This predominance of *K. marxianus* was confirmed by observations during continuous operation that in the separator the yeast flocs were small and suspended ie. typical of *K. marxianus* flocs. They were different to the glutinous and very flocculent yeast cells inside the tower.

It was also observed, during operation at the two higher velocities, that the concentrated yeast plug had expanded to the bottom of the separator.

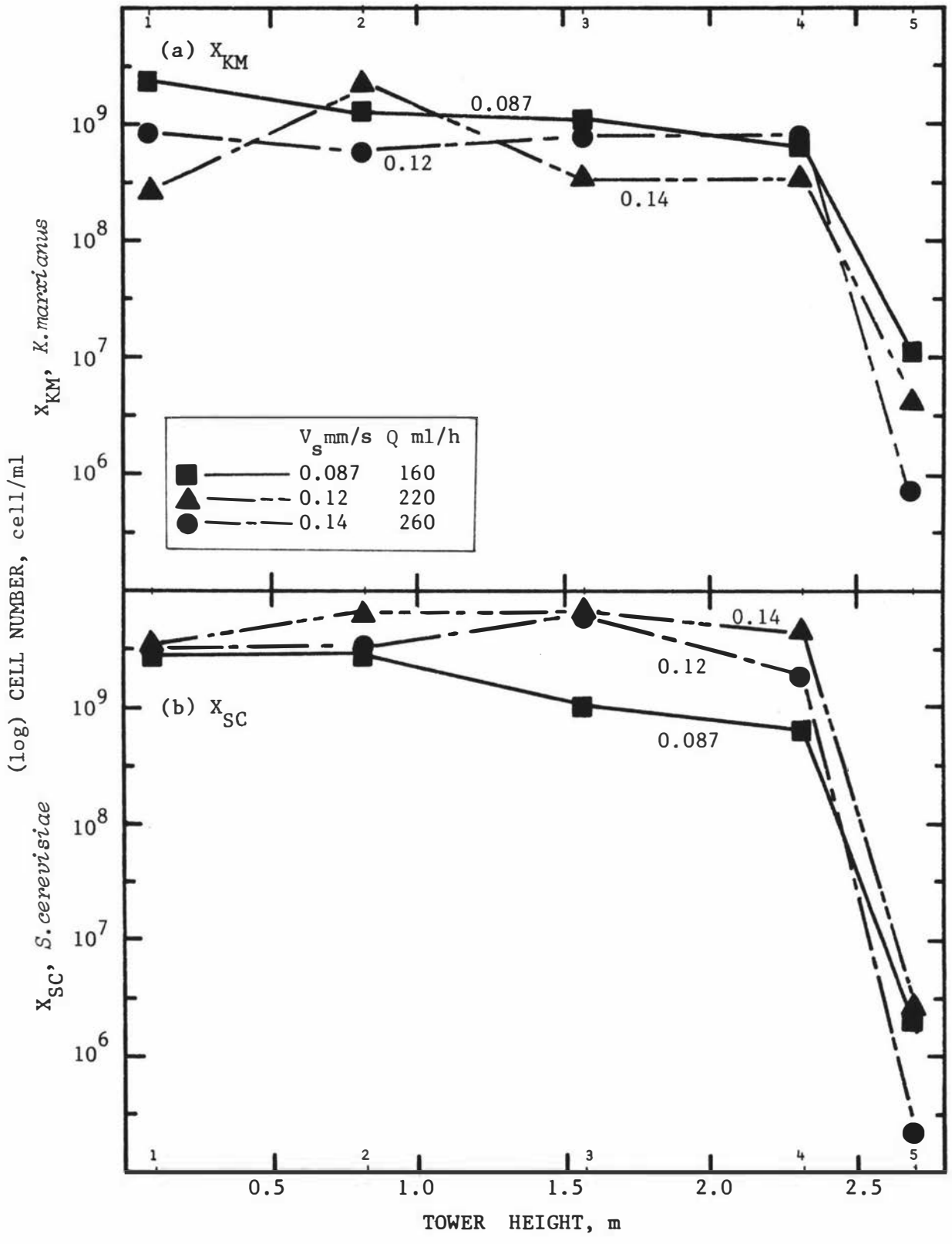


Fig. 5.7 Cell numbers vs tower height at various superficial liquid velocities. (a) *K.marxianus*, (b) *S.cerevisiae*. The corresponding estimated cell concentrations are given in table 5.4.

Table 5.4 The concentration of *K. marxianus* Y42 and *S. cerevisiae* CFCC39 at various heights in the tower and superficial liquid velocities.

		whey permeate + molasses						whey permeate*	
V_s, mm/s		0.087		0.12		0.14		0.080	0.17
sample point	height m	cell concentration, g/l DW							
		X_{KM}	X_{SC}	X_{KM}	X_{SC}	X_{KM}	X_{SC}	X_{KM}	X_{SC}
1	0.096	170	0	66	70	23	99	101	96
2	0.82	98	66	47	95	162	17	10	30
3	1.57	81	94	62	112	28	93	6	26
4	2.32	51	7	65	9	29	85	4	22
5	2.69	1	0.1	0.1	0.1	0.5	0.1	0.2	0.2

X_{KM} - *K. marxianus* concentration was estimated by using fig.B.1 to convert the *K. marxianus* plate count number (Appendix B.4) to cell dried weight.

X_{SC} - *S. cerevisiae* concentration was the difference between the measured total cell dried weight (Appendix B.4) and X_{KM} above.

* - The *K. marxianus* cell dried weights obtained in the tower fermentation of whey permeate (Appendix B.3). These are for comparison.

The results showed that a high concentration of *K. marxianus* throughout the tower was essential for improved lactose utilization in mixed sucrose and lactose medium by a mixed culture of *K. marxianus* and *S. cerevisiae*. This was because at the lowest velocity (0.087 mm/s), the *K. marxianus* concentration was high throughout the tower and this resulted in lactose being utilized throughout the tower (table 5.4). However, at a higher velocity of 0.12 mm/s, the concentration was moderate throughout the tower but was not high enough in the first 0.82 m of the tower to give a noticeable utilization of lactose within these heights. So there was an uneven reduction of the lactose concentration and when the effluent liquor left the tower only 33% of lactose was utilized (table 5.4). Finally, at the highest velocity (0.14 mm/s), there was a moderate concentration of *K. marxianus* inside the tower and an extra high concentration at 0.82 m. This high concentration had enough impact on lactose utilization to give 49% lactose utilization at the exit even though the cell concentration at

other heights was lower than at the lower velocity of 0.12 mm/s. If the cell concentration were higher, it is likely that more lactose could be utilized.

The results also showed that *K. marxianus* occupied the separator and formed the majority of the biomass leaving the tower.

(b) *S. cerevisiae* (X_{SC}) The number of *S. cerevisiae* cells inside the tower was high (between 6.5×10^8 and 6.2×10^9 cells/ml) and was very low in the effluent liquor (between 2.2×10^5 and 2.6×10^6 cells/ml) (fig.5.7 b). Within the tower, the concentration was generally greater than that of *K. marxianus* at corresponding heights. Thus, these cells which were very flocculent caused the very high yeast cell concentrations reached in the tower (fig.5.8) and contributed to the rapid consumption of sucrose. Some of the values of *S. cerevisiae* cell dried weight listed in table 5.4 do not give a true representation of the cell concentration because these values were obtained as the difference between the measured total cell dried weight and the estimated *K. marxianus* cell dried weight. The uncertainty of the estimated *K. marxianus* weight was high (between 280 and 460% of the values reported (sect.E.6.4)).

(c) Total cell concentration At a constant velocity of 0.087 and 0.12 mm/s, the total cell concentration (fig.5.8) increased to a high value as the height in the tower increased from 0.096 to 1.57 m and then decreased rapidly as the height increased to 2.32 m.

There was a different profile at the highest velocity (0.14 mm/s). The cell concentration showed a general reduction as the height increased from 0.096 to 2.32 m. The concentration at 2.32 m was greater than for the two lower velocities.

The results showed a high cell concentration within the first 1.57 m of the tower. Within these sections, there was a greater concentration of the more flocculent yeast *S. cerevisiae* CFCC39. Thus, the cell concentration was high. It was considered that the chemicals present in whey and the slow velocities at which the tower was operated contributed to this high flocculence.

The initial increase in the cell concentration as the height increased to 1.57 m for the two lower velocities (0.087 and 0.12 mm/s) was considered to be the effect of gas production in the yeast flocs. More gas was produced in the lower tower sections up to 0.82 m and as a result the void volume increased. At 1.57 m, there was less gas

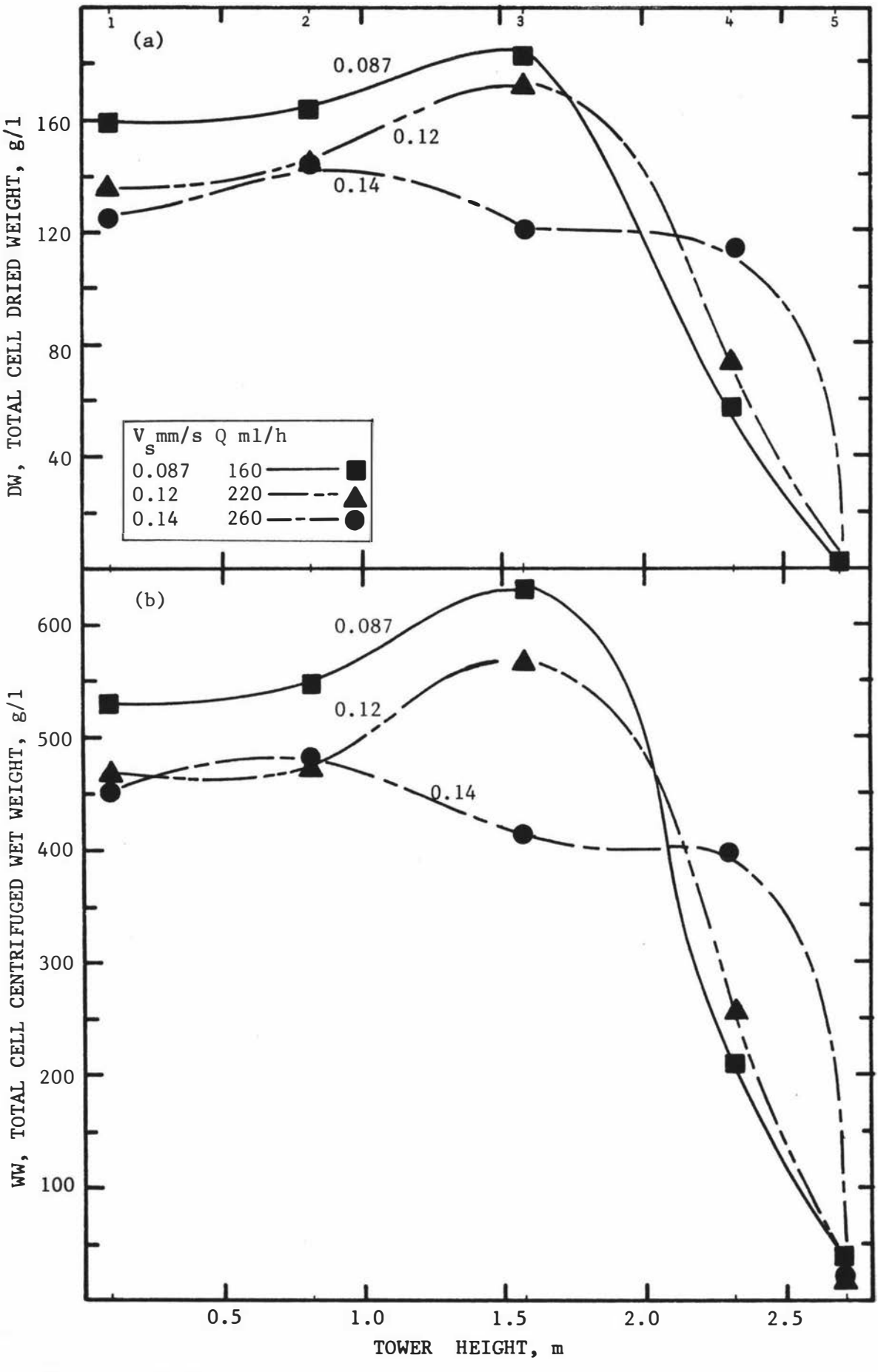


Fig. 5.8 Cell concentration vs tower height at various superficial liquid velocities. (a) Cell dried weight, (b) Cell centrifuged wet weight.

production and, thus, the cell concentration was greater.

The decrease in the cell concentration at 2.32 m was due to the highly flocculent nature of the yeast. The flocs were large and heavy and were able to settle to the bottom of the tower because the liquid velocity was low and was not able to suspend the flocs.

The different profile observed at the highest velocity of 0.14 mm/s was a result of the expansion of the yeast bed with an increase in the velocity, decreasing the cell concentration at heights between 0.82 and 2.32 m. It was observed that the concentrated cell mass had expanded to the bottom of the separator and blocked the return path of the flocs. Thus, the concentration was lower at 1.57 m, and was greater at 2.32 m, than for the other two velocities.

The total cell concentration obtained in this experiment was considerably greater than those reached in the tower fermentation of whey permeate (fig.4.9) and molasses (fig.C.5). This difference was due to the increased flocculence of the yeast strain CFCC39 which was used here.

The high cell concentration and glutinous yeast cell mass was found to cause considerable channelling inside the tower (fig.5.9). This was a result of the low velocity used and the extremely flocculent nature of the yeast strain CFCC39. A very high velocity would be required to suspend the yeast flocs. The high concentration of calcium ions in the whey permeate probably contributed to the good flocculation of the yeast cells inside the tower. The modified Burn's number (MBN*) for the tower yeast culture was 166 indicating that the yeasts were very flocculent. The continuous cell mass did not permit free movement of carbon dioxide and the medium up the tower but induced coalescence and thus channelling occurred. This in effect reduced the cell to sugar contact and created gas slugs which slowly washed out the less flocculent *K. marxianus* by creating turbulence in the separator. As the flow rate was increased the volume occupied by the yeast cells expanded. When the expansion reached the bottom of the separator, the yeast recycle path was blocked. This also created turbulence in the separator and cell washout.

Thus, it is possible that the use of a very highly flocculent yeast together with a moderately flocculent yeast as in this study may be undesirable. It may be more appropriate to use the less flocculent *S. cerevisiae* strain SC146, which was used in the tower fermentation of

Fig. 5.9 Channelling inside the tower during mixed culture and mixed substrate tower fermentation.

molasses (Appendix 6), either as mixed culture with KM Y42, or to use only KM Y42. There was, however, insufficient time to investigate this in the tower fermenter.

The nature of the cell mass also caused problems during sampling. The cell mass would not flow out through the sampling tubes easily. Each tube had to be squeezed like a toothpaste tube to obtain sufficient sample for analysis. An uncertainty allowance of 5% should be added to the cell concentration values for heights up to 1.57 m (sect.E.5.3). The sampling problems increased analysis errors by increasing time between sampling and separation of yeast cells from the liquor, which was particularly critical at the bottom sampling point (0.096 m) because of the rapid reduction in the sugar concentration in this region. Chemical agent could not be added to kill the yeast cells because the sample was required for a plate count. In addition, the chemical agent if added could interfere with the enzyme membranes used for sugar analysis.

5.1.9 Medium pH

The pH (fig.5.10) of the fermenting medium decreased from an initial value between pH 4.8 and 5.0 to between pH 4.6 and 4.8 over the first 0.096 m of the tower height. It then increased to between pH 5.0 and 5.1 as the height increased to 0.82 m and remained in this range up to the exit for all velocities used.

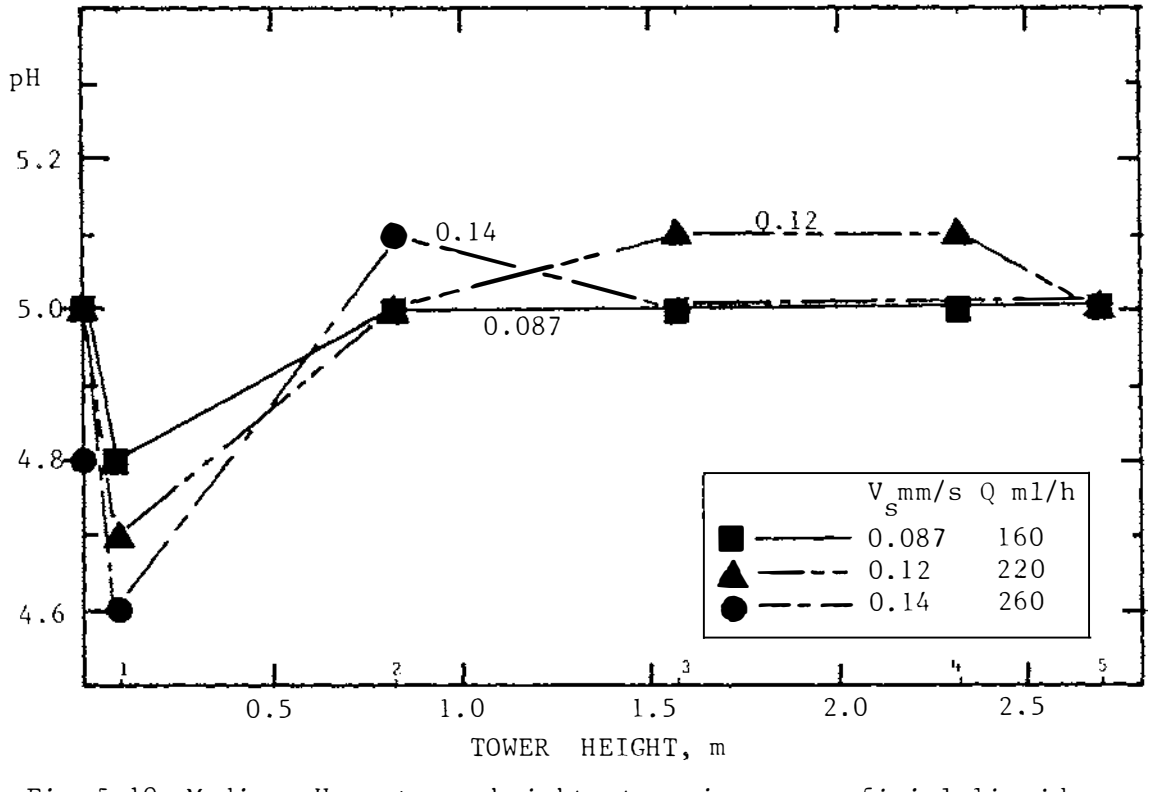


Fig. 5.10 Medium pH vs tower height at various superficial liquid velocities.

The trends observed here were similar to those in the tower fermentation of molasses (fig.C.6) in that there was an initial pH reduction over the first 0.096 m of tower height and the pH returned to between pH 4.8 and 5.0 as the height increased. The major pH change occurred in the tower sections where ethanol was produced and thus there was little change in pH at heights greater than 0.82 m. A similar pattern was observed during fermentation of whey permeate.

5.2 CONTAMINATION OF CONTINUOUS TOWER FERMENTATION CULTURE

Great care was required to ensure that the tower was not contaminated by allowing unfiltered air to enter through the sample ports and the feed inlet. Infection problems were compounded in that the high residual lactose concentration in the upper section of the tower permitted growth of bacteria. This occurred toward the end of the continuous operation at the velocity of 0.14 mm/s. Instead of abandoning the operation, it was decided that penicillin and streptomycin should be added to the medium to suppress the infection and permit the continuation of the fermentation.

5.3 TOWER FERMENTER PERFORMANCE

It was evident from the results described previously (sect.5.1) that the tower operation at the velocity of 0.087 mm/s was the only velocity at which there was 90% total sugar utilization at a tower height of 2.32 m. Thus, only the performance at this velocity could be used as a comparison with other tower fermentations.

This velocity was lower than the optimum velocities of 0.24 and 0.33 mm/s observed in the tower fermentation of whey permeate (sect.4.4) and molasses (sect.C.4), respectively. It was shown that this fermentation was limited by the diauxic behaviour of *K. marxianus* due to the presence of sucrose and lactose together and sucrose was utilized before lactose in the tower section below 0.82 m. At greater heights, the rate of fermentation of lactose was probably reduced by the presence of ethanol. Thus, it was shown that in order to achieve the same rapid rate of fermentation as for whey permeate fermentation, the *K. marxianus* cell concentration should be considerably higher than for whey permeate fermentation. *S. cerevisiae* cells did not take part

in the fermentation of lactose. This could be regarded as an inefficient use of the yeast cells and it would be more efficient to use only *K. marxianus* in the tower, provided that a very flocculent strain were available (sect.5.5).

The height of 2.32 m, required for 90% total sugar utilization, was greater than the 0.82 m required for the other two tower fermentations to allow longer residence time for lactose to be utilized.

The residence time of 7.4 hours was long when compared with the time of 1.0 and 0.7 hour for the tower fermentations of whey permeate (sect.4.4) and molasses (sect.C.4), respectively. The increased time was a direct result of the diauxic behaviour of the yeast and probably also ethanol inhibition.

The resulting ethanol productivity from this was 5.8 g/lh at 2.32 m. The productivity was lower than 16 and 67 g/lh obtained (excluding the separator) in the tower fermentation of whey permeate and molasses, respectively. Batch fermentation of a similar mixed sugar medium required 48 hours to produce 39 g/l ethanol (fig.7.3 a). This gave a productivity of 0.8 g/lh which was approximately one seventh of the rate observed in the present work. Thus, tower fermentation of mixed sugar did not achieve a very high productivity, but was better than batch fermentation of a similar substrate.

The factors which limited the performance of the tower fermenter in these experiments could be listed as follows :

1. The presence of sucrose and lactose together caused *K. marxianus* to exhibit diauxic behaviour. Lactose utilization was repressed by glucose produced from sucrose, so a longer residence time was required to achieve lactose fermentation and this fermentation occurred at heights within which there was high ethanol concentration. Thus, the maximum superficial velocity was limited and the height required increased in order to achieve a long fermentation time.

2. In the upper sections of the tower above 0.82 m, there was probably ethanol inhibition, because the rate of lactose utilization was low even though the cell concentration was comparable with that used in whey permeate fermentation. Thus, the fermentative activity of *K. marxianus* reduced considerably within these heights. This effect contributed to the incomplete lactose utilization. A greater *K. marxianus* concentration than that achieved in this fermentation

would be required to achieve the same degree of lactose utilization as rapidly as in the whey permeate fermentation.

3. There was a difficulty in maintaining a sufficiently high concentration of *K. marxianus* in the tower fermenter to compensate for the reduction in the lactose fermentation rate throughout the tower. The difficulty was caused by a combined effect of the moderately flocculent nature of this yeast strain compared with that of *S. cerevisiae* CFCC39 and the incompatability of the two yeasts in the tower. The highly flocculent yeast mass caused gas slug formation and channelling which created turbulence in the separator. Thus, the slow washout of the less flocculent yeast occurred even at the low velocities used.

It was desirable, therefore, to investigate whether the incomplete lactose utilization was caused by an insufficient concentration of *K. marxianus* as well as by the diauxic behaviour, or as a result of the interaction of molasses and whey permeate. This is described in sect.5.4. An investigation of the effect of the concentration of each culture in the inoculum on the final cell ratio was also considered desirable, because it could clarify whether the observed change in the *K. marxianus* concentration was caused by washout as described earlier. This investigation is described in sect.5.5.

5.4 COMPARISON BETWEEN FERMENTATION OF WHEY PERMEATE ENRICHED WITH MOLASSES AND WITH SUCROSE

In various fermentations of whey permeate enriched with molasses (sect.5.1 and 7.3) it was observed that there was always a greater amount of residual lactose than sucrose remaining at the end of the fermentation. An experiment was conducted to investigate the effect of the addition of molasses to whey permeate.

Molasses and pure sucrose were added to separate quantities of whey permeate to give a final sucrose concentration of 60 g/l. The fermentation was carried using 450 ml of media volume in 2 litre shake flasks. The media were inoculated with 50 ml of inoculum and incubated at 30°C with shaking at 150 rpm.

It was found that the sucrose concentration (table 5.5) was reduced rapidly in both media and was completely utilized in 24 hours. The residual concentrations were 0.4 and 0.1 g/l in molasses and

sucrose enriched media, respectively.

In contrast, lactose was reduced, after 48 hours, to 6.7 and 2.1 g/l (83 and 94% lactose utilization, respectively). After 72 hours, there was a very small decrease in the lactose concentration.

Table 5.5 Comparison between fermentation of whey permeate enriched with molasses and with sucrose by *K. marxianus* Y42 and *S. cerevisiae* CFCC39.

PM - Permeate + molasses ; PS - Permeate + sucrose

media	time h	S_s g/l	S_l g/l	S_t g/l	E g/l	$X_{KM/X}$ %	pH
PM	0	55	39	94	-	43	4.9
PS	0	58	37	95	-	44	5.1
PM	24	0.4	22	22	25	46	4.5
PS	24	0.1	11	11	29	55	3.8
PM	48	0.4	6.7	7.1	32	58	4.4
PS	48	0.1	2.1	2.2	34	66	4.2
PM	72	0.4	3.2	3.6	28	56	4.4
PS	72	0.1	1.7	1.8	32	63	4.3

S_s - Sucrose concentration ; S_t - Total sugar concentration
S_l - Lactose concentration ; E - Ethanol concentration
$X_{KM/X}$ - Ratio of *K. marxianus* to total cell number

The total residual sugar concentration was higher in the molasses-enriched whey permeate than for sucrose-enriched whey permeate, throughout the fermentation. After 48 hours, the concentrations were 7.1 and 2.2 g/l in molasses and sucrose-enriched whey (92 and 98% utilization, respectively). The major component of the residual total sugar was lactose.

There was less ethanol in the molasses-enriched whey throughout the fermentation. Maximum ethanol concentrations were reached after 48 hours and were 32 and 34 g/l, respectively. This was an indication of a slower fermentation in molasses-enriched whey.

The ratio of *K. marxianus* Y42 to the total cell count (X_{KM}/X) remained close to 50% throughout the fermentation, showing a small increase as the fermentation progressed.

The pH of whey enriched with sucrose decreased more than the pH of

whey permeate enriched with molasses. This was probably due to a greater rate of carbon dioxide production in whey permeate enriched with sucrose.

The results indicated that molasses had an inhibitory effect on fermentative activity of *K. marxianus* Y42 when used to enrich whey permeate.

It has been reported that lactose can be thermally rearranged to lactulose upon sterilization (Thayanithy et al 1982). The lactulose formed was not metabolized by a bacterial β-amylase enzyme. The amount of lactose (lactulose) remaining was reported to be between 20 and 36%. However, a shake-flask fermentation test using 20 g/l lactulose demonstrated that *K. marxianus* Y42 grew on lactulose. Therefore, the unmetabolized lactose detected in this study was unlikely to be due to the formation of lactulose.

5.5 FERMENTATION COMPARISON USING DIFFERENT RATIOS OF MIXED YEAST CULTURE IN THE INOCULUM

It was observed that during the tower fermentation by *K. marxianus* Y42 and *S. cerevisiae* CFCC39 of whey permeate enriched with molasses (sect.5.1.8), there was a decrease in the number of *K. marxianus* Y42 cells as the fermentation progressed. The experiment described here investigated the effect of the ratio of the two yeast strains in the inoculum on the cell ratio during fermentation of whey permeate enriched with molasses. Three cell ratios in the inoculum were used, 9:1, 1:1 and 1:9 (*K. marxianus* Y42 : *S. cerevisiae* CFCC39) The fermentation was carried out using 450 ml medium volume in 2 litre shake flasks. The flasks were inoculated with 50 ml of inoculum and were incubated at 30°C with shaking at 150 rpm.

The results of the fermentation are shown in table 5.6. The total cell counts for the three inoculum ratios were similar at inoculation and after 24 hours. However, after 48 hours, the 9:1 inoculum ratio had the lowest total cell number while the medium inoculated with 1:9 inoculum ratio contained the greatest cell concentration. For the 1:9 inoculum ratio, lactose uptake required longer time because of the lower initial *K. marxianus* Y42 concentration.

The ratio of *K. marxianus* Y42 to the total cell count (X_{KM}/X) was found to be close to the ratio at inoculation time for 9:1 and 1:1

Table 5.6 Fermentation comparison using different ratios of yeast culture in the inoculum.

KM:SC - Ratio of *K. marxianus* Y42 to *S. cerevisiae* CFCC39 in the inoculum.

KM:SC ratio	X cells/ml	X_{KM} cells/ml	$X_{KM/X}$ %	S_s g/l	S_1 g/l	S_{lu} %	S_t g/l	E g/l	pH
0 hour									
9:1	1.2×10^8	1.1×10^8	91	47.5	38.0	-	85.5	-	4.7
1:1	1.0×10^8	6.0×10^7	59	47.0	37.1	-	84.1	-	4.7
1:9	1.1×10^8	1.2×10^7	11	46.6	38.0	-	84.6	-	4.7
24 hours									
9:1	8.7×10^8	7.3×10^8	84	0.5	12.3	68	12.8	38.6	4.4
1:1	1.1×10^9	5.2×10^8	48	0.4	19.8	47	20.2	29.3	4.3
1:9	7.3×10^8	9.0×10^8	12	0.4	30.5	20	30.9	25.7	4.4
48 hours									
9:1	3.2×10^9	3.0×10^8	94	0.5	7.0	82	7.5	39.3	4.4
1:1	1.1×10^9	5.1×10^8	44	0.4	6.9	81	7.4	29.5	4.4
1:9	2.2×10^9	6.0×10^8	28	0.4	9.9	74	10.3	28.5	4.4

X - Total cell number ; X_{KM} - *K. marxianus* cell number

S_{lu} - percentage lactose utilization

flasks. However, in the case of the 1:9 ratio flask the cell ratio increased more than 2 fold after 48 hours.

Sucrose (S_s) was found to be completely utilized after 24 hours for all inoculum ratios. The greatest utilization of lactose (82%) occurred in the 9:1 ratio flask. The lowest utilization (74%) occurred at the 1:9 inoculum ratio. Thus, lactose utilization was affected by the concentration of *K. marxianus* Y42 in the inoculum for all inoculum ratios whereas sucrose consumption was not affected. There was more rapid total sugar utilization when the ratio of *K. marxianus* Y42 in the inoculum was higher. The inoculum ratio of 9:1 gave the lowest residual total sugar level of 7.3 g/l after 48 hours (92% utilization).

After 24 hours, the medium inoculated with the 9:1 ratio contained the highest concentration of ethanol (39 g/l). The lowest concentration of 26 g/l was in the 1:9 inoculum ratio. After 48 hours, the order was the same.

5.6 EFFECT ON FLOC MORPHOLOGY OF SPECIES RATIO IN INOCULUM

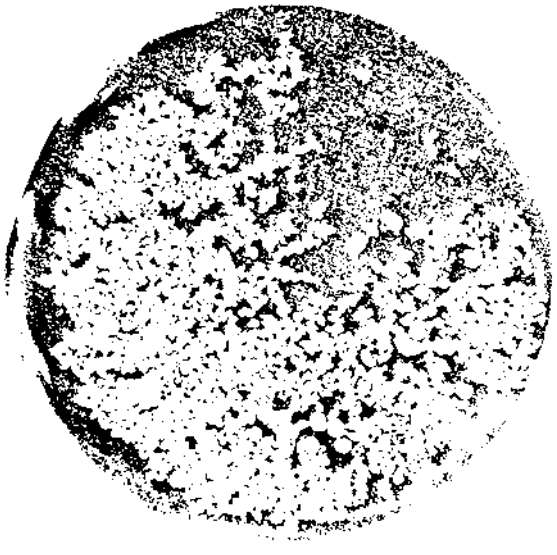
The morphology of the yeast flocs present in the medium at the end of the fermentation is shown in fig.5.11. In (a, 9:1), there were mainly small flocs of *K. marxianus* Y42, as it accounted for 90% of the total cell concentration. In (b, 1:1), there were more large flocs of diameter up to 1.5 mm. The two yeast strains had apparently formed mixed flocs because the floc size increase corresponded with an increase in *S. cerevisiae* CFCC39. In (c, 1:9), there were many larger flocs up to 4 mm in diameter. This was a result of the higher ratio of *S. cerevisiae* CFCC39.

It is evident that the higher concentration of *K. marxianus* Y42 in the inoculum resulted in faster sugar utilization and ethanol production during the early stage of the fermentation. This also resulted in greater ethanol production over the same fermentation time when compared with the fermentation using a lower initial concentration of *K. marxianus* Y42. There was an increase in the ratio of *K. marxianus* Y42 when its concentration in the inoculum was low. This occurred after sucrose was used up and lactose was being utilized. Thus, the cell ratio changed from the level in the inoculum since *S. cerevisiae* was unable to utilize the remaining substrate. This, however, occurred when the ethanol concentration was high and the activity of *K. marxianus* was therefore reduced. This resulted in slow sugar uptake and ethanol production.

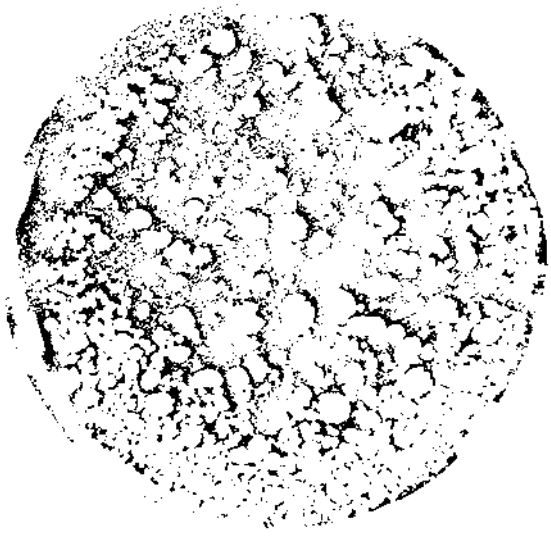
Hence, there was evidence that it may be more desirable to use a concentration of *K. marxianus* Y42 greater than a 50:50 ratio in the tower. According to the results observed here, it should be the dominant cell population throughout the tower fermenter. There would then be a more rapid lactose uptake. Higher concentration of *S. cerevisiae* CFCC39 resulted in larger floc formation and this is desirable in the tower fermenter.

The composition of the mixed culture is therefore determined by a compromise between maximum sugar utilization and floc retention at high superficial liquid velocity.

(a) 9 : 1



(b) 1 : 1



(c) 1 : 9

K.marxianus : *S.cerevisiae*

actual size

Fig.5.11 The effect of mixed culture ratio on floc morphology in shake flask culture.

5.7 CONCLUSIONS

1. There was incomplete lactose utilization in the tower fermentation of whey permeate enriched with molasses by mixed culture of *K. marxianus* Y42 and *S. cerevisiae* CFCC39. This was found to be a result of many factors :

Diauxic behaviour of *K. marxianus* Y42 which caused sucrose to be utilized before lactose,

Ethanol and molasses inhibition of *K. marxianus* Y42 fermentative activity,

The difficulty in maintaining a sufficiently high *K. marxianus* Y42 population in the tower due to cell washout caused by channelling, gas slug formation and the only moderately flocculent nature of *K. marxianus* Y42,

2. Channelling and gas slugging occurred because the yeast bed was too dense (not fluidized) at the low superficial liquid velocities used.

3. The two yeast strains employed were not compatible for use in this fermentation because the mixed culture flocs that were formed could be broken down easily by gas bubbles, and the less flocculent *K. marxianus* was slowly washed out of the tower.

4. An increased concentration of *K. marxianus* in the mixed culture improved both the rate and yield of ethanol when grown on the mixed subtrate.

5. It may be desirable to use a pure culture of *K. marxianus* for fermentation of this substrate. This area of the work was not taken further, but it is clear that more experiments could be designed to study the behaviour of mixed cultures of various strains in completely fluidized beds, examining the effects of population ratios on floc size and operating at greater superficial velocity.

5.8 SUMMARY

There was incomplete lactose utilization in the tower fermentation of whey permeate enriched with molasses by mixed culture of *K. marxianus* Y42 and *S. cerevisiae* CFCC39. This was shown to be the result of many factors : The diauxic behaviour of *K. marxianus* Y42 in the presence of sucrose and lactose together caused sucrose to be

utilized first ; ethanol inhibition slowed down lactose uptake ; fermentation was inhibited by molasses and there was difficulty in maintaining a high population of *K. marxianus* Y42 because of cell washout, caused by channelling, gas slug formation and the only moderately flocculent nature of *K. marxianus* Y42.

CHAPTER 6

FLOCCULATION TESTS

6.1 INTRODUCTION

Tower fermentation of whey permeate to ethanol requires a flocculent lactose-fermenting yeast. There are no published reports of flocculent lactose-fermenting yeast species. There was a need, therefore, to select for such an organism. Thus, the experiments described in the following sections were performed. Once the desired yeast was obtained, its flocculent behaviour in various media was studied, including its behaviour in the presence of *S.cerevisiae* in the same medium. It was intended to utilize both yeast species together to ferment a mixed substrate consisting of whey permeate enriched with molasses.

6.2 TEST MEDIA

The compositions of the various media used in these experiments have been included here (table 6.1 to 6.6) for convenience during the presentation and discussion of results. All media were sterilized as described in sect.3.3.1.

The pH value was adjusted by the addition of sulphuric acid or calcium hydroxide. Sodium hydroxide was not used for pH adjustment because sodium ions may interfere with certain sites on yeast cell walls which normally complex with calcium ions to form flocs with other cells (Stewart & Goring 1976).

6.2.1 Glossary of abbreviations used in flocculation tests

A	Aluminium sulphate, $Al_2(SO_4)_3$
B	Broth
Ca	Calcium sulphate, $CaSO_4$
CB	Yeast cleaning buffer ($CaSO_4$ wash)
CC39	*S. cerevisiae* CFCC39
EF	Extremely flocculent
F	Medium was membrane filtered (0.45µ)
FM	Flocculation medium (acetate buffer)
G	Glucose

KM Y42	*K. marxianus* Y42
M	Malt exract broth (Difco)
M*	Malt extract broth (Oxoid)
Ma	Maltose
MBN	Modified Burn's number
MBN*	Non-standard modified Burn's number
Me	Malt extract powder medium
MF	Moderately flocculent
Mo	Molasses
Ms	Malt extract syrup (Maltexo)
na	Data not available
NF	Non-flocculent
P	Whey permeate
Pe	Peptone
P4.6	Whey permeate with no pH correction
R	Rough
SC146	*S. cerevisiae* FT146
SM	Spent malt extract broth
TS	Subcultured from the bottom of the tower fermenter
VF	Very flocculent
WF	Weakly flocculent
Y	Yeast extract powder
YM	Yeast-malt extract broth
10	10●g/l whey extract solution
44	Ratio of lactose to sucrose of 4●:40 g/l
46	Ratio of lactose to sucrose of 4C:60 g/l
5	pH 5.0

Table 6.1 Whey permeate as the basic medium.

1. P Whey permeate containing approximately 40 g/l lactose ; initial pH was 4.6 ; adjusted to 5.0.

1F. PF Whey permeate, membrane filtered after autoclaving to extract precipitate ; pH adjusted to 5.0 before autoclaving.

2. P4.6 Whey permeate at pH 4.6 ; no pH adjustment or precipitate removal after autoclaving.

2F. P4.6F As for 2.P4.6 but membrane filtered after autoclaving.

3. PCa Whey permeate plus 0.5 g/l calcium chloride which was added after autoclaving ; pH adjusted to 5.0.

3F. PCaF As for 3.PCa but membrane filtered after autoclaving and before calcium chloride was added.

4. PY Whey permeate plus yeast extract powder (1 g/l) ; pH adjusted to 5.0.

5. PYM Whey permeate plus yeast extract powder (1 g/l) and malt extract powder (1 g/l) ; pH was 4.7 not adjusted.

6. PYMCa Whey permeate plus yeast extract powder (1 g/l) ; malt extract powder (1 g/l) ; and calcium chloride (0.5 g/l) ; pH 4.6 ; no adjustment.

7. PXM Whey permeate plus malt extract broth powder (15 g/l) ; no pH adjustment.

8. PXM5 As for 7.PXM but pH adjusted to 5.0.

8F. PXM5F As for 8.PXM5 but membrane filtered after autoclaving.

9. PMs Whey permeate plus malt extract syrup (20 g/l) (Maltexo), Vegemite (20 g/l) ; and 0.5 g/l of each of calcium chloride ; diammonium sulphate ; diammonium hydrogenphosphate ; pH adjusted to 5.0 .

10. PPe Whey permeate plus peptone powder (3 g/l) ; pH not adjusted ; membrane filtered.

11. PUAm Whey permeate plus urea (1 g/l) and diammonium hydrogenphosphate (0.5 g/l) ; pH adjusted to 5.0 ; membrane filtered.

12. P10 Whey permeate with added lactose to 100 g/l ; pH adjusted to 5.0.

13. PMo46 Whey permeate plus molasses ; lactose to sucrose ratio of 40 to 60 g/l ; 1 g/l urea ; 0.5 g/l diammonium hydrogenphosphate ; pH adjusted to 5.0.

13F. PMo46F As for 13.PMo46 but membrane filtered after autoclaving.

14. PMo44 As for 13.PMo46 but the sucrose concentration was 4.0 g/l.

15. PB Whey permeate containing 40 g/l sucrose ; 1 g/l malt extract powder ; 1 g/l yeast extract ; 0.5 g/l of each of diammonium sulphate ; diammonium hydrogenphosphate ; & calcium chloride ; no pH adjustment .

16. PB5 As for 15.PB but pH adjusted to 5.0.

16F. PB5F As for 16.PB but membrane filtered after autoclaving.

17. PMoYMCa As for 6.PYMCa but molasses was added to give 60 g/l

sucrose ; pH was not adjusted.

17F.PMoYMCaF As for 17.PMoYMCa but membrane filtered afted autoclaving.

Table 6.2 Molasses medium.

18. Mo Molasses medium containing 100 g/l sucrose ; 1 g/l urea ; 0.5 g/l diammonium hydrogenphosphate ; pH adjusted to 5.0.

Table 6.3 Lactose as the sole sugar source.

19. LYA Lactose (40 g/l) plus 0.5 g/l aluminium sulphate ; 0.5 g/l dihydrogen sulphate ; 0.5 g/l dipotassium hydrogenphosphate ; 1 g/l yeast extract powder ; 2 g/l yeast nitrogen base ; pH adjusted to 5.0.

20. LYCa As for 19.LYA with calcium chloride (0.5 g/l) instead of aluminium sulphate ; pH 4.8 no adjustment.

21. LSM Lactose (40 g/l) was added to spent malt extract broth ; pH adjusted to 5.0.

Table 6.4 Maltose as the sole sugar source.

22. Ma Maltose (10 g/l) plus yeast nitrogen base (2 g/l) ; ammonium chloride (1 g/l) and dipotassium hydrogenphosphate (0.5 g/l) ; pH adjusted to 5.0.

23. MaCa As for 22.Ma but calcium chloride (0.5 g/l) was added.

24. MaCa4 As for 23.MaCa but maltose content was increased to 40 g/l.

Table 6.5 Glucose as the sole sugar source.

25. G As for 24.MaCa4 but glucose (40 g/l) replaced maltose.

26. GCa As for 25.G but with calcium chloride (0.5 g/l).

Table 6.6 Prepared media.

27. YM Yeast-malt extract broth ; pH adjusted to 5.0.
28. YMCa As for 27.YM but with calcium chloride (0.5 g/l).
29. YMA As for 27.YM but with aluminium sulphate (0.5 g/l).
29F. YMCaF As for 29.YMA but membrane filtered after autoclaving.
30. M Malt extract broth (Difco) ; pH adjusted to 5.0.
31. M* Malt extract broth (Oxoid Ltd., Basingstoke, UK) (40 g/l) ; pH adjusted to 5.0.
32. MCa As for 30.M but with calcium chloride (0.5 g/l).
33. Ms Malt extract syrup (Maltexo) (50 g/l) ; pH adjusted to 5.0 ; membrane filtered after autoclaving.
34. Me Malt extract powder (15 g/l) ; pH adjusted to 5.0.

Table 6.7 Flocculence measurement media.

35. CB Yeast cleaning buffer (calcium sulphate wash) ; aqueous solution of calcium sulphate (0.5 g/l).
36. FM Flocculation medium (acetate buffer) contained calcium sulphate (0.5 g/l) ; sodium acetate (6.8 g/l) ; glacial acetic acid (4.05 g/l) in deionized water ; pH 4.9.

6.3 FLOCCULATION TEST RESULTS

These experiments were intended to select flocculent yeasts suitable for tower fermentation of whey permeate.

6.3.1 Modified Burn's number and flocculation testing methods used

(a) Flocculation test media The standard method for measuring modified Burn's number (MBN) (Greenshields et al 1972) requires the yeast to be grown in a very concentrated malt extract solution (200 g/l) which has been treated with pepsin and trypsin to make the solution protein free and then filtered to extract precipitates which could interfere with the flocculation test. This medium was found to be unsuitable for lactose fermenting yeasts which generally cannot utilize the maltose present in the malt extract. *K. marxianus* Y42 is an exception to this, since it can ferment both maltose and lactose.

Malt extract solution was found to give poor growth and to be difficult to prepare.

Normal malt extract broth (15 g/l), using 100 ml volume in a 250 ml flask, yielded approximately 1 gm of wet yeast which was sufficient for the flocculation test. This medium was reasonably clear after autoclaving. If more yeast cells were required, they could be prepared easily by increasing the number of shake flasks. The modified Burn's numbers determined using normal malt extract broth were of the same order as those attained using malt extract solution. This study obtained MBN = 172 for strain CC39 while Coote's (1974) value for a strain of *S. cerevisiae* (CFCC54), the most flocculating yeast tested by him, was 170. The main advantage of malt extract is that it is easier to prepare and to standardize since it is a commercially prepared medium.

In order to carry out flocculation tests on lactose fermenting yeasts, it was found to be more appropriate to grow them in whey permeate rather than in malt extract broth. The disadvantage of whey permeate was its tendency to precipitate upon autoclaving and its residual protein content. It also has a high content of calcium ions. It was found during the test that membrane filtration extracted components important for good growth and flocculence of KM Y42. However, flocculence tests using whey permeate were considered to be appropriate to the aim of this work, that is identifying flocculent lactose fermenting yeasts for growth in the tower fermenter.

The standard method required MBN measurement to be carried out using the flocculation medium (acetate buffer or 36.FM). This medium was found to reduce flocculence of KM Y42 grown in malt extract broth. In order to have a better indication of the yeast settling ability, it may be best to measure the flocculence in the medium that will be used for fermentation. The standard medium could be retained or used for comparing the flocculating ability of different yeast strains. It was with this concept in mind that many tests were carried out using the growth media or media of potential interest as the flocculation testing medium.

The summation of the slope of the yeast settling volume was defined as MBN* (sect.3.4.5 b). This, with an appropriate multiplication factor, should more correctly be called "the rate of yeast settlement" having a unit of "ml/minute".

(b) Technique for the determination of modified Burn's number Another shortcoming of the Sharp's modified Burn's method (Greenshields et al 1972) is the summation method for the slope of the settling volume curve. This involves the addition of the average slopes between 0-1, 1-5, 5-10, and 10-15 minutes which were taken from a curve plotted to the scale of 1 inch/2 ml and 1 inch/5 minutes. Another time zone which was found to be important was between 1-2 minutes. During this period some very flocculent yeasts settled at a much greater rate than between 2-5 minutes (see curves for strains CC39 in comparison with strains SC146 and KM Y42 in fig.6.1). In this situation it would be difficult to obtain a reasonably accurate average slope from the curve between 1-5 minutes. Consideration of this time zone would help to refine this method further.

(c) Alternative technique The calculation of the average slopes between 0-1, 1-5, 5-10, and 10-15 minutes using the yeast settled volume measured at 0,1,5, and 15 minutes, summation and then multiplication by 25 to convert the sum to Sharp's graphical scale (Greenshields et al 1972) will give the same MBN value without having to plot and estimate the slope as described for the original method.

(d) Flocculent scale method The method used to determine the flocculent scale from 0-5 is not an accurate method as it is subjective. However, it would be difficult to determine MBN value for each of tests carried out in this study, since The Sharp's modified Burn's number method is not sensitive enough for weakly flocculent yeasts. There were many sets of test conditions in which the strain KM Y42 was weakly flocculent. Thus, this subjective scale was used to grade quickly the flocculence of yeast after growth in various media, while the MBN values were determined only when the cells were very flocculent.

6.3.2 Flocculating ability of some lactose-fermenting yeast strains

Thirteen lactose-fermenting yeast strains, grown in whey permeate (medium 2.P4.6)), were tested for flocculation in the flocculation medium (medium 36.FM).

It was found that all strains except KM Y42 were non-flocculent (table 6.8). The strain KM Y42 was found to be weakly flocculent, forming small flocs which did not settle very rapidly. Thus, the

flocculence of KM Y42 in various media was selected for further investigation.

Table 6.8 Flocculating ability of some lactose-fermenting yeasts.

The yeasts were grown in whey permeate (medium 2.P4.6)
They were tested in flocculation medium (medium 36.FM)

yeast strains	flocculent scale	yeast strains	flocculent scale
1.CP 2234	NF 0	8. KM Y1109	NF 0
2.KL 416	NF 0	9. KM 7158	NF 0
3.KL 469	NF 0	10.KM XDRI	NF 0
4.KM 10022	NF ●	11.KM Y18	NF 0
5.KM 397	NF 0	12.KM Y42	WF 2
6.KM 100	NF 0	13.KM 10[illegible]1●	NF ●
7.KM 587	NF 0		

Yeast number and species are as in table 3.1.

6.3.3 Observation of flocculence during shake flask fermentation

The yeasts were grown in various media using 100 ml of medium in 250 ml shake flasks. The culture was studied after 12, 24 and 48 hours fermentation time. The observed results are given in Appendix D.1.

(a) KM Y42 The flocculating behaviour of KM Y42 during growth in various media (Appendix D.1.1) was different from its behaviour when tested in the flocculation medium (36.FM). During early fermentation, there was no visible floc formation.

When cultivated in media in which this yeast showed good flocculation, however, flocs could be seen after 48 hours or at completion of fermentation. Media in which this was observed were :
4. PY ; 5. PYM ; 7. PXM ; 8. PXM5 ; 9. PMs ; 15.PB ; 16.PB5
27.YM ; 28.YMCa ; 29.YMA ; 30.M ; 32.MCa ; and 33.Ms.
The flocs formed were small, less than 0.5 mm in diameter.

As a general observation, if a medium supported good yeast growth then some degree of flocculence was observed. This varied from poorly

flocculent to very flocculent except in the following media in which flocculence was not seen :

1. PF ; 3F.PCaF ; 10.PPe ; 12.P10 ; 13.PMo46F
21.LSM ; 22.Ma ; 23.MaCa ; 24.MaCa4

A heat precipitated component removed on filtration appeared necessary for good flocculence since those media which had been filtered (name code ending in F) supported poor flocculation.

When comparing the results observed in whey permeate (1.P) and in whey permeate with added peptone (10.PPe), it was observed that the presence of peptone appeared to discourage flocculation.

The spent malt extract broth with added lactose (21.LSM) also supported poor flocculation. This was attributed to a lack of nutrients necessary for yeast growth and flocculation.

It was considered that glucose or maltose might have an important role on the flocculence of KM Y42, since the yeast was highly flocculent when grown in malt extract broth. Thus, tests were carried out in minimal media with either of these two sugars as the only sugar source. The growth and the yield was found to be poor (22.Ma ; 23.MaCa ; 24.MaCa4 ; 25.G ; and 26.GCa). The cells showed poor flocculence in the growth media.

(b) KM Y42(TS) This strain was isolated from a tower fermentation of KM Y42 in whey permeate. When tested in a medium consisting of whey permeate with added malt extract syrup (9.PMs) it showed very good flocculation, forming flocs after 24 hours fermentation time. It also showed good flocculation when grown in whey permeate (1.P and 2.P4.6).

(c) *S. cerevisiae* FT146 (SC146) This yeast strain showed good growth and flocculence in molasses (18.Mo) and malt extract broth (30.M) (Appendix D.1.2). The flocs formed rapidly after 12 hours fermentation time. Their size was slightly larger than those KM Y42, but less than 0.5 mm in diameter.

(d) *S. cerevisiae* CFCC39 (CC39) This yeast strain was found to be very flocculent in molasses (18.Mo) and in malt extract broth (30.M). Large spherical flocs (0.5 mm in diameter) formed rapidly after 12 hours fermentation time (Appendix D.1.3).

(e) SC146 + KM Y42 These two yeast strains when grown together in whey permeate enriched with molasses (13.PMo46) were found to flocculate moderately (Appendix D.1.2). The floc sizes were less than

0.5 mm in diameter.

(f) CC39 + KM Y42 Flocculation occurred within 12 hours when these two strains were grown together in whey permeate enriched with molasses (13.PMo46). The floc size varied from 0.1 to 0.5 mm in diameter and some were larger than those formed by the mixture of SC146 + KM Y42 ((e) above).

In summary, KM Y42 is moderately flocculent when grown in certain media. This flocculence varied considerably between different media. SC146 was also moderately flocculent. In contrast, CC39 was very flocculent. The mixed culture of SC146 and KM Y42 was only moderately flocculent while the mixed culture of CC39 and KM Y42 was very flocculent.

6.3.4 Flocculation of KM Y42 grown in whey permeate with no additive

The results described here and in the remaining sections as far as section 6.3.13 were condensed from a full description of the results given in Appendix D.2. The flocculent scales are given for tests in the standard flocculation testing medium (36.FM) and in the growth medium where data are available.

The original KM Y42 culture was weakly flocculent in whey permeate (1.P and 2.P4.6) (table 6.9).

Table 6.9 Flocculation of KM Y42 grown in whey permeate with no additive.

pH_i = initial pH ; 36.FM = flocculation medium
(TS) = strain subcultured from tower fermenter

growth medium	KM Y42			KM Y42(TS)		
	pH_i	flocculent scale		pH_i	flocculent scale	
		36.FM	growth medium		36.FM	growth medium
1. P	5.0	1	1	5.0	5	4
1F.PF	5.0	1	na	5.0	1	na
2. P4.6	4.6	1	1	4.6	5	5
				4.7	4	4
2F.P4.6F	4.6	1	1	4.6	1	1

However, the subculture (KM Y42(TS)) taken from the tower fermenter was very flocculent in similar media. This subculture was initially prepared in whey permeate with added malt extract (9.PMs) before it was inoculated into the tower.

Cells of both strains that were cultured in membrane filtered whey permeate (1F.PF and 2F.P4.6F) showed poor flocculation.

6.3.5 The effect of initial medium pH on flocculation of KM Y42 grown in whey permeate

A comparison can be made in only two cases, where the difference between media was solely one of pH value (table 6.10). That is , between media 2.P4.6 and 1.P ; and between 2F.P4.6F and 1F.PF. No differences in flocculence between the cells grown at these two pH (pH 4.6 and pH 5.0) were observed.

Table 6.10 Flocculation of KM Y42 grown in whey permeate with additives at pH of 4.6 and 5.0

pH 4.6			pH 5.0		
growth medium	flocculent scale		growth medium	flocculent scale	
	36.FM	growth medium		36.FM	growth medium
2. P4.6	1	1	1. P	1	1
2. P4.6(TS)	5	5	1. P(TS)	5	4
2. P4.6(TS)	4	4			
2F.P4.6F	1	1	1F.PF	1	na
2F.P4.6(TS)	1	1	1F.P4.6F(TS)	1	na

(TS) indicates subculture KM Y42(TS)

6.3.6 The effect of membrane filtration on flocculation of KM Y42 grown in whey permeate with additives

Media containing whey permeate formed a precipitate on autoclaving. This also occurred with a number of other media. It was considered that the presence of a precipitate might interfere with the measurement of flocculence. The effect of the removal of this precipitate by membrane filtration (0.45 μm) was, therefore, examined

(table 6.11).

Table 6.11 Flocculation of KM Y42 grown in whey permeate and additive : with and without membrane filtration

FILTRATION			NO FILTRATION		
growth medium	flocculent scale		growth medium	flocculent scale	
	36.FM	growth medium		36.FM	growth medium
1F. PF	1	na	1. P	1	1
2F. P4.6F	1	1	2. P4.6	1	1
3F. PCaF	1	1	3. PCa	1	1
8F. PXM5F	2	2	8. PXM5	4	na
13F.PMo46F	3	na	13.PMo46	4	na
15F.PB5F	1	1	15.PB5	5	5
17F.PMoYMCaF	2	na	17.PMoYMCa	2	1
Tower subculture KM Y42(TS)					
1F. PF(TS)	1	na	1. P(TS)	5	4
2F. P4.6F(TS)	1	1	2. P4.6(TS)	4,5	4,5
3F. PCaF(TS)	1	2	3. PCa(TS)	5	5

There was no difference in flocculation of the cells of KM Y42 cultivated in whey permeate (1.P vs 1F.PF ; 2.P4.6 vs 2F.P4.6F ; and 3.PCa vs 3F.PCaF). There was a reduction in the degree of flocculence of the cells that were grown in other media after filtration (8.PXM5 vs 8F.PXM5F ; 13.PMo46 vs 13F.PMo46F ; 15.PB5 vs 15F.PB5F).

This was also the case for the subculture KM Y42(TS) that was grown in whey permeate (1.P(TS) vs 1F.PF(TS) ; 2.P4.6(TS) vs 2F.P4.6F(TS)) and in whey permeate with added calcium (3.PCa(TS) vs 3.PCaF(TS)).

6.3.7 Flocculation of KM Y42 grown in whey permeate supplemented with organic nutrients

The addition of organic nutrients to whey permeate improved the flocculation of the original KM Y42 culture (table 6.12).

Table 6.12 Flocculation of KM Y42 grown in whey permeate supplemented with organic nutrients

ADDITION				NO ADDITION		
growth medium	organic nutrient	flocculent scale		growth medium	flocculent scale	
		36.FM	growth medium		36.FM	growth medium
4. PY	Y	3	na	1. P	1	1
5. PYM	YM	3	na			
7. PXM	M	4	na			
8. PXM5	M	4	na			
9. PMs	M	4,5	na			
10. PPe	Pe	2	na			
6. PYMCa	YM	3	na	3. PCa	1	1

The improvement was small when the additive was peptone.

6.3.8 Flocculation of KM Y42 grown in whey permeate supplemented with inorganic nutrients

The effects of added ammonium phosphate and urea were examined (table 6.13). The flocculation of cells grown in whey permeate with added urea and ammonium sulphate (11.PUAm) was slightly better than the cells grown on unsupplemented whey permeate (1.P).

Thus, the presence of these two inorganic nutrients gave a small improvement.

Table 6.13 Flocculation of KM Y42 grown in whey permeate supplemented with inorganic nutrients

growth medium	nutrient added	flocculent scale	
		36.FM	growth medium
1. P	none	1	1
11. PUAm	Am,U	2	na

6.3.9 Flocculation of KM Y42 grown in media supplemented with flocculation aids

Calcium and aluminium ions were added to some media as flocculation aids (table 6.14).

Table 6.14 Flocculation of KM Y42 grown in media supplemented with flocculation aids

WITH ADDITION				NO ADDITION		
growth medium	aid	flocculent scale		growth medium	flocculent scale	
		36.FM	growth medium		36.FM	growth medium
3. PCa	Ca	1	1	1. P	1	1
3F.PCaF	Ca	1	1	1F.PF	1	na
6. PYMCa	Ca	3	na	5. PYM	3	na
23.MaCa	Ca	0	na	22.Ma	0	na
26.GCa	Ca	2	na	25.G	3	na
28.YMCa	Ca	1	3	27.YM	1	2
29.YMA	A	5	3			
32.MCa	Ca	4	1	30.M	2	na

The addition of calcium ions to various media (3.PCa ; 3F.PCaF ; 6.PYMCa ; 23.MaCa ; 28.YMCa ; and 32.MCa) caused no significant change to flocculation behaviour of KM Y42. A slight improvement in flocculence was observed when calcium was added to malt extract medium (32.MCa vs 30.M). A small decrease in flocculence occurred when calcium was added to the glucose medium (26.GCa vs 25.G). The addition of aluminium ions to yeast-malt extract medium (29.YMA vs 27. YM) caused a marked improvement in flocculation when compared with both the yeast-malt extract alone (27.YM) or 28.YMCa in which calcium ions had been added.

On the other hand, the effects of addition of flocculation aids to some media (23.MaCa vs 22.Ma ; 26.GCa vs 25.G) were masked by poor growth, so the effects of the addition of flocculation aids on flocculation could not be observed independently.

6.3.10 Flocculation of KM Y42 grown in double-sugar substrates

Two sugars (maltose from malt extract and sucrose from molasses) were added to whey permeate. Flocculation of the cells grown in these media was generally good (table 6.15).

Table 6.15 Flocculation of KM Y42 grown in double-sugar substrates

growth medium	lactose plus	flocculent scale	
		36.FM	growth medium
5. PYM	maltose	3	na
6. PYMCa	maltose	3	na
7. PXM	maltose	4	na
8. PXM5	maltose	4	na
9. PMs	maltose	4,5	na
13.PMo46	maltose	4	1
15.PB	sucrose	5	na
16.PB5	sucrose	5	na
17.PMoYMCa	maltose sucrose	2	na

6.3.11 Flocculation of KM Y42 in different media

Flocculation medium (36.FM) was used as a testing medium for all yeast cells cultivated in different media. The most important media were whey permeate (2.P4.6) and whey permeate enriched with molasses (13.PMo46), since these were the media to be used in tower fermentations. Table 6.16 contains the results of tests in a variety of media, including the three test media described above.

When the cells were tested in whey permeate enriched with molasses (13.PMo46), there was a slight reduction in the degree of flocculence when compared with tests in whey permeate (2.P4.6) or flocculation medium (36.FM). Cells that were grown in certain media showed better flocculence when tested in the flocculation medium (36.FM) than in whey permeate (2.P4.6). (The growth media were : 1.P(TS) ; 4.PY ; 5.PYM ; 9.PMs ; 30.M ; 32.MCa ; and 34.Me) However, cells that were grown

in other media showed similar flocculence in both flocculation medium and whey permeate. One exception to this was the cells grown in a lactose broth (19.LYA), in which flocculation was better in whey permeate (2.P4.6) than in the flocculation medium.

The cells grown in malt extract broth and syrup (30.M ; 30.M(TS) ; 33.Ms) showed good flocculation during fermentation but flocculated poorly when tested in the flocculation medium (36.FM). This was not so for the cells grown in such media as 7.PXM ; 8.PXM5 ; 9.PMs ; 13.PMo46 ; and 16.PB5 which all contained whey permeate. These cells showed good flocculation in their growth media as well as in the flocculation medium (36.FM).

Table 6.16 Flocculation of KM Y42 in different media

growth medium	flocculent scale			growth medium	flocculent scale		
	36.FM	2.P4.6	13.PMo46		36.FM	2.P4.6	13.PMo46
1. P	1	1	na	19.LYA	1	2	na
1. P(TS)	5	4	na	19.LYA(TS)	4	4	na
2. P4.6	1	1	1	20.LYCa	3	3	2
2. P4.6(TS)	5,4	5,4	na	21.LSM	0	0	na
3. PCa	1	1	1	22.Ma	0	0	na
3. PCa(TS)	5	5	na	23.MaCa	0	0	na
4. PY	3	2	0	24.MaCa	0	0	na
5. PYM	3	2	0	25.G	3	3	na
6. PYMCa	3	3	0	26.GCa	2	2	na
7. PXM	4	4	2	27.YM	1	1	na
8. PXM5	4	0	na	28.YMCa	1	1	na
9. PMs	4,5	3	na	29.YMA	5	5	na
10.PPe	2	2	na	30.M	2	1	na
				30.M(TS)	3	2	na
13.PMo46	4	na	1	32.MCa	4	1	0
16.PB5	5	5	na	33.Ms	2	2	na
				30.Me	1	0	na

The cells grown in media 19.LYA, 21.LSM, 23.MaCa and MaCa4 showed poor flocculation in the testing media.

Thus, the flocculation medium (36.FM) was the most suitable medium for comparison of flocculence of KM Y42 cells grown on various media.

6.3.12 Flocculation of KM Y42 grown as mixed culture with either CC39 or SC146 in mixed substrate

These experiments were intended to study the flocculation of mixed cultures of KM Y42 + CC39 , and KM Y42 + SC146 when grown in whey permeate enriched with molasses (13.PMo46). The mixed culture of KM Y42 + CC39 was more flocculent than the mixed culture of KM Y42 + SC146, although both mixed cultures were very flocculent (table 6.17).

Table 6.17 Flocculation of KM Y42 grown as mixed culture with *S. cerevisiae* in mixed substrate

growth medium	KM Y42 and	36.FM		13.PMo46	
		scale	MBN*	scale	MBN*
13.PMo46	CC39	5	134	5	174
"	CC39	4	33	4	43
"	SC146	5	50	5	na
"	SC146	4	29	4	30
15.PB	SC146	5	na	na	na

The observed flocculence of both mixed cultures, as measured in the MBN*, was different in the growth medium compared with the flocculation medium, while the flocculent scale values were the same.

Using strain CC39, large differences in MBN* values were observed between tests carried out under apparently identical conditions. No explanation could be found for this behaviour.

6.3.13 Flocculation of strains CC39 and SC146

Yeast strain CC39 was found to be more flocculent than yeast strain SC146 (table 6.18). The observed flocculence of both yeast strains, as measured in the MBN*, was different in the growth medium compared with the flocculation medium. It should be noted here that

the MBN* values for cells grown in malt extract broth (30.M) that were tested in the flocculation medium are standard MBN values since the tests were carried out under standard conditions.

Both yeasts generally displayed good flocculence in molasses (18.Mo) and in malt extract broth (30.M and 31.M*) with some variations observed when different batches of cells were used. Yeast strain CC39 showed better flocculation if the inoculum were grown in either malt extract broth or yeast-malt extract broth before inoculation into molasses (18.Mo).

Thus, strain SC146 was only moderately flocculent while strain CC39 was extremely flocculent.

Table 6.18 Flocculation of strains CC39 and SC146

growth medium	CC39					SC146				
	pH_i	flocculence in				pH_i	flocculence in			
		36.FM		growth medium			36.FM		growth medium	
		scale	MBN*	scale	MBN*		scale	MBN*	scale	MBN*
18.Mo(M)	5.0	4	46	5	120					
18.Mo(M)	5.0	4	41	4	43					
18.Mo(Mo)	5.0	4	44	1	0	4.9	3	30	4	56
18.Mo(Mo)						5.0	3	25	3	31
18.Mo(YM)	5.0	5	154	4	40					
30.M	5.0	5	172	5	187	4.5	4	61	na	na
31.M	5.1	4	58	4	53	5.2	4	na	3	na
						4.9	3	52	4	47
						5.1	2	31	2	16

(M) - inoculum grown in malt extract broth
(Mo) - inoculum grown in molasses solution (100 g/l sucrose)
(YM) - inoculum grown in yeast malt extract broth

6.3.14 Flocculation curves

Sample flocculation curves are given in fig.6.1. Yeast strain CC39 settled to a small volume (2 ml) in 5 minutes (fig.6.1 a). The moderately flocculent strain SC146 showed a slower reduction in volume

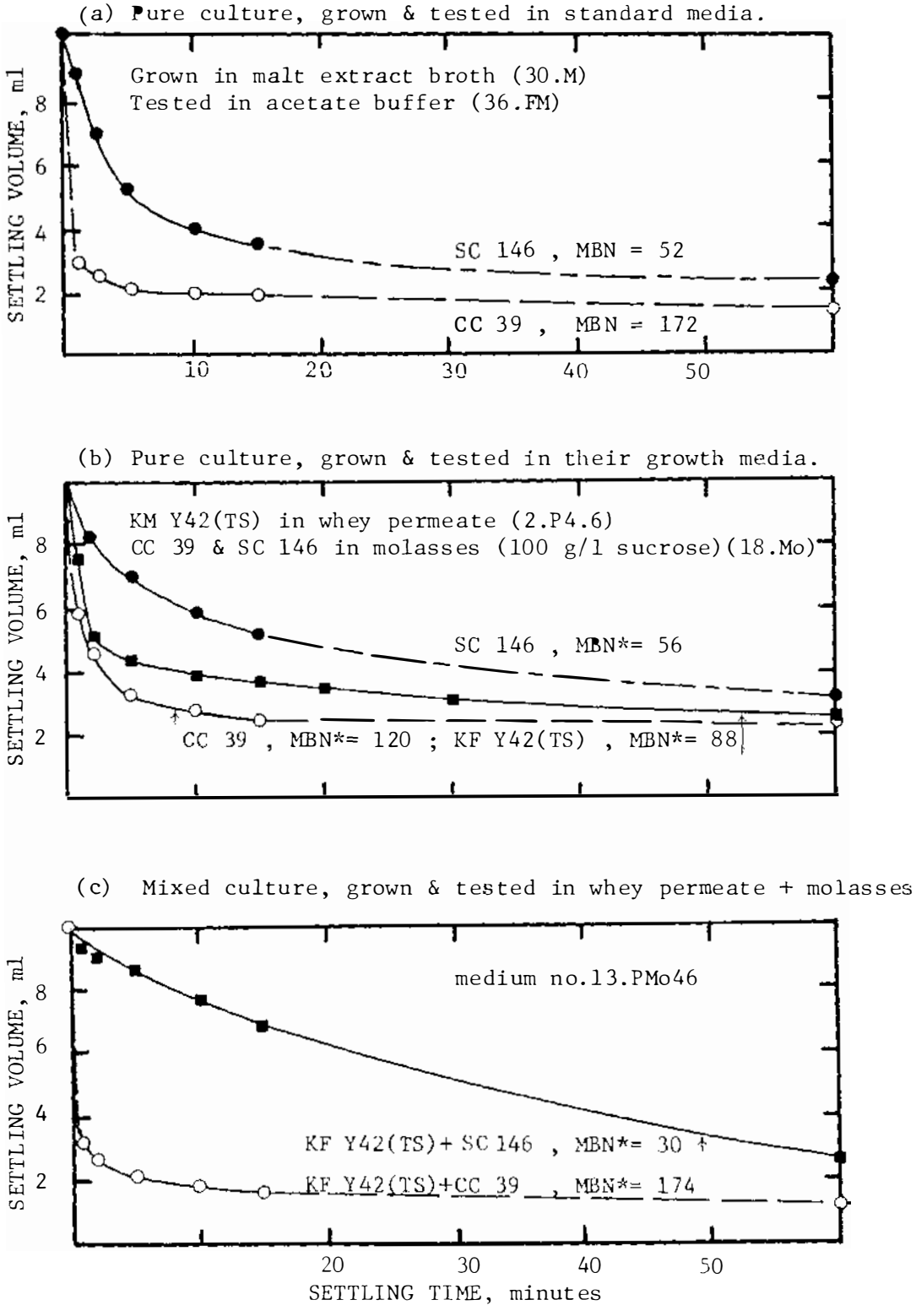


Fig.6.1 Flocculation of KM Y42(TS), SC146, and CC39 grown and tested in different media.

to 5.1 ml in 5 minutes. The MBN values were 172 and 52 for CC39 and SC146, respectively, when grown and tested in standard medium (A description of Sharp's modified Burn's number method is given in section 2.5.1 (c)).

Strain CC39 showed less flocculence (MBN 120) when it was grown and tested for flocculation in molasses (fig.6.1 b). The volume reduced to 3.1 ml after 5 minutes. Strain KM Y42(TS) gave an MBN value of 88 when grown and tested in whey permeate. The yeast settled volume was 4.2 ml after 5 minutes. Strain SC146 showed less flocculence in molasses giving an MBN* value of 56. Its volume reduced to 7.0 ml after 5 minutes.

A mixed culture of strains, CC39 and KM Y42, when grown and tested in whey permeate enriched with molasses gave a MBN* value of 174. The yeast volume reduced to 3 ml in 5 minutes. In comparison, a mixed culture of KM Y42 and SC146, when grown and tested in a similar medium gave a MBN* value of only 30. The yeast volume reduced to 9.5 ml after 5 minutes.

6.4 DISCUSSION

6.4.1 Flocculation of *K. marxianus* Y42

(a) Initial investigation During initial investigation, this yeast was not flocculent when grown in whey permeate. Further investigations showed that it flocculated strongly when grown in malt extract broth but cells from this medium flocculated rather poorly when tested in the flocculation medium. The addition of calcium chloride to malt extract broth improved flocculence slightly. The same behaviour was observed for cells grown in malt extract syrup. Malt extract powder by itself was not a very good growth medium for this yeast, and it flocculated poorly. If lactose were added to the spent malt extract broth, growth and yeast flocculence were found to be poor. These observations would tend to indicate that good flocculation of KM Y42 is related to rich media which contain many nutrients important for good growth.

(b) Membrane filtration Membrane filtration contributed toward the understanding of the flocculent behaviour of this yeast because the yeast grew and flocculated poorly in those media which had been

membrane filtered after autoclaving. This operation may extract some nutrients which are important for good growth and flocculation. Alternatively, the suspended particles, which would be removed by filtration could provide sites to trap cells during fermentation and thus eventually form flocs.

Thus, the flocculence of KM Y42 was affected by filtration of the media in which it originally showed good flocculence. This effect was probably a result of the removal of nutrients and precipitated particles that may be required for flocculation.

(c) The addition of yeast and malt extract broths to whey permeate The addition of yeast and malt extract to whey permeate resulted in good growth and moderate flocculation of KM Y42 in a number of media including the flocculation medium. The addition of calcium chloride did not result in better flocculation than that of cells grown in whey permeate enriched with yeast-malt extract broth. Flocculence of cells grown in whey permeate supplemented with either malt extract broth or malt extract syrup exhibited a reasonably stable flocculence when tested in the flocculation medium compared with those grown and flocculated in malt extract broth. The latter cells flocculated poorly in the flocculation medium.

(d) The addition of peptone, urea and diammonium hydrogen phosphate It was considered that peptone, which is generally added to the formulation of malt extract broth, may have made some contribution to the flocculence of this yeast. Its addition to the permeate resulted in weak flocculation both during fermentation and when tested in the flocculation medium.

The addition of urea and diammonium hydrogenphosphate to whey permeate produced cells of KM Y42 with similar flocculence to those grown in whey permeate with added peptone.

(e) Lactose, glucose or maltose as a carbon source The growth and the yield of cells in these media were found to be poor due to the low buffering capacity of these media. The final pH (approximately 3) was probably too acidic for good growth and flocculence of *K.marxianus* (Helm et al 1953). In general, cells cultivated in either glucose or lactose as the sole sugar source were found to have better flocculence than those grown in maltose. Thus, glucose and lactose are probably better substrates for production of flocculent yeast cells than maltose. It should be noted, however, that acidity of the media could

have exerted a great influence on the flocculence.

(f) Enriched whey permeate Flocculence of KM Y42 grown in whey permeate with added sucrose, malt extract, yeast extract and a few other nutrients was very good. When these cells were tested in flocculation medium and spent whey permeate, flocculence remained good. These cells formed more stable flocs than did the cells grown in malt extract broth and YM broth. This is an advantage since these cells would be able to tolerate changes in medium conditions. Growth and flocculence in whey permeate supplemented with molasses was good.

It should be noted, however, that these were rich media containing nutrients which contributed to good yeast growth. Thus, although flocculation was improved in these media, the effects of the presence of maltose and sucrose in whey permeate, on yeast flocculation could not be clearly isolated because of the presence of some other nutrients.

In contrast, when whey permeate was supplemented with yeast-malt extract and calcium as well as molasses flocculence decreased. This result could not be explained, although it could be an erroneous result in that the dark colour of the medium made flocculence difficult to observe.

(g) The addition of flocculation aids to the growth media The addition of aluminium sulphate or calcium chloride was found to improve flocculation in some media but this effect was not as profound as the influence of the medium composition. That is, the effect of the carbon source and non-specific growth factors source were more significant. The flocculation improvement observed after the addition of calcium to yeast or malt extract was probably because these media were deficient in calcium initially. Whey permeate contains sufficient quantity of calcium such that further addition of calcium caused no improvement to flocculation.

Thus, the addition of calcium as a flocculation aid provided no improvement on the flocculation of KM Y42 grown in whey permeate but improved flocculation when the base media were yeast or malt extract. Aluminium, on the other hand, improved the flocculation in yeast-malt extract medium.

(h) Medium pH The pH did not affect flocculence within the range used for most media (pH 4.6 - 5.0) but acidic pH was found to be associated with poor flocculence. The acidic pH (3) occurred because

of the poor buffering capacity of these media. The poor flocculence at low medium pH probably occurred as a result of the effect of the low pH on yeast growth.

(i) Subculture of KM Y42 The original strain KM Y42 was not as flocculent as the tower subculture (KM Y42(TS)) when grown in whey permeate or whey permeate supplemented with lactose (100 g/l).

Finally, whey permeate supplemented with malt extract syrup was the best medium for producing flocculent KM Y42. The tower fermenter subculture KM Y42(TS) was sufficiently flocculent to be used in the tower fermenter even though this ability varied considerably. Since none of the other lactose fermenting yeasts tested was flocculent, KM Y42 was the only choice of flocculent yeast available for use in the tower fermentation of whey permeate.

6.4.2 Flocculation of CC39 and SC146 grown as pure or mixed cultures with KM Y42

(a) SC146 The *S. cerevisiae* strain SC146 was found to be moderately flocculent. The mixed culture of this yeast strain and KM Y42 could be used in the tower fermentation since the MBN* obtained was in the same range as that obtained for SC146 alone, and this yeast had been used successfully in the tower fermenter (Appendix C). The flocs of the mixed culture were not spherical, but were serrated and small.

(b) CC39 The yeast strain CC39 was found to be an extremely flocculent yeast in most media tested including malt extract broth. The flocs were stable when tested in the flocculation medium and in the growth medium. It was also very flocculent when grown in a mixed culture with KM Y42 in whey permeate supplemented with molasses. The flocculence of the mixed culture was also stable in the flocculation medium. When CC39 was grown as a mixed culture with KM Y42 in whey permeate enriched with molasses, the flocculence was better than that of the mixed culture of KM Y42 + SC146. It was considered that mixed culture of KM Y42 + CC39 would be more suitable for tower fermentation of whey permeate/molasses mixtures since this mixed culture was very flocculent. This mixed culture would be able to remain in the tower fermenter at a higher medium feed rate than a mixed culture of KM Y42 + SC146.

(c) The effect of the inoculum-growth medium In some cases during flocculation tests, cells were prepared from the same medium on

different occasions and the resulting flocculence behaviour observed was variable. It was observed that the flocculence of CC39 was affected by the medium in which the inoculum was prepared. Flocculence observed for cells grown in molasses medium (18.Mo) was better if the inoculum were grown up in either malt extract broth (30.M) or yeast-malt extract broth (27.YM) rather than in molasses solution (18.Mo). This was a good indication of the influence that the growth environment, in this case substrate and nutrients, has on yeast flocculation.

6.5 CONCLUSIONS

K. marxianus Y42 was the only flocculent lactose-fermenting yeast identified. It was found to be moderately flocculent when grown in media which support good growth but showed poor flocculence when grown in acidic media or media which did not support good growth. It showed poor flocculence when grown in whey permeate and showed moderate flocculence when grown in whey permeate enriched with molasses. However, its subculture showed good flocculence in whey permeate.

6.6 SUMMARY

1. The hybrid yeast *K. marxianus* Y42 (KM Y42) was the only flocculent yeast found amongst the lactose-fermenting yeasts tested.

2. Flocculence of KM Y42 grown in acidic media or media which did not support good growth was poor but the yeast was moderately flocculent in many media which supported good growth.

3. The flocculence of KM Y42 cells grown in whey permeate supplemented with yeast extract or malt extract or malt extract syrup, to which ammonium sulphate, diammonium hydrogensulphate, and calcium chloride were added, was more stable than that of cells grown in malt extract or yeast-malt extract broth, even though the cells grown in the last two media flocculated readily.

4. The cells of KM Y42 grown in whey permeate were weakly flocculent. The subculture of KM Y42 taken from the tower fermenter was more flocculent than the parent strain. This subculture was initially prepared in whey permeate supplemented with malt extract syrup.

5. *S. cerevisiae* CC39 was an extremely flocculent yeast. It was more flocculent than the strains SC146 and KM Y42.

6. The mixed culture of KM Y42 + CC39 grown in whey permeate enriched with molasses (lactose to sucrose ratio of 40:60) was more flocculent than the mixed culture of KM Y42 + SC146, but not as flocculent as the strain CC39 grown in pure culture.

CHAPTER 7

MEDIUM OPTIMIZATION AND CULTURE IMPROVEMENT

7.1 INTRODUCTION

Medium optimization and batch fermentations were carried out using a different strain of *K.marxianus* from the one used for tower fermentation of whey permeate. This resulted in further investigations to improve ethanol tolerance of *K.marxianus*, to select for an isolate which showed no diauxic behaviour and to mutate *K.marxianus* using UV radiation in order to isolate a mutant which could not utilize sucrose. The resulting improved strain could then be used to provide genetic materials to produce a flocculent *K.marxianus* mutant which could tolerate high concentrations of ethanol, and was diauxic-negative or sucrose negative. The strain KM Y42 was not used in the mutation studies because it was not available at the time that these experiments were performed.

7.2 MEDIUM OPTIMIZATION

A factorial experiment was performed using a 3^3 design consisting of 14 runs (Webb 1971). Whey permeate (40g/l lactose) of volume 250ml was used in 500ml shake flasks (agitated at 5 rpm) and the yeast used was *K.marxianus* UCD FST 7158. Three variables were investigated: $(NH_4)_2SO_4$, K_2HPO_4 and yeast extract. A summary of fermentation results is given in appendix B.1. There was very little noticeable difference between each nutrient condition.

Statistical analysis of the results gave the following correlation:

$$E = 1.78 - .03N - .021K - .024Y + .0375NK + .035KY + .025NKY$$

Where E is ethanol concentration. The t-ratio of each nutrient is given in table 7.1.

Table 7.1: t Ratio of parameters

$N=(NH_4)_2SO_4$, $K=K_2HPO_4$, Y =Yeast extract. The null hypothesis was to consider whether N, K, and Y have any effect on ethanol production.

Parameter	N	K	Y	NK	KY	NKY
t-ratio	-.92	-.64	-.74	1.03	.96	.69

t-ratios at 90 & 95% confidence level for 7 degrees of freedom are 1.9 and 2.37 respectively. Regressed against ethanol concentrations at 30 h.

Further regression by dropping progressively the parameter with the lowest t-ratio did not yield a t-ratio greater than the 90% confidence level.

At zero nutrient addition level, the ethanol concentration was 19 g/l and the corresponding yield was 89%, after 30 hours which in this fermentation test was slightly greater than for other nutrient addition levels.

The shake flasks were agitated because it was found that still culture required too long a fermentation time and partially aerobic fermentation resulted in rapid ethanol production (Burgess & Kelly 1974).

Thus, nutrient supplementation of whey permeate was not necessary for ethanol fermentation by this *K.marxianus* strain .

7.3 ISOLATION OF AN ETHANOL-TOLERANT *K.marxianus*

7.3.1 Preliminary batch fermentation

(a) Whey permeate containing 40 g/l lactose It was found that *K.marxianus* UCD FST 7158 was able to ferment 40 g/l lactose in whey permeate (using 10 litres medium volume) to completion in 16 hours (fig.7.1). The residual lactose was 3 g/l (93% lactose utilization) and 18 g/l ethanol was produced in that time giving a yield of 92% ethanol on lactose utilized.

The results obtained were in good agreement with those obtained by other workers. Burgess & Kelly (1979) reported complete utilization of 50 g/l lactose in cheddar cheese whey permeate by two strains of *K.marxianus* (NRRL-Y-1109 and CBS 5795) in 18 and 12 hours, respectively ($28^{o}C$, in 250 ml shake flask). Industrial batch fermentation time of 16 hours was reported for the fermentation of deproteinated whey containing 44 g/l lactose (Howell & Tichbon 1980).

(b) Whey permeate containing 100 g/l lactose As a result of the previous fermentation, a further 10 litres batch fermentation was carried out using the same yeast strain but the whey permeate contained a greater lactose concentration of 100 g/l.

K.marxianus had not utilized all lactose available after 48 hours (fig.7.2) Lactose was reduced from 100 to 15 g/l during this time but only a small amount of lactose (1.6 g/l) was utilized between 35 and 48 hours. At 35 hours, 29 g/l of ethanol had been produced and then increased to 33 g/l after 48 hours. The final concentration of ethanol was produced after 69% of lactose was utilized resulting in 92% ethanol yield based on lactose utilized.

The result above showed that when the ethanol concentration reached 30 g/l, the fermentation activity of *K.marxianus* UCD FST 7158 reduced considerably. This was considered to have been a result of ethanol inhibition. It has been reported that 32 g/l ethanol could reduce the

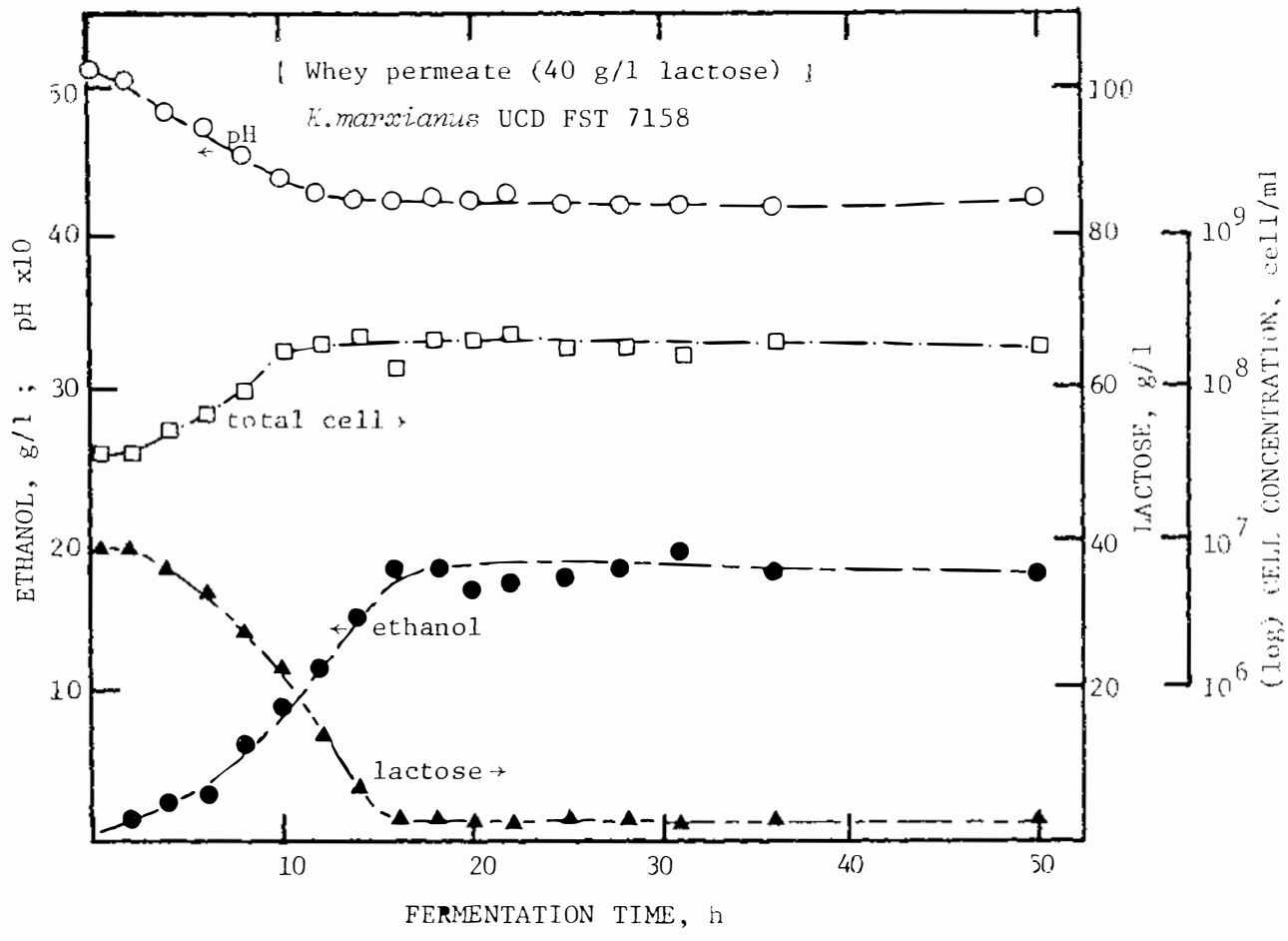


Fig.7.1 Batch fermentation of whey permeate (40 g/l lactose).

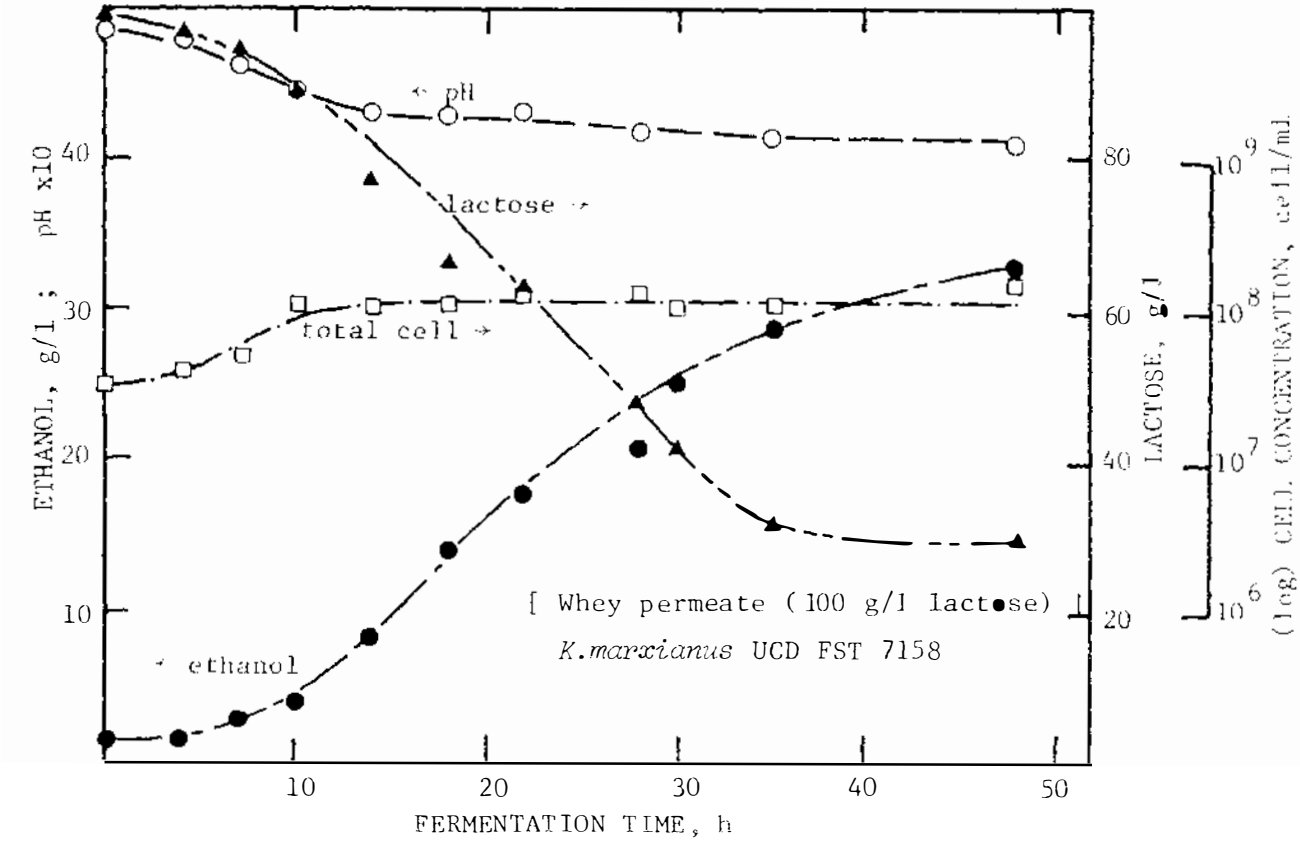


Fig.7.2 Batch fermentation of whey permeate (100 g/l lactose).

activity of β-galactosidase in *K.marxianus* NRRL Y-1109 by 77%. The inability of some *K.marxianus* strains to ferment concentrated lactose solutions of greater than 100 g/l has been reported by a number of workers (Yoo 1974 ; O'Leary et al 1977 ; Gawel & Kosikowski 1978). There have been reports of *K.marxianus* strains which withstand greater ethanol concentrations than 30 g/l during fermentation, but the fermentation conditions used were partially aerobic (Burgess & Kelly 1979 ; Moulin et al 1980).

(c) Whey permeate enriched with molasses

It was intended that whey permeate enriched with molasses would be used as a feed medium for tower fermentation. A batch fermentation study was necessary to observe the behaviour of *K.marxianus* in mixed culture with *S.cerevisiae* when the total sugar concentration was 100 g/l and to study whether ethanol inhibition would occur as found in the previous fermentation (sect. 7.3.1 b).

One 10 l batch fermentation was carried out using a lactose to sucrose ratio of 20:80 g/l (fig.7.3 a). It was found that little lactose was utilized. After 24 h, lactose had been reduced from 20 to 16 g/l (21% lactose utilization) whereas sucrose was reduced from 75 to 1 g/l (99% utilization). At this time, 35 g/l of ethanol had been produced. As the fermentation time increased to 50 h, lactose was reduced further to 11 g/l (45% lactose utilization). The resulting total sugar concentration reduced from 95 to 1.2 g/l (87% sugar utilization). The residual sugar was mainly lactose (93% lactose). By this time 38 g/l ethanol had been produced (87% ethanol yield on sugar utilized).

Similar results were obtained for another fermentation using a lactose to sucrose ratio of 10:90 g/l (fig.7.3). In this fermentation, lactose decreased from 10 to 7.3 g/l (30% lactose utilization) while sucrose reduced from 84 to 0.9 g/l (99% sucrose utilization) to produce 38 g/l ethanol, after 24 h. As the fermentation time increased to 48 h, lactose was reduced further to 5.5 g/l (47% lactose utilization) and the total sugar reduced from 94 to 6.3 g/l (93% sugar utilization). By this time, 39 g/l ethanol had been produced. This was 83% yield on sugar utilized.

The results presented here indicated that when sucrose and lactose were available simultaneously, sucrose would be utilized first at a very much faster rate than lactose. Lactose utilization did not occur until all sucrose was utilized, but by this time the ethanol concentration was greater than 30 g/l which could reduce β-galactosidase activity considerably (Wendorf et al 1970a). Hence, even though lactose utilization occurred,

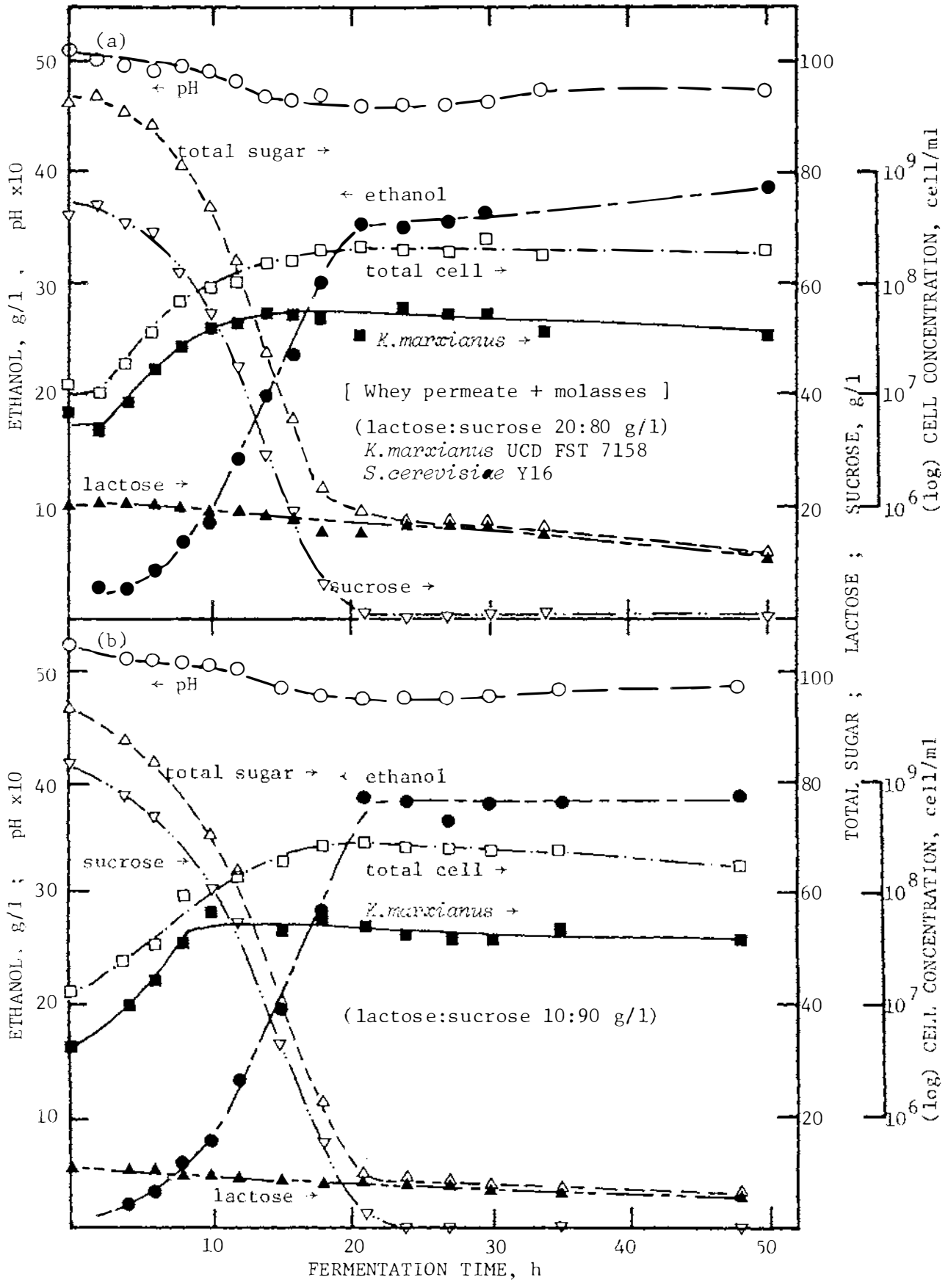


Fig. 7.3 Batch fermentation of whey permeate enriched with molasses using lactose : sucrose ratio of (a) 20:80, and (b) 10:90 g/l by *K.marxianus* and *S.cerevisiae*.

the rate of utilization was very slow due to ethanol inhibition. This resulted in only 45 to 47 % lactose utilization. Thus, there was only 87 to 93 % sugar utilization. Even though 99% sucrose uptake occurred after 24 hours, during the next 24 hours *K.marxianus* utilized only 45 to 47 % of the available 10 to 20 g/l lactose, respectively. In the batch fermentation of 40 g/l lactose, complete lactose utilization required only 16 hours. The lower *K.marxianus* concentration of only 4 to 6×10^7 cell/ml,in the mixed culture fermentation, when compared with a pure culture fermentation concentration of 1 to 2×10^8 cell/ml also contributed to this slower uptake rate.

It was considered that further 10 litre batch fermentation should be performed using equal concentrations of lactose and sucrose. The initial total sugar concentration should be low so that ethanol produced after the sucrose was all utilized would be low and would not therefore interfere with growth while the remaining lactose was utilized. The total sugar concentration used was 40 g/l as this was equivalent to the normal lactose concentration in whey permeate. *K. marxianus* was found to be able to ferment this concentration of sugar rapidly (sect.7.3.1 a).

When only *K. marxianus* was used (fig.7.4 a), sucrose was reduced from 19 to 1 g/l (96% utilization) while lactose was reduced from 24 to 21 g/l (16% utilization). after 15 hours. As fermentation time reached 27 hours, lactose was reduced further to 3 g/l (88% utilization). This gave 92% total sugar utilization and the residual sugar of 3.6 g/l was mainly lactose (83%). There was no further reduction in lactose concentration, after 48 hours. Ethanol produced was 18 g/l which was 85% yield on sugar utilized.

Similar results were obtained when *K. marxianus* and *S. cerevisiae* were used to ferment a similar medium (fig.7.4 b). Sucrose was reduced from 19 to 1.1 g/l (94% utilization) while lactose was reduced from 24 to 23 g/l (6% utilization), after 12 hours. As the fermentation time approached 27 hours, lactose was reduced further to 2.3 g/l (90% utilization). This gave 93% total sugar utilization. The total residual sugar of 2.9 g/l was 79% lactose. Ethanol produced was 18 g/l, a yield of 85% on sugar utilized.

In mixed culture fermentation, the ratio of *K. marxianus* to the total cell number could contribute considerably to the understanding of fermentation progress. Figure 7.5 shows the cell ratios of three fermentations using different lactose to sucrose ratios of (1) 20:80 (from sect. 7.3.1 (c) fig.7.3 a), (2) 10:90 (from sect. 7.3.1 (c) fig.7.3 b),

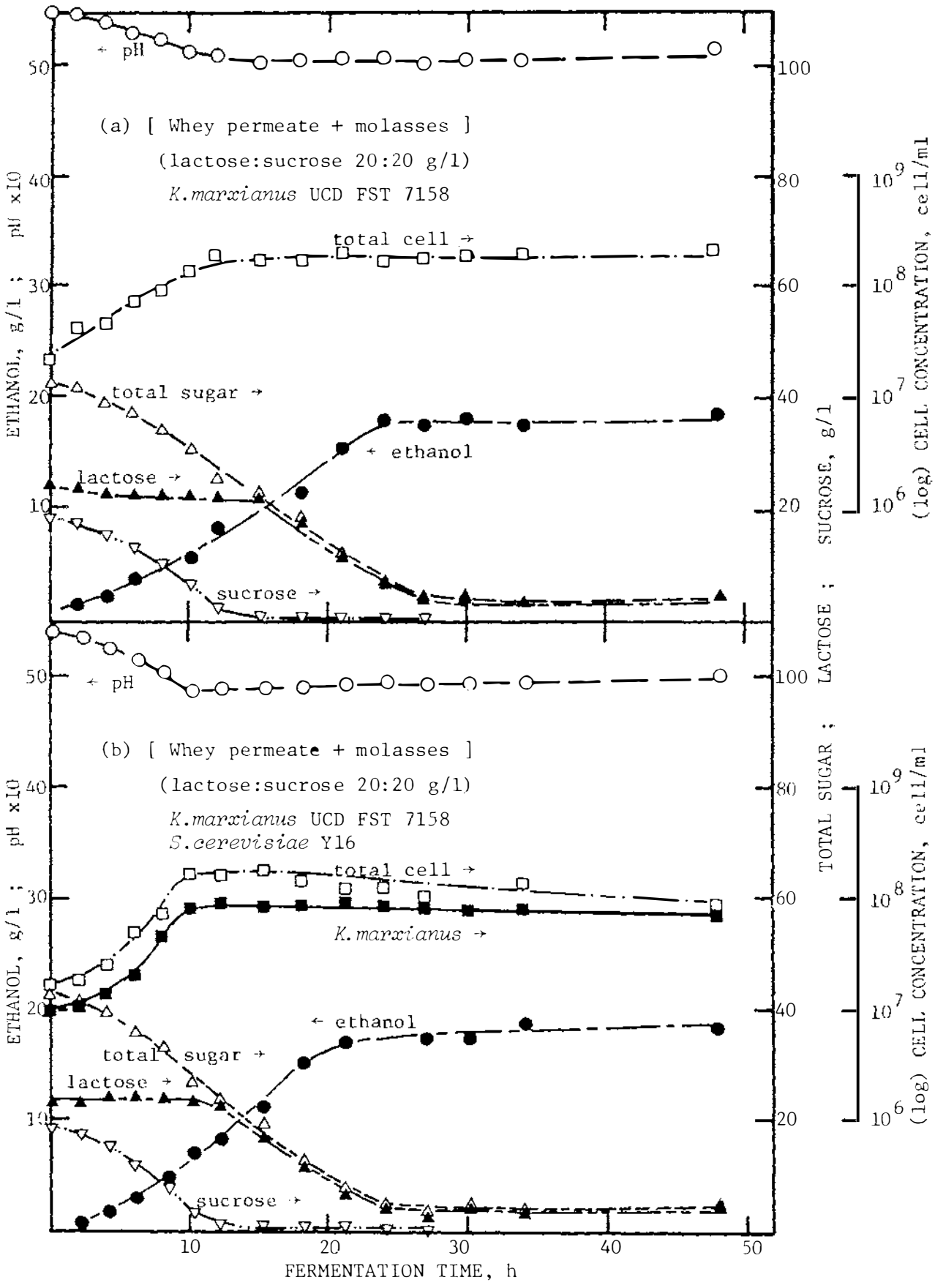


Fig.7.4 Diauxic behaviour study in the fermentation of whey permeate enriched with molasses. (a) *K.marxianus*, (b) *K.marxianus* & *S.cerevisiae*.

and (3) 20:20 (cf. fig.7.4 b). Curve 1 (20:80) shows that the initial *K.marxianus* was between 40 and 60 % of the total cell during the initial 12 hours. The ratio decreased slowly to 17% as fermentation time increased. In this fermentation (fig.7.3 a), sucrose was 98% utilized after 20 hours, from this time on *K.marxianus* was expected to consume the remaining lactose. It, however, failed to do so as shown by the very slow lactose uptake rate (fig. 7.3 a). The poor growth showed up as a decreasing cell ratio as fermentation proceeded in curve 1 of fig.7.5.

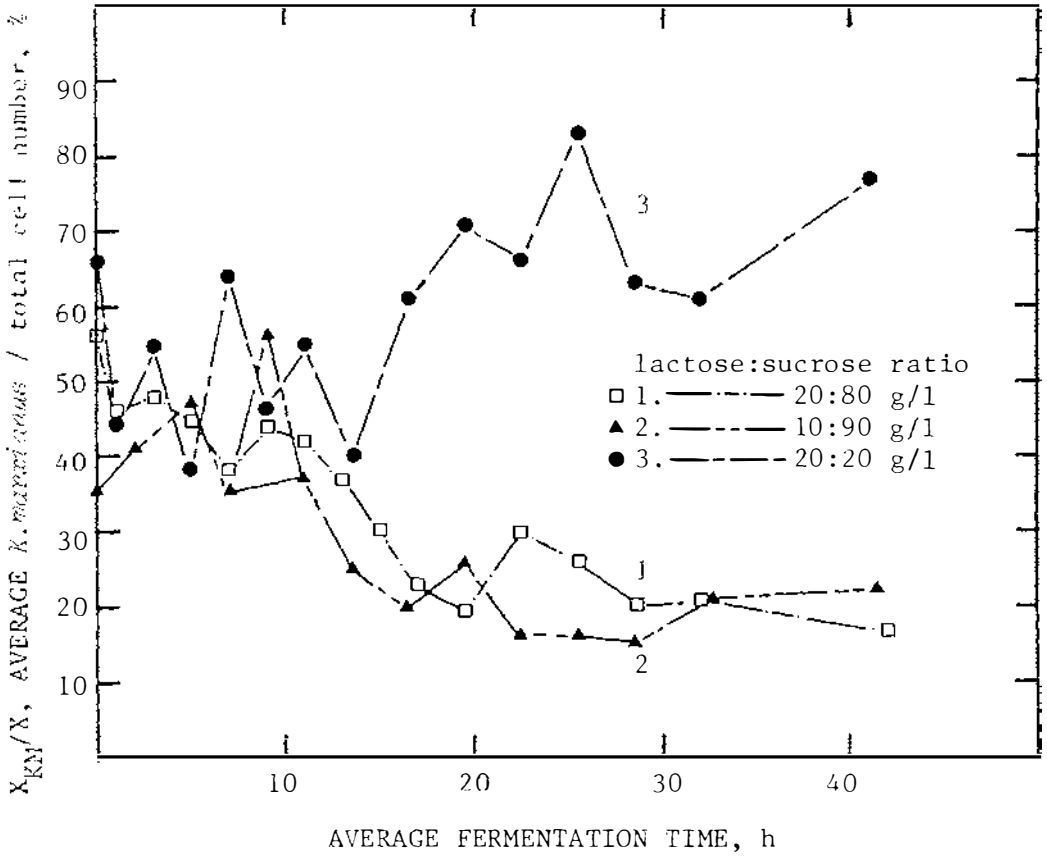


Fig. 7.5 *K.marxianus* : total cell number ratio vs fermentation time for mixed culture fermentation of whey permeate enriched with molasses using various lactose to sucrose ratios.

Curve 2 (10:90) followed a similar pattern. During the initial 10 hours, the ratio was between 35 to 56 %. It then decrease slowly to 22% after 48 hours. It was shown that there was 97% sucrose utilization after 20 hours (fig. 7.3 b) and there was very little lactose consumption after this time. In both cases, it was clear that ethanol was inhibiting the activity of *K.marxianus* and this in turn decreased the ratio of *K.marxianus* in the medium.

The cell ratio shown by curve 3 (20:20) increased from between 40 to 66 % during the first 14 hours to a maximum of 83% after 25 hours. This ratio remained greater than 60% for the rest of the fermentation when lactose uptake was high. Thus, the lower ethanol concentration of 18 g/l did not inhibit lactose uptake by *K.marxianus* after lactose was utilized.

The results have clearly shown that *K.marxianus* exhibited diauxic behaviour in the presence of sucrose and lactose. Sucrose was utilized first before lactose. When *K.marxianus* was used on the mixed substrate alone, it required 15 hours to consume 20 g/l lactose after sucrose was utilized. This was approximately the same length of time as the 16 hours required to consume 40 g/l lactose in the fermentation of whey permeate. Considering that *K.marxianus* made up only a portion of the total cell population the 15 hours required to consume half the lactose concentration was reasonable. By providing less sugar than the previous mixed substrate fermentation, there was less ethanol to inhibit fermentation and growth. Thus, there was an increase in the *K.marxianus* cell ratio when *K.marxianus* was consuming lactose after sucrose was used up. Sucrose was used up two hours earlier by mixed culture fermentation than by pure culture fermentation indicating that *S.cerevisiae* could utilize sucrose faster than *K.marxianus* .

Therefore, there was a need to isolate a *K.marxianus* strain which was less inhibited by ethanol and which did not exhibit diauxic behaviour.

7.3.2 Selection of ethanol tolerating isolate

It was found during initial batch fermentations (sect.7.3.1) that ethanol inhibited fermentative activity of *K.marxianus*, resulting in incomplete lactose utilization. Thus, an experiment was proposed to improve the ethanol tolerance of *K.marxianus*.

The experiment was carried out by serial subculturing of *K.marxianus* UCD FST 7158 on gradient whey agar plates and in whey permeate broths which contained an increasing amount of ethanol as the subculturing progressed (the procedure was described in sect.3.10.1). From this experiment, 4 yeast isolates were obtained, called KM10A, KM10B, KM10C, and KM10D. A fermentation comparison was then carried out in order to select the strain with the fastest lactose fermentation and greatest ethanol tolerance. The fermentation comparison was carried out in shake flask using whey permeate (100 ml) containing 100 g/l lactose.

Table 7.2 contains a summary of the results of the fermentation comparison. After 24 hours fermentation, the cell level of all strains reached between 3.3×10^8 and 4.5×10^8 cell/ml. Lactose was reduced to between

1.5 & 2.8 g/l, and all showed greater than 97% lactose utilization compared with the parent strain which utilized only 65% of the lactose present. All isolated strains utilized lactose at rates between 3.8 and 4.1 g/lh while the parent strain utilized lactose at a rate of 2.6 g/lh. At this time, all isolates produced more ethanol than the parent strain.

After 48 hours, the ethanol produced by the parent strain reached 44 g/l which was the same level as those produced by the isolates, 24 hours earlier.

Table 7.2 Comparison of fermentation ability of 4 ethanol tolerant isolates of *K.marxianus* UCD FST 7158.

isolate	pH	X cells/ml	lactose g/l	S_{lu} %	E g/l	Y %
0 hour						
parent	4.7	1.9×10^7	94			
KM10A	4.8	1.1×10^7	97			
KM10B	4.7	1.2×10^7	94			
KM10C	4.7	1.4×10^7	100			
KM10D	4.7	2.6×10^6	102			
24 hour						
parent	4.1	4.5×10^8	33	65	33	99
KM10A	4.0	3.4×10^8	2.0	98	39	71
KM10B	3.9	3.3×10^8	1.5	98	45	91
KM10C	3.9	4.2×10^8	2.2	98	38	73
KM10D	3.9	4.0×10^8	2.8	97	44	83
48 hour						
parent	4.1	4.8×10^8	6.4	93	43	93
KM10A	4.9	4.4×10^8	1.6	98	36	70
KM10B	4.8	3.8×10^8	1.4	99	38	76
KM10C	5.1	3.8×10^8	2.2	98	37	70
KM10D	4.3	3.8×10^8	2.6	97	37	70

S_{lu} - lactose utilization

The results show that the isolates utilized lactose at a rate which was almost double that of the parent. Strains KM10B and KM10D were considered to be better than the other two strains because they produced more ethanol after 24 hours. KM10D was chosen for further improvement.

KM10D was serially subcultured in whey broth containing 100 g/l lactose and 35 g/l added ethanol. The tenth and final culture was called KM10D10. The stability of the ethanol tolerance of this culture was then tested.

7.3.3 The stability of ethanol-tolerant isolate KM10D10

If the ethanol tolerance of strain KM10D10 were merely a phenotypic change, then it might be expected that tolerance would be lost when the organism was cultured in conditions which would lead to production of low concentrations of ethanol. A genotypic change would be expected to be stable. To test this hypothesis the isolate KM10D10 and the parent strains were serially subcultured onto a series of sucrose agar plates using a single colony to give approximately 60 generations of growth. A single colony from the last agar plate culture was used to inoculate whey permeate broth (containing 40 g/l ethanol) in shake flask incubated under the usual conditions. (Details of method are given in sect. 3.2.6 b.)

The results are shown in table 7.3. After 48 hours fermentation, all 4 KM10D10 samples had reduced the lactose to a residual level of 2.2 to 2.4 g/l, while only one parent sample reached the same level. Thus, the isolate obtained from the culture improvement appeared to be stable.

Table 7.3 Test of the stability of ethanol tolerating ability of *K.marxianus* mutant, KM10D10.

culture	pH	X cells/ml	lactose g/l	ethanol g/l
0 hour				
1.parent	5.0	$2.5\times10^{4}_{5}$	51	36
2. "	5.0	1.8×10^{5}	50	36
3. "	5.0	1.0×10^{5}	49	38
4. "	5.0	2.5×10^{4}	51	38
1.KM10D10	5.0	$2.5\times10^{4}_{5}$	51	35
2. "	5.0	1.0×10^{5}	53	38
3. "	5.0	2.5×10^{4}	52	39
4. "	5.0	2.5×10^{4}	53	37
24 hour				
1.parent	4.8	$4.0\times10^{7}_{7}$	51	36
2. "	4.8	5.2×10^{7}	50	37
3. "	4.8	1.5×10^{7}	48	38
4. "	4.8	2.2×10^{7}	50	35
1.KM10D10	4.7	$6.6\times10^{7}_{7}$	47	40
2. "	4.7	7.1×10^{7}	48	34
3. "	4.7	8.1×10^{7}	50	37
4. "	4.7	7.7×10^{7}	50	35
48 hour				
1.parent	4.4	$6.8\times10^{7}_{8}$	11	42
2. "	4.3	2.1×10^{8}	2.3	49
3. "	4.5	1.2×10^{8}	30	39
4. "	4.5	6.9×10^{7}	25	42
1.KM10D10	4.3	$3.4\times10^{8}_{8}$	2.3	47
2. "	4.3	3.4×10^{8}	2.2	46
3. "	4.3	2.3×10^{8}	2.2	44
4. "	4.3	2.7×10^{8}	2.4	47

7.3.4 Fermentation comparison of some lactose fermenting yeasts.

In this experiment, the ability of the isolate KM10D10 to ferment lactose was further compared with other lactose-fermenting yeasts. The comparison was carried out using 12 other yeast strains including the mutant parent strain. The names of these yeasts have been listed in sect. 3.1.4. Other yeast strains that have been reported to be rapid lactose fermenters but were not used in this fermentation comparison are : *C.pseudotropicalis* ATCC 8619 (Izaguirre & Castillo 1982), *C.pseudotropicalis* IP 513 (Moulin et al 1980), *K.marxianus* CBS 5795 (Burgess & Kelly 1979), and *K.marxianus* NRRL Y2415 (Moulin et al 1980).

The fermentation comparison was carried out in shake flask using whey permeate (100 g/l lactose) with 5% v/v inoculum. Samples were collected at 12 and 24 hours. Table 7.4 gives the results obtained after 24 hours fermentation.

Table 7.4 Summary of fermentation comparison of lactose fermenting yeasts after 24 hours fermentation.

Initial pH = 5.0, lactose = 100 g/l, and average cell number = 5.7×10^6 cells/ml
KM - *K.marxianus*, KL - *K.lactis*, CP - *C.pseudotropicalis*

no	yeast strain	pH	X cells/ml	ΔS_1 g/l	S_1' g/lh	E g/l	E' g/lh	Y %
1	KM10D10	3.7	4.2×10^8	81	3.4	42	1.7	84
2	KM7518	4.1	3.2×10^8	81	3.4	25	1.1	68
3	KMY42	4.0	3.2×10^8	82	3.4	29	1.2	61
4	KMY18	3.9	4.3×10^8	71	3.0	28	1.2	72
5	KMY1109	3.9	4.9×10^8	79	3.3	33	1.4	77
6	KM100	3.9	5.4×10^8	92	3.8	30	1.3	61
7	KM397	3.6	4.1×10^8	95	3.9	34	1.4	67
8	KM587	5.7	5.9×10^8	12	0.5	0.5	0.02	8
9	KM10022	4.0	4.6×10^8	81	3.4	39	1.6	88
10	KMXDRI	4.3	2.2×10^8	41	1.7	17	0.7	67
11	KL416	4.2	2.6×10^8	41	1.7	12	0.5	52
12	KL469	4.5	3.6×10^8	19	0.8	5.4	0.2	5.4
13	CP2234	4.3	3.2×10^8	51	2.1	18	0.8	66

ΔS_1 - lactose consumed
S_1' - volumetric rate of lactose utilization
E' - volumetric rate of ethanol production

At this time, the cell concentrations for all yeasts were between 2 and $6x10^8$ cells/ml. Most *K.marxianus* strains utilized between 70 and 95 g/l lactose, except for yeast no.8 *K.marxianus* NCYC 587 and no. 10 *K.marxianus* XDRI.

The strain KM10D10 (no.1) produced the greatest amount of ethanol (42 g/l) at a rate of 1.7 g/lh, and a yield of 84%. *K.marxianus* ATCC 10022 (no.9) was the next highest ethanol producer. It produced 39 g/l ethanol, at a rate of 1.6 g/lh and a yield of 88%.

After 48 hours fermentation, the same trend remained. All *K.marxianus* strains and *C.pseudotropicalis* utilized 96-98% of lactose available except yeasts no.8 & no.10. The two *K.lactis* strains utilized only 50-56% of the lactose available.

In summary, most of the *K.marxianus* strains tested were rapid lactose fermenters. The *C.pseudotropicalis* strain tested was a moderately rapid lactose fermenter,while the two *K.lactis* strains were slow lactose fermenters. The mutant, KM10D10, was a rapid lactose fermenter and produced more ethanol than the other yeasts tested.

7.3.5 10 l batch fermentations of ethanol-tolerant isolate

For purposes of comparison with the parent strain, it was considered necessary to study the performance of the isolate KM10D10 in the larger scale fermenter and in the joint presence of lactose and sucrose.

In this experiment two 10 litre batch fermentations were carried out using two different media. These were :

1. Whey permeate (100 g/l lactose)
2. Whey permeate enriched with molasses, using lactose to sucrose ratio of 40:60.

Samples were collected at various time intervals up to 48 hours.

(a) Whey permeate containing 100 g/l lactose Figure 7.6 shows the behaviour of the fermentation with time. There was 91% lactose utilization after 48 hours and the residual lactose was 9 g/l. Ethanol was produced steadily, reaching a concentration of 41 g/l after 48 hours. The cell level at inoculation was $2x10^6$ cells/ml and reached $2x10^8$ cells/ml after 24 hours.

In contrast, at 48 hours, the parent strain had utilized only 69% lactose, 33 g/l ethanol was produced (table 7.5)(sect.7.3.1 b).

It is evident from the results that in the 10 l scale fermentation, KM10D10 utilized lactose to produce ethanol more rapidly than the parent.

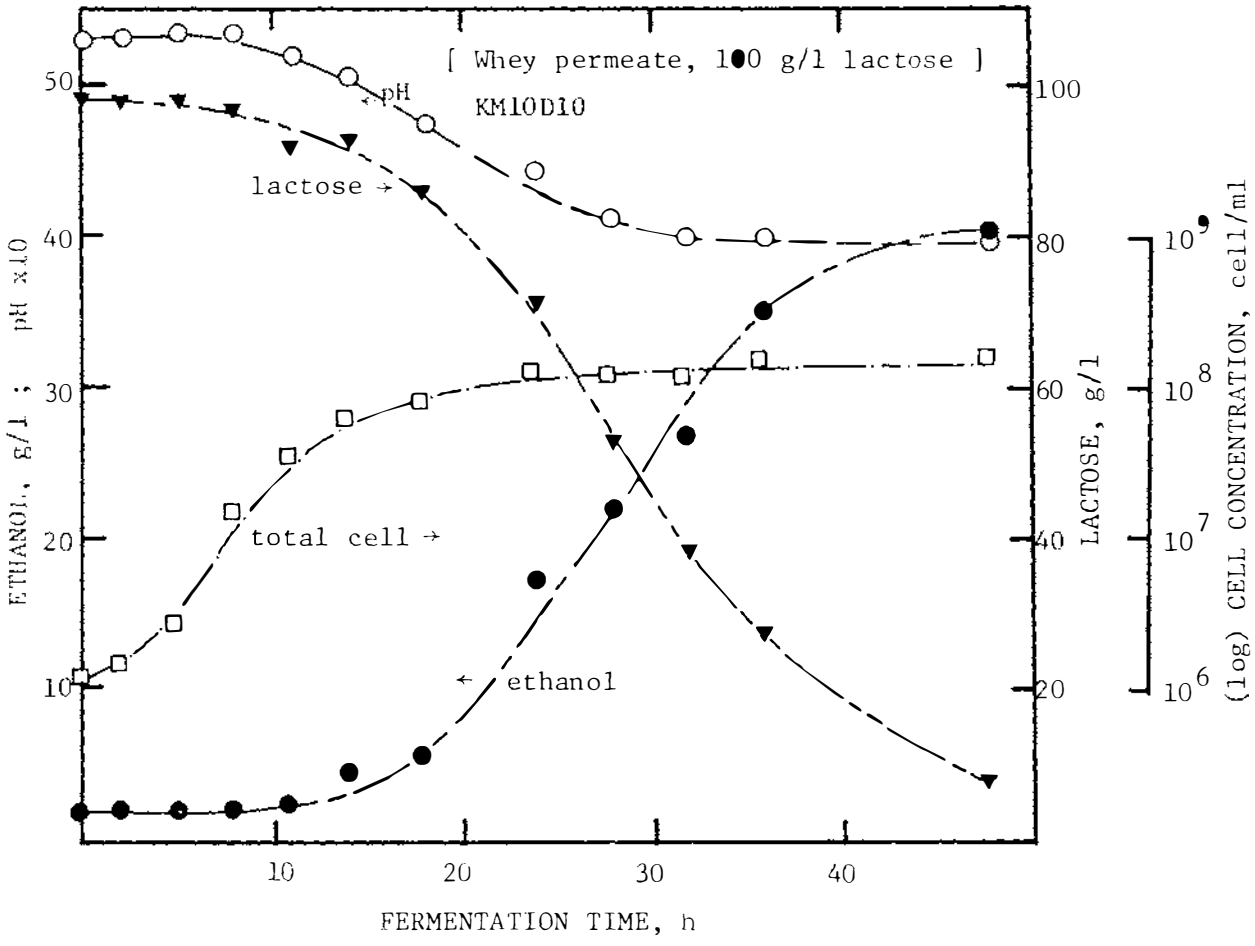


Fig.7.6 10 litre batch fermentation of whey permeate (100 g/l lactose) by KM10D10.

Table 7.5 Comparison of lactose utilization and ethanol production of 10 litre batch fermentation of whey permeate (100 g/l lactose) by KM10D10 and parent strain.

strain	S_1 (g/l) 0 hour	S_1 (g/l) 48 hour	ΔS_1 (g/l)	E (g/l)	Y (%)
parent	98	30	68	33	87
KM10D10	99	9.0	90	40	79

(b) Whey permeate enriched molasses (lactose:sucrose 40:60 g/l)

The ratio 40:60 g/l lactose to sucrose was chosen because this was the sugar ratio used during mixed substrate tower fermentation (Chapter 5). The results of this batch fermentation are shown in fig.7.7.

Sucrose was completely utilized after 32 hours while lactose, during this period, was utilized very little. Consumption of lactose began after 32 hours. After 48 hours, only 14% of lactose had been utilized.

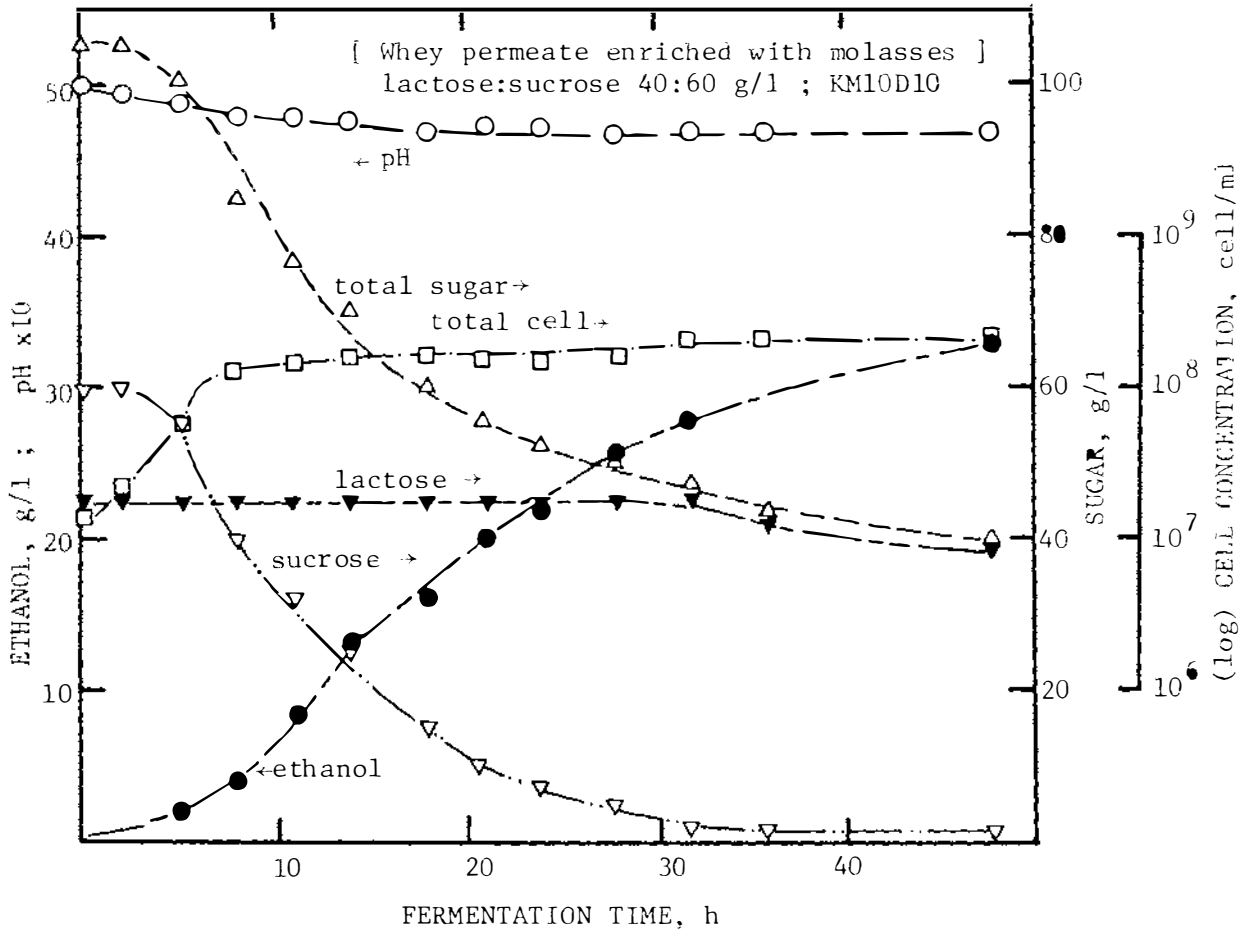


Fig.7.7 10 litre batch fermantation of whey permeate enriched with molasses by KM10D10 (lactose:sucrose 40:60 g/l).

Thus, only 62% of the total sugar was utilized and most of this was due to sucrose utilization. Ethanol produced during this time was 33 g/l, a yield on sugar utilized of only 93%.

The results indicates that KM10D10 retained the diauxic behaviour of the parent yeast, utilizing sucrose before lactose. It was not able to fully utilize 100 g/l of mixed sucrose and lactose within 48 hours. It could utilize either one of these sugars in a single substrate fermentation using the same sugar concentration within 48 hours.

7.3.6 Conclusions

K.marxianus UCD FST 7158 could ferment whey permeate without any additional nutrient in batch fermentation. In a 10 litre batch fermentation of whey permeate it required 16 hours to utilize 93% of 40 g/l lactose producing 18 g/l ethanol. This was 92% yield of ethanol on lactose utilized. It was, however, inhibited by ethanol concentration greater than 30 g/l and exhibited diauxic behaviour when it was given a choice of both lactose and sucrose at the same time. Sucrose was always

utilized first. This was followed by poor lactose uptake. A culture improvement programme was successful in isolating a more ethanol-tolerant strain, KM10D10.

Comparison of the improved yeast culture with its parent and other lactose-fermenting yeasts showed it to be a rapid lactose fermenter and more rapid ethanol producer.

The isolate yeast strain, KM10D10, was found to have a stable ethanol tolerance. However, in the presence of two sugars, in this case lactose and sucrose, it retained the diauxic behaviour of its parent, utilizing sucrose before lactose. This was an undesirable characteristic, because in order for lactose and sucrose to be rapidly converted to ethanol in in the tower fermenter, the two sugars must be metabolized simultaneously.

There was a need to attempt to isolate a mutant which showed no preference for sugar (diauxie-negative) or a mutant which utilized only lactose (sucrose-negative), so that it could be used in mixed substrate, mixed culture, for production of ethanol in economic concentrations from supplemented whey permeate.

7.4 AN ATTEMPT TO ISOLATE DIAUXIE-NEGATIVE *K.marxianus* strains

7.4.1 Introduction

In the batch fermentation of mixed substrate of sucrose and lactose by *K.marxianus* UCD FST 7158 and KM10D10 (sect. 7.3.1 & 7.3.5), sucrose was found to repress lactose utilization. This is an undesirable characteristic in tower fermentation.

Thus, the aim of this experiment was to carry out an isolation experiment for diauxie-negative *K.marxianus* strains, using D-glucosamine (DGA) as a gratuitous catabolite repressor.

DGA has been used by a number of investigators as a glucose analogue in carbohydrate metabolism of yeasts (Furst & Michels 1977 ; Michels & Romanowski 1980). DGA at a concentration of 0.5 g/l was found to repress completely the growth of a strain of *S.cerevisiae* (Elliot & Ball 1973). Further study found that DGA repressed the respiration rate more rapidly than glucose,could repress the level of cytochrome oxidase to the same level as glucose and repressed fermentation of maltose and galactose, but not sucrose. In all, DGA produced a repressed state very similar to glucose in many aspects, but did not affect growth on glucose (Furst & Michels 1977). DGA has no generalized growth repressive effects as does 2-deoxyglucose, another glucose analogue that has been used for isolation of mutants in *S.cerevisiae* insensitive to glucose repression (Zimmerman & Scheel 1977).

Thus, the presence of DGA in a medium which contained lactose as the sole sugar source, would inhibit growth of *K.marxianus* only if DGA could repress the synthesis of the enzyme(s) required for lactose utilization. The growth of *K.marxianus* in the lactose medium with added DGA would be affected by the concentration of DGA. When the level of DGA was greater than a minimum limiting level, there would be reduced growth. Those colonies that could grow at this level of DGA or greater should be cells that could utilize both glucose and lactose simultaneously (Dunn 1981). They are not repressed by the analogue, DGA, hence,these are the required diauxie-negative *K.marxianus* strains.

7.4.2 Isolation experiment

In this experiment, two sets of lactose and glucose agar with DGA added at different concentrations were prepared. One set each of lactose and glucose agar was plated with 0.1 ml of KM10D10 yeast suspension, while the remaining sets were streaked with KM10D10. After incubation,

the growth in the lactose agar plates was compared with the control glucose agar plates. KM10D10 cells which had diauxic behaviour should not grow in the presence of DGA in lactose agar plates, but grow in glucose agar plates. KM10D10 cells which had no diauxic behaviour should grow in both agars. Colonies with this behaviour were isolated and investigated further.

Two isolation attempts were carried out. In the first attempt DGA levels used were .1, .5, 1, 2, and 5 g/l based on the finding that 0.15 g/l DGA could cause inhibition and 1.5 g/l DGA had an effect equivalent to 5 g/l glucose on the growth of *S.cerevisiae* (Furst and Michels 1977). The KM10D10 yeast culture used was grown in whey permeate for 24 hours and received no further treatment prior to inoculation.

(a) First attempt

The results from the first attempt are given in table 7.6 while fig. 7.8 provides a visual representation of the effect of DGA on growth.

Table 7.6 Observation of growth of KM10D10 in glucose and lactose agars with added DGA (First attempt, cf. fig. 7.8)

DGA g/l	growth after 48 h (25°C) in	
	glucose agar	lactose agar
0.1	Moderate growth, large colonies (~.5mm)	Moderate growth, large colonies (.5-.6mm)
0.5	Moderate growth, colony size .2-.4mm	Moderate growth, colony size ~.2-.3mm
1	Moderate growth, colony size .2-.3mm	Moderate growth, colony size ~.5-.8mm
2	Poorer growth, smaller colonies ~.1-.2mm	Poorer growth, smaller colonies ~.1mm
5	Moderate growth, colony size ~.5-.6mm	Poorer growth, smaller colonies ~.1-.2mm

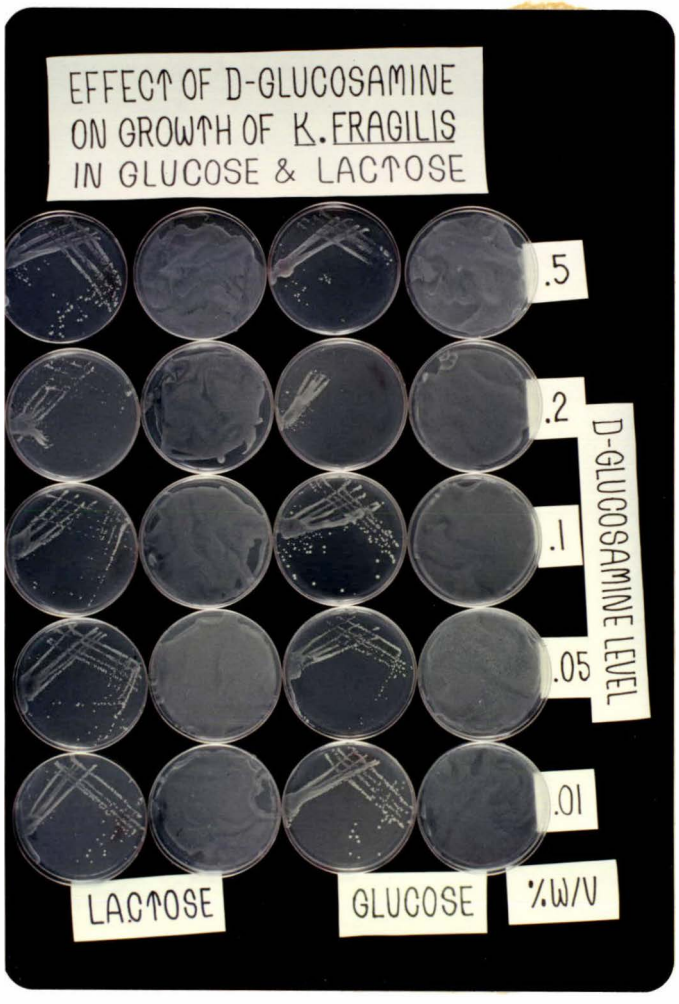


Fig.7.8 Growth of KM10D10 in lactose and glucose agars which contained different levels of D-glucosamine. (cf. table 7.5)

KM10D10 grew on lactose agar which contained DGA up to 5 g/l. Both plated and streaked agar plates showed similar growth patterns to those obtained for the corresponding control glucose agar plates.

It was concluded that DGA concentrations up to 5 g/l did not repress growth of KM10D10.

(b) Second attempt

The concentration of DGA was increased to 10 and 20 g/l. The results are given in table 7.7. The growth in agar plates containing DGA up to 5 g/l was similar to those described previously (table 7.6 and fig. 7.8). There was very little growth in plates with 10 & 20 g/l DGA both on glucose and lactose agar plates.

Table 7.7 Observation of growth of KM10D10 in glucose and lactose agars which contained up to 20 g/l DGA (Second attempt)

DGA g/l	growth after 48 h (25°C) in	
	glucose agar	lactose agar
10	Very little growth	Very little growth, few isolated colonies.
20	Very little growth	Very little growth, very few colonies

At this point, it was considered that a level of DGA at which the growth of KM10D10 was repressed had been reached. The control glucose agar plates showed the same level of poor growth. This may be an indication of toxicity, (rather than repression of enzyme synthesis by DGA). In order to resolve this problem, a fermentation test was carried out, using whey permeate enriched with sucrose.

(c) Fermentation test

A colony growing on the 10 g/l DGA lactose agar plate was used in the test. The whey permeate used contained equal concentration of sucrose and lactose (40:40 g/l) to check whether or not the yeast was diauxie-negative.

The results from this fermentation are given in table 7.8, which shows that sucrose was utilized first during the initial 24 hours fermentation. A small amount of lactose was utilized during this initial period. The amount of sucrose and lactose utilized were 43 and 12 g/l corresponding to 96 and 24% utilization respectively.

Table 7.8 Fermentation test of isolate obtained from lactose agar containing 10 g/l DGA.

The medium used was whey permeate enriched with 40 g/l sucrose (100 ml medium)

time h	pH	X Cells/ml	sucrose g/l	ΔS_s g/l	lactose g/l	ΔS_l g/l	ethanol g/l
0	4.9	5.0×10^4	45	-	50	-	-
10	4.9	3.0×10^7	37	8.7	48	2	-
17	4.5	2.9×10^8	13	32	47	3.2	5.8
24	4.2	3.7×10^8	1.9	43	38	12	22
48	4.6	5.0×10^8	1.1	44	6.6	44	33

ΔS_s = sucrose used

After 48 h, the utilizations were 98 and 87% respectively. In section 7.3.5(a), KM10D10 was shown to be able to utilize 97% of 100 g/l lactose present in whey permeate in 48 hours. In this fermentation, there was 92% total sugar utilization after 48 hours. This indicates that the presence of two sugars in the same medium had delayed the rapid utilization of lactose.

Comparison with the parent strain can be made further. From Table 7.11, in a similar fermentation, KM10D10 reduced sucrose and lactose from 41 and 40 g/l to .8 and 1.8 g/l in 24 hours. This corresponded to 98 and 96% utilization respectively. This shows that the isolate obtained here has shown no improvement over the parent strain.

Thus, the isolate was not diauxie-negative. The poor growth on 10 and 20 g/l DGA agar plates was a result of DGA inhibition on growth, not repression of specific enzyme synthesis.

7.4.3 The effect of D-glucosamine on growth of *K.marxianus*

It was apparent from the results of the two isolation attempts that DGA did not repress the utilization of lactose by KM10D10 at levels up to 5 g/l. However, at 10 and 20 g/l, growth was inhibited.

The fermentation test of a colony from the 10 g/l DGA lactose agar plate showed that the diauxic behaviour was still present when the two sugars, sucrose and lactose, were available. Sucrose was utilized before lactose. Thus, the cells which grew on lactose agar plates which contained 10 and 20 g/l DGA were those that could tolerate DGA rather than diauxie-negative strains.

Other workers, had found that DGA repressed maltose and galactose fermentation but not sucrose, while glucose repressed fermentation of all three sugars (Furst and Michels 1977). It is generally known that sucrose is hydrolyzed at the cell wall whereas maltose and lactose are hydrolyzed inside the cells (Sutton & Lampen 1962; Yoo 1974).

From these studies, one would expect lactose to be repressed in a similar way to maltose by DGA since they are both hydrolyzed inside the yeast cells. In this study, lactose was found to be affected in a similar manner to sucrose in that it was utilized by KM10D10 in the presence of DGA up to 5 g/l. It was pointed out earlier that only 5 g/l of DGA was needed to completely repress the growth of a strain of *S.cerevisiae*. Thus, the inhibition of growth on the 10 and 20 g/l DGA lactose and glucose agar plates was probably a result of toxicity of DGA (due to its very high concentration) and not enzyme synthesis repression.

7.4.4 Conclusions

K.marxianus utilized lactose in the presence of concentrations of D-glucosamine up to 5 g/l. At higher concentrations (up to 10 and 20 g/l), the growth of *K.marxianus* was poor. This was considered to be due to the toxicity of D-glucosamine. Diauxie-negative strains were not isolated.

7.5 A MUTATION ATTEMPT TO ISOLATE SUCROSE-NEGATIVE *K.marxianus*

7.5.1 Introduction

In the previous sections, it was established that *K.marxianus* and its mutant KM10D10 (sect.7.3.1, 7.3.5, 7.4.2) showed diauxic behaviour in the presence of sucrose and lactose. Sucrose was utilized before lactose, prolonging the fermentation time of a mixture of these sugars. There was a need to isolate a yeast strain which could utilize both sugars simultaneously, or utilize lactose only so that it could be used in conjunction with *S.cerevisiae* to ferment a mixture of sucrose and lactose. An attempt to isolate a diauxie-negative mutant was unsuccessful (sect.7.4).

This experiment investigated the second alternative, which was to isolate a lactose-fermenting yeast incapable of utilizing sucrose. The mutant could be used in conjunction with *S.cerevisiae* to ferment a mixture of lactose and sucrose in the tower fermenter.

7.5.2 First mutation attempt

In an attempt to produce a sucrose-negative *K.marxianus* from KM10D10, two experiments were carried out, using UV light as mutagen.

The first attempt yielded 2 mutants which showed very little growth in sucrose agar but had a very much better growth in whey agar. Typical growth comparison is given in fig. 7.9. These were designated as FSN 1 and FSN 2. FSN 1 were found to be large round cells which tended to collapse after 24 to 48 hours fermentation in liquid culture. FSN 2 had the normal oblong shape of *K.marxianus* in a similar medium.

(a) Fermentation of whey permeate by mutant FSN 1 & 2 A fermentation test in whey permeate was carried out to study the abilities of the mutants to ferment lactose and to select the fastest lactose fermenter for tests in the presence of sucrose and lactose.

The fermentation used 100 ml medium in a shake flask. Table 7.9 shows results of the fermentation by 10 isolate colonies after 48 hours. Isolates no.1-4 (from FSN 1), utilized on average 60% of the 76 g/l lactose available. They gave an average ethanol production of 14 g/l. Isolates no.5-10 (from FSN 2), utilized on average 26% of the lactose available to produce 6.8 g/l ethanol. For all isolates, The final pH was 4.5, a normal value for *K.marxianus*, while the final cell number was about half that expected from the parental strain.

The results showed that FSN 1 isolates could ferment more than twice the amount of lactose utilized by isolates of FSN 2, but that this was a poor fermentation performance. It was decided that the behaviour of these isolates in the presence of both sucrose and lactose should be investigated.

Fig.7.9 Comparison of growth of possible sucrose-negative mutants in whey and sucrose agars.

Table 7.9 Fermentation of whey permeate by two possible sucrose-negative mutants of KM10D10 (after 48 hours).

(No.1-4 were colonies taken from FSN 1 agar plate ;
no.5-10 were colonies taken from FSN 2 agar plate)

Initial pH = 4.9, cell concentration = 2.5×10^4 cells/ml, lactose = 76 g/l.

colony number	X cells/ml	ΔS_1 g/l	S_{1u} %	ethanol g/l
FSN 1				
1	1.4×10^8	45	58	13
2	9.2×10^7	43	54	13
3	6.3×10^7	54	70	14
4	1.8×10^8	45	60	15
FSN 2				
5	9.8×10^7	16	22	6.5
6	1.2×10^8	19	26	7.2
7	9.4×10^7	16	21	7.5
8	1.4×10^8	20	26	6.7
9	1.4×10^8	20	27	4.8
10	1.2×10^8	24	30	5.8

ΔS_1 - lactose consumed.

(b) Fermentation of mixed substrate of lactose and sucrose by mutants FSN 1 and 2. The inocula for these fermentation tests were obtained from the previously described fermentation of whey permeate by FSN 1 and 2 (table 7.9). The tests were carried out to study the behaviour of the mutants in mixed substrate of lactose and sucrose. Nomenclature of isolates and fermentation conditions were as shown in table 7.8.

The results of the fermentation at 0 and 48 hours are shown in table 7.10. Both sets of isolates showed poor lactose and sucrose utilization and ethanol production. More sucrose than lactose was utilized by isolates (1,2,3,5,6,7) while the remaining isolates utilized more lactose than sucrose. Isolates no. 2 and 3 produced the greatest amount of ethanol (3.1 g/l), but this was a much lower yield than that expected of the parent strain. There was also very little increase in cell number from inoculation. This was another indication of poor growth.

Isolates no.4,9, & 10 were selected for fermentation comparison with the parent yeast, KM10D10, on the basis of their ability to utilize more lactose than sucrose .

Table 7.10 Fermentation of whey permeate enriched with sucrose by isolates FSN 1 & 2. (Nomenclature as for table 7.9)

	0 hour					48 hours											
no	pH	X cell/ml	S_l g/l	S_s g/l	S_t g/l	pH	X cell/ml	S_l g/l	S_s g/l	S_t g/l	E g/l	ΔS_l g/l	ΔS_s g/l	ΔS_t g/l	S_{lu} %	S_{su} %	S_{tu} %
FSN 1																	
1	5.4	7.7×10^5	42	36	78	2.7	4.4×10^7	40	28	67	0.8	22	8.0	10	5	22	3
2	5.5	8.3×10^5	44	39	83	2.7	5.2×10^7	41	21	61	3.1	3.0	19	22	7	48	26
3	5.5	1.2×10^6	42	39	82	2.7	5.0×10^7	40	20	60	3.1	2.2	19	22	5	49	26
4	5.7	1.2×10^6	46	22	48	3.0	3.0×10^7	13	11	23	1.1	13	12	25	51	53	52
FSN 2																	
5	5.7	4.0×10^5	24	22	46	2.7	4.5×10^7	22	15	37	0	0	7.2	5.6	0	33	9
6	5.7	1.1×10^6	25	29	53	2.7	6.7×10^7	21	22	43	1.1	3.4	6.8	10	15	24	19
7	5.8	6.8×10^5	25	22	47	2.7	6.1×10^7	24	19	43	0.6	1.2	3.0	4.6	5	13	9
8	5.5	3.0×10^5	43	34	82	2.7	4.4×10^7	38	37	75	0	4.6	2.3	6.9	11	6	9
9	5.5	3.0×10^5	43	37	80	2.7	5.0×10^7	36	35	72	0.6	6.6	2.2	8.8	15	6	11
10	5.8	3.8×10^5	24	18	42	2.7	6.0×10^7	21	17	38	0.4	3.2	0.9	4.1	13	5	10

ΔS_t - total sugar utilization ; S_{tu} - percentage total sugar utilization ;
S_{su} - percentage sucrose utilization.

(c) Fermentation comparison of mutants FSN 1 and 2 with parent strain KM10D10 These fermentations were carried out to compare the mutants with the parent strain, KM10D10. Similar conditions to the previous fermentation were used.

The results are shown in table 7.11. At 24 hours, KM10D10 had reduced both sugars to residual level and produced 31 g/l ethanol, while the three mutants had utilized only sucrose. By 48 hours, they utilized lactose and sucrose to residual levels of 2 and 0.8 g/l, respectively.

Finally, it is evident that all mutants were sucrose-positive in liquid medium and still retained the diauxic behaviour. They showed no improvement over the ability of the parent strain. It was decided that further mutation should be carried out.

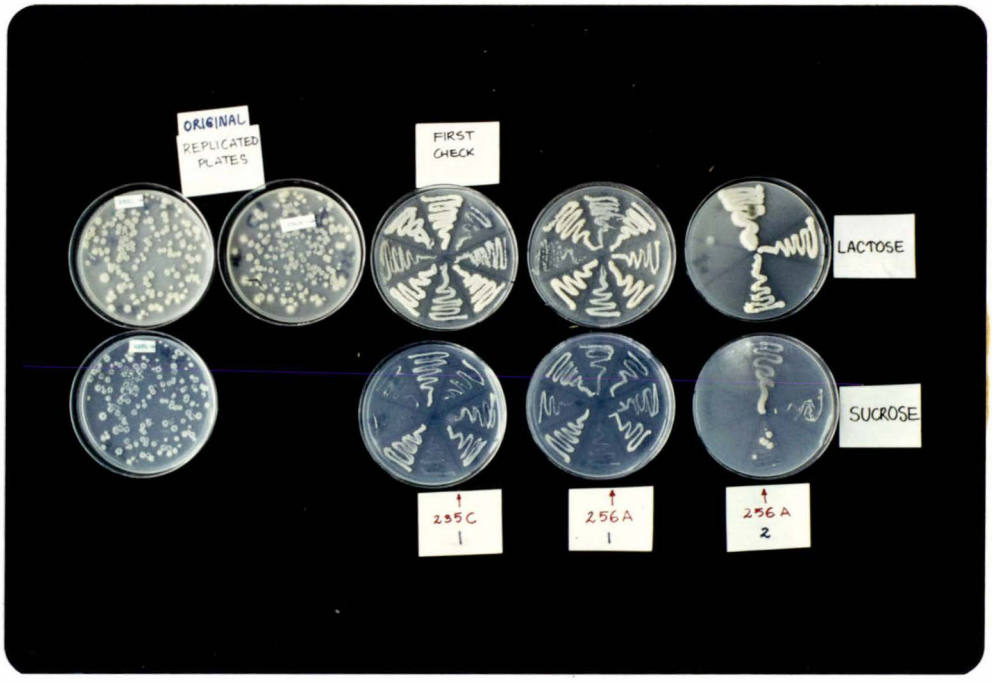
Table 7.11 Fermentation comparison of mutants FSN 1 and 2 with parent strain KM10D10 in whey broth enriched with sucrose (40:40 g/l lactose: sucrose.

(Similar fermentation conditions to those used in table 7.10)

time h	organisms	pH	X cells/ml	lactose g/l	sucrose g/l	ethanol g/l
0	KM10D10	5.2	1.0×10^5	41	41	
	4 (FSN 1)	5.2	1.1×10^6	41	39	
	11(FSN 2)	5.2	2.4×10^6	40	41	
	12(FSN 2)	5.2	3.0×10^6	39	39	
7	KM10D10	5.2	1.4×10^6	39	40	
24	KM10D10	3.4	8.0×10^8	1.8	0.8	31
	4	4.2	1.4×10^8	41	1.5	4.7
	11	4.1	1.4×10^8	40	0.8	9.0
	12	3.9	2.7×10^8	39	0.4	11
48	KM10D10	4.2	7.8×10^8	1.8	0.8	27
	4	3.9	3.3×10^8	2.4	0.8	22
	11	4.3	2.4×10^8	2.0	0.8	28
	12	4.4	2.4×10^8	2.1	0.8	29

7.5.3 Second mutation experiment

(a) Isolation of mutant In this attempt, 300 whey agar plates were inoculated with UV irradiated KM10D10 culture. After replication onto sucrose agar, 16 isolates were found to be unable to grow on sucrose. These were checked using a pair of sucrose and whey agar plates which had been divided into 8 sections (the 3rd & 4th pair of plates in fig.7.10). Each isolate was streaked onto one of these sections on both whey and sucrose agar plates. The isolates that grew on both plates were rejected, while those isolates that grew only on whey agar were retained. The photograph shows that isolates no. 235C and 256A showed very little growth in sucrose agar but good growth in whey agar. Isolate 256A was checked further by streaking onto whey and sucrose agars (the 5th pair of plates in fig.7.10). There were few large colonies growing in sucrose agar (agar plates no.5). A colony from lactose agar was used to inoculate the first set of plates (set no. 1 in fig.7.11). From this set, a colony was taken from whey agar to inoculate the next set of plates (no.2) in



1 2 3 4 5

Fig.7.10 Sequence of isolation of mutant 235C (FSN 3) and 256A. From original replicated plates and two subculturing checks.

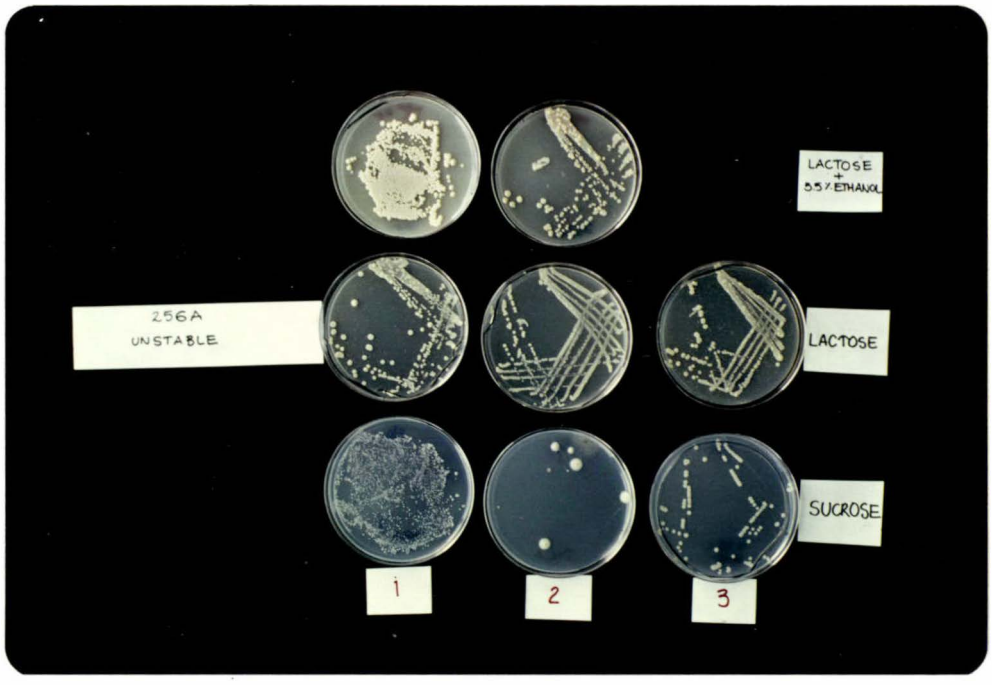


Fig.7.11 Sequence of plating and streaking to check the stability of mutant 256A. Growth in sucrose agars indicates instability.

in order to check for purity and stability of the isolate. A few colonies grew on the sucrose agar plate. The final subculture onto set number 3 showed more colonies on sucrose agar. At this point, this isolate was rejected because of the growth on sucrose agar.

Isolate no.235C from the plate shown in fig.7.10 (the middle pair, 3rd set from right) was used to inoculate the first set of plates (from left) in fig.7.12. There was no growth on the sucrose agar plate. An isolated colony from whey agar was used to inoculate the next set of plates (no.2 from left). There were 3 large colonies growing on the sucrose agar plate. At this point, there was a need to isolate a mutant which grew in lactose only because there was contamination by sucrose utilizing strains.

This was done by selecting a few isolated colonies from the whey agar plate and using them to inoculate a separate whey agar plate. After incubation they were replicated onto sucrose agar plates. These plates are shown in fig. 7.12 (no.1,2,3,in red, 3 sets of plates in the far right). There was no growth in the sucrose agar plates. The markings on these plates were a result of replication pressing on the agar.

Thus, it appeared that, the isolate 235C was pure and stable (It did not grow on sucrose agar). It was assigned a new name of FSN 3.

(b) Culture improvement of mutant FSN 3 It was shown by the previous experiment that FSN 3 was a stable mutant when tested for growth in whey and sucrose agar. In addition the mutants FSN 1 & 2 were slow fermenters which reverted to sucrose utilization in liquid medium. In order to improve the FSN 3 culture, it was decided that FSN 3 should be repeatedly subcultured into whey permeate broth with added ethanol, the ethanol concentration being increased after each subculture so that the isolate would have an improved fermentation rate similar to the result obtained in the isolation of the ethanol tolerant KM10D10 (sect.7.2). The inoculum size used was 10% v/v of the final medium volume to allow for cell loss due to ethanol toxicity.

Fig.7.13 illustrates the first subculturing sequence. Six subcultures were made. There was progressively poorer growth as the ethanol level increased (5 g/l per subculture) to 20 g/l. The fifth and sixth flasks both contained 20 g/l ethanol.(Note the clearer medium in these two flasks. Each flask was thoroughly shaken before the photograph was taken in order to show the extent of yeast growth).

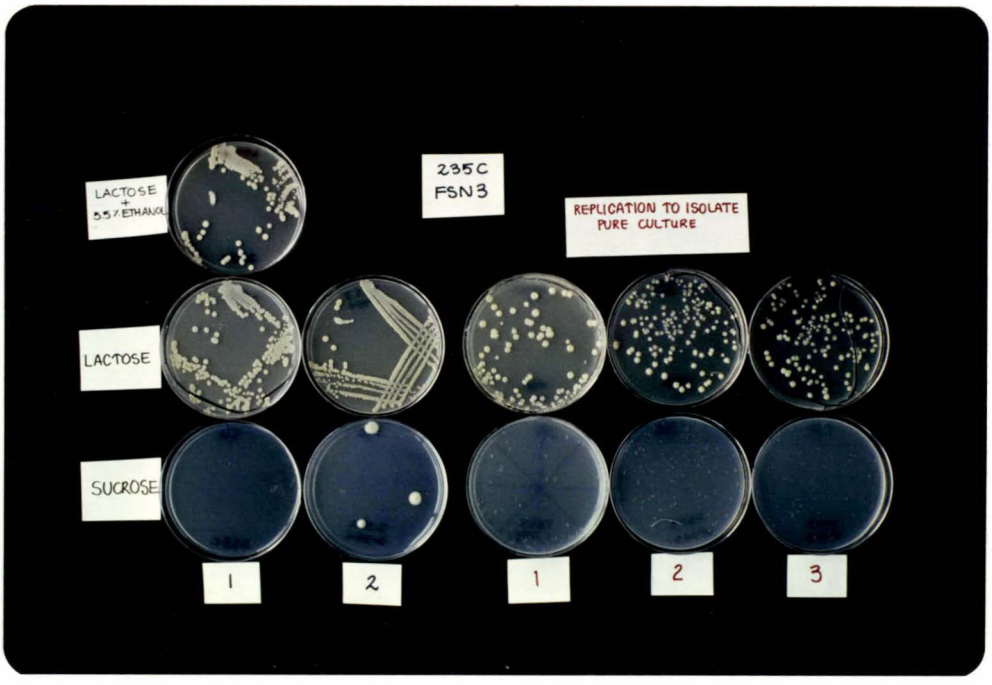


Fig.7.12 Streaking sequence to check stability of 235C (FSN 3) and replication to isolate pure culture.

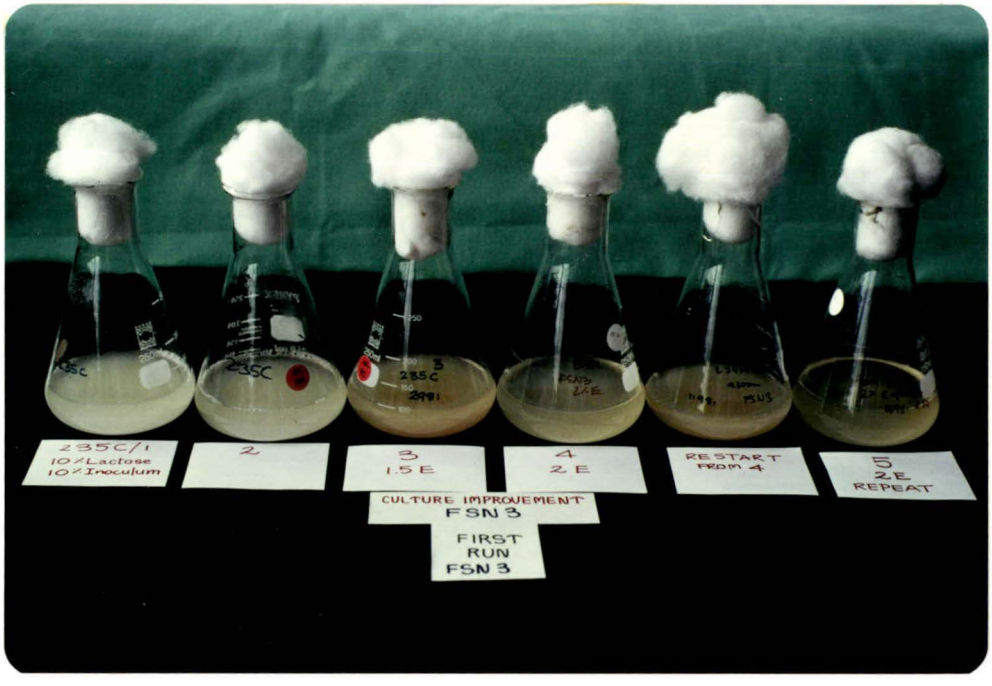


Fig.7.13 First culture improvement sequence of mutant FSN 3 to improve growth rate and ethanol-tolerance by growing in whey broth (100 g/l lactose) with added ethanol (stepwise increase).

This run was unsuccessful because the isolate was unable to grow in presence of ethanol up to 20 g/l. Hence, the subculturing was restarted from the initial culture.

In the second run, 5 g/l ethanol increase was used for each subculturing step and the results are illustrated in fig.7.14. The extent of growth was also monitored on lactose and sucrose agar plates (fig.7.14 b). The ethanol level of 35 g/l was reached after 6 subcultures. There was good growth in all of these flasks (up to no.6). However,as shown by fig.7.14(b), the growth on sucrose agar plates increased progressively as the ethanol concentration increased, while the growth in the whey agar plates was better and improved very slightly as ethanol increased.

The growth improvement on the sucrose agar did not approach that of the parent strain. There would be more growth also if there was a complete reversion to sucrose utilization.

At this point, culture from flask no.6 (fig.7.14 a) was used to ferment whey permeate (100 g/l lactose). This fermentation was carried out to study the ability of the isolate to ferment lactose.

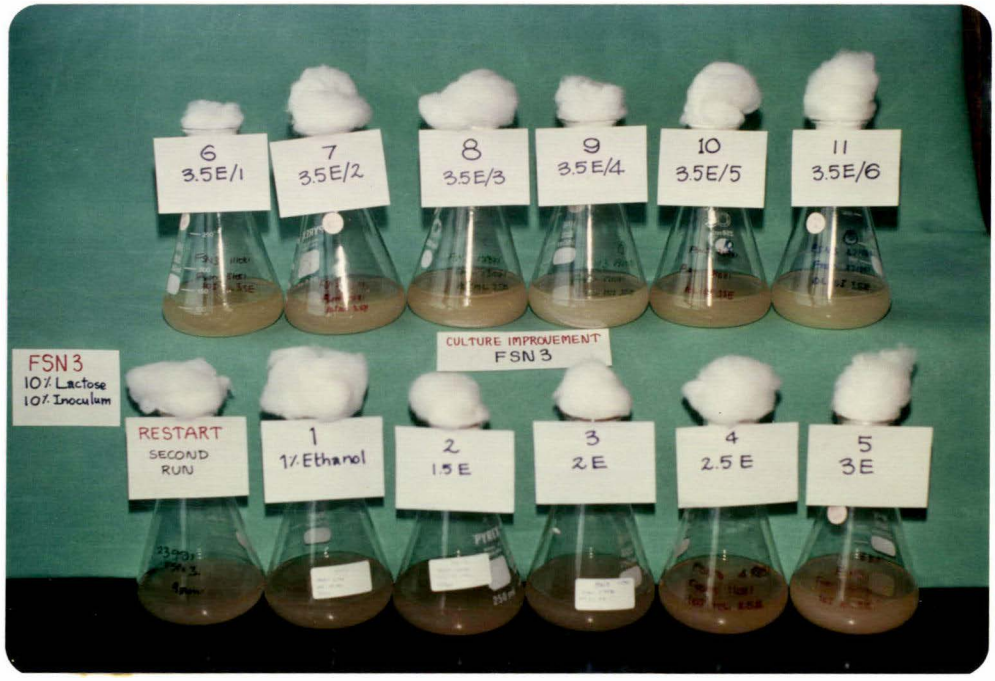
Table 7.12 shows the fermentation results. The initial cell concentration of $1x10^6$ cells/ml was of normal inoculation level but the cell levels at 24 and 48 hours of $1x10^7$ cells/ml were low and lactose utilization was poor.

Table 7.12 Fermentation of whey permeate (100 g/l lactose) by mutant FSN 3 (100 ml volume).

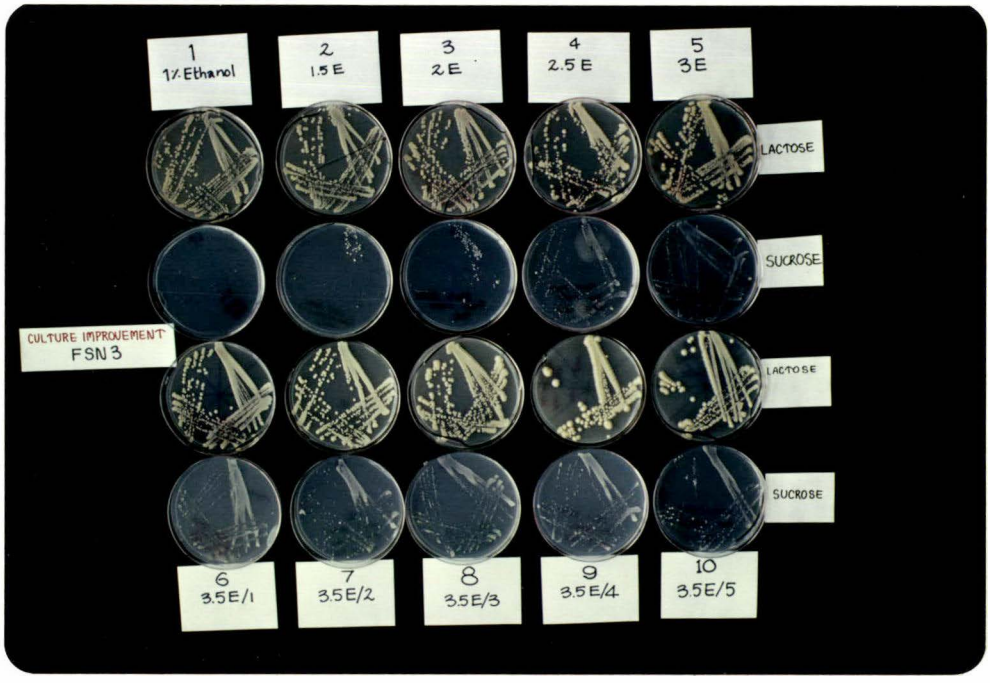
After a series of 6 subcultures in whey broth (100 g/l lactose) with up to 35 g/l added ethanol (no.6-3.5E/1)(cf. fig.7.14)

time h	pH	X cells/ml	lactose g/l
0	5.1	$4.1x10^6$	110
24	4.8	$1.5x10^7$	103
48	4.5	$1.4x10^7$	55

From these results it was decided that culture improvement should be carried out further. The added ethanol level of 35 g/l was maintained. This was considered to be a sufficiently high concentration because it was found that a strain of *K.marxianus* was inhibited by ethanol at a concentration of 32 g/l (Wendorf et al 1970). The results of this last subculturing series were also illustrated in fig.7.14 (from no.7-3.5E/2



(a)



(b)

Fig.7.14 Second culture improvement sequence of mutant FSN 3. (a) In whey broth, (b) Corresponding lactose and sucrose agar plates to monitor mutant stability. Note increasing growth in sucrose agar as the ethanol content increased.

to 11-E/6). There was good growth in all flasks (fig.7.14 a). The growth in both whey and sucrose agars improved slightly (fig.7.14 b).

Finally, a fermentation test for the last culture was carried out using whey permeate with added sucrose (lactose:sucrose 40:40)(in 100 ml medium). Table 7.13 shows the fermentation results at 0 and 48 hours.

Table 7.13 Fermentation of whey permeate enriched with sucrose by mutant FSN 3 which was passed through a series of 11 subcultures in whey broth (100 g/l lactose) with added ethanol (35 g/l).
(This was from the sixth subculture which was grown in presence of 35 g/l ethanol. No.11-3.5E/6) (cf. fig.7.14 a).

time h	pH	sucrose g/l	lactose g/l	ethanol g/l
0	4.6	40	43	0
48	4.4	1.4	12	22

Sucrose was all utilized but only 31 g/l of lactose was utilized. There was 12 g/l remaining. From table 7.11, the parent strain was able to completly utilize a similar amount of mixed sugars in 24 hours.

7.5.4 Conclusions

The results showed that even though the fermentation capability of the mutant FSN 3 was improved over its initial culture, it still reverted to utilizing sucrose before lactose when both sugars were available simultaneously. The growth of the mutant on sucrose agar increased with improvement, although growth on whey agar was more profuse than on sucrose agar. The final fermentation test also showed that the improved ability of the mutant was not as good as that of the parent strain.

Thus, the three mutants isolated were sucrose-negative when they were grown on sucrose and whey agars. However, all mutants reverted to utilizing sucrose before lactose when the two sugars were available simultaneously in liquid medium. The last mutant (FSN 3) was passed through a series of subculturing in the presence of increasing concentrations of ethanol to improve its fermentation ability. This improved the culture but abilty to utilize sucrose also improved. Sucrose was also utilized before lactose when tested for growth in mixed substrate of lactose and sucrose.

7.6 SUMMARY

1. Fermentation of whey permeate by *K.marxianus* UCD FST 7158 could be carried out without nutrient addition. This yeast strain was able to utilize 40 g/l lactose in whey permeate in 16 hours but was not able to utilize completely 100 g/l lactose. This was shown to be due to inhibition of growth by ethanol. In the fermentation of whey permeate enriched with molasses, the culture exhibited diauxic behaviour, utilizing sucrose before lactose. The overall fermentation time was increased compared with that for unsupplemented whey fermentation.

2. A simple culture improvement technique was used to improve ethanol tolerance of *K.marxianus*. An improved yeast strain, KM10D10, was isolated. It was a rapid lactose fermenter and better producer of ethanol from lactose than its parent, *K.marxianus* UCD FST 7158 and other lactose fermenting yeasts under the same fermentation conditions and medium used. It was found to be stable.

3. An investigation was carried out to isolate a diauxie-negative *K.marxianus* using D-glucosamine as the glucose analogue. *K.marxianus* was found to be able to grow on lactose in the presence of D-glucosamine up to 5 g/l. At higher D-glucosamine concentrations (10 and 20 g/l), the growth was inhibited. Fermentation tests of cells that could grow at these concentrations showed them to be diauxie-positive.

4. UV irradiation and agar plate replication techniques were used to produce a sucrose-negative strain of *K.marxianus*. Three mutants were obtained and all were sucrose-negative in sucrose agar but always reverted to sucrose utilization when grown in a broth medium that contained both lactose and sucrose.

CHAPTER 8

FINAL DISCUSSION AND CONCLUSION

The feasibility of fermenting whey permeate to ethanol using a tower fermenter has been investigated. The commercial advantage in using such a process is considerable, since the tower fermentation process provides continuous treatment of whey permeate to ethanol requiring low energy input, plant cost, and maintainance but can give a high rate of ethanol production.

Initial screening and investigations to obtain a lactose-fermenting and flocculating yeast found that there was only one strain of *K. marxianus* available that was flocculent. This yeast strain, *K. marxianus* Y42, showed lactose-fermenting ability comparable with other *K. marxianus* strains. Further investigations of the behaviour of this yeast species showed it to be unable to ferment completely a high concentration of lactose in whey permeate (100 g/l lactose). This was found to be due to inhibition by ethanol. The optimum operating conditions for tower fermentation of whey permeate by *K. marxianus* Y42 were found at a superficial liquid velocity of 0.24 mm/s, and pH between 4.2 and 4.6 when the fermentation temperature was 30 C. The minimum tower height was 0.82 m (excluding separator section) which corresponded to a residence time of 1 hour. The concentration of ethanol produced under these conditions was 16 g/l at productivity of 16 g/lh from 45 g/l of lactose (94% lactose utilization) and 71% yield on lactose utilized. If the separator section were included in the consideration, the productivity reduced to 5 g/lh, and the effluent liquor contained 18 g/l of ethanol. The overall retention time was 3.7 hours. The cell concentration inside the tower varied between 10 and 100 g/l dried weight (54 and 350 g/l wet weight) being greatest at the bottom of the tower.

The fermentation was limited by the moderately flocculent nature of *K. marxianus* Y42. The productivity was found to be lower than that observed for tower fermentation using sucrose based media, but was found to be more than 13 times that of current commercial fermentation of whey to ethanol.

During continuous tower operation, bacterial contamination occurred. This lowered lactose utilization, ethanol production and the medium pH. When this occurred, the fermenter was emptied, cleaned and

sterilized before restarting the fermentation.

Tower fermentation using mixed culture of *K. marxianus* and *S. cerevisiae* to ferment whey permeate enriched with molasses was difficult. There was incomplete lactose utilization even at the low feed rates used (up to 0.14 mm/s). Sucrose was completely utilized at the bottom of the tower fermenter. The incomplete lactose utilization was found to be a result of the diauxic behaviour of *K. marxianus* in the simultaneous presence of sucrose and lactose. Sucrose was utilized first then lactose was utilized, but by this time the ethanol concentration was high. The high concentration of ethanol inhibited lactose uptake in the tower. Thus, lactose utilization took place at a low rate. It was also found that molasses, when mixed with whey permeate, could slow down the rate of fermentation and contributed to the incomplete sugar utilization. There was also some incompatability of the two yeast strains used. *S. cerevisiae* CFCC39 was very flocculent in whey permeate enriched with molasses. The medium feed rate to the tower was very slow. This resulted in a very dense cell population causing the blockage of the separator and the formation of gas slugs. The less flocculent *K. marxianus* Y42 was slowly washed out of the tower and lactose utilization was therefore reduced.

Investigation of the flocculent nature of *K. marxianus* Y42 indicated that this yeast strain exhibited different flocculating behaviour when grown in different media. It was found to have good flocculence when grown in media prepared from yeast and malt extract powder, malt extract broth powder, malt extract syrup or in whey permeate enriched with these nutrients. It showed poor flocculence when it was grown in acidic media or media which did not support good growth. During the start up of the tower for fermentation of whey permeate and whey permeate enriched with molasses, the whey permeate feed was enriched further with malt extract syrup in order to enhance the flocculence of *K. marxianus* for rapid build up of cell concentration.

An attempt to improve the ethanol tolerance of a strain of *K. marxianus* was successful. The isolate, called KM10D10, fermented lactose in whey permeate rapidly and grew in presence of ethanol up to 50 g/l. The isolate was found to be able to produce ethanol at a faster rate than all of the *K. marxianus* strains tested. The isolate was found to be stable upon repeated subculture. However, it could not

be used in the tower fermenter as it was not flocculent.

A similar culture improvement technique could be used to improve the ethanol tolerance of a flocculent parent strain and this could reasonably be expected to produce a suitable culture for use in the tower fermentation process.

An attempt to isolate a diauxie-negative strain of *K. marxianus*, using D-glucosamine as a glucose analogue, was unsuccessful. This yeast utilized lactose in the presence of the analogue in the growth medium.

An attempt to isolate a strain of *K. marxianus*, which was unable to utilize sucrose, using UV radiation as a mutagen was only partially successful. Mutants that showed no growth in sucrose agar plates were isolated. These mutants, when tested for growth in liquid medium containing mixed substrate of sucrose and lactose, reverted to utilizing sucrose before lactose. Further attempts were carried out, followed by a culture improvement subculturing sequence similar to that used in the isolation of an ethanol toleratant strain. The mutant obtained from this later attempt reverted to sucrose utilization in liquid medium as in the first attempt.

In summary, it has been shown that the fermentation of whey permeate to ethanol using a tower fermenter is feasible and requiring no nutrient addition to the whey permeate except for the requirement of malt extract syrup during start up and periodically thereafter to maintain high yeast flocculence. The use of a mixed yeast culture to ferment whey permeate enriched with molasses in a tower fermenter was difficult. There remained some problems to be solved. These are the diauxic behaviour of *K. marxianus* and the incompatibility of the flocculence of the two yeast strains used. The flocculent behaviour of *K. marxianus* Y42 needs to be investigated further.

Suggestions for future investigations are listed below.

1. Further investigation of KM Y42 flocculence properties

It was found that this yeast showed variable flocculence in different media but it was not possible to isolate the factors that caused this behaviour. KM Y42 is the only lactose fermenting yeast that is known to flocculate. Thus, further investigation to establish the cause of its flocculence would be of great benefit to the study of tower fermentation of whey to ethanol.

2. Mutation to isolate flocculent lactose-fermenting yeast

Flocculation is a genetically controlled behaviour. Transformation mutation could be employed to transfer gene information for lactose transport and fermentation to a highly flocculent yeast such as *S. cerevisiae*. The transformation of β -galactosidase information from *K. lactis* to *S. cerevisiae* has been successfully carried out but the mutant could not utilize lactose because lactose transport information was lacking (Dickson 1980). Recombinants of *S. cerevisiae* and *K. lactis* through spheroplast fusion were found to be unstable (Stewart 1981). Thus, presently fusion is an unsuitable approach because of the incompatability of the two yeasts. It is generally accepted that flocculation is a cell wall phenomenon (Stewart 1975). Thus, an attempt to transfer flocculation genes into *K. marxianus* spheroplasts may not be successful because there may be up to four genes controlling flocculence and the existing cell wall may not be suitable for the exhibition of flocculence.

3. Tower fermentation of whey containing 100 to 200 g/l lactose

In this study, the concentration of lactose in the whey permeate was 40 g/l. Fermentation of higher concentrations of lactose in whey (up to 200 g/l) has been reported using either *K. marxianus* or *C. pseudotropicalis* in batch fermentation (Moulin et al 1980 ; Burgess & Kelly 1979). A high lactose concentration means greater ethanol productivity and improved economy on the utilization of fermentation and distillation equipment.

4. Mixed culture fermentation of whey permeate enriched with molasses

It was found that the yeast strains CFCC39 and KM Y42 did not have compatible flocculence for use together in the tower fermenter. The less flocculent KM Y42 was washed out of the tower slowly because the highly flocculent CFCC39 caused blockage of the yeast recycling path and the formation of gas slugs. The yeast strain SC146 may be more compatible with KM Y42 since it is moderately flocculent.

It may also be more desirable to use only *K. marxianus* in the tower since it was found that the mixture of sucrose and lactose was utilized more rapidly when the ratio of *K. marxianus* to total cell concentration was greater than 90%.

5. Fermentation of whey permeate containing high concentration of lactose with added nutrient that induces ethanol tolerance

It was found that high ethanol concentration exerted considerable inhibition on the fermentative activity of *K. marxianus*, causing incomplete lactose utilization when lactose and sucrose were available together. It has been shown for a mumber of *S. cerevisiae* strains that the addition of proteolipid at the early stage of the fermentation can improve ethanol tolerance, (Hayashida & Ohta 1981). The addition of such a nutrient to whey permeate containing a high sugar concentration and studying its effect on ethanol tolerance could contribute to the understanding of the behaviour of *K. marxianus*.

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APPENDIX A

FEED MEDIUM PUMP CAPACITY AND SAMPLING DATA SHEETS

A.1 Capacity of the feed medium pump for the tower fermenter

Fig.A.1 provides pumping capacity data for the Cole Parmer Masterflex pump used for pumping medium into the tower. Two pump head sizes were used, they were sizes 13 & 14.

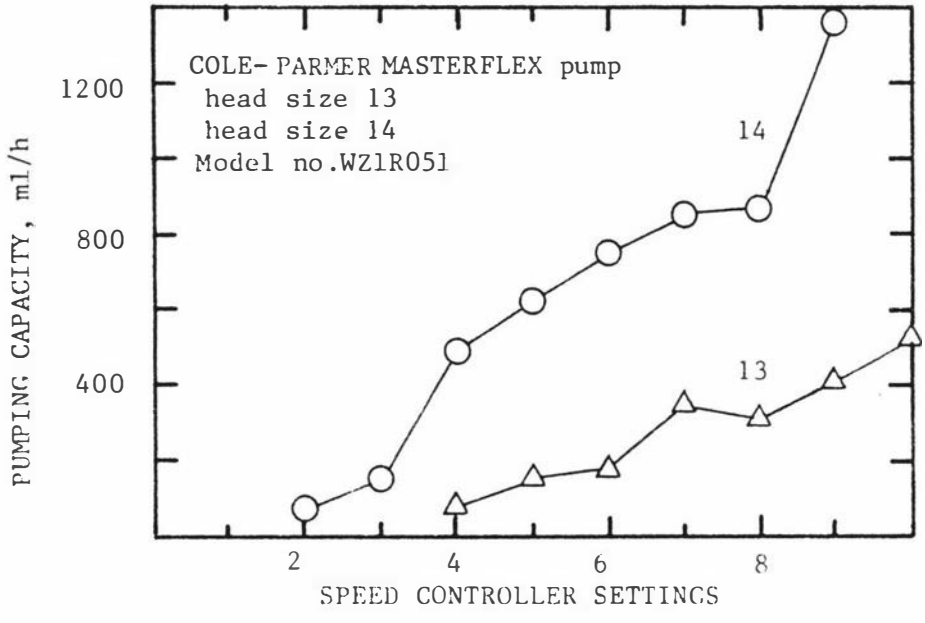


Fig.A.1 Pump capacity curves for 25 mmϕ tower fermenter.

A.2 Sampling data sheets

The following data sheets were used to record data collected for a set of samples at a particular medium flow rate.

Tower Fermentation

C. Boontanjai

Run .....

Date.....

Medium .....

Run duration from .....to .....

Sampling time .....

Tower aeration .....ml/min. Line pressure .....kPa.

Flow rate .....ml/hr. Pump head no./motor controller setting.....

Duration of sampling .....hr. Start ..... Finish .....

Volume beer collected .....ml. Ev.....

Feed wort aeration .....hr. at.....ml/hr.....kPa.

Residence time, T_R .....hr.

Liquid superficial velocity, Vs .....cm/s (flow (ml/hr) x 5.4257 x 10^{-5})

Dilution rate, D.....hr^{-1} ($1/T_R$)

Collect samples from the top of column fist.

	H_A	H_E	V_E
0 - Feed wort	0	0	
1 - Bottom of tower	95	96	49
2 - Lower mid-tower	817	817	418
3 - Upper mid-tower	1573	1573	805
4 - Top of 3rd section	2316	2316	1186
5 - Effluent beer	2693	2693	2921

% w/v g/100 ml.

tower cross sectional area = 5.1195 cm^2

H_A - Actual height, mm

H_B - Effective height, mm

V_E - Effective volume, ml

RUN NO. .....

Parameters	0 feed	1 bottom	2 mid-lower	3 mid-upper	4 top	5 effluent
Volume of sample, ml.						
Temperature, ^oC						
pH						
SG						
Total cell weight						
Centrifuge tube wt.(CT), g.						
CT + wet weight, g.						
CT + dried weight, g.						
Centrifuged wet wt., % w/v						
Cell dried weight, % w/v						
K. fragilis						
Dilution						
Cell/ml						
K.F. cell dried wt., % w/v						
Sucrose Dilution						
Sugar analyzer						
% w/v						
Lactose Dilution						
Sugar analyzer						
% w/v						
Ethanol Internal std.% w/v						
Peaks						
% w/v						
Ethanol parallel std. % w/v						

Tower Fermentation — C. Boontanjai

Run.....

Date.....

Medium.....

Run duration.....days.....hrs.

Tower aeration.....ml/min. .....kPa.

Feed wort aeration.....hr. .....ml/min. .....kPa.

Flow rate.....ml/hr. E_v .....

Residence time, T_R.....hr.

Superficial liquid velocity, V_s .....cm/s.

Dilution rate, D .....hr^{-1}

sample	pH	SG	total cell		*K. fragilis*		T_R	sucrose	lactose	ethanol
			WW %w/v	DW %w/v	cell/ml	DW %w/v	hr	%w/v	%w/v	%w/v
0										
1										
2										
3										
4										
5										

0 - Feed wort

1 - Bottom of tower

2 - Lower mid-tower

3 - Upper mid tower

4 - Top of 3rd section

5 - Effluent beer

WW - Centrifuged wet weight, g/100 ml. DW - Dried weight, g/100 ml.

Tower Fermentation — C. Boontanjai

Run.....

Date.....

Medium.....

Run duration.....days.....hrs.

Tower aeration.....ml/min. .....kPa.

Feed wort aeration.....hr. .....ml/min. .....kPa.

Flow rate.....ml/hr. E_v .....

Residence time, T_R.....hr.

Superficial liquid velocity, V_s .....cm/s.

Dilution rate, D .....hr^{-1}

sample	pH	SG	total cell		*K. fragilis*		T_R	sucrose	lactose	ethanol
			WW %w/v	DW %w/v	cell/ml	DW %w/v	hr	%w/v	%w/v	%w/v
0										
1										
2										
3										
4										
5										

0 - Feed wort

1 - Bottom of tower

2 - Lower mid-tower

3 - Upper mid tower

4 - Top of 3rd section

5 - Effluent beer

WW - Centrifuged wet weight, g/100 ml. DW - Dried weight, g/100 ml.

APPENDIX B

FERMENTATION DATA

B.1 MEDIUM OPTIMIZATION DATA

Variables and levels Three variables were used at three different levels of concentrations (3^3 design, 14 runs).

Table B.1 Variables and their concentrations used at various RUNS.

level		0	1	2
$(NH_4)_2SO_4$	N	0	0.5	1.0
K_2HPO_4	K	0	0.5	1.0
Yeast extract	Y	0	0.1	0.2

All concentrations in g/l

RUNS : 000 002 011 020 022 101 110 112 121 200 202 211 220 222

Table B.2 Fermentation results of factorial experiment on nutrient requirement of *K.marxianus* UCD FST 7158 growing on whey permeate.

	lactose concentration (g/l) after					ethanol concentration (g/l) after				
runs	7.5	19.5	30	48	66.5 (h)	7.5	19.5	30	48	66.5
000	33	14	4.0	3.6	2.7	3.3	11	19	19	18
002	33	17	4.3	3.3	2.6	3.0	8.7	19	19	18
011	36	12	3.9	3.6	2.7	3.3	11	18	18	18
020	33	14	3.9	3.7	2.8	3.3	12	18	18	17
022	34	17	4.4	3.5	2.7	3.1	11	18	18	17
101	29	19	3.6	3.4	2.6	2.8	8.3	18	17	19
110	33	13	4.0	3.8	2.9	3.2	11	18	18	18
112	34	13	3.7	3.5	2.9	3.7	11	17	17	19
121	33	15	3.9	3.6	2.9	3.1	11	18	19	18
200	34	14	4.2	3.7	2.9	3.3	9.8	19	18	19
202	35	16	4.3	3.5	2.8	3.3	9.5	17	18	19
211	33	18	4.8	3.6	2.8	3.2	9.1	15	18	18
220	35	15	4.2	3.0	2.0	2.8	10	18	19	18
222	32	15	3.9	3.7	2.0	2.6	10	19	19	19

The initial lactose concentration was 43 g/l.

B.2 10 l BATCH FERMENTATION OF WHEY PERMEATE (40 g/l lactose) by *K.marxianus* Y42

E ethanol concentration, g/l

S_1 lactose concentration, g/l

ΔS_1 lactose utilization, g/l

S_{1u} lactose utilization, %

T fermentation time, h

X total cell number, cell/ml

Y yield of ethanol on lactose utilized, %

Table B.3 Batch fermentation of whey permeate by *K.marxianus* Y42

T h	pH	X cell/ml	S_1 g/l	ΔS_1 g/l	S_{1u} %	E g/l	Y %
0	5.0	1.1×10^7	40	0	0	-	-
3	5.0	2.4×10^7	37	3.1	7.8	1.6	96
6	4.9	4.8×10^7	34	6.2	16	3.8	95
9	4.8	8.0×10^7	23	17	41	7.8	89
12	4.7	1.1×10^8	14	26	65	14	98
16	4.7	1.1×10^8	4.1	36	90	18	94
24	4.6	1.1×10^8	2.0	38	95	18	90

Fermentation was completed after 16 h. There was 90 % lactose utilization to produce 18 g/l ethanol. This was 94 % yield of ethanol. The average rate of lactose utilization was 2.2 g/lh and ethanol productivity was 1.1 g/lh. The maximum specific rate of ethanol production occurred between 9 and 12 h and was 1.4 g/gh (cell dried weight = 1.4 g/l from figure B.1).

B.3 TOWER FERMENTATION OF WHEY PERMEATE (40 g/l lactose)

Data for the superficial liquid velocity(V_s) of 0.044, 0.080, 0.17, & 0.24 mm/s were averaged from 5 separate sets of samples. For the superficial liquid velocity of 0.30 mm/s, the data were averaged from only 2 sets of samples.

D Dilution rate based on tower straight section, h^{-1}

H Tower height, m

H* Mean tower height at the mid-point of each tower section, m

Q Feed medium volumetric flow rate, ml/h

SG_e Exit specific gravity

T_r Residence time at various tower height, h

T_r^* Mean residence time at height of H*

T_{ri} Residence time at height H, h

X_a Mean cell concentration in a tower section, g/l DW

H*,T_r^* and X_a are used in conjunction with S_1', q_1,E',and ν.

Table B.4 A summary of the dimensions of the tower fermenter.

sample point	V_E ml	H_E m	average between	H* m	ΔV ml
0 (inlet)	0	0			
1	49	0.096	0-1	0.048	49
2	418	0.82	1-2	0.46	369
3	805	1.57	2-3	1.20	387
4	1186	2.32	3-4	1.95	381
5 (exit)	2921	2.69	4-5	2.51	1735

In tables B.5 and B.6, the standard deviations (SD) for the data are given after a slash following each datum.

Table B.5 Tower fermentation of whey permeate, data at various sampling points

H m	T_{ri} h	pH	WW g/l	DW g/l	S_1 g/l	S_{su} %	E g/l	Y %
V_s= 0.044, Q = 81, D = 0.063, T_r= 15.9, SG_e= 1.003								
0	0	5.4/0.04	25/ 2.1	2/ 0.1	47/0.5	0	0	-
0.096	0.6	4.6/0.13	348/ 6.6	97/20.1	7.0/3.0	85	20 /2.7	97
0.82	5.2	4.6/0.13	92/14.1	21/ 4.5	1.9/1.3	96	22 /0.9	93
1.57	10.0	4.6/0.15	78/14.8	16/ 4.0	1.3/0.7	97	23 /0.6	92
2.32	14.7	4.6/0.16	54/11.6	10/ 3.0	1.3/0.6	97	23 /1.3	95
2.69	36.3	4.6/0.12	18/ 2.0	0.2/ 0.1	1.3/0.4	97	22 /0.8	90
V_s= 0.080, Q = 150, D = 0.12, T_r= 8.7, SG_e= 1.004								
0	0	5.1/0.18	19/ 4.0	1/ 0.2	43/2.3	0	0	-
0.096	0.3	4.7/0.06	344/22.0	101/11.8	28/2.9	35	5.6/1.0	75
0.82	2.8	4.5/0.02	55/ 8.5	10/ 2.3	3.9/1.1	91	17 /0.6	82
1.57	5.5	4.5/0.06	39/ 5.0	6/ 1.2	3.4/0.8	92	18 /1.3	84
2.32	8.1	4.5/0.04	28/ 2.8	4/ 0.8	3.3/0.9	92	18 /1.4	83
2.69	19.8	4.6/0.06	16/ 0	0.2/ 0	3.2/0.9	93	17 /1.5	82
V_s= 0.17, Q = 310, D = 0.25, T_r= 4.1, SG_e= 1.004								
0	0	4.9/0.15	10/ 2.0	0.6/ 0.1	46/7.3	0	0	-
0.096	0.2	4.8/0.11	341/71.7	96/21.8	32/5.3	32	6 /1.4	77
0.82	1.3	4.6/0.05	120/ 8.4	30/ 1.6	4.8/0.9	90	18 /0.9	77
1.57	2.6	4.6/0.07	108/10.6	26/ 2.5	3.1/0.6	93	18 /1.7	80
2.32	3.8	4.5/0.07	92/ 9.1	22/ 2.4	2.7/0.3	94	19 /2.0	84
2.69	9.3	4.6/0.09	10/ 2.6	0.2/ 0.1	2.5/0.4	95	19 /1.5	82
V_s= 0.24, Q = 440, D = 0.34, T_r= 3.0, SG_e= 1.004								
0	0	4.9/0.05	8/ 2.6	0.5/ 0.2	45/1.4	0	0	-
0.096	0.1	4.5/0.04	244/ 4.1	66/11.4	33/1.9	27	4.1/1.3	63
0.82	1.0	4.2/0.06	140/10.0	36/ 2.3	2.8/0.1	94	16 /1.6	71
1.57	1.8	4.2/0.06	136/ 7.6	35/ 1.6	2.8/0.6	94	18 /0.3	78
2.32	2.7	4.2/0.04	140/11.6	36/ 2.5	2.2/0.9	95	17 /0.6	80
2.69	6.7	4.3/0.04	10/ 4.0	0.4/ 0.2	1.9/0.9	97	19 /1.0	75
V_s= 0.30, Q = 550, D = 0.43, T_r= 2.3, SG_e= 1.005								
0	0	4.7/0.02	8/ 1.9	0.4/ 0.1	38/1.3	0	0	-
0.096	0.1	4.4/0.03	219/12.8	56/ 6.0	33/4.3	14	3.2/0.2	150
0.82	0.8	4.3/0.01	106/ 3.1	24/ 0.4	16/3.1	57	11 /0.4	93
1.57	1.5	4.2/0.01	134/ 6.4	32/ 0.2	6.1/0.4	84	16 /1.1	91
2.32	2.2	4.2/0.01	140/ 5.9	33/ 0.2	3.8/0.5	90	17 /1.1	91
2.69	5.3	4.2/0.02	11/ 3.2	0.5/ 0	3.2/0.9	92	18 /0.7	96

Table B.6 Tower fermentation of whey permeate, data at various tower sections.

H* m	T_r^* h	X_a g/l DW	S_1' g/lh	q_1 g/gh	E' g/lh	ν g/lh
V_s = 0.044						
0.048	0.3	97	66	0.68	33	0.3
0.46	2.9	59	1.1	0.02	0.4	0
1.20	7.5	19	0.1	0.01	0.1	0
1.95	12.3	13	0	0	0	0
2.51	20.5	5	0	0	0	0
V_s = 0.080						
0.048	0.17	101	45	0.45	18	0.2
0.46	1.6	56	9.6	0.17	4.6	0.1
1.20	4.1	8	0.2	0.03	0.2	0
1.95	6.8	5	0	0	0	0
2.51	14.0	2	0	0	0	0
V_s = 0.17						
0.048	0.08	96	91	0.95	38	0.4
0.46	0.75	63	23	0.37	11	0.2
1.20	2.0	28	1.3	0.05	0	0
1.95	3.2	24	0.4	0.02	1	0
2.51	6.6	11	0	0	0	0
V_s = 0.24						
0.048	0.06	66	108	1.64	37	0.7
0.46	0.5	51	35	0.69	14	0.3
1.20	1.4	36	0	0	1.9	0.1
1.95	2.3	36	1.0	0.03	0	0
2.51	4.7	18	0	0	0.3	0
V_s = 0.30						
0.048	0.04	56	60	1.07	36	0.6
0.46	0.4	40	25	0.63	12	0.3
1.20	1.1	28	14	0.50	6.8	0.2
1.95	1.8	33	3.3	0.10	1.7	0.1
2.51	3.8	17	0.2	0	0.3	0

B.4 TOWER FERMENTATION OF WHEY PERMEATE ENRICHED WITH MOLASSES

(40:60 g/l lactose : sucrose)

The results for each V_s were averaged from two sets of samples.

e - The power of 10 of the cell concentration ; S' - Volumetric rate of total sugar utilization

$q_{,t}$ - Specific rate of total sugar utilization ; X_{KM} - *K.marxianus* cell concentration

S_t - Total sugar concentration ; X_{aKM} - Average *K.marxianus* cell concentration

Table B.7 Data for tower fermentation of whey permeate enriched with molasses at various sampling points.

H m	T_{ri} h	pH	WW g/l	DW g/l	X cell/ml	X_{KM} cell/ml	S_l g/l	S_s g/l	S_t g/l	S_{lu} %	S_{su} %	S_{tu} %	E g/l	Y %
V_s= 0.087, Q = 160, D = 0.13, T_r= 8.0, SG_e= 1.014														
0	0	5.0/0.05	23/ 9	9/ 1	-	-	40/1.1	58/1.0	98	-	-	-	-	-
0.096	0.3	4.8/0.05	530/26	160/ 1	(5.1/3.9)e9	(2.4/2.0)e9	35/4.9	13/1.3	48	12	78	51	17/1.3	64
0.82	2.6	5.0/0.05	550/23	164/ 8	(4.1/2.5)e9	(1.3/0.7)e9	28/0.8	0.5/0	29	29	99	71	36/5.3	97
1.57	5.0	5.0/0.05	634/77	185/21	(2.1/0.3)e9	(1.1/0.1)e9	13/2.0	0.4/0	13	68	99	87	41/1.2	90
2.32	7.4	5.0/0.10	209/11	58/ 4	(1.3/0.3)e9	(6.5/3.5)e8	9.3/0.7	0.4/0	9.7	77	99	90	43/0.3	90
2.69	18.2	5.0/0.20	40/18	2/ 1	(1.3/0)e7	(1.1/0.1)e7	8.1/0	0.4/0	8.5	80	99	91	41/0.9	86
V_s= 0.12, Q = 220, D = 0.17, T_r= 5.8, SG_e= 1.020														
0	0	5.0/0.05	18/ 1	2/ 0	-	-	41/0.9	57/7.8	98	-	-	-	-	-
0.096	0.2	4.7/0.03	466/50	136/11	(3.8/0.4)e9	(8.5/7.5)e8	40/0.8	22/2.8	62	1	62	37	15/0.4	75
0.82	1.9	5.0/0.08	474/ 2	142/ 1	(4.0/1.6)e9	(5.9/5.1)e8	39/1.4	2.3/0.7	41	3	96	58	25/1.5	83
1.57	3.6	5.1/0.15	568/78	174/42	(7.0/5.0)e9	(8.0/7.0)e8	34/1.6	1.3/0.2	35	17	98	64	30/0.3	87
2.32	5.3	5.1/0.08	258/47	74/15	(2.8/1.6)e9	(8.4/7.6)e8	29/1.6	1.3/0.1	30	30	98	70	34/1.3	92
2.69	13.2	5.0/0.08	17/ 1	1/ 0	(9.4/0.7)e5	(7.2/1.0)e5	27/1.4	1.3/0.1	28	33	98	71	33/0.5	88
V_s= 0.14, Q = 260, D = 0.20, T_r= 4.9, SG_e= 1.020														
0	0	4.8/0.05	18/ 1	3/ 0	-	-	46/6.8	62/3.5	108	-	-	-	-	-
0.096	0.2	4.6/0.03	450/64	124/22	(3.7/1.4)e9	(2.7/0.3)e8	44/5.3	23/0.8	67	4	62	37	13/0.7	58
0.82	1.6	5.1/0.05	484/22	145/ 8	(8.7/2.3)e9	(2.3/1.7)e9	39/3.6	2.2/0.3	41	16	97	62	28/1.1	76
1.57	3.1	5.0/0	416/ 8	121/ 2	(7.2/1.2)e9	(3.4/2.2)e8	32/1.1	1.3/0	33	31	98	70	30/2.5	74
2.32	4.5	5.0/0.08	400/18	114/ 5	(4.8/4.0)e9	(3.8/1.8)e8	24/2.1	1.2/0.1	25	47	98	76	33/3.5	74
2.69	11.1	5.0/0.05	17/ 2	2/ 1	(6.8/2.8)e6	(4.2/2.2)e6	23/3.7	1.1/0	24	49	98	77	34/4.3	76

Table B.8 Data for tower fermentation of whey permeate enriched with molasses at various tower sections.

H* m	T^*_r h	X_{aKM} g/l DW	X_a g/l DW	S'_l g/lh	q_l g/gh	S'_s g/lh	q_s g/gh	S'_t g/lh	q_t g/gh	E' g/lh	v g/lh
V_s= 0.087											
0.048	0.2	170	160	16	0.09	148	0.43	164	1.03	57	0.4
0.46	1.5	134	162	2.9	0.02	5.4	0.03	8.3	0.05	8.3	0.1
1.20	3.8	90	174	6.9	0.07	0	0	6.6	0.04	2.0	0
1.95	6.2	66	120	1.4	0.01	0	0	1.4	0.01	0.7	0
2.51	11.6	26	30	0.1	0	0	0	0.1	0	0	0
V_s= 0.12											
0.048	0.1	66	136	1.8	0.03	162	1.19	164	1.21	66	0.5
0.46	1.1	57	139	0.5	0.01	12	0.09	12	0.09	6.4	0
1.20	2.8	55	158	3.2	0.06	0.5	0	3.7	0.02	2.5	0
1.95	4.5	64	124	3.1	0.05	0.1	0	3.1	0.03	2.5	0
2.51	9.3	33	38	0.1	0	0	0	0.1	0	0	0
V_s= 0.14											
0.048	0.1	23	124	8.6	0.37	208	1.68	217	1.75	68	0.5
0.46	0.9	93	135	3.9	0.04	15	0.11	19	0.14	11	0.1
1.20	2.3	95	133	4.8	0.03	0.6	0	5.4	0.05	1.6	0
1.95	3.8	29	118	5.0	0.18	0.1	0	5.1	0.04	1.9	0
2.51	7.8	15	58	0.1	0	0	0	0.1	0	0.2	0

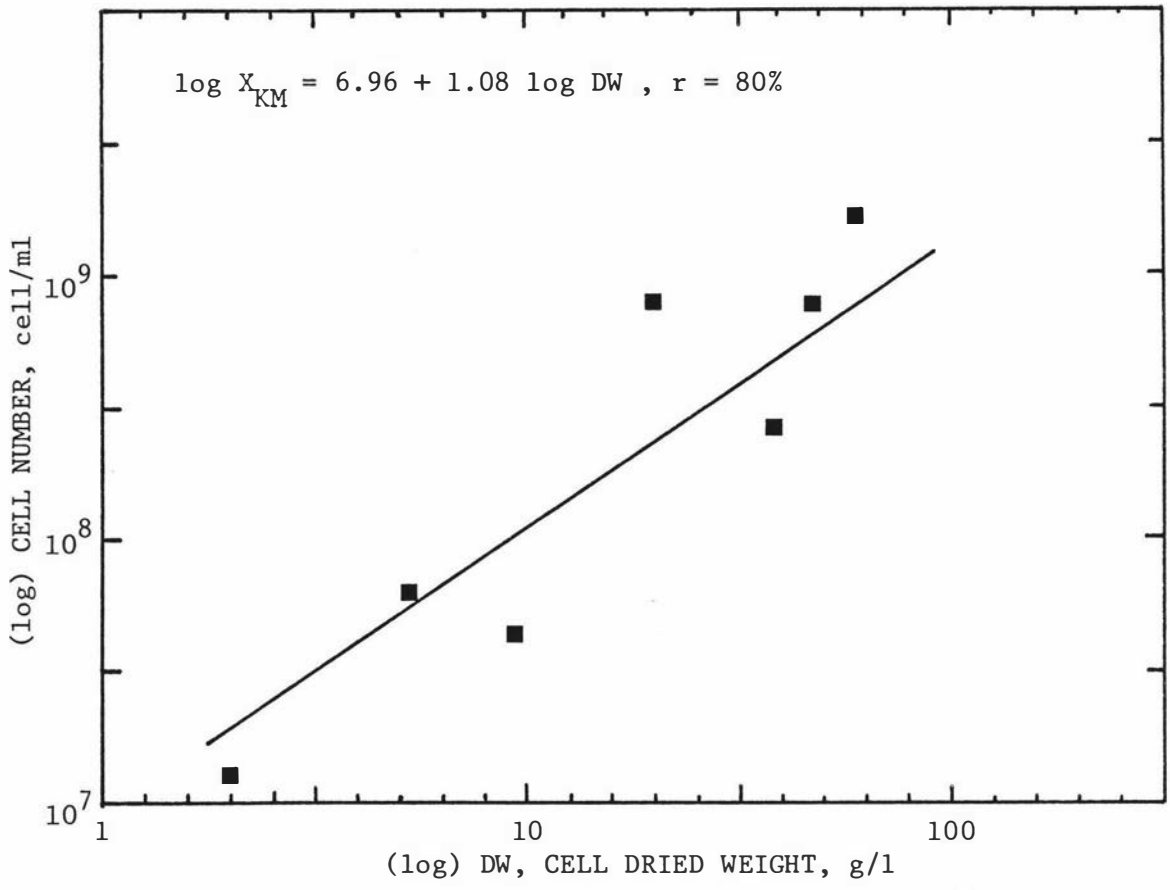


Fig.B.1 *K.marxianus* Y42 cell plate count number vs cell dried weight. Cells were obtained during the tower fermentation of whey permeate.

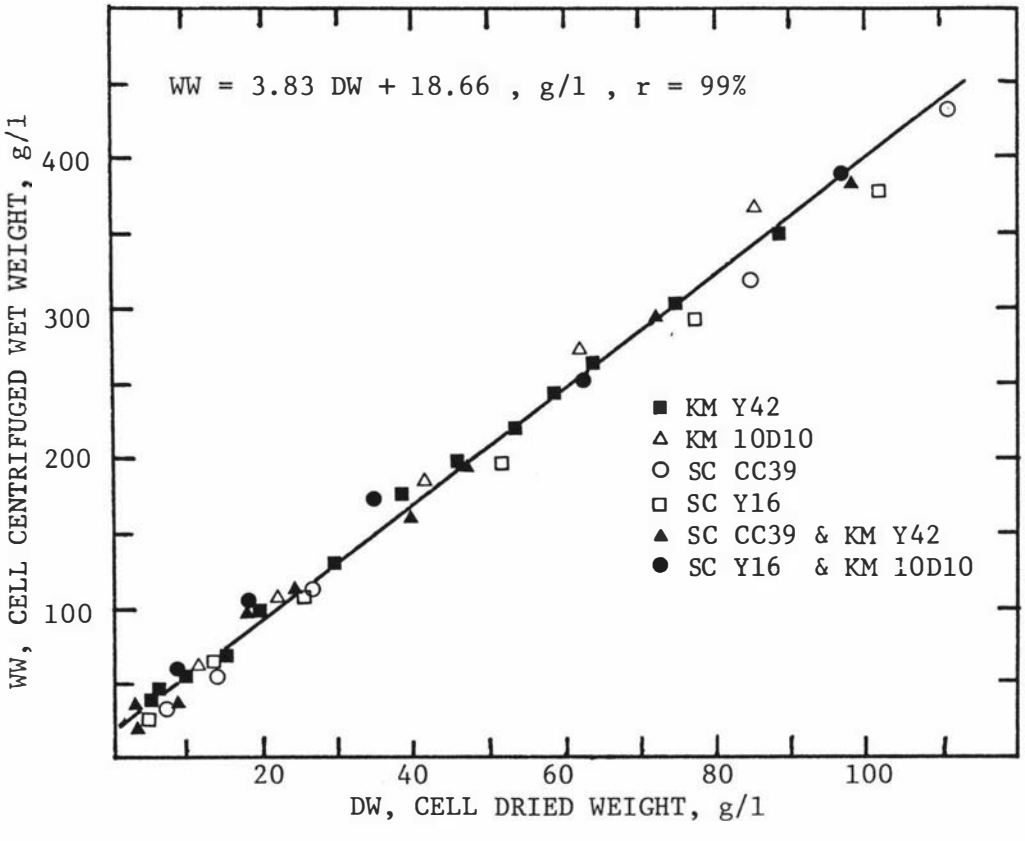


Fig.B.2 Cell centrifuged wet weight vs cell dried weight.

B.5 TOWER FERMENTATION OF MOLASSES (100 g/l sucrose)

Data for each superficial lquid velocity were averaged from 2 separate sets of samples except for the last two velocities at which there was only one set of samples.

Table B.9 Tower fermentation of molasses, data at various sampling points.

H m	T_{ri} h	pH	WW g/l	DW g/l	S_s g/l	S_{su} %	E g/l	Y %
V_s = 0.084, Q = 160, D = 0.12, T_r = 8.3, SG_e = 1.020								
0	0	5.1/0.10	14/ 0.4	2/0.4	97/12.5	0	0	-
0.096	0.3	5.0/0.01	171/ 7.7	57/1.4	3.4/ 0.3	97	51 /0.8	101
0.82	2.7	5.0/0.01	166/ 5.3	56/0.8	1.5/ 0.1	99	49 /1.3	95
1.57	5.2	5.0/0	164/ 2.2	55/0.2	1.3/ 0.1	99	49 /1.5	96
2.32	7.7	5.0/0	148/10.1	49/4.7	1.3/ 0.1	99	51 /2.6	98
2.69	18.9	5.0/0	17/ 2.2	2/0.1	1.3/ 0.1	99	49 /0	95
V_s = 0.17, Q = 310, D = 0.24, T_r = 4.2, SG_e = 1.020								
0	0	5.2/0.10	19/ 7.1	4/2.4	102/ 0.7	0	0	-
0.096	0.2	5.0/0.04	166/21.1	51/0.3	46/14.6	55	25 /0.4	82
0.82	1.4	4.9/0.02	225/11.2	72/3.8	2.5/ 0.5	98	43 /1.4	80
1.57	2.6	4.9/0.01	218/ 7.7	70/2.5	1.5/ 0	99	50 /6.5	92
2.32	3.8	5.0/0.01	195/ 6.3	62/2.2	1.4/ 0	99	47 /1.0	87
2.69	9.4	4.9/0.01	12/ 0.6	2/0.1	1.4/ 0	99	48 /0.2	89
V_s = 0.20, Q = 360, D = 0.28, T_r = 3.5, SG_e = 1.020								
0	0	5.2/0.02	14/ 0.1	3/0	104/ 2.1	0	0	-
0.096	0.1	5.0/0.01	163/17.5	49/6.4	57/ 9.9	45	23 /3.5	89
0.82	1.1	4.8/0.03	224/ 1.7	74/0.5	3.6/ 0.7	97	51 /0.4	94
1.57	2.2	4.9/0.01	228/ 1.4	76/0.6	1.7/ 0.1	98	53 /0.3	97
2.32	3.3	5.0/0.02	215/ 0.7	72/0.4	1.8/ 0.1	98	51 /2.3	94
2.69	8.0	5.0/0.03	16/ 0.2	3/0	1.6/ 0.1	99	50 /0.2	91
V_s = 0.33, Q = 600, D = 0.47, T_r = 2.4, SG_e = 1.020								
0	0	5.2/0.02	12/ 0.5	2/0.1	102/ 3.2	0	0	-
0.096	0.1	5.1/0.05	68/ 9.5	16/5.2	88/ 4.4	14	6.2/3.6	82
0.82	0.7	4.8/0.01	203/ 3.2	60/0.7	4.4/ 0.6	96	47 /1.0	89
1.57	1.3	4.8/0.01	197/ 2.0	57/0.6	1.8/ 0.5	98	46 /0.4	86
2.32	2.0	4.9/0.01	184/ 4.1	54/0.4	1.7/ 0.1	98	50 /1.5	92
2.69	4.9	4.9/0.02	31/ 1.0	7/0.2	1.6/ 0.1	98	47 /0.2	87
V_s = 0.46, Q = 850, D = 0.66, T_r = 1.5, SG_e = 1.020								
0	0	4.9	15	3	98	0	0	-
0.096	0.1	4.9	24	4	97	0.4	1.6	744
0.82	0.5	4.7	106	28	54	45	18	78
1.57	0.9	4.6	135	38	16	84	38	87
2.32	1.4	4.5	124	35	4.7	95	39	78
2.69	3.4	4.6	51	12	1.4	99	45	87
V_s = 0.56, Q = 1040, D = 0.80, T_r = 1.2, SG_e = 1.030								
0	0	5.0	17	3	97	0	0	-
0.096	0.1	4.9	16	3	96	0.4	0.6	279
0.82	0.4	4.9	28	5	96	0.7	3	637
1.57	0.8	4.7	86	22	43	56	12	40
2.32	1.2	4.6	103	26	27	72	25	66
2.69	2.8	4.5	48	11	10	90	39	83

Table B. 10 Tower fermentation of molasses, data at various tower sections.

H* m	T_r^* h	X_a g/l DW	S'_s g/lh	q_s g/gh	E' g/lh	ν g/lh
V_s = 0.084						
0.048	0.2	57	296	5.19	160	2.8
0.46	1.5	57	0.8	0.01	0	0
1.20	3.9	56	0.1	0	0.2	0
1.95	6.4	52	0	0	0.5	0
2.51	13.2	26	0	0	0	0
V_s = 0.17						
0.048	0.08	51	351	6.88	155	3.0
0.46	0.75	62	37	0.60	15	0.2
1.20	2.0	71	0.8	0.01	5.4	0.1
1.95	3.2	66	0.1	0	0	0
2.51	6.6	32	0	0	0	0
V_s = 0.20						
0.048	0.07	49	349	7.12	167	3.4
0.46	0.6	62	53	0.86	28	0.5
1.20	1.7	75	1.8	0.02	2.7	0
1.95	2.7	74	0	0	0	0
2.51	5.6	38	0	0	0	0
V_s = 0.33						
0.048	0.04	16	172	10.75	76	4.8
0.46	0.4	38	136	3.58	66	1.7
1.20	1.0	59	4.0	0.07	0	0
1.95	1.7	56	0.2	0	5.0	0.1
2.51	3.4	31	0	0	0	0
V_s = 0.46						
0.048	0.03	4	7.0	1.75	28	7.0
0.46	0.3	16	100	6.25	39	2.4
1.20	0.7	33	85	2.58	44	1.3
1.95	1.2	37	24	0.66	1.3	0
2.51	2.4	24	1.6	0.07	3.0	0.1
V_s = 0.56						
0.048	0.02	3	8.4	2.80	13	4.3
0.46	0.2	4	0.8	0.16	5.9	1.5
1.20	0.6	14	142	10.51	24	1.8
1.95	1.0	24	43	1.79	34	1.4
2.51	2.0	19	10	0.54	8.2	0.4

APPENDIX C

TOWER FERMENTATION OF MOLASSES

Tower fermentation of molasses was performed using *S. cerevisiae* AWRI 350 (FT146 or SC146). The results of the tower fermentation of molasses have been included here in order to provide a comparison for the tower fermentation of whey permeate (Chapter 4) and whey permeate enriched with molasses (Chapter 5). The data used for plotting the graphs are given in Appendix B.5.

C.1 THE RELATIONSHIP BETWEEN TOWER HEIGHT AND VARIOUS FERMENTATION PARAMETERS

C.1.1 Sucrose and ethanol concentrations(S_s, E)

Sucrose concentration (S_s) (fig.C.1 a) showed a general decrease as height increased at a constant superficial liquid velocity. The rate at which the concentration reduced with an increase in height decreased as the velocity increased. The height at which sucrose reduced to less than 10 g/l (90% utilization) was higher at greater velocity (table C.1). Sucrose concentration reduced to less than 4 g/l (96% utilization) at 0.82 m for all velocities up to 0.33 mm/s. Sucrose concentration decreased to less than 10 g/l at the height of 2.32 m and the exit when the velocities were 0.46 and 0.56 mm/s, respectively. Cell wash out was observed at these two velocities (sect. C.1.4 and C.3.3).

In contrast ethanol concentration (E) increased as the height increased, with some exceptions, at a constant velocity (fig.C.1 b). The concentration increased rapidly to 51 g/l as the height increased to 0.096 m at the lowest velocity and then remained relatively constant over the remaining heights in the tower. For velocities of 0.17 and 0.20 mm/s, the concentration increased rapidly to a value greater than 46 g/l as the height increased to 0.82 m and then remained relatively constant over the remaining heights. For velocity of 0.33 mm/s, the concentration increased to 46 g/l when the height was 1.57 m and was relatively constant over the remaining tower sections. Ethanol concentration increased slowly and peaked at 45 and 39 g/l at the tower exit when the velocities were 0.46 and 0.56 mm/s, respectively.

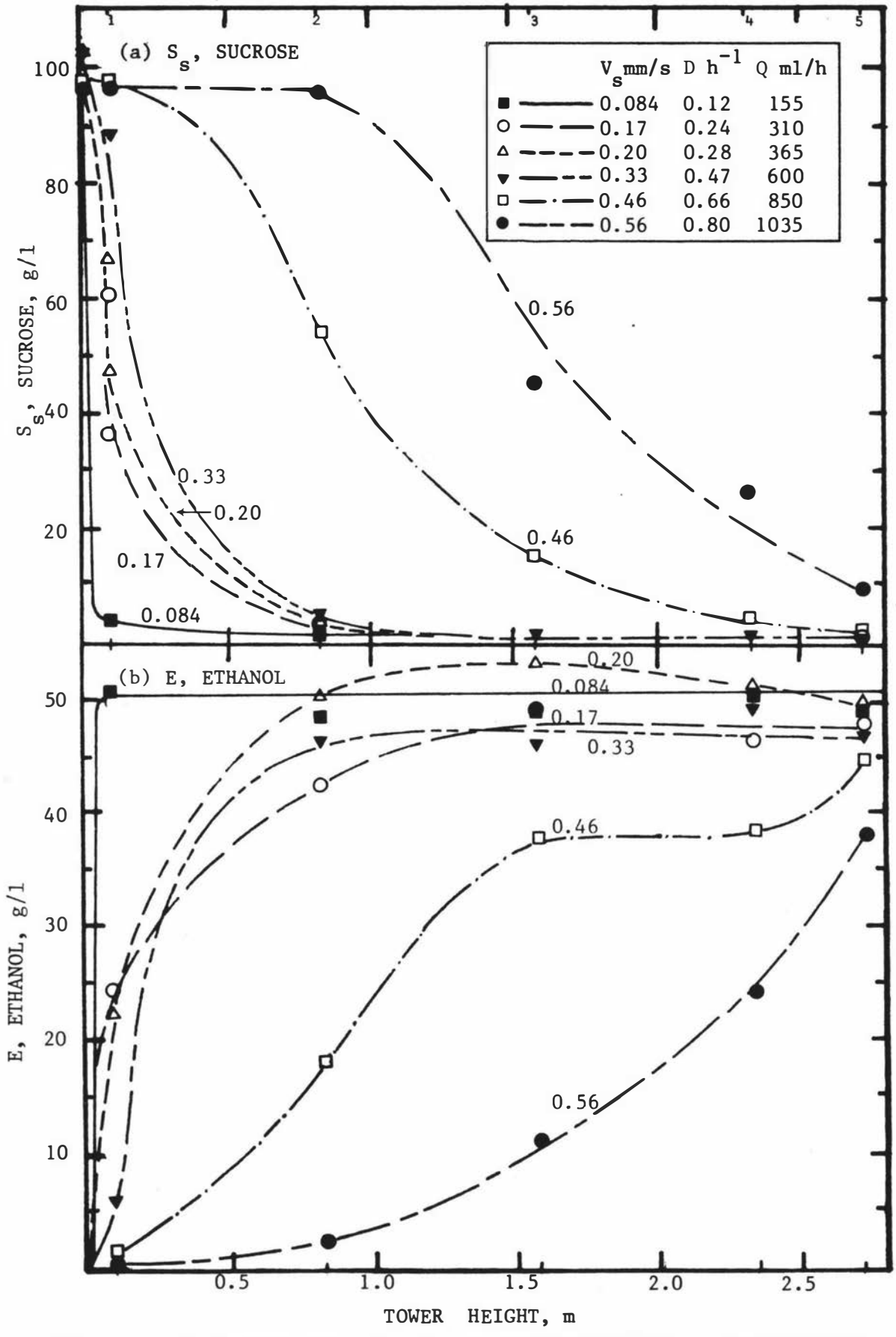


Fig.C.1 (a) Sucrose and (b) ethanol concentrations vs tower height at various superficial liquid velocities.

Table C.1 Percentage sucrose utilization at various heights in the tower and superficial liquid velocities.

location/height		S_{su}(%), at various V_s (mm/s)					
	m	0.084	0.17	0.20	0.33	0.46	0.56
1	0.096	97	55	45	14	0.4	0.4
2	0.82	99	98	97	96	45	0.7
3	1.57	99	99	98	98	84	56
4	2.32	99	99	98	98	95	72
5	2.69	99	99	99	99	99	90

The results showed that for velocities up to 0.33 mm/s the high cell concentration used reduced 100 g/l sucrose to less than 5 g/l within the first 0.82 m of the height in the tower. Most ethanol was also produced up to this height (0.82 m). At heights greater than this there was little fermentation taking place. At velocities greater than this (0.46 and 0.56 mm/s) fermentation occurred throughout the tower. The highest ethanol concentration was measured at the exit. At these two velocities cell wash out was observed and thus the data were not at steady state (sect. C.1.4).

C.1.2 Rates of sucrose utilization and ethanol production

(a) Volumetric rates There was a generally rapid reduction of the volumetric rates of sucrose utilization (S_s') and ethanol production (E') (fig.C.2) from high values, at 0.048 m, to less than 5 g/lh as the mean height increased for all velocities up to 0.33 mm/s. The mean height at which this occurred was 0.048 m for the lowest velocity (0.084 mm/s) and was 0.46 m for all other velocities up to 0.33 mm/s. The volumetric rates were less than 5 g/lh at heights greater than these.

For velocities greater than 0.33 mm/s (0.46 and 0.56 mm/s), the volumetric rates increased with the mean height to a peak and then decreased with further height increase. The maximum volumetric rate of sucrose utilization was 100 and 142 g/lh and occurred at mean heights of 0.46 and 1.20 m for the two respective velocities. The maximum volumetric rate of ethanol production of 44 and 34 g/lh for these respective velocities was observed at greater mean height of 1.20 and

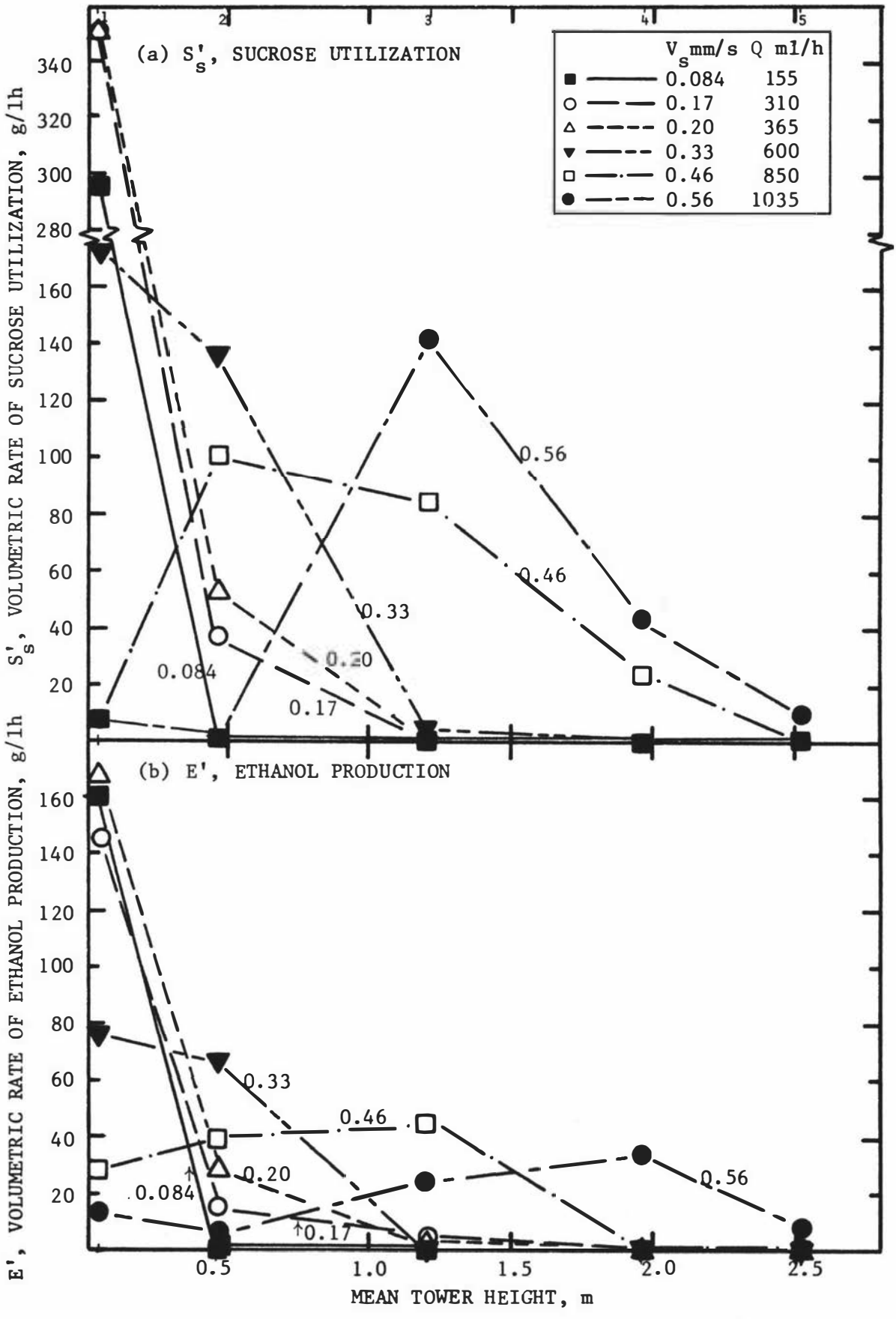


Fig.C.2 Volumetric rates of (a) sucrose utilization and (b) ethanol production vs mean tower height at various superficial liquid velocities.

1.95 m respectively. This showed that there was high fermentative activity inside the tower between 0.096 and 1.57 m as a result of the yeast mass being lifted up by the medium from lower heights.

The results showed that, for all velocities up to 0.33 mm/s, most sucrose utilization and most ethanol production occurred rapidly within heights up to 0.82 m. There was little sucrose utilization and ethanol production at heights greater than this. For velocities greater than 0.33 mm/s, there was sucrose utilization and ethanol production throughout the entire tower but the maximum volumetric rates were lower than those observed at lower velocities because of lower biomass concentration (sect. C.1.4).

(b) Specific rates The specific rates of sucrose utilization (q_s) and ethanol production (ν) (fig.C.3) decreased rapidly with mean height increase for all velocities up to 0.33 mm/s. The trend was similar to the corresponding volumetric rates (sect. C.1.2 a). The specific rates decreased from high values to less than 0.1 g/gh with mean height increase.

A slightly different profile was observed for the specific rate of ethanol production when the velocity was 0.46 mm/s. At this velocity, the specific rate of ethanol production was not zero until the mean height was 1.95 m. This was different from the profile observed for the volumetric rate at this velocity (fig.C.2 b).

Thus, there was a general trend of decreasing volumetric and specific rates with mean height increase for all velocities up to 0.33 mm/s and up to 0.82 m. Above 0.82 m, all rates were very slow.

C.1.3 Ethanol yield (Y)

For all superficial liquid velocities except the two highest velocities (0.46 and 0.56 mm/s), the ethanol yield (fig.C.4) showed only minor change between 0.096 and 2.32 m. It varied between 78 and 98%.

The yield at the lowest sampling point (0.096 m) was very high (between 280 and 740%) for the two velocities of 0.46 and 0.56 mm/s. For the velocity of 0.56 mm/s, the yield decreased to a low value of 40% at height of 1.57 m and then increased to 83% when the effluent left the tower (2.69 m). The fluctuations observed were due to back-mixing of ethanol into the feed medium which contained high concentration of sucrose, at the bottom of the tower, and analytical

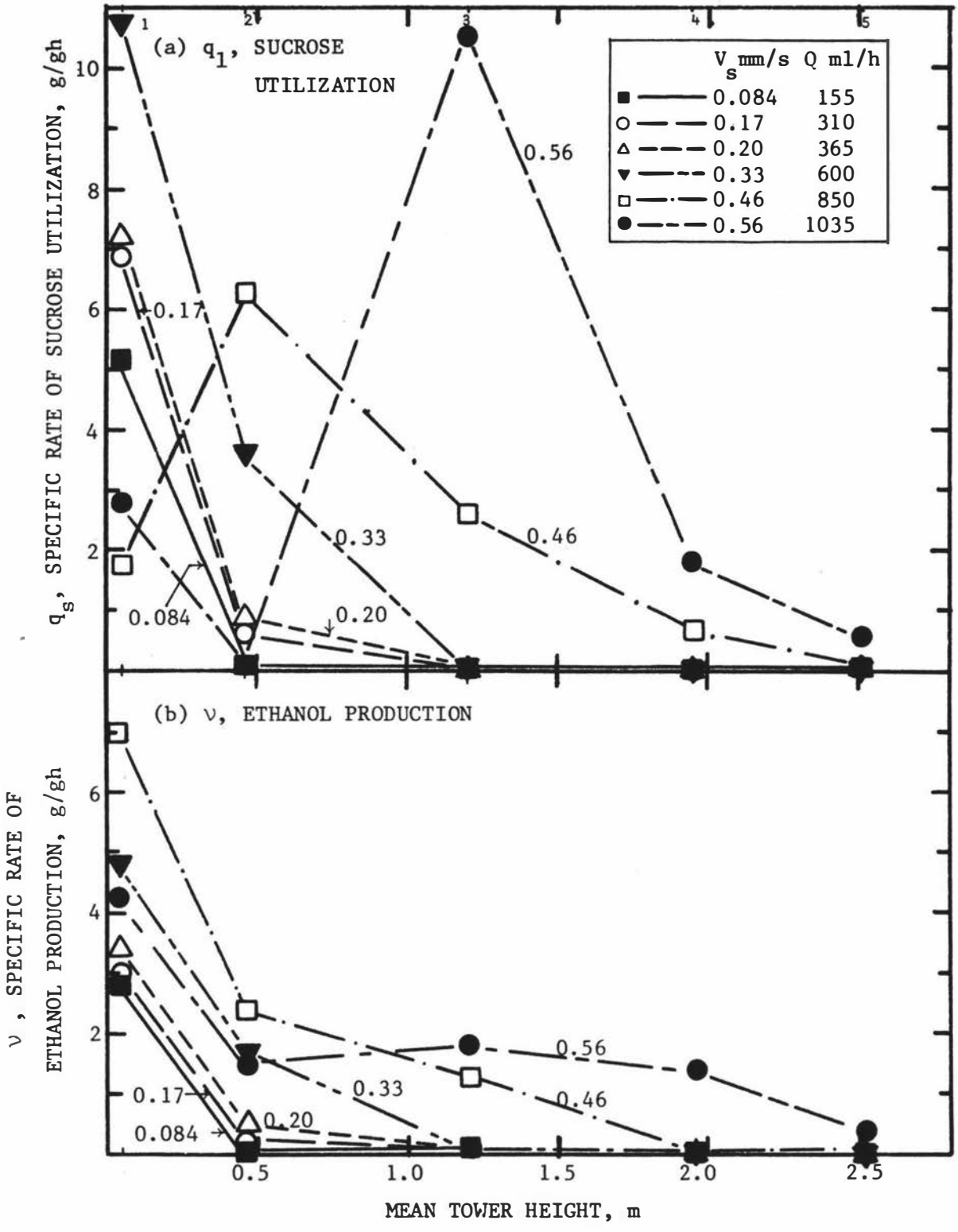


Fig.C.3 Specific rates of (a) sucrose utilization and (b) ethanol production vs mean tower height at various superficial liquid velocities.

uncertainty (between 12 and 85% of the yield value, sect. E.3) at high sugar and low ethanol concentrations.

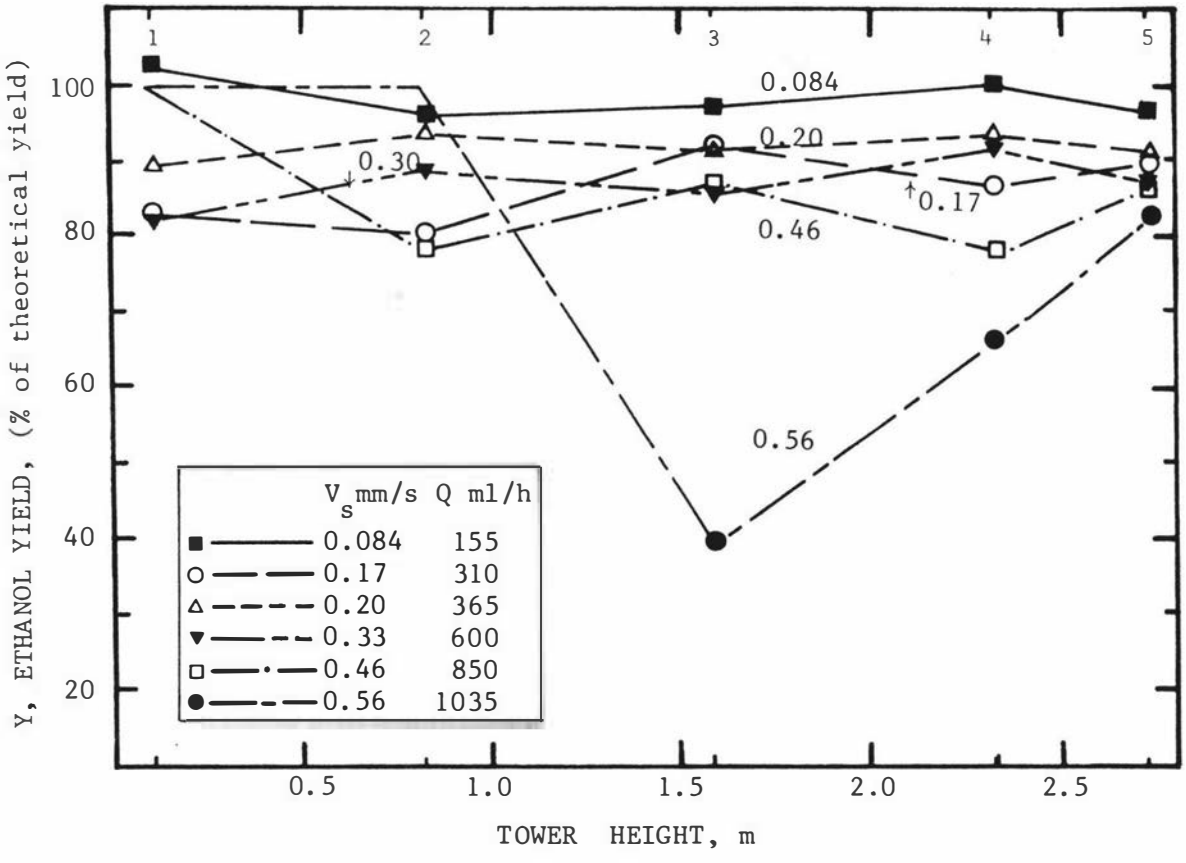


Fig.C.4 Ethanol yield (% of theoretical yield based of sucrose utilized) vs tower height at various superficial liquid velocities.

The results showed that the yield changed little as the height increased for all velocities below wash out. The variations that occurred were due to experimental uncertainties.

C.1.4 Cell concentration

The cell concentration (fig.C.5) increased as the height in the tower increased from 0.096 to 0.82 m for all velocities except the lowest velocity (0.084 mm/s). As the height increased further to 2.32 m, the concentration was relatively constant at 74, 74 and 57 g/l DW (210, 220 and 200 g/l WW) for velocities of 0.17, 0.20 and 0.33 mm/s, respectively. For a higher velocity of 0.46 mm/s, the concentration increased to a maximum of 38 g/l DW (135 g/l WW) at height of 1.57 m and then reduced to 35 g/l DW (124 g/l WW) as the height increased further to 2.32 m. The trend was slightly different for the highest

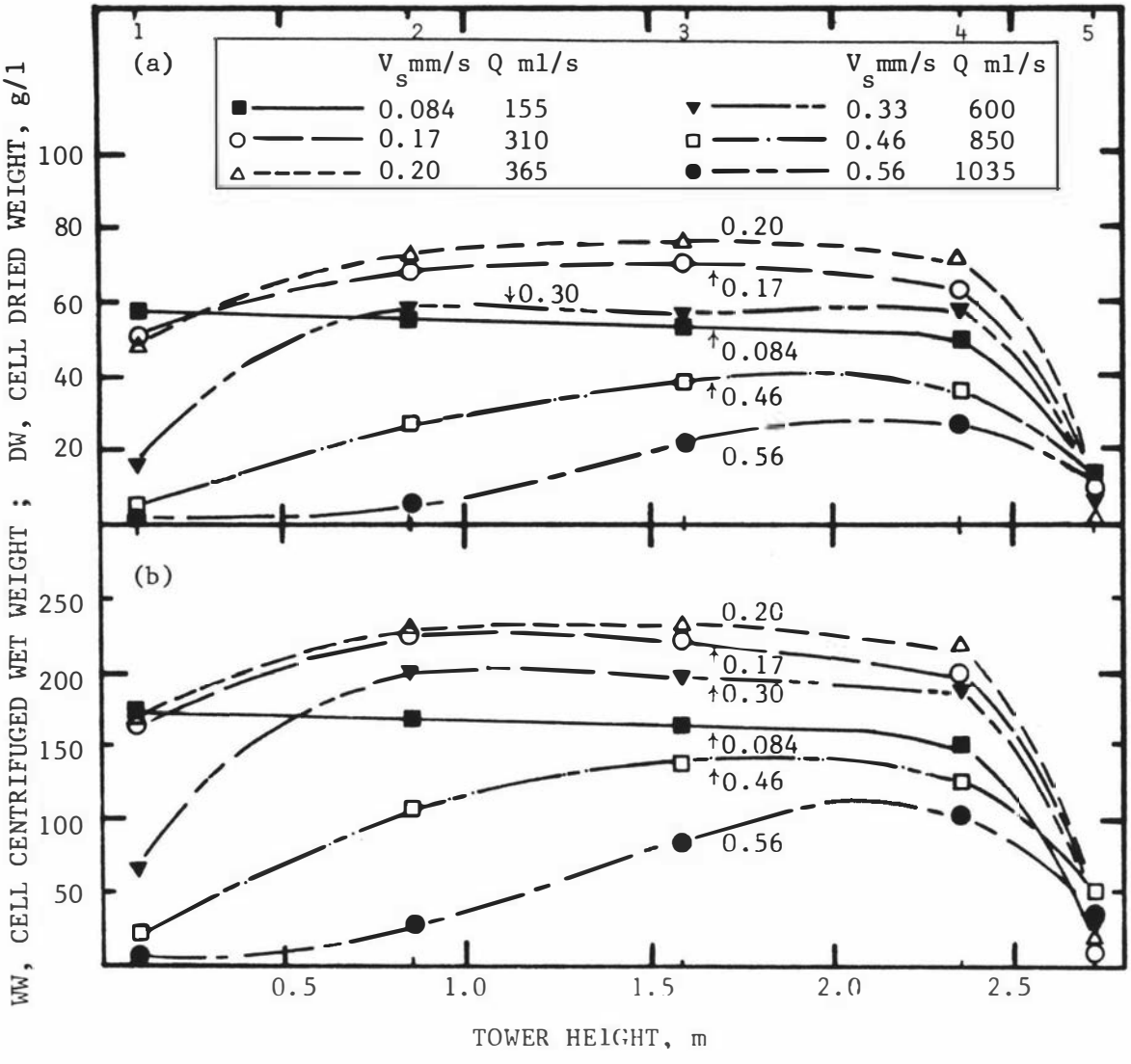


Fig.C.5 Cell concentration vs tower height at various superficial liquid velocities.

velocity of 0.56 mm/s, the concentration increased to the highest value of 26 g/l DW (103 g/l WW) at a height of 2.32 m. As the height increased further to the exit, there was a rapid reduction of the concentration to between 2 and 12 g/l DW (16 to 51 g/l WW) for all velocities.

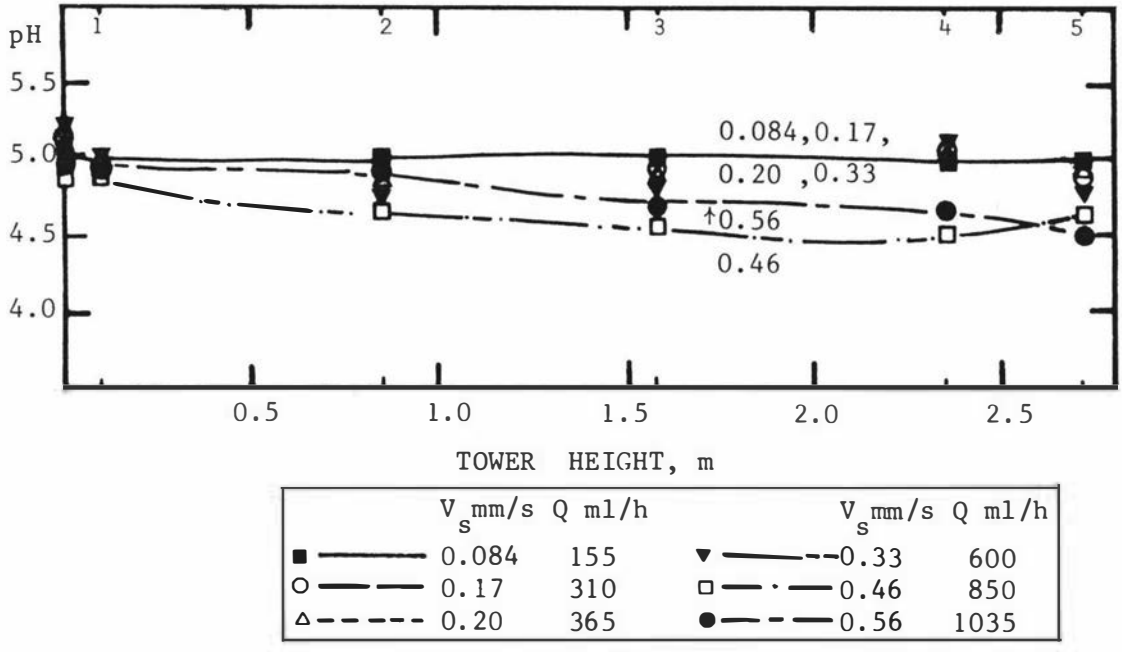
The cell concentration was greater within heights between 0.096 and 2.32 m than within the heights up to 0.096 m because of the moderately flocculent nature of the yeast *S.cerevisiae* FT146 (or SC146). The cell flocs were lifted up to greater tower heights by carbon dioxide and the upward movement of the medium. For these two higher velocities of 0.46 and 0.56 mm/s, a considerable number of the yeast flocs were lifted up from the bottom of the tower by the medium. This had resulted in the cell concentration being high between 1.57 and 2.32 m but lower than the greatest concentration at the lower velocity. There was a slow cell wash out. This was shown by the increase in the cell concentration at the exit to 12 g/l DW (51 g/l WW).

There was an almost linear decrease in the cell concentration from 0.096 to 0.82 m at the lowest velocity of 0.084 mm/s. The cell concentration followed the general trend by decreasing rapidly to 2 g/l DW (17 g/l WW) as the medium left the tower at the exit (2.69 m). This showed that the medium flow and the gas production had not lifted the yeast plug off the bottom of the tower at this velocity but the upflow movement was able to suspend a large number of the yeast flocs inside the tower as shown by the almost constant cell concentration between 0.096 and 2.32 m.

Thus, for all velocities up to 0.33 mm/s, the cell concentration between 0.82 and 2.32 m was constant and was greater than at the lowest height of 0.096 m. At velocities greater than this, the yeast bed was lifted by the medium and carbon dioxide to the upper section of the tower and cells were slowly washed out of the tower.

C.1.5 Medium pH

The fermentation broth pH (fig.C.6) showed a small decrease from between pH 4.9 and pH 5.1 as the height in the tower increased to 0.096 m for all velocities and then was relatively constant (between pH 4.8 and 5.0) as the height increased further to 2.32 m and the exit for all velocities except 0.46 and 0.56 mm/s.



	V_s mm/s	Q ml/h		V_s mm/s	Q ml/h
■ ———	0.084	155	▼ ————	0.33	600
○ — —	0.17	310	□ — · —	0.46	850
△ - - - -	0.20	365	● — - —	0.56	1035

Fig.C.6 Medium pH vs tower height at various superficial liquid velocities

The initial pH decrease indicated that there was high fermentative activity within these sections. At greater heights, there was little activity since most sucrose was utilized when the height of 0.82 m was reached so that there was no change in the pH.

At the two higher velocities of 0.46 and 0.56 mm/s, sucrose was utilized throughout the entire tower. Hence, there was a slow but continuous pH decrease to between pH 4.5 and 4.6 as the height increased to 2.32 m and the exit (2.69 m).

Thus, for all velocities below wash out, there was little change in the medium pH as the height increased except for the initial reduction over the first 0.096 m of the tower due to the high fermentative activity within this section. There was a slow pH decrease throughout the tower at the wash out velocities (0.46 and 0.56 mm/s) because there was active fermentation taking place over the entire tower.

C.2 THE EFFECT OF THE RESIDENCE TIME ON VARIOUS FERMENTATION PARAMETERS

C.2.1 Sucrose and ethanol concentration

Sucrose concentration (S_s) (fig.C.7 a) decreased rapidly as the residence time increased. The sucrose concentration was reduced to less than 4 g/l (greater than 96% utilization) in 1.5 hours for all velocities used. There was very small reduction after this time.

In contrast, ethanol concentration (E) increased rapidly from 0 g/l to greater than 49 g/l as the residence time increased from 0 to 2 hours (fig.C.7 b). The concentration increased slightly as the residence time increased further beyond this time.

The results showed that sucrose was almost all utilized in 1.5 hours and most ethanol was produced in 2 hours for all velocities used. This occurred as a result of the biomass concentration used.

C.2.2 Rates of sucrose utilization and ethanol production

The volumetric rates of sucrose utilization (S'_s) and ethanol production (E') (fig.C.8) reduced very rapidly from high values (350 and 167 g/lh respectively) to less than 1 g/lh as the mean residence time increased from 0.1 to to 1.5 hours for the volumetric rate of sucrose utilization (fig.C.8 a) and 1.2 hours for the volumetric rate of ethanol production (fig.C.8 b). The volumetric rates were less than 1 g/lh when the mean residence time was greater than 1.5 hours.

A similar trend was observed for the specific rates (q_s and ν) (fig.C.9). Both specific rates decreased rapidly from high values (10.8 and 7.0 g/gh respectively) to less than 0.1 g/gh during the same time period.

The results showed that most sucrose utilization and ethanol production occurred during the initial 1.5 hours in the tower. There was little sucrose utilization after 1.5 hours and little ethanol production after 1.2 hours for all velocities used. The very high cell concentration used had resulted in very high volumetric rates of sucrose utilization and ethanol production. The specific rates decreased with an increase in the mean residence time because the biomass was recycled and changed little while sucrose concentration decreased as the mean residence time increased.

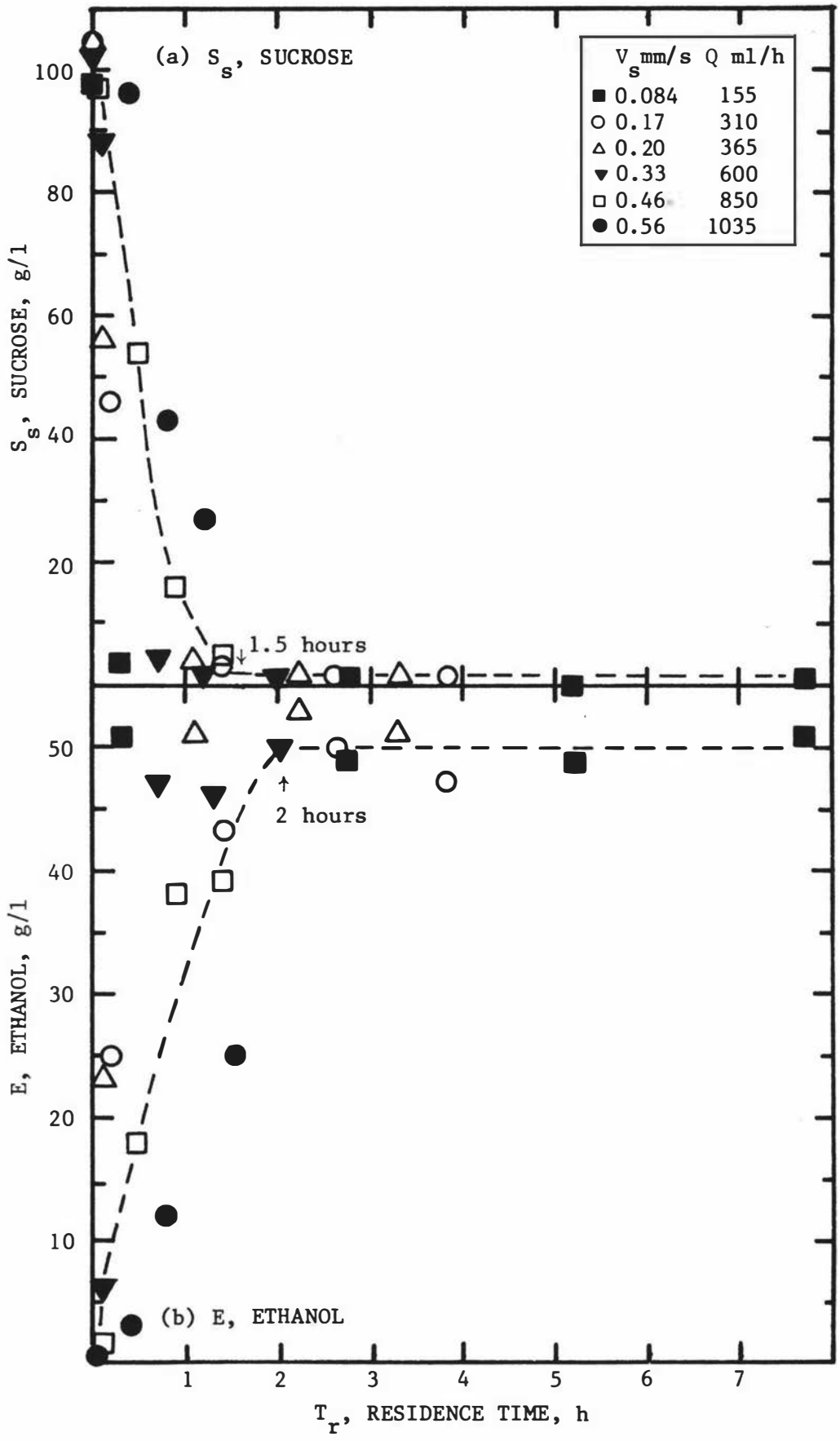


Fig.C.7 (a) Sucrose and (b) ethanol concentrations vs residence time at various superficial liquid velocities.

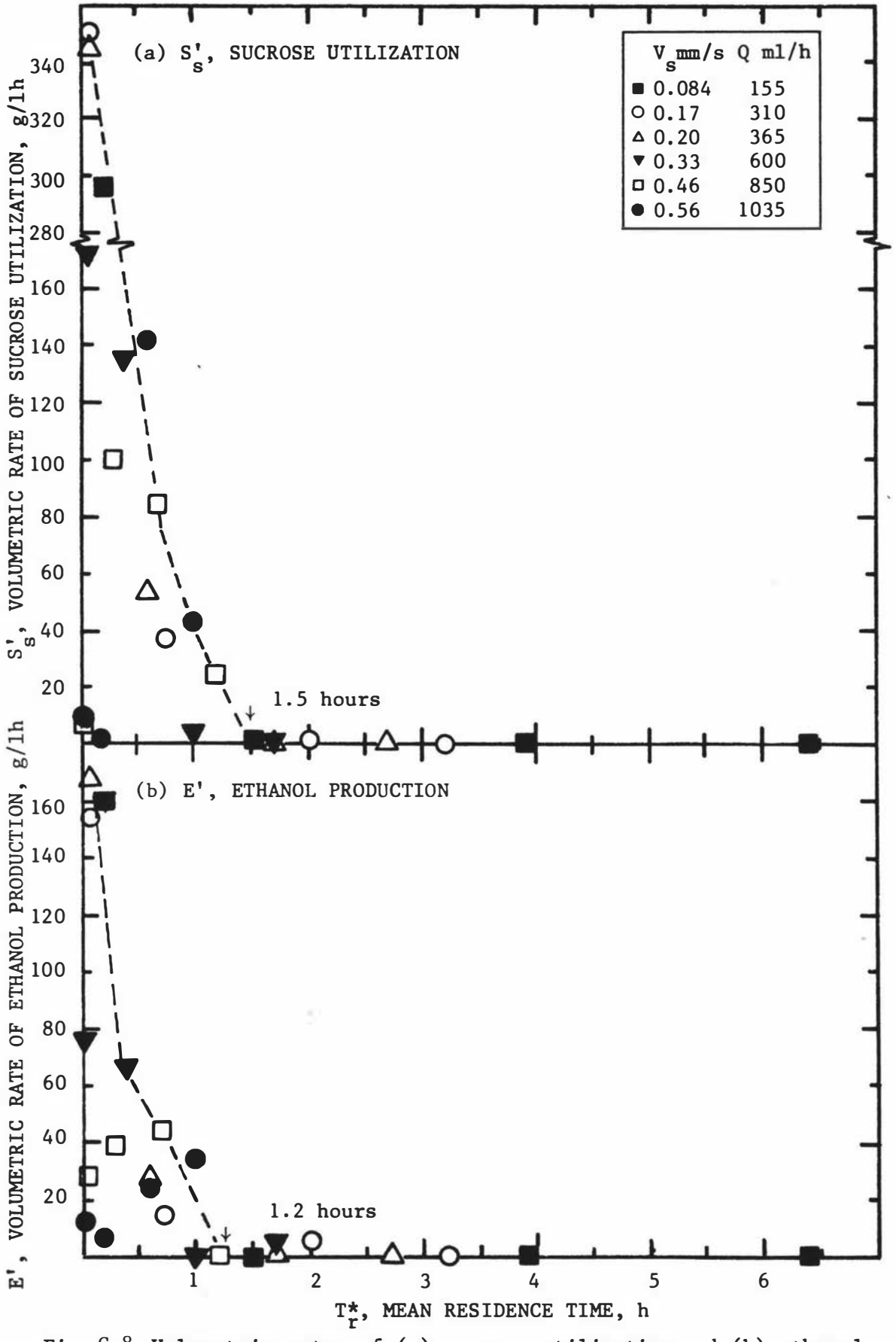


Fig. C.8 Volumetric rates of (a) sucrose utilization and (b) ethanol production vs mean residence time at various superficial liquid velocities

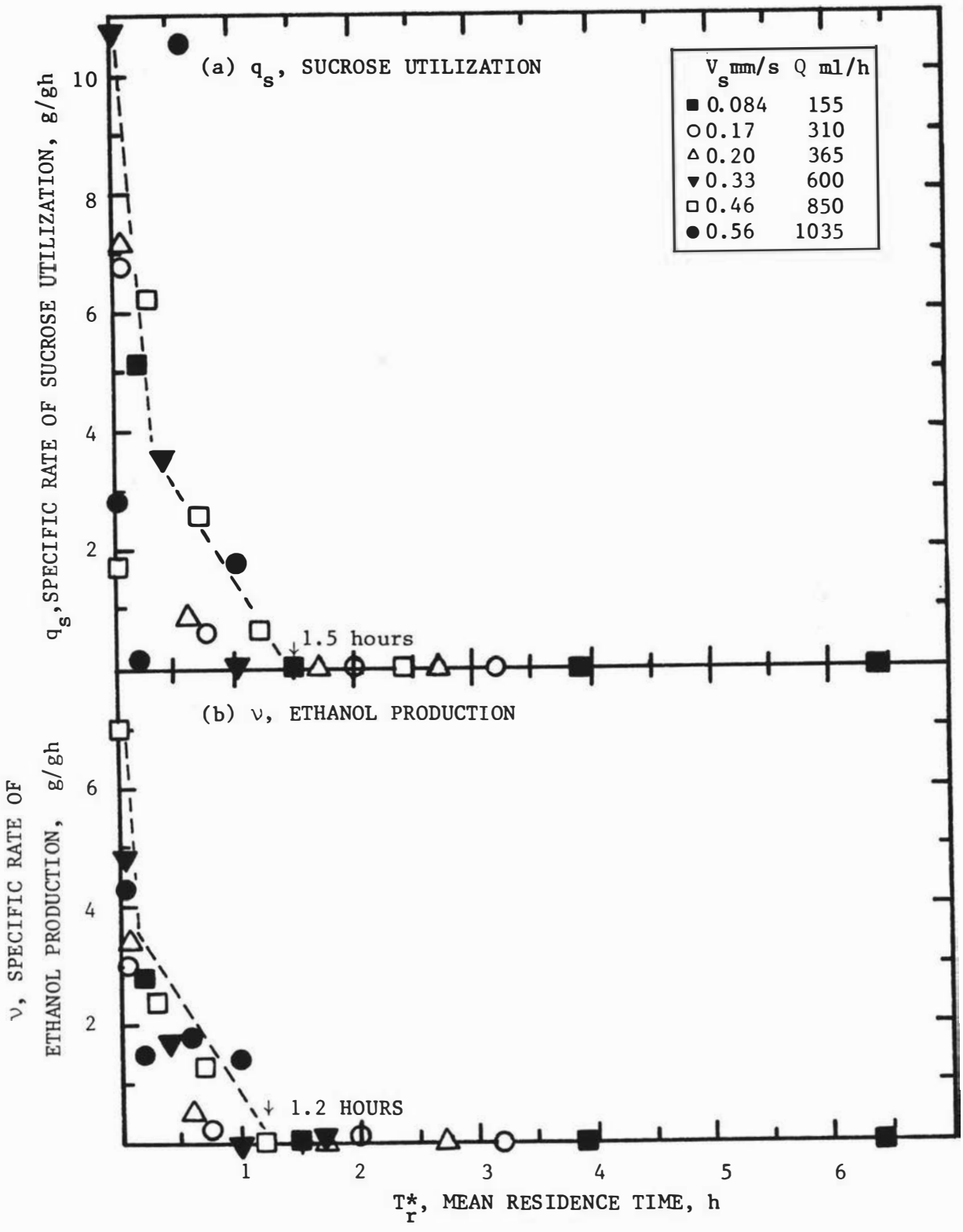


Fig. C.9 Specific rates of (a) sucrose utilization and (b) ethanol production vs mean residence time at various superficial liquid velocities.

C.3 THE EFFECT OF THE SUPERFICIAL LIQUID VELOCITY ON VARIOUS FERMENTATION PARAMETERS

C.3.1 Sucrose and ethanol concentrations

Sucrose concentration showed a general increase with velocity increase with some exceptions (fig.C.10 a). At a height of 0.096 m, the sucrose concentration increased from 3 to 96 g/l as the velocity increased from 0.084 to 0.56 mm/s (curve 1). At heights greater than this the concentration was less than 5 g/l as the velocity increased to 0.33 mm/s and then increased with further increase in the velocity to 0.56 mm/s (curve 2,3 and 4). At the exit (curve 5), there was an increase in the concentration at velocity greater than 0.46 mm/s.

In contrast, ethanol concentration (fig.C.10 b) at a constant tower height, decreased with an increase in the velocity with some exceptions. At a height of 0.096 m, ethanol concentration decreased rapidly from 51 to 0.6 g/l as the velocity increased from 0.084 to 0.56 mm/s as a result of the reduction of sucrose utilization with velocity increase.

At heights greater than this (curves 2,3 and 4), ethanol concentration was high (50 g/l) and was not affected by an increase in the velocity to 0.20 mm/s. As the velocity increased further to 0.56 mm/s, the ethanol concentration decreased with an increase in the velocity. The ethanol concentration was not affected by the velocity below 0.20 mm/s because at these velocities sucrose was almost all utilized below 0.82 m. Thus, ethanol concentration was at its highest value within the heights above 0.82 m. Then as the velocity increased further beyond 0.33 mm/s, ethanol was produced throughout the entire tower because sucrose was being utilized throughtout the tower.

At the exit (curve 5), the ethanol concentration did not decrease with velocity until a high velocity of 0.46 mm/s was reached.

The results showed that the reduction in the fermentation time and cell concentration with velocity increase (fig.C.13) had resulted in an increase in the sucrose concentration at the height of 0.096 m. The trend at greater heights followed that for 0.096 m when the velocity was greater than 0.33 mm/s because there was a reduction in cell concentration at points below 0.82 (fig.C.13). Hence, sucrose was not all utilized at the bottom of the tower and thus the sucrose concentration increased with velocity. As a result of this less

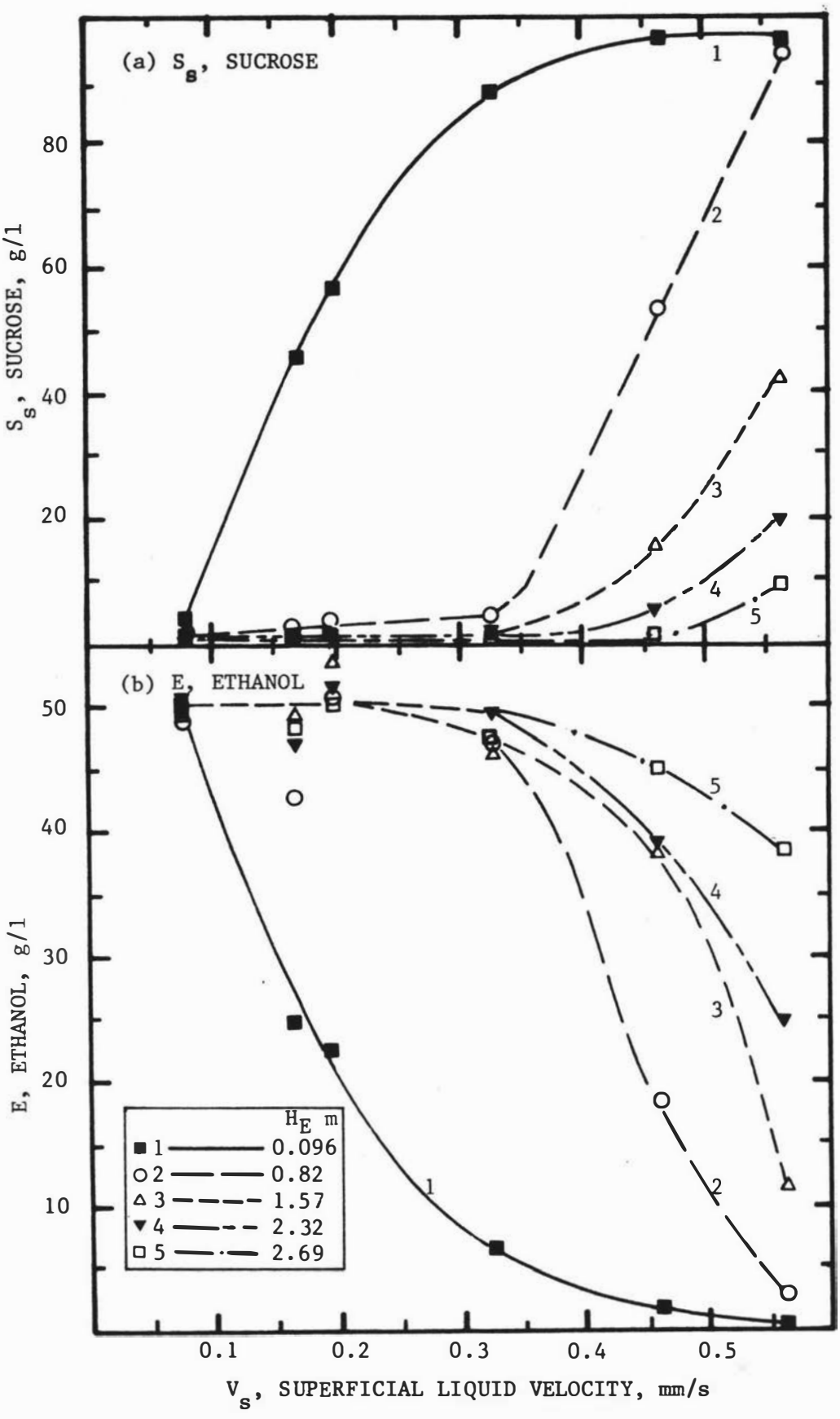


Fig. C.10 (a) Sucrose and (b) ethanol concentrations vs superficial liquid velocity at various tower heights.

ethanol was produced in the lower tower section as the velocity increased.

Thus, the velocity of 0.33 mm/s was the limiting velocity for complete utilization of sucrose (even though sucrose was reduced to less than 5 g/l at the height of 2.32 m for velocity of 0.46 mm/s) since cell wash out occurred at this velocity. The highest concentration of ethanol reached for velocities up to 0.33 mm/s and height of 0.82 m was 46 g/l. Ethanol concentration was less than this at greater heights and velocities.

C.3.2 Rates of sucrose utilization and ethanol production

(a) Volumetric rates (S_s' , E') The volumetric rates of sucrose utilization (S_s') and ethanol production (E')(fig.C.11), at heights below 0.82 m (curves 1 and 2), increased with an increase in the velocity to a peak and then decreased with further velocity increase. Maximum rates of sucrose utilization of 351 and 136 g/lh were observed at velocities of 0.17 and 0.33 mm/s for mean heights of 0.048 and 0.46 m respectively . Similarly maximum rates of ethanol production of 167 and 67 g/lh were observed at velocities of 0.20 and 0.33 mm/s for these two mean heights respectively.

For greater heights (curves 3 and 4), the volumetric rates were not affected until the velocity was greater than 0.33 mm/s. Above this velocity, the volumetric rates increased with velocity. At the exit (curve 5), this did not occur until a velocity of 0.46 mm/s was reached.

(b) Specific rates (q_s,ν) Similar trends were observed for the specific rates (fig.C.12) but the maximum specific rate of sucrose utilization occurred at higher velocities of 0.33 and 0.46 mm/s for mean heights of 0.048 and 0.46 m, respectively, and were 10.8 and 6.3 g/gh respectively (fig.C.12 a, curves 1 and 2). The maximum specific rate of ethanol production occurred at 0.46 m for both mean heights of 0.048 and 0.46 m (fig.C.12 b, curves 1 and 2) and were 7.0 and 2.4 g/gh respectively.

The results showed that volumetric and specific rates increased with an increase in the velocity within the tower sections where there was sucrose utilization and ethanol production. The velocity at which the peak rates occurred was related to the effect the velocity has on the cell concentration (sect. C.3.3) and fermentation time. The

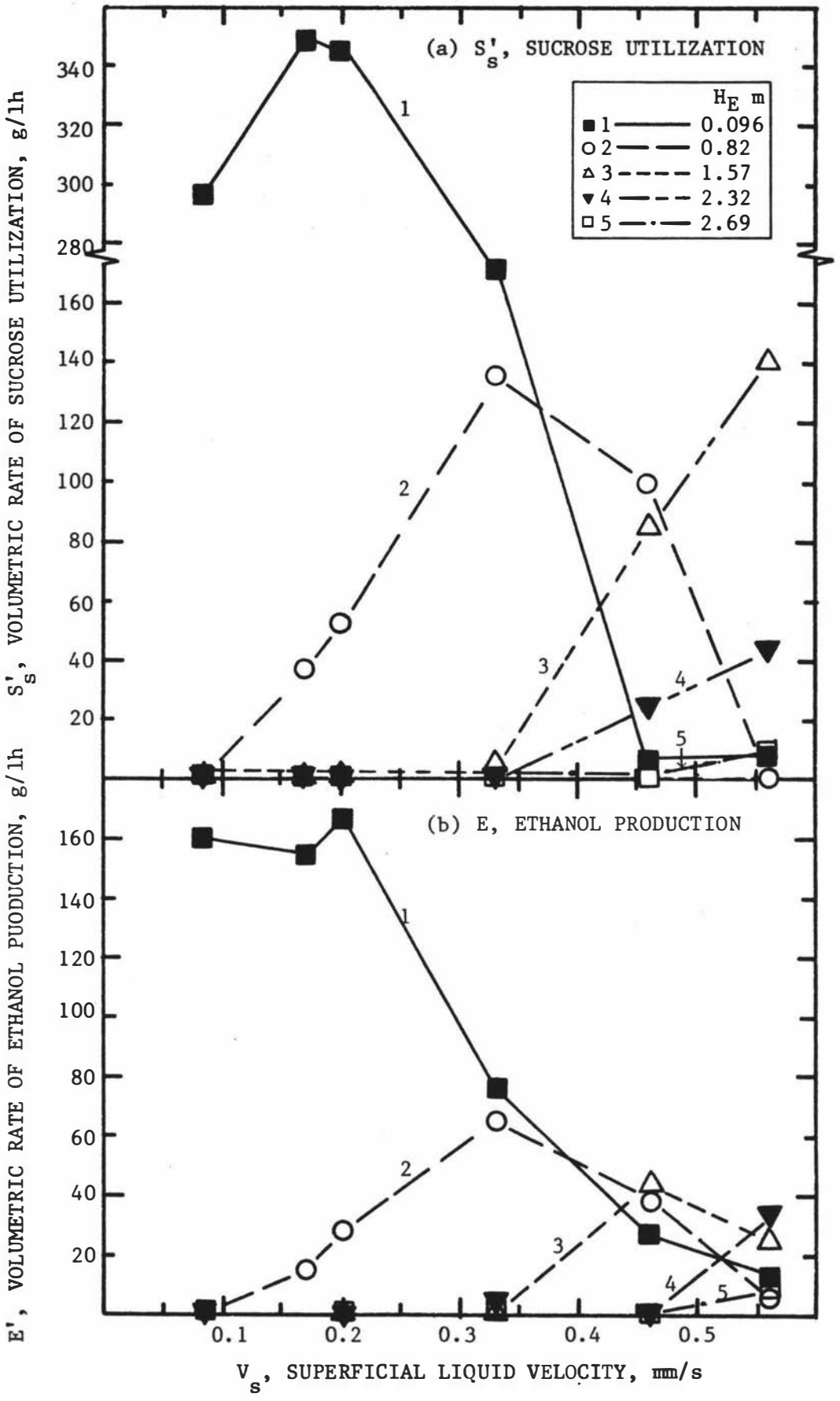


Fig. C.11 Volumetric rates of (a) sucrose utilization and (b) ethanol production vs superficial liquid velocity at various mean tower heights.

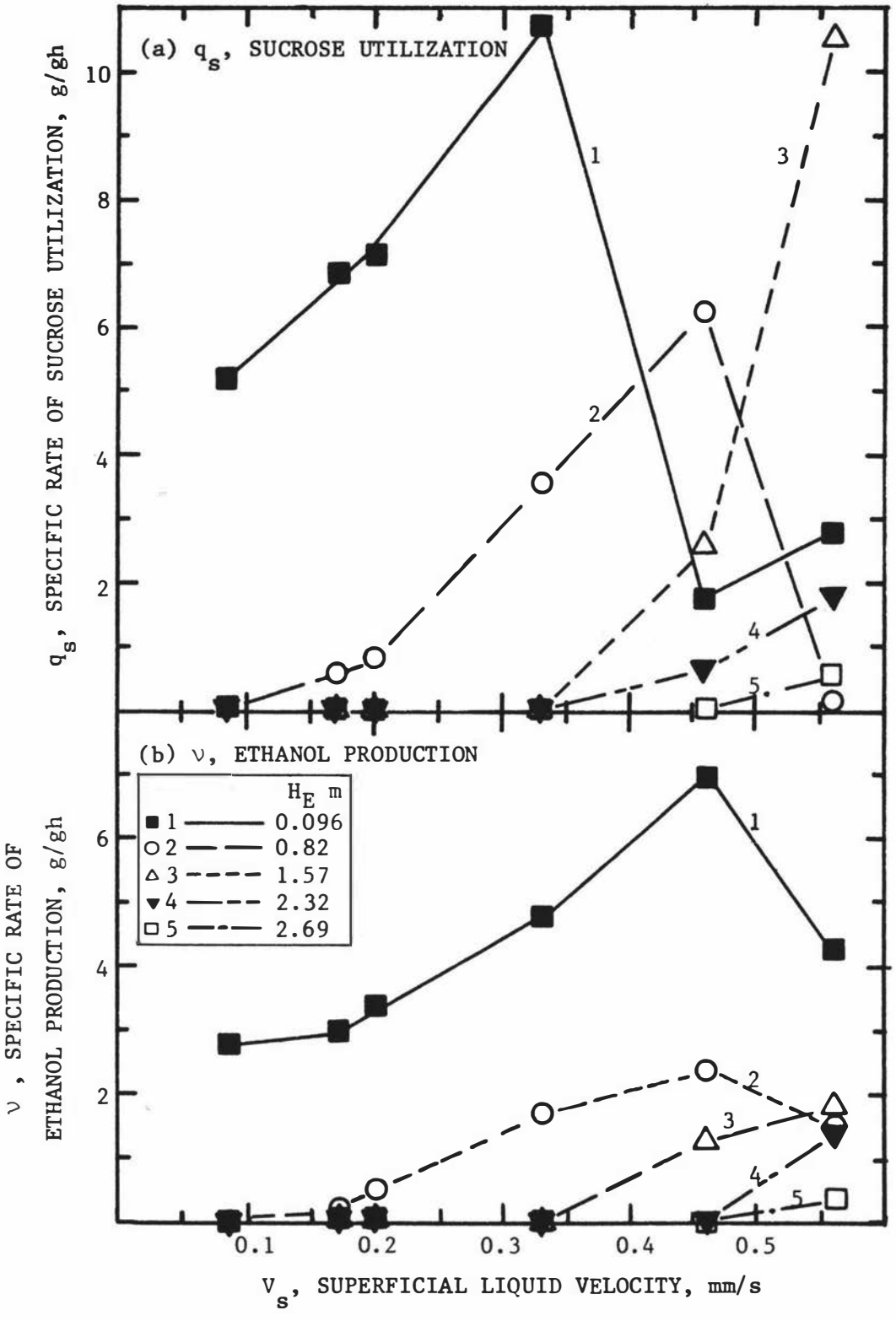


Fig. C.12 Specific rates of (a) sucrose utilization and (b) ethanol production vs superficial liquid velocity at various mean tower heights.

specific rates, for mean heights up to 0.46 m, increased with velocity as a result of the reduction in the cell concentration and fermentation time caused by an increase in the velocity. Further decrease in the specific rates with further increase in the velocity, after the peak rates were reached, was due to the yeast cells being lifted to greater heights.

Thus, the rates of sucrose utilization and ethanol production, at mean heights up to 0.46 m, increased to a maximum with an increase in the velocity and then decreased with further velocity increase. At greater mean heights, the rates were initially unaffected by the velocity increase until velocity reached 0.33 mm/s, above which the rates also increased with velocity. This occurred as a result of the effect the medium velocity had on the cell concentration and fermentation time at a particular height in the tower.

C.3.3 Cell concentration

The effect of the superficial liquid velocity on the cell concentration was closely associated with the height in the tower (fig.C.13).

At height of 0.096 m, the concentration decreased from 57 to 3 g/l DW (171 to 16 g/l WW) as the velocity increased from 0.084 to 0.56 mm/s. The increase in the velocity had resulted in yeast bed expansion and the cell flocs were lifted to greater heights. Hence, the reduction in the cell concentration.

Between 0.82 and 2.32 m (curves 2, 3 and 4), the cell concentration increased to a peak as the velocity increased from 0.084 to 0.20 mm/s. Then the concentration decreased with further increase in the velocity to 0.56 mm/s. The initial increase in the concentration was a result of the yeast cell bed expansion from the lower heights. The concentration decrease after the peak concentration was a result of the cell flocs being lifted to greater heights. At the exit, the cell concentration was low (3 g/l DW) and changed little as the velocity increased from 0.084 to 0.20 mm/s. It then increased to 11 g/l DW (48 g/l WW) with further velocity increase to 0.56 mm/s. reflecting a gradual increase in the amount of the cell flocs being washed out of the tower.

Thus, for all sections of the tower, the cell concentration decreased with an increase in the velocity from 0.20 mm/s.

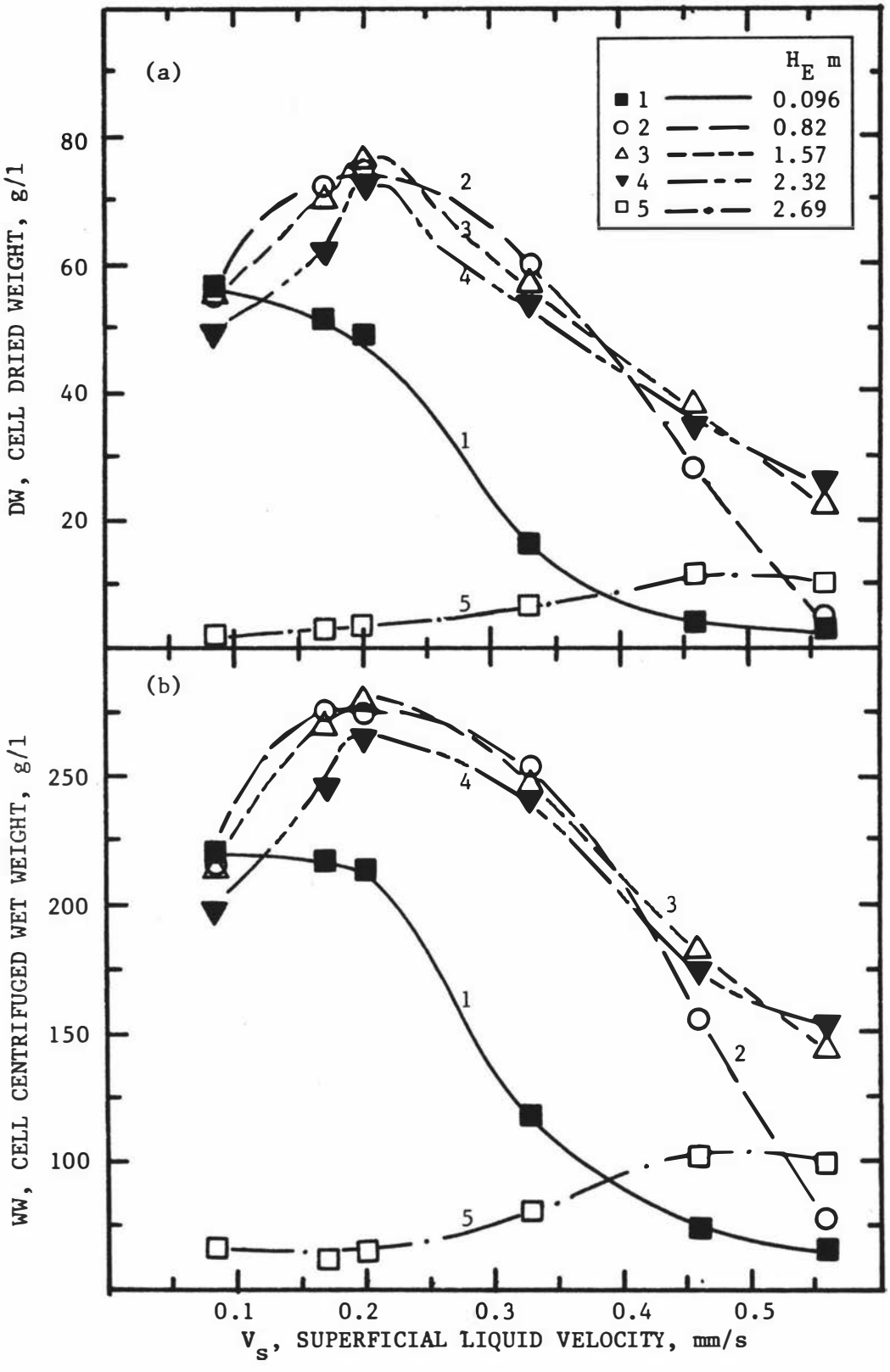


Fig.C.13 Cell concentration vs superficial liquid velocity at various tower heights.

C.3.4 Specific growth rate (μ_{sc})

The mean specific growth rate increased from 0.0044 to 0.67 g/gh as the velocity increased from 0.084 to 0.56 mm/s (table C.2).

Table C.2 Mean specific growth rate at various superficial liquid velocities.

V_s , mm/s	0.084	0.17	0.20	0.33	0.46	0.56
μ_{sc} , g/gh	0.0044	0.0072	0.012	0.059	0.29	0.67
X_a , g/l	54.8	65.7	69.5	49.4	27.6	13.8

The increase in the specific rate as the velocity increased to 0.20 mm/s was due mainly to the change in residence time, since the mean cell concentration in the tower was relatively constant during this increase. However, at greater velocities than this, the mean cell concentration was reduced and the exit cell concentration increased.

Thus, there was an increase in the mean specific growth rate as the velocity increased due to the reduction in the residence time with velocity. For velocities increase greater than 0.20 mm/s, the increase was also caused by a reduction in biomass in these sections.

C.4 TOWER PERFORMANCE

The results showed that up to 99% sucrose utilization occurred. The residual sucrose concentration at the exit varied from 1 to 10 g/l depending on the velocity. Most sucrose was utilized in the lower tower sections up to 0.82 m for all velocities up to 0.33 mm/s. For all velocities, sucrose was more than 95% utilized in 1.5 hours. More than 46 g/l of ethanol was produced within the first 0.82 m at a velocity of 0.33 mm/s. For all velocities, ethanol concentrations greater than 49 g/l were produced in 2 hours. The yield of ethanol was between 80 and 97% for heights between 0.82 and 2.32 m and for velocities up to 0.33 mm/s. The cell concentration attained inside the tower ranged from 20 to 80 g/l DW (depending of the velocity and height) for velocities up to 0.33 mm/s. The continuous operation was free from contamination.

It is evident from the results that the optimum velocity for this

fermentation was 0.33 mm/s. If the total tower height were considered, 47 g/l of ethanol was produced from 102 g/l of sucrose in the molasses solution (98% utilization) and ethanol yield was 87%. The overall ethanol productivity was 10 g/lh. It was, however, shown that the greatest fermentative activity occurred in the first 0.82 m of the tower. Therefore, the upper sections of the tower (except for the separator) were not essential. At 0.82 m and 0.33 mm/s, there was 96% sucrose utilization. Sucrose concentration reduced to 4 g/l in 0.7 hour and 47 g/l of ethanol was produced. The corresponding ethanol yield was 89% based on sucrose utilized and the theoretical yield. The mean cell concentration was 60 g/l DW (200 g/l WW). The overall hold up time (including 2.7 hours in the separator) was 3.4 hours. The ethanol productivity (E') was 67 g/lh while the overall productivity (E'_o) including the separator was 14 g/lh. The hold up time in the separator could increase sucrose utilization by a small amount to 98% (table C.1) and ethanol concentration could increase to 50 g/l (fig.C.7 b). The ethanol productivity (E') was approximately 33 times the productivity reported for batch fermentation of molasses (sect.2.3).

The results obtained were comparable to those obtained for other tower fermentations using sucrose based media (table C.3).

Table C.3 Comparison of tower fermentation of 100 g/l sucrose media.

	S_{su} %	E g/l	Y %	E' g/lh	E'_o g/lh	V_s mm/s	D h^{-1}	T_r h	T_{ro} h
1. Cane molasses	96	50	92	67	14	0.33	1.4	0.7	3.4
2. Beet molasses	90	39	81	41	10	0.53	1.1	0.9	3.8
3. Cane juice	90	43	89	15	13	0.26	0.45	2.2	2.5
4. Fodder beet extract	93	49	97	12	11	0.05	0.24	4.2	na

1. This study
2. Coote 1974
3. Prince & Barford 1982
4. Henderson & Smith 1982

The productivity was better than other investigations (no.2 to 4 in table C.3) because these investigators did not optimise their tower heights, even though they used more flocculent yeasts.

C.5 CONCLUSIONS AND SUMMARY

In tower fermentation of molasses using 100 g/l sucrose in the feed, sucrose was more than 96% utilized within the first 0.82 m of the tower height and at superficial liquid velocities up to 0.33 mm/s. Thus, the optimum operating conditions using a moderately flocculent *S. cerevisiae* were at a superficial liquid velocity of 0.33 mm/s, tower straight section height of 0.82 m and the resultant residence time was 0.7 hour. The resulting ethanol concentration was 47 g/l from 96% sucrose utilization and the corresponding ethanol yield on the sucrose utilized was 89%. Optimization of the tower height has resulted in a productivity comparable to other investigations using more flocculent yeasts in tower fermenters. A major limiting factor of the performance of this tower fermentation was the flocculating ability of the yeast used.

APPENDIX D
FLOCCULATION TEST OBSERVATIONS AND DATA

D.1 Observation of flocculating behaviour during fermentation

Observations of the flocculating behaviour of the yeasts during fermentation. The abbreviations and the medium numbers used are the same as those used in section 6.2.

D.1.1 *K.marxianus* Y42 (or KMY42)

Flocculating behaviour of this yeast strain observed during fermentation in some media was different from the behaviour displayed in the flocculating medium. The following is a brief description of the flocculating behaviour of this yeast during fermentation in various media.

1.P In whey permeate at pH 5.0, the growth was good but flocculation was poor. Even after fermentation was completed.

1F.PF Whey permeate which was membrane filtered after autoclaving to remove the precipitate did not encourage flocculation of KMY42. Flocculation was poor than in 1.P.

2.P4.6 There was no difference between flocculation in this medium and in 1.P.

2F.P4.6F Growth was moderate, poorly flocculating as very few flocs were seen.

3.PCa In whey permeate with $CaCl_2$ the yeast showed generous growth but still flocculated poorly.

3F.PCaF In whey permeate with $CaCl_2$ added after autoclaving and membrane filtration, the growth was poor and flocculation was poor.

4.PY The addition of yeast extract powder resulted in improved flocculation of KMY42. Few flocs could be seen when fermentation was completed.

5.PYM Yeast-malt extract powder in whey permeate improved flocculation of KMY42. Large flocs (0.2 mm diameter) could be seen when fermentation was completed.

6.PYMCa The addition of $CaCl_2$ to the above medium (5.PYM) did not result in marked difference in the flocculation of KMY42.

7.PXM Growth on this medium was good. When the cells were allowed to settle they tended to stick to the bottom of the flasks and not all the cells flocculated readily. Flocculation was good.

8.PXM5 The growth in this medium was good. Floc formation was similar to 7.PXM.

8F.PXM5F Membrane filtration had removed some nutrient from this medium which encourage good flocculation. There was poor flocculation when fermentation was completed.

9.PMS KMY42 grown in whey permeate with added malt extract syrup flocculated readily when fermentation was complete. The floc size was large (0.2-0.3mm). The floc once formed was reasonably stable. Part of the cell population did not flocculate readily.

10.PPe The addition of peptone powder to whey permeate did not result in good flocculation of KMY42 in this medium. The growth was moderate and some cells were sticky.

11.PuAm The addition of urea and $(NH_4)_2 HPO_4$ to whey permeate did not result in good flocculation of KMY42 in this medium. Only poor flocculation was observed.

12.P10 KMY42 did not flocculate in whey permeate with added lactose (to 100g/l) even after the completion of fermentation. Very few small flocs(0.1 mm) formed.

13.PMO46 Generous growth and moderate flocculation, some small flocs could be seen.

13F.PMo46F The colour of whey permeate enriched with molasses was rather dark and it was difficult to observe flocculation. Few large flocs (0.2mm) could be seen but most of the cells remained in suspension.

15.PB This was a very rich medium. KMY42 flocculated well, growth was generous and cells did not stick to the bottom of the flask. Sucrose, malt extract and yeast extract were added to this medium.

16.PB5 Growth was moderate, moderately flocculating small flocs could be seen.

16F.PB5F This was as for the above medium (15.PB5) but was membrane filtered. KMY42 flocculated moderately and part of cell population remained in suspension.

17.PMoYMCa Growth was good, moderate flocculation few small flocs.

17F.PMoYmCaF The growth in this medium was generous but flocculation was difficult to observe because of the dark colour. Flocculation was similar to 13.PMo46, i.e. moderate flocculation.

19.LYA Growth in this medium was very poor, the medium buffering capacity was not good and the pH dropped to below 3. KMY42 showed poor flocculation.

20.LYCa Behaviour of KMY42 in this medium was similar to that in 19.LYA.

21.LSM Growth in spent malt extract broth with added lactose was poor but better than in 19.LYA. There was no flocculation in the medium.

22.Ma Growth in maltose (10g/l) was poor, the medium pH dropped to 2.9 and no flocculation.

23.MaCa Similar results as for 22.Ma.

24.MaCa4 Similar results to those for 22.Ma and 23.MaCa.

25.G Growth in glucose (40g/l), was better than in maltose but the poor buffering capacity of the medium resulted in rather acidic medium after fermentation. Flocculation was very poor.

26.GCa Similar results as for 25.G.

27.YM Growth in yeast malt extract broth was generous and KMY42 flocculated rapidly by 24 hours. Very flocculant and cells did not stick to the bottom of the flask when the flask was shaken, i.e. could be loosened easily.

28.YMCa The behaviour was similar to that in 27.YM.

29.YMA The behaviour was similar to that in 27.YM and 28.YMCa.

29F.YMAF Membrane filtration has taken some nutrients away, growth was poor and poor flocculation.

30.M KMY42 showed generous growth and very good flocculation which occurred rapidly by 24 hours. Cells loosen from the bottom of the flask easily after having settled to the bottom when flask was allowed to stand.

32.MCa Similar to the behaviour in 30.M.

33.Ms Growth in malt extract syrup was generous even though no other nutrient was added. The yeast, KMY42, was very flocculant and did not stick to flask bottom when the flask was shaken after having been left standing.

34.Me Growth of KMY42 in malt extract powder alone was poor and the yeast flocculated poorly.

D.1.2 *K.marxianus* Y42(TS) (or KMY42(TS))

The subculture of KMY42, which was originally grown in whey permeate enriched with malt extract syrup and then used in the tower fermenter, was found to be very flocculent in whey permeate (1.P and 2.P4.6) and also in whey permeate with added calcium chloride (3.PCa). Cell flocs (0.1 to 0.3 mm in diameter) formed after 24 hours fermentation. In later tests the strain was found to be only moderately flocculent in these media. When these media were membrane filtered, the strain showed weak flocculence in these media (1F.PF, 2F.P4.6F, and 3F.PCaF).

D.1.3 *S.cerevisiae* FT146 (or SC146)

This yeast strain was used in the commissioning of the tower fermenter using cane molasses (Appendix C).

13.PMo46 The strain SC146 was grown as mixed culture with the strain KMY42, in this medium. Moderately flocculating flocswere formed rapidly after 12 hours fermentation. The floc size was approximately 0.2-0.3mm in diameter.

18.Mo The strain SC146 was very flocculent in this molasses medium. The cells flocculated well in fresh whey permeate (2.P4.6), whey permeate supplemented with molasses (13.PMo46) and the molasses medium (18.Mo).

30.M The strain SC146 was very flocculent in malt extract broth. The flocs were observed when the fermentation times was 12 hours.

D.1.4 *S.cerevisiae* CFCC39 (or CC39)

13.PMo46 This strain was also grown as mixed culture with the strain KMY42, in this medium. The cells flocculated very rapidly in 12 hours. The floc size varied from 0.1 to 0.5 mm in diameter. Later tests found the cells to be only moderately flocculent but this was later found to be due to the medium used to prepare the inoculum.

18.Mo The strain showed good growth and very good flocculation in the medium. The cells flocculated rapidly after 12 hours fermentation. After 48 hours, the flocs were spherical about 0.5mm in diameter. Cells stuck to the bottom of the flask after the flask was left standing for a while. Later tests found the cells to be only moderately flocculent but this was found to be due to the inoculum medium used.

30.M The strain showed good growth and was extremely flocculent in malt extract broth. The behaviour was the same as described above for 18.Mo.

D.2 FLOCCULATION TEST DATA

The flocculation test data of various yeast strains in different media and at different pH have been tabulated. The data which are given in each column are arranged as follows :

column title — data

1 - yeast - The abbreviated name of the yeast being tested.
2 - grown in - The growth medium in which the yeast was grown.
3 - pHi - Initial pH of the growth medium.
4 - pHf - Final pH of the growth medium.
5 - test medium - The medium in which the yeast was tested for flocculation.
6 - pHt - The pH of the flocculation test medium.
7 - scale - The degree of flocculence of the yeast measured in the test medium (after Stewart 1975).

NF 0 - Non-flocculent
R 1 - Rough
WF 2 - Weakly flocculent
MF 3 - Moderately flocculent
VF 4 - Very flocculent
EF 5 - Extremely flocculent

8 - vol.ml.10 - The settled volume of the yeast cells in ml. after 10 min.
9 - vol.ml.60 - The settled volume of the yeast cells in ml. after 60 min.
10 - MBN - Modified Burn's number, * indicates non-standard MBN value because the cells were not grown and tested in standard media.

1 yeast	2 3 4 growth medium			5 6 test medium		7 scale	8 9 vol(ml/min)		10 MBN
	grown in	pHi	pHf	test in	pHt		10	60	
Y42	1.P	5.0	5.1	1.P	5.1	R 1	0.4	1.1	na
				36.FM	4.9	R 1	0.4	1.0	na
Y42(TS)	1.P	5.0	6.2	1.P	6.2	VF 4	4.9	3.0	50*
				36.FM	5.0	EF 5	3.8	2.3	91*
Y42	1F.PF	5.0	5.8	36.FM	4.9	R 1	0.1	1.0	na
Y42(TS)	1F.PF	5.0	5.9	36.FM	4.9	R 1	0.1	1.1	na
Y42	2.P4.6	4.6	4.7	2.P4.6	4.7	R 1	9.6	9.2	na
				36.FM	4.6	R 1	9.5	9.3	na
				13.PMo46	4.7	R 1	10.0	9.6	na
Y42(TS)	2.P4.6	4.6	4.6	2.P4.6	4.6	EF 5	3.9	2.6	88*
				36.FM	4.9	EF 5	4.1	2.8	83*

1 yeast	2 3 4 growth medium			5 6 test medium		7 scale	8 9 vol(ml/min)		10 MBN
	grown in	pHi	pHf	test in	pHt		10	60	
Y42	2F.P4.6F	4.6	5.8	2F.P4.6F	5.8	R 1	9.5	9.1	na
				36.FM	4.8	R 1	9.5	9.0	na
Y42(TS)	2F.P4.6F	4.6	4.7	2F.P4.6F	4.7	R 1	9.3	8.9	na
				36.FM	4.9	R 1	9.4	9.0	na
Y42	3.PCa	5.3	5.9	3.PCa	5.9	R 1	9.4	8.9	na
				36.FM	4.8	R 1	9.4	9.0	na
				13.PMo46	4.7	R 1	9.6	9.3	na
Y42(TS)	3.PCa	5.0	5.5	3.PCa	5.5	EF 5	2.9	2.2	na
				36.FM	4.9	EF 5	3.3	2.5	na
Y42	3F.PCaF	5.0	5.8	3F.PCaF	5.8	R 1	9.7	9.4	na
				36.FM	4.8	R 1	9.6	9.1	na
Y42(TS)	3F.PCaF	5.3	4.5	3F.PCaF	4.5	WF 2	10.0	8.1	na
				36.FM	4.9	R 1	10.0	9.3	na
Y42	4.PY	5.0	na	2.P4.6	4.7	WF 2	1.8	1.7	na
				36.FM	4.9	MF 3	3.2	2.6	na
				13.PMo46	4.7	NF 0	0.1	0.2	na
				32.MCa	4.3	MF 3	1.8	2.2	na
Y42	5.PYM	4.7	na	2.P4.6	4.7	WF 2	1.1	1.4	na
				36.FM	4.9	MF 3	2.5	2.2	na
				13.PMo46	4.7	NF 0	0.1	0.2	na
Y42	6.PYMCa	4.6	na	2.P4.6	4.7	WF 2	1.6	1.7	na
				36.FM	4.9	MF 3	2.6	2.2	na
				13.PMo46	4.7	NF 0	0	0.2	na
				32.MCa	4.3	MF 3	1.9	2.4	na
Y42	7.PXM	4.5	4.4	2.P4.6	4.0	VF 4	1.9	1.3	na
					4.9	VF 4	1.9	1.4	na
					5.3	VF 4	1.9	1.3	na
				36.FM	4.9	VF 4	1.9	1.5	na
				32.MCa	4.9	EF 5	3.3	2.6	na
		5.1	na	2.P4.6 (fresh)**	4.7	VF 4	2.4	1.7	na
				13.PMo46	5.0	WF 2	0.1	1.6	na
				18.Mo	5.3	VF 4	1.9	1.3	na
Y42	8.PXM5	5.0	5.3	2.P4.6	4.2	NF 0	0	0.6	na
				36.FM	4.9	VF 4	1.3	1.2	na
				27.YM	5.2	VF 4	2.0	2.1	na
Y42	8F.PXM5F	5.0	4.7	2.P4.6	4.6	WF 2	0	1.9	na
				36.FM	4.9	WF 2	0	1.4	na
				8F.PXM5F	4.7	WF 2	1.4	1.2	na
				28.YMCa	5.3	VF 4	2.9	2.1	na

** (fresh) - freshly prepared medium instead of the spent medium.

1 yeast	2 3 4 growth medium			5 6 test medium		7 scale	8 9 vol(ml/min)		10 MBN
	grown in	pHi	pHf	test in	pHt		10	60	
Y42	9.PMs	5.0	4.6	2. P4.6	4.2	MF 3	1.3	1.9	na
				36.FM	4.9	VF 4	1.7	1.9	na
				27.YM	5.2	VF 4	0.2	2.1	na
Y42(TS)	9.PMs	5.0	4.6	36.FM	4.9	EF 5	3.8	2.6	118*
Y42	10.PPe	4.5	4.6	2. P4.6	4.0	NF 0	0.1	0.2	na
					4.9	WF 2	0.7	0.5	na
					5.3	WF 2	0.6	0.5	na
				36.FM	4.9	WF 2	0.6	0.6	na
				32.MCa	4.9	VF 4	2.8	2.5	na
Y42	11.PUAm	5.0	na	36.FM	4.9	WF 2	2.2	1.9	na
Y42	12.P10	4.9	4.5	36.FM	4.9	R 1	9.7	9.3	na
Y42(TS)	12.P10	4.7	4.3	12.P10	4.3	VF 4	3.7	2.6	44*
				36.FM	4.9	VF 4	3.8	2.6	49*
Y42	13.PMo46	5.0	4.7	13.PMo46	4.7	R 1	9.8	9.5	na
				36.FM	4.7	VF 4	2.8	1.7	45*
CC39 & Y42(TS)	13.PMo46	5.0	5.3	13.PMo46	5.3	EF 5	1.9	1.4	174*
				36.FM	4.9	EF 5	2.7	2.2	134*
CC39 & Y42	13.PMo46	4.9	5.5	13.PMo46	5.5	VF 4	3.8	2.4	43*
				36.FM	4.9	VF 4	6.1	3.4	33*
SC146 & Y42	13.PMo46	5.0	na	36.FM	4.9	EF 5	4.5	3.0	50*
				2. P4.6 (fresh)	4.7	EF 5	4.5	3.3	na
				13.PMo46 (fresh)	5.0	EF 5	5.8	2.0	na
				18.Mo (fresh)	5.3	EF 5	6.0	3.1	na
SC146 & Y42	13.PMo46	5.0	5.4	13.PMo46	5.4	VF 4	7.7	3.6	30*
				36.FM	4.9	VF 4	7.5	3.9	29*
Y42	13F.PMo46F	5.0	na	36.FM	4.9	MF 3	2.5	2.1	na
SC146 & Y42	15.PB	4.7	4.1	2. P4.6	4.2	EF 5	6.0	2.9	na
				36.FM	4.9	EF 5	5.3	2.7	na
				27.YM	5.2	EF 5	5.3	3.1	na
Y42	16.PB5	5.3	3.2	2. P4.6	4.9	EF 5	7.7	3.5	na
				36.FM	4.9	EF 5	3.9	2.5	na
				16.PB5	3.2	EF 5	4.3	2.7	na
				28.YMCa	5.3	EF 5	5.2	3.0	na
Y42	16F.PB5F	5.3	3.0	16F.PB5F	3.0	R 1	9.8	9.4	na
				36.FM	4.8	R 1	9.6	9.2	na

1 yeast	2 3 4 growth medium			5 6 test medium		7 scale	8 9 vol(ml/min)		10 MBN
	grown in	pHi	pHf	test in	pHt		10	60	
Y42	17.PMoYMCa	5.6	5.2	17.PMoYMCa	5.2	R 1	9.7	9.4	na
				36.FM	4.9	WF 2	9.6	9.4	na
Y42	17F.PMoYMCaF	4.9	na	2. P4.6	4.7	R 1	0.1	0.2	na
				36.FM	4.9	WF 2	1.6	1.7	na
				13.PMo46	4.7	NF 0	0	0.1	na
SC146	18.Mo	5.0	na	36.FM	4.9	EF 5	8.1	3.4	21*
				36.FM	4.9	EF 5	5.1	2.9	46*
				2. P4.6 (fresh)	4.7	EF 5	5.5	2.6	na
				13.PMo46 (fresh)	5.0	VF 4	4.3	1.1	na
				18.Mo (fresh)	5.3	VF 4	4.1	0.8	na
CC39	18.Mo	5.0	4.8	18.Mo	4.8	EF 5	2.9	2.5	120*
				36.FM	4.9	EF 5	5.1	3.0	46*
	18.Mo(M)***	5.0	4.9	18.Mo	4.8	VF 4	4.1	3.0	43*
				36.FM	4.8	VF 4	4.6	2.7	41*
	18.Mo(Mo)***	5.0	5.6	18.Mo	5.6	R 1	9.5	9.0	0
				36.FM	4.8	VF 4	4.0	2.5	44*
	18.Mo(YM)***	5.0	4.8	18.Mo	4.8	VF 4	4.8	2.9	40*
				36.FM	4.8	EF 5	2.6	1.8	154*
SC146	18.Mo(Mo)***	4.9	4.9	18.Mo	4.9	VF 4	5.9	3.3	56*
				36.FM	4.8	MF 3	7.3	3.7	30*
	18.Mo(Mo)***	5.0	4.9	18.Mo	4.9	MF 3	7.6	3.8	25*
				36.FM	4.8	MF 3	7.6	4.1	31*
Y42(TS)	19.LYA	5.0	3.6	2. P4.6	4.2	VF 4	1.9	1.3	na
				36.FM	4.9	VF 4	1.6	1.3	na
				27.YM	5.2	VF 4	1.6	1.3	na
Y42	19.LYA	5.1	3.5	2. P4.6	4.7	WF 2	0	1.9	na
				36.FM	4.9	R 1	0	0.7	na
				28.YMCa	5.3	R 1	0	0.5	na
Y42	20.LYCa	4.8	na	2. P4.6	4.7	MF 3	1.9	2.4	na
				36.FM	4.9	MF 3	2.7	2.9	na
				13.PMo46	4.7	WF 2	0.1	2.2	na
				32.MCa	4.3	MF 3	2.3	2.6	na
Y42	21.LSM	5.0	3.9	2. P4.6	4.2	NF 0	0	0.1	na
				36.FM	4.9	NF 0	0	0.2	na
				27.YM	5.2	R 1	0.2	0.2	na
Y42	22.Ma	4.8	2.9	2. P4.6	4.0	NF 0	0	0.2	na
					4.9	NF 0	0	0.2	na
					5.3	NF 0	0	0.1	na
				36.FM	4.9	NF 0	0	0.2	na
				32.MCa	4.9	NF 0	0	0.2	na

*** The medium in the bracket indicates the inoculum growth medium.

1 yeast	2 3 4 growth medium			5 6 test medium		7 scale	8 9 vol(ml/min)		10 MBN
	grown in	pHi	pHf	test in	pHt		10	60	
Y42	23. MaCa	4.7	2.9	2. P4.6	4.0	NF 0	0	0.1	na
					4.9	NF 0	0	0.1	na
					5.3	NF 0	0	0.1	na
				36.FM	4.9	NF 0	0	0.1	na
				32.MCa	4.9	NF 0	0.1	0.2	na
Y42	24. MaCa4	4.4	2.9	2. P4.6	4.0	NF 0	0	0.1	na
					4.9	NF 0	0	0.1	na
					5.3	NF 0	0	0.1	na
				36.FM	4.9	NF 0	0	0.1	na
				32.MCa	4.9	NF 0	0.1	0.2	na
Y42	25. G	4.6	2.6	2. P4.6	4.0	MF 3	1.0	1.1	na
					4.9	MF 3	0.9	1.0	na
					5.3	MF 3	1.0	1.1	na
				36.FM	4.9	MF 3	1.0	1.1	na
				32.MCa	4.9	MF 3	1.2	1.2	na
Y42	26. GCa	5.1	2.6	2. P4.6	4.0	WF 2	0.1	0.9	na
					4.9	WF 2	0.4	0.9	na
					5.3	WF 2	0.4	0.9	na
				36.FM	4.9	WF 2	0.4	0.9	na
				32.MCa	4.9	MF 3	1.0	1.0	na
Y42	27. YM	5.0	5.2	2. P4.6	4.2	R 1	0	0.6	na
				36.FM	4.9	R 1	0	0.7	na
				27.YM	5.2	WF 2	0	1.1	na
Y42	28. YMCa	4.9	5.1	2. P4.6	4.2	R 1	0	0.6	na
				36.FM	4.9	R 1	0.1	1.7	na
				27.YM	5.2	MF 3	1.0	1.6	na
Y42	29. YMA	5.1	5.4	2. P4.6	4.2	EF 5	4.5	2.1	na
				36.FM	4.9	EF 5	4.3	2.5	na
				27.YM	5.2	MF 3	2.0	1.7	na
Y42	29F.YMAF	5.0	5.6	2. P4.6	4.9	WF 2	0	1.2	na
				36.FM	4.9	WF 2	0	1.6	na
				28.YMCa	5.3	WF 2	0	1.9	na
				29F.YMAF	5.6	WF 2	0	1.4	na
Y42	30. M	4.9	5.4	2. P4.6	4.0	R 1	0.1	0.4	na
					4.9	WF 2	0.6	0.6	na
					5.3	WF 2	0.4	0.4	na
				36.FM	4.9	WF 2	0.6	0.6	na
				32.MCa	4.9	VF 4	2.6	2.2	na
Y42(TS)	30. M	5.2	5.2	30.M	5.2	MF 3	3.0	2.6	na
				36.FM	4.9	WF 2	9.5	3.5	4
CC39	30. M	5.0	4.3	30.M	4.3	EF 5	2.1	1.5	187*
				36.FM	4.9	EF 5	2.3	1.6	172
SC146	30. M	4.5	na	36.FM	4.9	VF 4	4.6	2.7	61

1 yeast	2 3 4 growth medium			5 6 test medium		7 scale	8 9 vol(ml/min)		10 MBN
	grown in	pHi	pHf	test in	pHt		10	60	
CC39	31. M*	5.1	4.7	31.M*	4.7	VF 4	4.2	2.9	53*
				36.FM	4.8	VF 4	3.9	1.7	58
SC146	31. M*	5.2	3.9	31.M*	3.9	MF 3	1.5	1.1	na
				36.FM	4.8	MF 3	2.6	1.7	na
	31. M*	4.9	4.7	31.M*	4.7	VF 4	4.0	2.3	47*
				36.FM	4.9	VF 4	3.9	2.3	52
	31. M*	5.1	4.7	31.M*	4.7	WF 2	7.5	3.0	16*
				36.FM	4.8	WF 2	6.5	3.4	31
Y42	32. MCa	4.7	na	2. P4.6	4.7	R 1	0.1	0.3	na
				36.FM	4.9	VF 4	2.2	1.9	na
				13.PMo46	4.7	R 1	0.1	0.2	na
				32.MCa	4.3	R 1	0.1	0.4	na
Y42	33. Ms	5.0	4.8	2. P4.6	4.2	WF 2	0	1.1	na
				36.FM	4.9	WF 2	0.3	1.3	na
				27.YM	5.2	MF 2	1.0	1.7	na
Y42	34. Me	5.0	3.8	2. P4.6	4.2	NF 0	0	0.3	na
				36.FM	4.9	R 1	0.1	0.2	na
				27.YM	5.2	R 1	0.2	0.4	na

APPENDIX E

ESTIMATION OF DATA UNCERTAINTIES

E.1 SUGAR CONCENTRATIONS

The lactose concentration readings were obtained for a set of replicate dilutions (50x) of a whey solution. The readings were 107.2, 107.5, 110.5, 111.8, 114.0, 114.8, 116.5 and 116.5 g/l. The mean ($\bar{x}$) and the standard deviation (SD) were 112.4 and 3.7 g/l, respectively.

The 95% confidence interval uncertainty in each reading was

$B = \pm t \times SD$

Where t is a constant multiplication factor for various sample size (n) at the 95% level of confidence.

$SD = \sqrt{[\Sigma(x-x)^2 \div (n-1)]}$

and x is an individual reading (Cleland 1983).

Thus, the uncertainty for each lactose reading is (t = 2.37 for n = 8)

$B_s = 2.37 \times 3.7 = 8.77 = 9$ g/l

$P_s = 8.77 / 112.4 = 7.8 = 8$ %

(P = percentage uncertainty)

Thus, there was approximately 95% certainty that one lactose value was ±8% of the true value. The glucose membrane of the sugar analyzer was found to give more consistent readings of sucrose concentration than the lactose membrane. Thus, the uncertainty in the sucrose measurement would be less than 8%.

In the tower fermentation of whey permeate, each datum point was generally averaged from 5 readings. The uncertainty in each mean value was = $8 / \sqrt{5} = 3.58$ %.

When the lactose concentration was lower the uncertainty would be lower since less dilution was required. The high lactose concentration of 100 g/l was chosen to estimate the uncertainty because the uncertainty at this concentration would be greater than at lower concentrations since more dilution was required for the higher concentration.

E.2 ETHANOL CONCENTRATION

Replicate ethanol analyses were made using solutions containing equal concentrations of ethanol and isopropanol (20 g/l). The ratios of the peaks were 0.9130. 0.9616, 0.9677, 0.9902, 1.0162, 1,0544 and 1.1180. The mean and the standard deviation were 1.0030 and 0.0674, respectively. The value of t for 7 replicate readings is 2.45 for 95% confidence level.

Thus, the uncertainty in each injection was

B_E = 2.45 x 0.0674 = 0.1651

P_E = 0.1651 / 1.0030 = 16.46 = 17 %

Each ethanol measurement was obtained from an average of generally two sets of peaks and multiplied by the peak ratio of the external standard. Thus, the uncertainty of the ethanol measurement was

$P_E = \sqrt{[(16.5)^2 \times 2 / \sqrt{2}) + (16.5)^2]}$ = 25.6 = 26 %

Assuming the mean ratio of the two peaks was approximately equal to 1.

Thus, the uncertainty of the average of 5 samples

= 26 / $\sqrt{5}$ = 11.6 = 12 %

The percentage uncertainty was the same for high or low ethanol concentration since no dilution was used and the standards used were designed to give a ratio as close to 1:1 as possible.

E.3 ETHANOL YIELD

The yield was calculated based on the ratio of ethanol produced to the amount of sugar utilized. Thus, the uncertainty in the ethanol yield is

$P_Y = \sqrt{[(P_E)^2 + (P_s)^2]}$ %

where P_E (12%) is the percentage uncertainty of the average ethanol concentration while P_s (4%) is that of the average sugar utilized. The value of P_s varied from 4 to 550% of the sugar utilized for 99 and 1 % utilization of 100 g/l sugar, respectively. This was because P_s was calculated as follows :

$P_s = [\sqrt{(B^2_{so} + B^2_{si})}] \times 100 / \Delta S$ %

where B_{so} and B_{si} are the uncertainties of the initial and instantaneous sugar concentrations, respectively. ΔS is the change in the sugar concentration.

Thus, for these two extremes of 4 and 550% uncertainty in the

value of the sugar utilized (using B_s = 4 g/l for 100 g/l sugar and P_E of 12% for average ethanol concentration) the magnitude of the uncertainty of the ethanol yield was between 12 and 490% for 99 and 1% sugar utilization, respectively.

Thus, there was considerable uncertainty in the yield value when there was low sugar consumption at high concentration of sugar.

E.4 RATES OF SUGAR UTILIZATION

E.4.1 Volumetric rate (S')

The rate of sugar utilization (S') was calculated from the amount of sugar utilized within a particular tower section during the time that the medium was within that tower section.

The uncertainty is

$$P_{s'} = [\sqrt{(B_{si}^2 + B_{si+1}^2)}] \times 100 / \Delta S \quad \%$$

Where B_{si} and B_{si+1} are the uncertainties (4%) of the sugar concentrations at the bottom and the top of the section, respectively. Assuming that there was negligible uncertainty in the value of the residence time.

Thus, the value of $P_{s'}$ varied in proportion to the mean sugar concentrations present being approximately 6% for a very high rate of sugar utilization (350 g/lh) and as high as 80% for a very low rate of sugar utilization (0.1 g/lh).

E.4.2 Specific rate (q)

The specific rate was calculated by dividing the volumetric rate (S') in each tower section by the mean cell concentration (X_a) in that particular section. Thus, its uncertainty incorporated the uncertainty of the cell concentration. The percentage uncertainty of the specific rate (q) is

$$P_q = \{[(B_{DWi}^2 + B_{DWi+1}^2)^{\frac{1}{2}} \times 100 \div X_a]^2 + P_{s'}^2\}^{\frac{1}{2}} \quad \%$$

Where B_{DWi} and B_{DWi+1} are the uncertainties of the cell concentration (DW) (4%) at the bottom and the top of the tower section, respectively, and X_a is the mean cell concentration in this tower section. $P_{s'}$ was shown previously to be between 6 and 80%.

High cell concentration (200 g/l DW), generally occurred at the bottom of the tower where the volumetric rate (S') was high. The uncertainty of the volumetric rate ($P_{s'}$) was 6%. Thus, the uncertainty of the specific rate (P_q) at this cell concentration was approximately

7%.

However, when the cell concentration was low (between 0.2 and 10 g/l DW), the volumetric rate (S') was low because this occurred in the upper sections of the tower. The uncertainty associated with this low volumetric rate (P_s) was 80%. Thus, the uncertainty of the specific rate (P_q) was approximately 80%.

This showed that the uncertainties of the two rates of sugar utilization were of similar magnitude (between 7 and 80%).

E.5 RATE OF ETHANOL PRODUCTION

E.5.1 Volumetric rate (E')

The rate of ethanol production was calculated from the amount of ethanol produced within a particular tower section during the time that the medium was within that tower section. Thus, the uncertainty is

$$P_{E'} = [\sqrt{(B^2_{Ei+1} + B^2_{Ei})}] \times 100 / \Delta E \ \%$$

Where B_{Ei} and B_{Ei+1} are the uncertainties (12%) due to the mean ethanol concentration at the bottom and the top of the tower section, respectively.

Thus, the value of $P_{E'}$ varied in proportion to the ethanol concentration present assuming that there was negligible uncertainty in the value of the residence time. The uncertainty was approximately 12% of the value of the volumetric rate calculated in the tower sections where there was ethanol production. It is clear that in the upper sections of the tower, where ethanol concentration was high, but ethanol production was negligible, the resulting confidence limits will be very wide.

E.5.2 Specific rate (ν)

The specific rate was calculated by dividing the volumetric rate (E') in each tower section by the mean cell concentration (X_a) in that particular tower section. Thus, its uncertainty incorporated the uncertainty of the cell concentration. The percentage uncertainty of the specific rate (ν) is

$$P_\nu = \{[(B^2_{DWi} + B^2_{DWi+1})^{\frac{1}{2}} \times 100 \div X_a]^2 + P^2_{E'}\}^{\frac{1}{2}}$$

B_{DWi} and B_{DWi+1} are both 4%.

For high cell concentration (200 g/l DW), the volumetric rate (E') was high at the bottom of the tower. The uncertainty in the volumetric rate (E') was shown to be 12%. Thus, using the above equation, the

uncertainty of the specific rate (ν) at this cell concentration was approximately 13%.

When the cell concentration was low (between 0.2 and 10 g/l DW), the volumetric rate (E') was low because this occurred in the upper section of the tower. The confidence limits will be wide as discussed above (sect.E.5.1).

This showed that the uncertainties of the two rate of sugar utilization were of similar magnitude.

E.6 CELL CONCENTRATION

E.6.1 Haemacytometer cell count

A set of replicate readings of a yeast cell suspension using the Haemacytometer was determined. The cell number readings obtained were (1.6, 1.6, 1.6, 1.7, 1.9, 1.9, 2.0, 2.0, 2.1, 2.3) x 10^8 cells/ml. The mean and the standard deviation were 1.87×10^8 and 2.41×10^7 cells/ml, respectively. Thus, the 95% confidence interval uncertainty in each Haemacytometer cell count was

$B_X = 2.26 \times 2.41 \times 10^7 = 5.4 \times 10^7$ cells/ml

$P_X = 5.4 \times 10^7 / 1.87 \times 10^8 = 29$ %

(t = 2.26 for n = 10)

E.6.2 Plate count

Ten replicate readings of the cell plate count number of a cell suspension of *K. marxianus* Y42 (24 hours culture) were determined. The readings were (1.0, 1.1, 1.1, 1.2, 1.4, 1.4, 1.5, 1.5, 1.7, 2.5)$\times 10^9$ cells/ml. The mean and the standard deviation were 1.44×10^9 and 4.33×10^8 cells/ml, respectively. Thus, the 95% confidence interval uncertainty in each cell plate count number was

$B_X = 2.26 \times 4.33 \times 10^8 = 9.78 \times 10^8$ cells/ml

$P_X = 9.78 \times 10^8 / 1.44 \times 10^9 = 68$ %

E.6.3 Cell dried weight and centrifuged wet weight

Ten replicate readings of the cell concentration of a cell suspension of *K. marxianus* Y42 obtained during the tower fermentation of whey permeate were determined. The readings were 274.0, 278.7, 281.0, 283.2, 283.5, 283.5, 284.1, 284.6, 285.8, 292.0 g/l WW for the centrifuged wet weight. The mean and the standard deviation were 283.1 and 4.7 g/l WW, respectively.

B_{WW} = 2.26 x 4.7 = 10.62 g/l WW

P_{WW} = 10.62 / 283.1 = 4 %

The corresponding cell dried weight readings were 69.0, 69.3, 69.9, 70.1, 71.3, 71.4, 71.5, 71.5, 71.7, 73.0 g/l DW. The mean and the standard deviation were 70.9 and 1.2 g/l DW, respectively.

B_{DW} = 2.26 x 1.2 = 2.71 g/l DW

P_{DW} = 2.71 / 70.9 = 4 %

This showed that the uncertainty in the cell weight determination was small. However, at sample point 1 (0.096 m), there was considerable carbon dioxide production for all tower fermentation runs. This introduced more uncertatinty into the measurement than usual. The sampling difficulties encountered during the tower fermentation of whey permeate enriched with molasses were already discussed (sect.5.1.7). For the cell concentration measurements obtained under these conditions, the uncertainty should be higher at approximately 5%.

E.6.4 Estimation of the cell dried weight of *K. marxianus* Y42 from cell plate count number

The linear regression line used is from fig.B.1 in Appendix B.4.

$\log_{KM}$ = 6.96 + 1.08 logDW

The standard deviation (SD) of the intercept is 0.5864.

$B_{intercept}$ = 2.45 x 0.5864 = 1.436 = 21 %

t = 2.45 for n = 7

The SD of the slope is 0.4106,

B_{slope} = 2.45 x 0.4106 = 1.003 = 93 %

Now logDW = $(\log X_{KM} - 6.96) / 1.08$

The uncertainty in X_{KM} is 68% (sect.E.6.2) and for X_{KM} between 1×10^6 and 2×10^9 cells/ml, the log of the uncertainty in the estimated value of the cell dried weight is

$$B_{DW} = [\{(4.08^2 + 1.436^2)^{\frac{1}{2}} \times 100 / (6 - 6.96)\}^2 + 93^2]^{\frac{1}{2}}$$

= 460 % for $X_{KM} = 10^6$ cells/ml

$$B_{DW} = [\{(6.12^2 + 1.436^2)^{\frac{1}{2}} \times 100 / (9.03 - 6.96)\}^2 + 93^2]^{\frac{1}{2}}$$

= 280 % for $X_{KM} = 2 \times 10^9$ cells/ml

Thus, the uncertainty in the estimation of the cell dried weight of *K. marxianus* Y42 from fig.B.1 is between 280 and 460% for cell dried weight between 0.13 and 147 g/l DW.