THE SOLID STATE FERMENTATION OF APPLE POMACE USING YEASTS TO PRODUCE AN IMPROVED STOCK FEED SUPPLEMENT

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Abstract

Apple pomace is a waste stream generated from the apple juice extraction process and constitutes about 25% of the original fruit (Walter and Sherman, 1976). It contains a large amount of water and sugars, has a low pH and a small amount of protein. The total production of apple pomace in New Zealand is 2.7×10^4 tonnes/year. At present, it is mainly disposed of by transportation to landfill areas, with a minor portion being used locally as a pig feed supplement. However, this main disposal method is a major cost and is also of considerable environmental concern. As the quantity of pomace produced is forecasted to increase gradually over the next five years, alternative treatments and disposal options will become necessary.

This project involved the solid state fermentation of apple pomace with the aim of producing an improved stock feed supplement. The fermentations were conducted using a variety of yeasts with the purpose of improving the nutritional value by increasing the crude protein content. The effects of unsterilised media, moisture content and nitrogen addition were also addressed.

Sterilisation of the apple pomace medium prior to yeast inoculation was found to be necessary due to the superior growth characteristics of a yeast from the natural biota. This yeast was isolated and identified as *Kloeckera apiculata*.

The growth of *Kloeckera apiculata* on sterilised apple pomace was superior to that exhibited by *Candida utilis* Y15, *Saccharomyces cerevisiae* Y10 and *Yarrowia lipolytica* IFO1659. *Schizosaccharomyces pombe* H115 grew poorly on the apple pomace medium.

A reduction in the moisture content of the apple pomace medium from 80% to 65% was found to have little effect upon the growth characteristics of *C. utilis*, *Kl. apiculata* and *Sacch. cerevisiae*.

Ammonium hydroxide was the most effective nitrogeous growth substrate at improving the growth of *Kl. apiculata*, when used as a medium supplement. The growth of *C. utilis* benefited most from the addition of ammonium sulphate.

Kl. apiculata growth on apple pomace supplemented with 1% v/w 2 N ammonium hydroxide achieved a maximum crude protein content of 3.5%, measured on a dry weight basis, after 48 hours. *Kl. apiculata* growth on pomace supplemented with 1% v/w 7.8 N ammonium hydroxide achieved a maximum protein content of 7.2%, measured on a dry weight basis, after 72 hours.

Comparison of the amino acid profile of the microbially modified apple pomace (7.2% protein) with amino acid profiles recommended for growing pigs and breeding pigs revealed a deficiency in nearly all amino acids.

This research indicates that the increased protein content of the apple pomace, due to yeast propagation,

is still insufficient to qualify it as a suitable stock feed supplement. However, research into the effects of other fermentation parameters may lead to further improvements in yeast growth.

As the pig industry is potentially the greatest market in New Zealand for a microbial biomass product such as this, feeding trials with growing and breeding pigs are essential to determine its nutritional value. These trials would have a major bearing on determining the commercial prospects of this apple pomace feed product.

However, before any further research is conducted, consideration must be given to a new process which has been proposed for the extraction of apple juice. This process would result in an altered apple pomace waste stream and, if it was adopted for future commercial use, may reduce the applicability in New Zealand of the research results described in this thesis.

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Apple pomace is the residue left after juice extraction and represents about 25% of the original fruit (Walter and Sherman, 1976). It contains a large amount of water and sugars, has a low pH and a small amount of protein.

The New Zealand Apple and Pear Marketing Board processes approximately 1.5×10^5 tonnes of apples a year. From this apple pomace waste streams of 1.1×10^4 and 1.6×10^4 tonnes/year are generated from the juice extraction operations in Nelson and Hastings, respectively. This quantity is forecasted to increase gradually over the next five years to 3.5×10^4 tonnes/year (J. Marks, personal communication).

At present the pomace is disposed of by trucking to landfill sites with a small amount being used as pig food. However, disposal in landfill areas is costly and poses a serious environmental problem considering the volume produced and the high chemical oxygen demand, which ranges between 250 and 300 g/kg (Hours *et al.*, 1985). With increasing quantities being produced, alternative treatments and disposal options will become necessary.

A number of options have been studied but no literature has been found concerning their actual use in the apple processing industry.

The use of apple pomace as an animal feed has generally been regarded as unfeasible due to its low protein content (Hours *et al.*, 1985) and digestive troubles produced in some animals (Fontenot *et al.*, 1977; Rumsey, 1979). However, the chemical composition of pomace (Table 2) suggests that with the large concentrations of soluble and insoluble sugars available, protein enrichment by microbial growth would be possible.

The work undertaken in this thesis was aimed at improving the feed value of apple pomace by increasing its protein content. The protein enrichment was achieved by solid state fermentation of the pomace with a variety of yeasts. The effects of unsterilised media , moisture content and nitrogen addition were also monitored.

1.2 COMPOSITION OF APPLE POMACE

Apple pomace at a moisture level of approximately 80% is a solid, having no free water. Apple pomace composition (Table 2) will vary with apple varieties, growth conditions, harvesting, storage and juice extraction processes.

Table 2. Composition of Apple Pomace

	% Dry Weight	% Wet Weight
Moisture Content:	-	78-82
Carbohydrate:		
Total	72	14.4
Soluble		
(glucose and fructose)	46.2	9.2
Insoluble		
(hexose)	13.2	2.6
(pentose)	15.2	3.0
Protein (N × 6.25):	5.0	1.0
Fat:	4.0	0.8
Pectins:	8.0	1.6
Ash:	1.0	0.2
pH:	3.5	
Minerals:		
N	0.870	0.174
K	0.650	
P	0.105	
Ca	0.082	

(Table 2. cont.....)

	% Dry Weight	% Wet Weight
Mg	0.056	
Cl	0.060	
S	0.047	
Na	0.005	
Fe	0.004	
Al	0.001	
Mn	0.001	
Zn	0.001	
Cu	0.001	

(Figures supplied by the New Zealand Apple and Pear Marketing Board, Hastings, New Zealand, 1988).

1.3 PROCESSING AND DISPOSAL OPTIONS

As has already been mentioned, the major disposal method of apple pomace in New Zealand has been the transportation to landfill sites. This is also true for pomace produced in foreign countries (Etheridge and Jewell, 1988; Hours *et al.*, 1985). However, this disposal option is of major environmental concern due to the increasing quantities of pomace being produced and its high COD. As other disposal methods are also limited, the processing of apple pomace to produce a marketable and profitable product is most desirable. A summary of some processing options follows, with the processes divided into non-fermentative and

fermentative catagories. Although many of these operations still produce waste streams of utilised pomace these effluents will have a reduced COD and often a large reduction in bulk.

1.4 NON-FERMENTATIVE PROCESSES

1.4.1 Apple Press Cake Powder

As a food ingredient, apple press cake powder (apple pomace minus the pips and stalks) may have some useful attributes. The peel contains considerable apple aroma (Guadagni *et al.*, 1971) and the high pectin content (approximately 1.6% on a wet weight basis) makes the press cake potentially suitable as a thickening and texturising agent.

Bomben *et al.* (1971) described a process in which apple press cake is converted into a thickening and flavouring agent suitable for use in apple pies in place of starch. This process involved drying the press cake then grinding it into a fine powder. Drum-drying and air-drying showed poor aroma retention and, as a result, the flavour of pies made with the press cake powder often required the addition of apple aroma concentrate. In addition, the press cake powder gave the pie filling a brownish colour and, with some apple varieties, a grainy texture in contrast to the smooth, glossy appearance of the starch-thickened fillings. Bomben and Guadagni (1973) suggested that the problems associated with the grainy texture may be alleviated

by grinding the press cake finer. With the necessary use of apple aroma concentrate in conjunction with the apple press cake powder this is unlikely to be feasible product.

Factors influencing the development of aroma in apple peels were found to be harvest maturity, variety, cold storage history, room temperature ripening and storage temperature (Guadagni *et al.*, 1971).

1.4.2 Extraction of Edible Fibres

The composition of apple pomace and the known hydrocolloidal ability of some of its components, such as pectins and pentosans, make it a likely source of general purpose hydrocolloids for use in low-fibre, fabricated foods. A study of the isolation of such fractions from apple pomace and an evaluation of their hydrocolloidal functionality, by viscometry, was conducted by Walter *et al.* (1985).

Air-dried and milled apple pomace was subjected to mild alkaline degradation, yielding an α-cellulose fraction of approximately 26% of the untreated dry matter. Extraction by various aqueous solvents yielded water-dispersible, uronide fractions comprising 10-18% w/v, on a dry weight basis. These fibres had various viscosometric characteristics, depending on the extractant used. All the fibres produced had the potential to provide non-nutritive bulk to low fibre, constructed foods. It was also suggested that they may provide a high concentration of solid matter to an

aqueous food system without affecting its rheological properties significantly.

1.4.3 Apple Cellulose Gel

The development and characterisation of a cellulose gel from apple pomace was outlined in a paper by Walter *et al.* (1977). Cellulose was isolated from apple pomace in the form of a hydrogel, using dilute sodium hydroxide and hydrogen peroxide. Sodium hyroxide and hydrogen peroxide treatments have the effect of degrading lignin and other non-cellulosic constituents, leaving the cellulose fibres substantially intact.

The gel was characterised as a pseudo-plastic fluid with fluid properties similar to those of furcellan, guar and xanthan gums. Freeze-drying and milling of apple cellulose gel was found to impair its hydrophilic character, although the particles still showed a tendency to wet and redisperse.

1.4.4 Carbon Briquets

Low temperature pyrolysis as a method of recovering the fuel value of apple processing wastes was studied by Walter and Sherman (1976). Moist apple press cake was air-dried in a fume hood and then heated at 180^{o} $\pm 20^{o}C$ for 1.5 hours. A portion of the pyrolysate was further treated for conversion to granulated charcoal. This was achieved by suspending the pyrolysed presscake in a solution of sulphuric acid to produce

charred intermediates, which were easily separated from the aqueous medium. This mixture was then pyrolysed at 160-200^{o}C until smoking ceased (Walter and Sherman, 1975). It was found that the gross heats of combustion of apple charcoal briquets and apple pyrolysate were approximately 90% of that for a commercial barbeque briquet. The air-dried press cake contained approximately 62% compared to the commercial barbeque briquet.

At 180^{o}C, the sugar concentrated upon air-drying was transformed to thermoplastic caramels which had, with the addition of carbohydrate polymers, enough adhesive strength to bind the secondary reaction products and the ligno-cellulose fibres that escaped thermolysis. However, it is highly unlikely that the production of apple pyrolysate briquets would be economical due to the high energy requirements.

1.4.5 Apple Pomace as a Stock Feed

1) Cattle

Ruminant animals, with their ability to efficiently convert cellulosic substances to nutrients and waste, seem a likely target for apple pomace feeding trials.

A series of trials were conducted to determine the feasibility of supplementing apple pomace with nonprotein nitrogen (NPN) for winter feeding pregnant beef cows (Fontenot *et al.*, 1977). Feeding a combination of apple pomace with urea, biuret or a

combination of both lowered feed consumption and increased body weight losses in the cows, compared to supplementing corn silage with NPN or supplementing apple pomace with protein. Although, no signs of toxicosis were observed in the cows, feeding pomace and NPN in combination resulted in the birth of small, deformed dead or weak calves. Increasing the level of dietary energy did not prevent this occurrence, but feeding a small amount of coarse hay appeared to alleviate the effects.

Trials were conducted with gestating beef cows to determine whether the dietary addition of a commercial trace mineral premix, corn starch, or wheat straw would reduce the adverse effects of apple pomace-urea diets on weight gain and calving performance (Rumsey, 1979). Half the cows in each trial were orally administered copper. Dietary addition of a trace mineral premix or copper did not consistently improve cow weight gain or calving performance. Dietary starch improved weight gain but intensified the poor calving performance. In another series of trials gestating beef cows were fed apple pomace supplemented with cottonseed meal or urea and straw. Supplementing with cottonseed meal resulted in improved weight gain and normal calving performance. Supplementing with urea and straw resulted in poor weight gain but a normal calving performance. These results suggested that neither a lack of trace minerals nor ammonia toxicity was a major factor causing poor performance when gestating beef cattle were fed apple pomace-urea diets.

Although additional research was conducted on steers fed apple pomace-urea diets with respect to ruminal fermentation products, plasma ammonia (Rumsey, 1978), rumen microbial population, movement of ingesta from the rumen and water intake (Rumsey *et al.*, 1979), no causative factor was determined. However, it is apparent that apple pomace can be utilised as a cattle feed supplement if incorporated into a properly formulated diet.

Unfortunately, an apple pomace feed product may be of only limited value to the New Zealand ruminant industry due to the predominance of pasture feeding during virtually the whole year.

2) Pigs

The effects of dried apple pomace in swine rations on gowth rate, feed efficiency and carcass quality was studied by Bowden and Berry (1959). Experiments were conducted to test rations containing various levels of apple pomace. At pomace levels up to 20% no significant change occurred in daily gain, dressing percentage or carcass quality. Increasing the pomace level to 40% resulted in significantly slower growth, low dressing percentage, greater feed consumption per unit gain and a leaner carcass. In one series of trials, an estimated difference of 2 - 3% crude protein in the two ration feeds at each pomace level had no significant effect on any of the characteristics studied.

Snowden (1984) researched a least cost feed analysis

to determine the potential of dried apple pomace as a feed supplement for grower and breeder pigs. At that time, it was found that dried apple pomace with a value of NZ $180 per tonne had a possible 12.8% inclusion by weight in grower pig diets. The maximum inclusion rate in breeder pig diets was 12.5% at NZ $150 per tonne. A hypothetical demand curve for dried apple pomace in grower and breeder pig feeds gave a potential demand of 21,000 tonnes and 4,500 tonnes respectively, assuming that the product was fully utilised in all pig feeds throughout New Zealand. If all the apple pomace produced at present (27,000 tonnes/year) was dried to a microbially stable product of approximately 10% moisture, an annual production of about 6,000 tonnes/year would result. This suggests that the entire apple pomace waste stream could be utilised as a pig feed supplement if drying and distribution costs were not prohibitive.

1.5 FERMENTATION PROCESSES

Submerged fermentation, either by a direct one-stage process or by a multi-stage process, is generally considered the most efficient form of bioconversion of substrate. Present bioconversion schemes , however, are not applicable to all substrates under all conditions. This is true for apple pomace. An attractive alternative to submerged fermentation is solid state fermentation. Solid state fermentation is defined as microbial growth on solid particles in the absence of free fluid (Aidoo *et al.*, 1982). For the microbial transformation of marginally valuable

agricultural residues, solid state fermentation holds a number of advantages over the traditional submerged fermentation processes. Among the advantages are (Aidoo *et al.*, 1982; Durand and Chereau, 1988):

1) low level technology
2) reduced reactor volume per unit substrate converted
3) high productivity per unit volume of reactor
4) reduced energy requirement
5) low wastewater output
6) facility of product recovery

and 7) low capital investment.

Some disadvantages of solid state fermentation are (Aidoo *et al.*,1982; Durand and Chereau,1988):

1) difficulties in regulating fermentation parameters such as moisture, aeration, pH and heat removal
2) lack of knowledge concerning the physiology and growth mechanisms of micro-organisms on solid materials
3) types of micro-organisms are limited to those which can grow at reduced moisture levels

and 4) problems associated with scale up.

Although Durand and Chereau (1988) suggested that solid state fermentation would facilitate product recovery, no research on the recovery of fermentation products, especially secondary metabolites, generated

from solid materials has been found. Normal product recovery methods such as filtration, precipitation and distillation could be adversely affected by the particulate nature of the fermented material.

The following sections address some of the fermentation processes which have been proposed for the microbial transformation of apple pomace.

1.5.1 Acetone and Butanol

Currently, butanol is manufactured from ethylene and triethylaluminium and is used in the manufacture of lacquers, rayon, detergents, brake fluids and also finds use as an industrial solvent. Acetone is a by-product from the manufacture of phenol from cumene, or it can be manufactured by chemical reduction of iso-propanol. Acetone is used primarily as an industrial solvent (Linden *et al.*, 1985).

The acetone-butanol fermentation is a well known process which is awakening renewed interest due to the fluctuating values, strategic uncertainty and unsustainability of oil products. Traditional substrates used for acetone and butanol production by fermentation are a variety of starchy materials and molasses. With the cost of raw materials being a key factor in determining the viability of the acetone-butanol fermentation, the use of waste carbon sources may be beneficial (Jones and Woods, 1986).

The use of supplemented apple pomace as a substrate source for the production of acetone and butanol in a submerged fermentation system was studied by employing strains of *Clostridium acetobutylicum* and *Cl. butylicum* (Voget *et al.*,1985). Yields of butanol between 1.9% and 2.2% w/w of fresh apple pomace were reported, these results equating to reactor volume concentrations of approximately 13-15 g/l. The butanol:acetone ratio observed was 2:1 which is typical for acetone-butanol fermentations (Jones and Woods, 1986) on traditional substrates.

However, as is the case for all acetone-butanol fermentations, problems exist with low volumetric productivity, processing of biomass wastes, high water requirements, coproduct separation processes and butanol toxicity (Linden *et al.*, 1985).

1.5.2 Biodrying

The concept of biodrying involves a two-stage aerobic digestion and drying process combining high rate thermophilic aerobic digestion and low energy input drying. A predetermined amount of organic matter oxidation by thermophilic bacteria can be achieved by efficient management of substrate, water, energy and air for microbial use and drying (Jewell and Cummings, 1984). The exothermic heat of substrate oxidation provides the energy required to raise the temperature to thermophilic conditions and to preheat the drying air.

A biodryer feasibility study for the stabilisation and drying of apple pomace was conducted using a pilot scale prototype unit by Jewell and Cummings (1984). In a continuous feed study conducted over a 5 day period, a total mass and volume balance was maintained. In this system the pomace wet mass was reduced by 73% ; the volume of the pomace was reduced by 63% ; the biodegradable organics were reduced by 83% ; and approximately 1.3 times more energy was biologically produced than was consumed. The end product was dry, stable, odourless and remained quite high in digestible organics.

This process would undoubtedly produce a more environmentally sound product for waste disposal. In addition it would have some benefit in reducing the costs of transporting the apple pomace to landfill sites, due to the volumetric and mass reductions, but whether this would vindicate the necessary investment requires more study. Biodried apple pomace may find some use as a stock feed supplement but feeding and digestibility trials would first be required. If found to be unacceptable as a feed supplement the dry material may find application in the horticultural sector as a soil conditioning compost.

1.5.3 Anaerobic Digestion

A variety of technologies have been developed for energy production from biological fuels and these include combustion, fermentation, gasification and pyrolysis. Anaerobic digestion is an attractive

option, yielding a high value natural gas substitute as well as a stable residue which may have some market potential.

Using bench scale mesophilic (35°C) and thermophilic (55°C) reactors and a pilot scale thermophilic reactor with an apple pomace substrate, Jewell and Cummings (1984) achieved an average biodegradability of 76.3% at a loading rate of 5 g/l/day. This resulted in a biogas production of 3 v/v-d (volume of gas per volume of reactor per day). The methane content of the gas effluent was 60%.

In a full scale system Etheridge and Jewell (1988) used a flexible liner digester for the anaerobic digestion of fresh and ensiled pomace. It was found that the biogas potential of ensiled pomace was similar to fresh pomace, demonstrating that ensiling of apple pomace is feasible at full scale. Little difference between mesophilic and thermophilic operations, in relation to biogas production and removal efficiency, was noted except that stable mesophilic operation was difficult to maintain. Mesophilic systems were discovered to fail at organic loading rates greater than approximately 3.6 g/l/day. Run under thermophilic conditions at 55°C a 90% conversion of biodegradable volatile solids at a loading rate of 5 g/l/day was achieved, with a concomitant biogas production of 1.9 v/v-d.

1.5.4 Citric Acid

For the last 60 years citric acid has been produced by fermentation of carbohydrates. Originally, the surface process utilising the fungus *Aspergillus niger* was used. Latterly, submerged fermentations of beet or cane molasses or glucose syrups by *A. niger* have been introduced. A recent development has been the replacement of *A. niger* by strains of yeast which exhibit higher productivity and are less sensitive to variations in crude carbohydrate media (Milsom and Meers, 1985).

An evaluation of the suitability of apple pomace as a substrate source for citric acid production was conducted by Hang and Woodams (1984). In this study five strains of *A. niger* were used for the production of citric acid from apple pomace in a solid state fermentation. *A. niger* NRRL567 was found to produce the greatest amount of citric acid in the presence of 4% methanol. A yield of 88%, based on the amount of sugar consumed, was achieved. This compares favourably with yields of citric acid from a sucrose or molasses medium impregnated in a sugarcane bagasse carrier (Kapoor *et al.*, 1982).

A subsequent report by Hang and Woodams (1987) determined the effect of apple pomace moisture on fungal production of citric acid in a solid state system. It was found that the stimulatory effect of methanol on fungal production of citric acid decreased markedly as the pomace moisture decreased. Conversely, the citric acid production in the absence of methanol

addition increased as the pomace moisture level decreased.

The recovery of citric acid from the solid state fermentation would require a solvent extraction step, to remove the acid produced from the fibrous medium. To what extent this would effect downstream processing has not been addressed.

1.5.5 Ethanol

A solid state fermentation system for the production of ethanol from apple pomace using *Saccharomyces cerevisiae* was devised by Hang *et al.*, (1982). Pomace inoculated without pasteurisation or autoclaving with the yeast strain fermented rapidly at 30°C and had an ethanol content of more than 4% in 24 hours. It was shown that temperature had a marked influence on the rate of ethanol production. The fermentation times required for maximal production of alcohol at 30°C, room temperature (22-25°C) and 15°C were 24, 48, and 96 hours respectively. Of the alcohols analysed, ethanol was present in the highest concentration with the other alcohols being produced in much smaller quantities. The separation of alcohol from the pomace was achieved using a Buchi rotary vacuum evaporator. The efficiency of this operation ranged from 92-99% with an ethanol concentration in the final condensate as high as 13% v/v.

The protein content of the spent apple pomace was found to have increased from 4% to 9%, on a dry weight

basis. Hang *et al.* (1982) suggested that it may be used as a stock feed supplement although the still minimal protein content and combined drying costs required to form a microbially stable product would possibly make this option unfeasible.

The production of ethanol by solid state fermentation of apple pomace using the yeasts *Saccharomyces cerevisiae*, *Sacch. diastaticus*, *Pichia fermentans*, *Candida utilis* and *C. tropicalis* was conducted by Gupta *et al.* (1989). *Sacch. cerevisiae* and *Sacch. diastaticus* were found to have the greatest alcohol yields (2.6 and 2.8 g/100 g, respectively) of the five yeasts used. The effect of nitrogen, phosphate and trace element supplementation was compared in *Sacch. cerevisiae* and *Sacch. diastaticus* on the basis of the fermentation efficiency of apple pomace. The addition of various nitrogenous sources, phosphates or trace elements resulted in an improved fermentation efficiency for both yeasts.

Of the nitrogenous sources added ammonium sulphate effected the greatest increase in fermentation efficiency at an inclusion rate of 0.2% w/v. The addition of dipotassium hydrogen phosphate (0.2% w/v) resulted in the greatest increase in fermentation efficiency of the phosphate sources added. Although a greater increase was achieved with ammonium dihydrogen phosphate supplementation, this increase would have been largely due to the additional nitrogen content. The addition of individual trace elements (0.002% w/v) was found to improve fermentation efficiency, with the addition of zinc sulphate resulting in the greatest

addition of zinc sulphate resulting in the greatest increase.

The greatest alcohol yield obtained (Gupta *et al.*, 1989) was 5.7% using the yeast *Sacch. diastaticus* for the solid state fermentation of apple pomace supplemented with 0.2% w/v ammonium dihydrogen phosphate.

Industrial alcohol is produced from whey in New Zealand at Reporoa, Tirau and Clandeboye (Mawson, 1987). Potable ethanol is supplied by the New Zealand Distillery Co. Ltd., Edgecumbe, but limited quantities are also produced at Reporoa and Clandeboye. These four distilleries have a combined annual output of approximately 11 million litres absolute alcohol (Mawson, personnal communication) and supply both the N.Z. and limited export markets. As a result, any future for the production of ethanol from apple pomace in N. Z. would depend on its competitiveness with the established whey ethanol process.

1.5.6 Pectinases

The possible use of apple pomace for the production of pectinases in a solid state culture using an *Aspergillus foetidus* strain was investigated by Hours *et al.* (1988).

Using small scale solid state cultures, pectinase production reached viscosometric enzyme activities as high as 1062 U/g. These results were obtained after 48

fermentation, the maximum value achieved in this case was more than seven times higher than the values reported per millilitre by Tuttobello and Mill (1961). The period for obtaining the maximum enzyme levels was also reduced from 120 hours to 48 hours. The pool of pectinases obtained was characterised as containing endopectinylase and a polygalacturonase. The presence of amylases, cellulases and hemicellulases was also shown, but no proteases or lipases were detected.

Although apple pomace appears to be an adequate raw material for pectinases production, problems associated with the extraction of the enzymic pool have yet to be addressed.

1.5.7 Microbial Biomass Product

Present day processes for producing yeasts for food and feed protein originated in Germany during World War I and II, where both *Saccharomyces cerevisiae* and *Candida utilis* were grown on molasses and sulphite-waste liquor, respectively (Litchfield, 1980). During the last two decades, numerous processes have been developed for producing cells of micro-organisms for use as protein sources for human food or animal feed. It appears that food grade single cell protein (SCP) products may not enter the human nutritional markets to any great extent in the short term due to the relatively lower costs of alternative proteins from plant and animal sources, and the reqirements of regulatory agencies for extensive safety studies (Litchfield, 1985). However, Rank, Hovis and McDougal

have successfully marketed a fungal protein meat substitute filling for pastries recently. Also, microbial protein products for animal feed application are quite competitive with conventional plant and animal sources. A large number of micro-organisms and substrates have been analysed for their suitability for microbial protein production. Yeast protein is largely limited to the production of *Candida utilis*, with smaller quantities of protein produced from the *Saccharomyces cerevisiae* generated as a waste stream from wine and beer fermentations. British Petroleum and Liquichimica plants did produce a *Candida* sp. from purified n-alkanes (Kanazawa, 1975) and a plant in Oregon, U.S.A. produces *C. utilis* from sulfite waste liquor (Anderson *et al.*, 1974). *Kluyveromyces fragilis* has been produced from cheese whey in Wisconsin, U.S.A. (Bernstein *et al.*, 1977). With the volatility of petrochemical prices and the increasing values of traditional starch substrates much attention is now being focused on waste materials as carbon sources, including apple pomace.

Apple pomace has generally been regarded as a poor animal feed supplement due to its low protein content. However, the chemical composition of pomace suggests that it may be used for the production of a protein-enriched microbial biomass product (Hours *et al.*, 1985), a term used to denote the complex mixture of substrate source and micro-organisms obtained by the fermentation of undefined agricultural wastes. Studies on the formation of microbial biomass products from a number of substrate sources including annual ryegrass straw (Han *et al.*, 1978), rice straw (Han and

Anderson, 1974), wheat straw (Viesturs *et al*, 1981; Laukevics *et al.*, 1984), sugar-beet (Cochet *et al.*, 1988; Durand and Chereau, 1988) and apple pomace (Hours *et al.*, 1985; Hang, 1988) have been conducted using bacterial, yeast and fungal cultures. With respect to apple pomace, both submerged and solid state fermentation trials have been conducted, under various conditions and using different micro-organisms, to produce a protein enriched feed.

Small scale batch and fed-batch submerged fermentation processes using *Saccharomycopsis* (now *Yarrowia*) *lipolytica* and *Trichoderma reesei* in single and mixed cultures were analysed by Hours *et al.* (1985).

Batch fermentations in 200 ml Erlenmeyer flasks using a single culture of *Y. lipolytica* on a supplemented pomace substrate attained a protein yield (protein produced/sugar consumed) value of 0.45 after 34.6 hours. This corresponded to a protein content, on a dry weight basis, in the final product of 13%.

A 4-l fermenter trial using *Y. lipolytica* and pomace diluted with 33% water produced a protein content of 12.5% with the advantage of reducing the time of the process by approximately 50%. This was explained by the greater aeration efficiency at fermenter scale and it was therefore considered that the kinetics of the operation were controlled by the oxygen transfer rate. However, it seems that no consideration was given to the differences in fermentation procedure whereby pH in the Erlenmeyer flask was maintained using 1 M NaOH whilst pH in the fermenter was adjusted using 7% v/v

NH_4OH. It is highly likely that the nitrogen addition in the fermenter trials had a major bearing on the reduction in maximum protein production time.

Preliminary experiments using *T. reesei* in Erlenmeyer flasks (Hours *et al.*, 1985) revealed that the normal flora in the apple pomace competed strongly with the fungi, resulting in a decreased yield. For this reason, a medium containing apple pomace treated with sulphuric acid at 100°C for 2 hours was used. The combination of acid and heat treatment reduced the content of the normal flora, decreased the viscosity of the medium and produced a partial hydrolysis of the pectic substances and crude fibre, increasing the amount of substrate available for growth by 20%.

Experiments carried out in Erlenmeyer flasks with *T. reesei* using the acid/temperature-treated media showed that the protein level of the fermented material at 48 hours corresponded to 15% on a dry weight basis. As no trials were conducted on the acid/temperature-treated pomace using *Y. lipolytica*, no accurate comparison between the yeast and fungal protein production could be made for this medium.

Although *T. reesei* was found to have a lower growth rate than *Y. lipolytica* the pectolytic and cellulolytic activities of the fungus were considered beneficial in further reducing pectic substances and increasing soluble sugars in the treated pomace. As a result, fed-batch fermentation trials using a mixed culture of *T. reesei* and *Y. lipolytica* were conducted in order to increase the amount of dry matter at the

end of the process. A protein content of 15.6% was achieved which equated to a volumetric value of 15 g/l, which was double the values obtained in other experiments.

A study to improve the nutritional value of apple pomace by solid state fermentation with the food yeast *Candida utilis* was conducted by Hang (1988). Contrary to results obtained by Hours *et al.* (1985), *C. utilis* was shown to grow well on an ammonium sulphate-supplemented pomace substrate and significantly improved its nutritional value. Yeast growth resulted in a protein content of 15% on a dry weight basis. Significant increases in niacin and pantothenic acid, and small increases in both riboflavin and thiamine were observed. The enhancing effect of yeast fermentation on protein and vitamin yields was found to be dependent on the amount of ammonium sulphate added, up to a concentration of 1% w/w.

1.5.8 Summary

With the production of apple pomace wastes being such a disposal problem, research to find a constructive solution is necessary. Improved disposal methods and treatments that would be less harmful to the environment are plentiful but costly. However, apple pomace has compositional characteristics which suggest that some profitable use could be found for it. Research into possible productive uses for apple

pomace has been going on for the last three decades and yet none of the research has progessed from the laboratory to the factory, in N.Z. at least. Whether this is due to the lack of cost effectiveness of the processes or an unwillingness on the part of the apple industry to invest in new technologies due to the financial risks involved is not always clear. However, with the increasing environmental concern being shown by the populace, opposition to the dumping of apple pomace may also increase, forcing the apple industries to find some other method of disposal or usage.

A significant amount of research has been conducted into the processing of waste apple pomace from the development of human food products, ie. edible fibres, press cake powder and cellulose gels, to animal feed products, fermented and unfermented, to fuels, ie. carbon briquets, anaerobic digestion (biogas), ethanol production and biodrying, to the production of valuable secondary products, ie. acetone, butanol, citric acid and pectinases. Of the options covered in this literature the most promising treatments would appear to be anaerobic digestion, citric acid production and the use of pomace as an animal feed in either a fermented or unfermented, dried state. The following research to be described addresses aspects of producing a nutritionally improved apple pomace stockfeed by solid state fermentation using yeasts.

CHAPTER 2

MATERIALS AND METHODS

2.1 FERMENTATION STUDIES

2.2.1 CHEMICALS

All chemicals were of analytical grade and were supplied by either:

Ajax Chemicals Pty. Ltd., Auburn, N.S.W., Australia;

BDH Chemicals Ltd., Poole, England;

or M&B Laboratory Chemicals, May and Baker Ltd., Dagenham, England.

2.1.2 MEDIA COMPONENTS

All media components used were of laboratory grade and supplied by either:

Oxoid Ltd., Basingstoke, Hampshire, England;

Difco Laboratories, Detroit, Michigan, U.S.A.;

or Gibco Laboratories, Madison, Wisconsin, U.S.A..

2.1.3 MALT EXTRACT (ME) BROTH

Supplied as a powder by Oxoid Ltd..

Composition (g/l):

Malt extract	17 g
Mycological peptone	3 g

Dissolve the powder in distilled water and sterilise by autoclaving at 115°C for 10 minutes.

2.1.4 ME AGAR

Add 15 g agar to 1-l ME broth and sterilise by autoclaving at 115°C for 10 minutes.

2.1.5 PEPTONE WATER

Peptone water was prepared by dissolving 5 g peptone powder in 1-l distilled water and sterilised by autoclaving at 121°C for 15 minutes.

2.1.6 pH MEASUREMENT

The pH of the apple pomace samples was measured on a sample (1:10) blended in deionized water. Measurement of pH was conducted with an Orion Research Digital Ionanalyzer, Orion Research Incorporated, Cambridge, Mass., U.S.A..

2.1.7 pH ADJUSTMENT

The pH adjustment of apple pomace was conducted after drying and sterilisation, using either 2 N NaOH or 2 N NH_4OH.

2.1.8 MOISTURE CONTENT MEASUREMENT

Moisture was determined by drying to a constant weight at 105°C. The weight loss was calculated as the percent of water content in the original sample, expressed as % (w/w).

2.1.9 MOISTURE CONTENT ADJUSTMENT

The moisture content of apple pomace was adjusted in a tunnel dryer operating at 105°C and an air speed of 0.8 m/s. The pomace was dried in a basket attached to a set of scales. The final moisture content was determined from the weight loss during the drying and a sample of the dried pomace was finally checked for its moisture content as in section 2.2.10.

2.1.10 MICROBIAL COUNTS

Microbial counts were made from a 1 g apple pomace sample blended for 20 seconds in 9 ml peptone water. The sample was then serially diluted in peptone water, plated on ME agar and incubated at 30°C for 48 hours.

2.1.11 APPLE POMACE

Apple pomace was obtained as a waste stream from the New Zealand Apple and Pear Marketing Board's apple juice extraction plant, Hastings. The pomace was stored at -20°C. Two batches of apple pomace were supplied and each contained different varieties of apples. The first batch contained a predominance of red-skinned apples and the second was composed largely of green-skinned apples. Delivery times from the factory meant that batch pooling could not be practiced.

2.1.12 STERILISATION

All glassware was sterilised in an oven at 160°C for 1 hour.

Media was sterilised by either autoclaving under the appropriate conditions stated or by filtration using a Whatman cellulose nitrate membrane filter (0.45μm) in a Buchner funnel.

2.1.13 STERILISATION OF APPLE POMACE (SUBSTRATE)

Apple pomace was sterilised, prior to fermentation, by autoclaving at 121°C for 15 minutes. The first batch of apple pomace was used directly for the solid state fermentations. However, the second batch of apple pomace was found to release juice during the autoclaving process. This juice was drained from the

sterilised pomace prior to fermentation.

2.21.14 ADDITION OF AMMONIUM SALTS AND UREA

Ammonium salts or urea (1% [w/w]) were added to the apple pomace and mixed well after sterilisation.

2.1.15 CULTURE MAINTENANCE

The yeast cultures were maintained on ME agar slopes and subcultured every month. In addition to monthly subculturing, each culture was streaked for single colonies on ME agar.

2.1.16 INOCULUM PREPARATION

Sufficient ME broth was inoculated with yeast from a maintenance slope and incubated at 30°C overnight. The yeast culture was centrifuged at 1300 g for 10 minutes in a Clandon T52.1 benchtop centrifuge. The supernatant was discarded and the cells resuspended in an equivalent volume of deionized water.

2.1.17 FERMENTATIONS

Fermentations were conducted at ambient temperature in sterile 1-l Agee jars with a mutton cloth top to allow air circulation. The jars were loaded with 150 g of apple pomace and held in a horizontal position on a

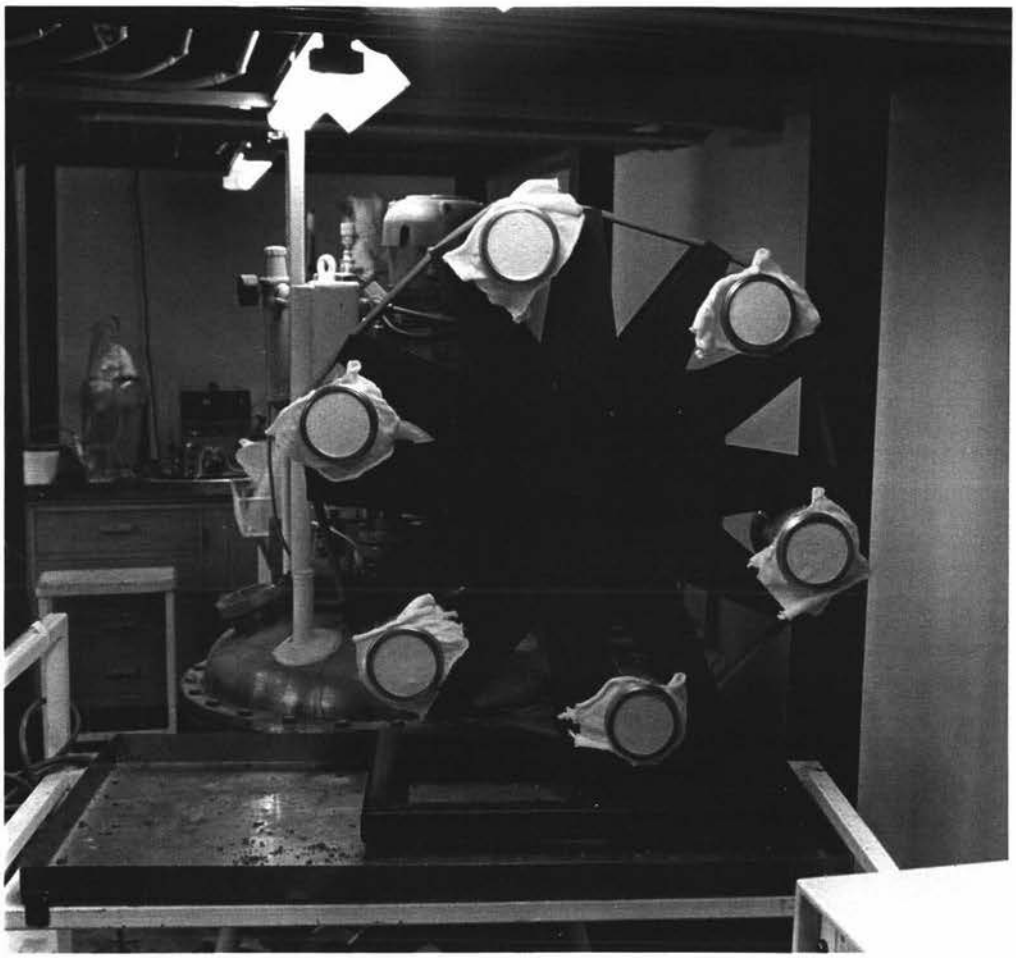


Figure 1. Solid state fermentation equipment.

wooden plate (Figure 1) rotating at 20 rpm. A 1% (v/w) inoculum of actively growing yeast was used. Samples were withdrawn periodically for plate counts and other analyses. Fermentation samples requiring storage were kept at -20°C.

2.1.18 EXTRACTION OF NON-PROTEIN NITROGEN (NPN) AND SOLUBLE SUGARS

The method for the extraction of NPN and soluble sugars was adapted from the method of Southgate (1976).

Method:

1) Apple pomace (10 g) samples was weighed out accurately into a 150 ml beaker and 90 ml of 60% methanol added.
2) Samples were mixed well for 30 minutes at ambient temperature.
3) Supernatant (20 ml) was withdrawn and centrifuged at 1300 g for 10 minutes in a Clandon T52.1 benchtop centrifuge.

a) Soluble Sugars

1) Supernatant (1 ml) was drawn off and filtered firstly through a Millipore AP prefilter then through a Millipore HA filter (0.45 µm). The filtered samples were stored at -20°C until analysed.
2) Standard solutions of 0, 0.625, 1.25, 2.5 and 5 mg/ml fructose, glucose and sucrose were prepared in

60% methanol.

3) Samples and standards analysed by High Performance Liquid Chromatography (see Section 2.1.21) used 50μm of undiluted solution.

b) Non-protein Nitrogen

1) Supernatant (1 ml) was diluted 1:10 with deionized water then quantified as in Section 2.1.20.

2.1.19 PROTEIN DIGESTION

Total nitrogen was determined by a modified version of the micro-Kjeldahl method of Perrin (1953). Crude protein was calculated by multiplying the total nitrogen by 6.25.

Method:

1) Samples (5 g) were weighed out accurately and transferred to a Kjeldahl digestion flask.
2) About 1.6 g K_2SO_4, 0.3 g $CuSO_4$ and 0.012 g Na_2SeO_4 and 20 ml concentrated H_2SO_4 were added. Also add 2 glass beads to prevent bumping.
3) The samples were then gently heated in a fume cupboard and when frothing had ceased the heat was turn up and simmered until the solution cleared.
4) The digests were cooled, the necks of the flasks washed down with hot distilled water and the heating continued until again clear and for a further 1 hour.
5) The digests were cooled again and the solid residues dissolved in distilled water, then

transferred to a 100 ml volumetric flask and made up to 100 ml with distilled water.

6) Diluted digest (10 ml) was withdrawn and the pH adjusted to 2.5 with 5 M potassium hydroxide. The digest was then made up to 100 ml with distilled water.

2.1.20 PROTEIN AND EXTRACTABLE NPN DETERMINATION

Protein and extractable NPN concentrations were determined by a modified method using Berthelot Reagent (Rock *et al.*, 1986).

Method:

1) Duplicate 0.25, 0.5 and 1.0 ml aliquots of the neutalised protein digests or NPN extractions were pipetted into labelled test-tubes and made up to 1 ml with distilled water. A series of standard $(NH_4)_2SO_4$ solutions (5 µg/ml) were also prepared and made up to 1 ml.

2) Phenol-nitroprusside solution (2.5 ml) was added to each tube and mixed thoroughly.

3) Alkaline-hypochlorite solution (2.5 ml) was then added and mixed thoroughly.

4) The tubes were placed in a water bath at 37°C for 20 minutes.

5) The absorbance of the standards and unknowns was then read at 560 nm on a Philips PU8625 Series UV/Vis Spectrophotometer (Philips Scientific, York St., Cambridge, Great Britain) and a standard curve prepared for each series of analyses.

2.1.21 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Isocratic High Performance Liquid Chromatography (HPLC) was used to determine the glucose, fructose and sucrose concentrations of the apple pomace fermentation samples. The HPLC used was a Waters Associates ALC 200 Series Chromatograph (Millipore Corporation, Milford, Massachusetts, U.S.A.) equipped with a Model 590 Solvent Delivery System and a Model 440 Absorbance Detector. The column used was a Waters Sugar-PAK 1 Column (Millipore Corporation).

The standard operating conditions were:

Flow rate	: 0.5 ml/min
Column temperature	: 90^{o}C
Back pressure	: < 1500 psi

A 20 mg/l solution of calcium acetate in deionized water, filtered and degassed, was used as the mobile phase.

A Waters 740 Data Module (Millipore Corporation) was used to record the peak heights of the samples. Standard curves of peak height (mm) vs fructose, glucose or sucrose concentration (mg/ml) were generated using duplicate standard sugar solutions. The sugar concentrations of the samples, determined from the standard curve, were converted to units of g/100g apple pomace, ie. percentage on a wet weight basis.

2.1.22 EXTRACTION OF VOLATILE FATTY ACIDS AND ETHANOL

The extraction process employed here was for the low molecular weight (C_2-C_4) water soluble fatty acids and ethanol (Rogosa and Love, 1968).

Method:

1) An apple pomace sample (1 g) was mixed with 9 ml of deionized water in a clean stoppered glass bottle and stored at 4°C for 6 days.
2) The slurry was centrifuged at ×1300 g for 10 minutes in a Clandon T52.1 benchtop centrifuge.
3) Concentrated HCl (0.2 ml) was then added to give a pH of approximately 2.
4) The precipitate was removed by recentrifugation at 1300 ×g for 10 minutes in a Clandon T52.1 benchtop centrifuge.
5) Volatile fatty acids and ethanol concentrations were analysed by gas chromatography.

2.1.23 GAS CHROMATOGRAPHY

Gas Chromatography (GC) was used to determine the ethanol, acetic acid, propionic acid, n-butyric acid and iso-butyric acid concentrations of the apple pomace fermentation samples. The GC used was a Shimadzu Gas Chromatograph GC-8A supplied by Sci. Med. Ltd., Wellington. The column used was a Propak Q Column.

The standard operating conditions were:

Air	: 0.2 kg/cm^2
Hydrogen	: 0.5 kg/cm^2
Nitrogen (carrier gas)	: 5.0 kg/cm^2
Primary	: 6.0 kg/cm^2
Injection temperature	: 220°C
Column temperature	: 200°C
Injection volume	: 3
Range	: 10^2
Attenuation	: 4

A Sekonic SS-250F Recorder (supplied by E.C. Gough Ltd., Wellington) was used to record the peak heights of the samples and standards. Single-point calibrations were used to calculate the ethanol and volatile fatty acid concentrations.

2.1.24 PREPARATION OF YEASTS FOR AMINO ACID ANALYSIS

1) Malt extract broth (200 ml) was inoculated aseptically from an isolated yeast colony on a malt extract agar plate and incubated at 30°C overnight.
2) The yeast cells were centrifuged at 2000×g for 10 minutes in a Sorvall RC5C centrifuge (Sorvall Industries, Du Pont).
3) The supernatant was discarded and the cells resuspended in 100 ml of deionized water.
4) The yeast cells were filtered using a Buchner funnel containing a Whatman cellulose nitrate membrane filter (0.45 µm pore size).

5) The cells were washed three times with deionized water then filtered as dry as possible.

6) The filter pad and yeast cells were then freeze-dried on a VirTis freeze-drier (The Virtis Company Inc., Gardiner, New York, U.S.A.) at 100 millitorr and -60^{o}C. The freeze-dried yeast cells were then transferred to a sterile 25 ml bottle and stored at room temperature.

2.1.25 PREPARATION OF FERMENTED POMACE FOR AMINO ACID ANALYSIS

The fermented apple pomace sample was frozen then freeze-dried on a VirTis freeze-drier at 100 millitorr and -60^{o}C. The freeze-dried fermented pomace was then blended and transferred to a sterile 25 ml bottle and stored at room temperature until amino acid analysis.

2.1.26 AMINO ACID ANALYSIS

Amino acid analyses were carried out using a Beckman 119BL amino acid analyser after hydrolysis of the samples *in vacuo* in 6 M HCl for 24 hours at 110 ^{o}C.

2.1.27 GRAPHICS

Graphical representation of data was achieved using the *EnerGraphics*^{TM} computing package, a trademark of Enertronics Research, Inc., Saint Louis, Missouri,

U.S.A.. This computer package was also used to calculate equations for the standard curves constructed for soluble glucose, fructose and sucrose analysis, and for N analysis.

2.2 MICRO-ORGANISMS

TABLE 2. List of Yeast Strains

YEAST	SOURCE
Candida utilis Y15	Department of Biotechnology Culture Collection, Massey University, Palmerston North.
Kloeckera apiculata	Isolated from apple pomace supplied by the New Zealand Apple and Pear Marketing Board, Hastings, New Zealand.
Saccharomyces cerevisiae Y10	Department of Biotechnology Culture Collection, Massey University, Palmerston North.
Yarrowia lipolytica IFO1659	Institute of Fermentation, Osaka, Japan.
Schizosaccharomyces pombe H115	Bayerische Landensanstalt, für Weinbau und Gartenbau, Warzburg, West Germany.

2.3 YEAST IDENTIFICATION

The procedures, unless stated otherwise, described by Lodder (1970) were used throughout for all yeast identification tests conducted on the isolate from the apple pomace.

2.3.1 ACTIDIONE AGAR

Supplied in a powdered form by Difco Laboratories.

Composition (g/l):

Bacto-Soytone	10 g
Bacto-Dextrose	10 g
Bacto-Agar	15 g
Actidione	0.5 g
Chloromycetin	0.05 g

Wickerham's Nitrogen Free Base (11.7 g) was rehydrated in distilled water and boiled to dissolve the powder. Sterilised by autoclaving at 120^{o}C for 10 minutes, removed from the autoclave and cooled quickly.

2.3.2 ARBUTIN AGAR SLOPES

Composition (g/l):

Yeast extract	5 g
Arbutin	5 g
Agar	20 g

The ingredients were dissolved by heating in 1-l distilled water, 7 ml aliquots dispensed in tubes and sterilised at 121°C for 15 minutes. While the tubes of agar were molten 2-3 drops of sterile 1% (w/v) ferric ammonium citrate solution were added aseptically and shaken carefully to disperse, then slanted and set.

2.3.3 CARROT WEDGE (SPORULATION)

A carrot was thoroughly washed and a long cylinder of about 1 cm diameter cut with a cork borer. The wedge was prepared by cutting the cylinder obliquely. The wedge was then rinsed with distilled water and placed in a suitable test tube containing some distilled water to prevent dessication. Sterilised by autoclaving at 110°C for 15 miutes.

2.3.4 CORN-MEAL AGAR

Yellow corn meal (12.5 g) was stirred into 300 ml of distilled water and heated in a water bath at 60°C for 1 hour. The solution was filtered through Whatman 1 filter paper (Whatman International Ltd., Maidstone, England). The volume was restored to 300 ml. Agar (3.8 g) was added and autoclave at 121°C for 15 minutes. The hot liquid was re-filtered through absorbent cotton wool, dispensed and sterilised again at 121°C for 15 minutes.

2.3.5 GYPSUM BLOCK (SPORULATION)

Gypsum hemihydrate (8 parts) was mixed with 3 parts of distilled water. The paste was caste into a rectangular form about 5 mm high. After setting, the block was placed in a sterile glass petri dish and sterilised at 121°C for 2.5 hours in an oven.

Prior to use , a sterile solution of mannitol (0.005% w/v) and dipotassium phosphate (0.05% w/v) was added to the dish to keep the block moist.

2.3.6 Mc CLARY'S ACETATE AGAR (SPORULATION)

Composition (g/l):

Glucose	1.0 g
KCl	1.8 g
Yeast extract	2.5 g
Sodium acetate trihydrate	8.2 g
Agar	15.0 g

Dissolved by heating. Dispensed in test tubes and sterilised by autoclaving at 121°C for 15 minutes.

2.3.7 OSMOPHILE AGAR SLOPES

Yeast extract (1 g) was dissolved in 100 ml of distilled water and then 50 ml of it used to dissolve 50 g glucose. Agar (3 g) was also dissolved in the distilled water, then the volume adjusted to 100 ml

with hot yeast extract solution. Aliquots (6 ml) were dispensed in 16 ml cotton plugged tubes and sterilised by autoclaving at 110°C for 15 minutes, slanted and set.

2.3.8 UREA AGAR

Supplied in a powdered form by Difco Laboratories.

Composition (g/l):

Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Potassium Phosphate	2.0
Urea	20.0
Phenol Red	0.012

Urea agar base (29 g) was dissolved in 100 ml of deionized water and filter sterilised.

Agar (15 g) was suspended in 900 ml of deionized water and heated to boiling with constant stirring to dissolve the components. The agar solution was sterilised by autoclaving at 121°C for 15 minutes, then cooled to 50°C and the filter-sterilised urea agar base added aseptically. The urea agar was mixed thoroughly and dispensed into sterile 25 ml bottles. The bottles were then cooled in a slanted position.

2.3.9 WICKERHAM'S FERMENTATION BASE MEDIUM

Composition (g/l):

Yeast extract	4.5 g
Peptone	7.5 g

A sufficient quantity of a 0.1% solution of Bromothymol blue was added to give a distinct green colour to the medium. Aliquots (4 ml) were dispensed in test tubes carrying Durham tubes and sterilise at 121^{o}C for 15 minutes.

On cooling 2 ml of filter sterilised sugar solution was added aseptically. The sugar solutions were prepared as follows:

Glucose, galactose, sucrose, maltose and lactose were 6% (w/v) solutions in 0.5% (w/v) yeast extract broth;

Raffinose was a 12% (w/v) solution in 0.5% (w/v) yeast extract broth.

2.3.10 WICKERHAM'S MALT EXTRACT AGAR (SPORULATION)

Agar (12 g) was dissolved in 400 ml of distilled water by steaming. Then 20 g powdered malt extract was dissolved in the hot solution, dispensed in tubes and sterilised by autoclaving at 100^{o}C for 15 minutes. The agar was allowed to solidify in tilted tubes to form a slant and refrigerated until used.

2.3.11 WICKERHAM'S NITROGEN FREE BASE

Supplied in powdered form by Difco Laboratories.

Wickerham's Nitrogen Free Base (11.7 g) was dissolved in 100 ml distilled water to give a 10 times strength solution and sterilised by membrane filtration.

2.3.12 WICKERHAM'S VITAMIN FREE BASE

Supplied in powdered form by Difco Laboratories.

Wickerham's Vitamin Free Base (16.7 g) was dissolved in 100 ml distilled water to give a 10 times strength solution and sterilised by membrane filtration.

2.3.13 YEAST EXTRACT - MALT EXTRACT (YM) BROTH

Composition (g/l):

Yeast extract	3 g
Malt extract	3 g
Peptone	5 g
Glucose	10 g

Adjusted to pH 3.7 with 1 N HCl and autoclaved at 121^{o}C for 15 minutes.

2.3.14 YM AGAR

YM broth with the addition of 2% agar. Autoclaved at 121°C for 15 minutes.

2.3.15 GROWTH IN MEDIA OF HIGH OSMOTIC PRESSURE

A suspension of yeast cells was lightly inoculated onto an osmophile agar slope and incubated at 30°C for 21 days. The slope was then examined for growth.

2.3.16 GROWTH IN VITAMIN FREE MEDIUM

A fresh tube of Wickerham's vitamin free medium and control were inoculated with 0.1 ml of an actively growing culture of yeast. The tubes were incubated at 30°C for 7 days.

A fresh set of vitamin free medium and control were inoculated with a loopful from the first set of equivalent tubes. The subcultures were incubated at 30°C for 21 days after which they were examined for growth.

2.3.17 GROWTH ON ACTIDIONE AGAR

A heavy inoculum was used to inoculate an actidione agar slope then the slope was incubated at 30°C for 14 days. The slope was examined for growth.

2.3.18 GROWTH ON UREA AGAR

A suspension of yeast cells was inoculated onto an urea agar slope and incubated at 30°C for 7 days. Urea hydrolysis was indicated by a change in the colour of the urea agar from yellow-brown to red.

2.3.19 MORPHOLOGICAL CHARACTERISTICS

1) Broth Culture

A 100 ml Erlenmeyer flask containing 30 ml of YM broth was inoculated with material from an actively growing culture and incubated at 30°C for 3 days then at room temperature for a further 21 days. The culture was examined after 3 days, 7 days and 21 days under a light microscope.

2) Rivalier and Seydal Slide Culture

Molten corn meal agar was poured into a sterile empty petri dish. A glass slide was dipped into the molten agar, using sterile forceps, then placed on a U-shaped glass rod in another sterile petri dish.

After solidification of the agar on the slides the agar was inoculated. Three streaks near the centre of the slide were made. A sterile coverslip was placed over part of the inoculated area.

The sterile chamber and slide were incubated at 30°C for 21 days then observed under a light microscope.

3) Dalmau-plate Method

A well dried, thickly poured plate of corn meal agar was inoculated with a single streak near one side of the plate. The inoculum was taken from a fresh YM slope culture. Two point inoculations were made near the other side. The central region of the streak and one of the point inoculations were covered with sterile cover slips.

The plate was incubated at 25°C for 3 weeks and examined at 3 days and 21 days under a light microscope.

4) Sporulation Studies

The sporulation media used were as follows:

Wickerham's malt extract agar
Mc Clary's acetate agar
Carrot wedge
Gypsum block with mannitol-phosphate

The media was inoculated with an active culture of yeast by spreading the yeast cells lightly over the whole surface, except for the gypsum block which was heavily inoculated.

The media were then incubated at 30°C for 28 days. Smears were prepared at 7 day, 14 day and 28 day intervals, stained for ascospores and examined under a light microscope.

2.3.20 SPLITTING OF ARBUTIN

A slope of arbutin agar was inoculated with an actively growing culture of yeast and incubated at 30°C for 7 days. The slopes were examined for growth.

2.3.21 STAINING OF ASCOSPORES

1) Heat fixed yeast slide preparations were flooded with 5% (w/v) aqueous malachite green for 30-40 seconds.
2) Heated to steaming, then for a further 3-4 minutes.
3) Rinsed in cold water for 30 seconds then counterstained with 0.5% safranine for 30 seconds.
4) Rinsed and dried.

Examined under oil at 1000 times magnification. Mature ascospres appeared green-blue and vegetative cells appeared red-pink.

2.3.22 PREPARATION OF CARBON SOURCE COMPOUNDS

Twenty organic compounds were selected for testing the oxidative utilisation of carbon compounds. The compounds were D-glucose, D-galactose, sucrose, maltose, cellobiose, trehalose, lactose, raffinose, inulin, starch, D-xylose, D-arabinose, D-ribose, L-rhamnose, glycerol, erythritol, ribitol, D-mannitol, citric acid and inulin.

The various carbon source solutions were prepared to give a carbon content equivalent to 5% glucose. Each solution was sterilised by membrane filtration.

Starch and inulin solutions of 0.5% were prepared by heating in distilled water over a low flame until dissolved. The solutions were distributed in 2.5 ml aliquots in test tubes then autoclaved at 120^{o}C for 20 minutes.

A 10% solution of raffinose was prepared by warming in distilled water then sterilised by membrane filtration.

The pH of the citric acid solution was adjusted to 5.6 with NaOH pellets then sterilised by membrane filtration.

2.3.23 PREPARATION OF CARBON ASSIMILATION MEDIA

1) Sterile glass distilled water was dispensed in 4.0 ml amounts into sterile test tubes.
2) Each tube was labelled with the name of the sugar.
3) Ten times strength Bacto-yeast nitrogen base (0.5 ml) was added to the tubes from 1) and to the starch and inulin tubes.
4) Prepared sugar solutions (0.5 ml) was added to the appropriate tubes.
5) A control tube was arranged, ie. basal medium with no sugar added but with the addition of 0.5 ml distilled water.

2.3.24 CARBON ASSIMILATION TESTS

The carbon assimilation tubes were inoculated aseptically with 1 ml of an actively growing culture of yeast. The tubes were incubated at 30°C for up to 21 days and examined at 3, 7 and 21 day intervals. After incubation the tubes were thoroughly shaken and examined for growth by comparing to the control tube.

2.3.25 FERMENTATION TESTS

The fermentation tubes were inoculated aseptically with 0.1 ml of an actively growing culture of yeast. the tubes were incubated at 30°C for up to 21 days. After incubation the tubes were lightly shaken and observed for gas production, acid production and the presence or absence of growth.

2.3.26 PREPARATION OF NITROGEN SOURCE COMPOUNDS

The compounds used for nitrogen assimilation were potassium nitrate, potassium nitrite and ethylamine hydrochloride. Each quantity was dissolved in 90 ml of sterile glass distilled water.

Potassium nitrate	0.078 g
Potassium nitrite	0.026 g
Ethylamine hydrochloride	0.064 g

Each solution was filter sterilised.

2.3.27 PREPARATION OF NITROGEN ASSIMILATION MEDIA

Wickerham's nitrogen free basal medium (sterile) was dispensed as 0.5 ml volumes in test tubes. Sterile nitrogen solution (4.5 ml) was added to each tube and mixed thoroughly. A blank was prepared containing 4.5 ml distilled water and 0.5 ml of basal medium.

2.3.28 NITROGEN ASSIMILATION TESTS

The nitrogen assimilation tubes were inoculated aseptically with 0.1 ml of an actively growing culture of yeast. The tubes were incubated at 30^{o}C for 7 days.

A fresh set of nitrogen assimilation tubes were inoculated with one loopful from the first set of equivalent tubes. The subcultures were incubated at 30^{o}C for 21 days after which they were examined for growth by comparing to the control tube.

CHAPTER 3

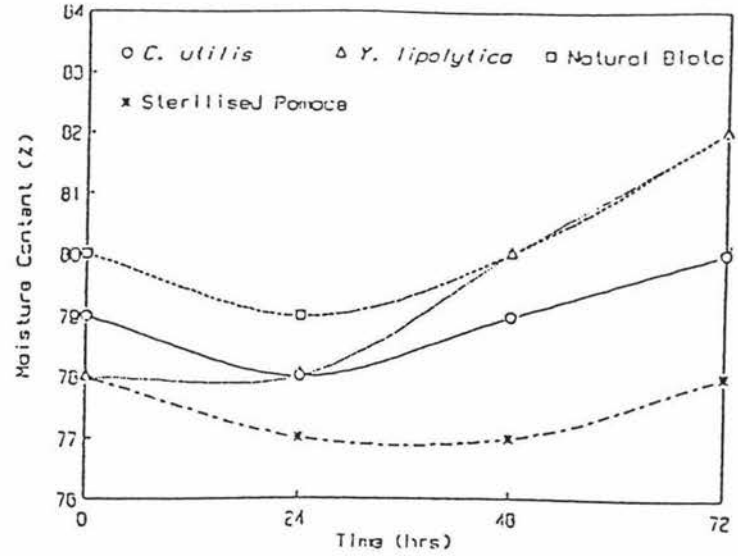
RESULTS

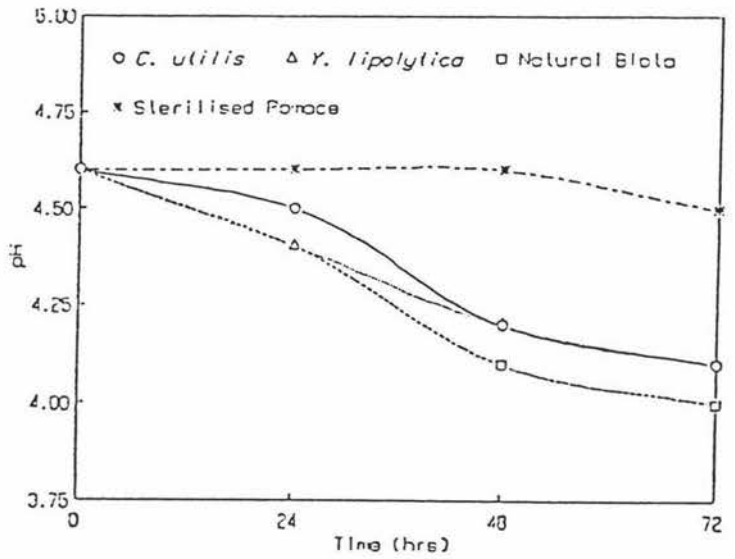
3.1 FERMENTATION STUDIES

3.1.1 YEAST GROWTH ON UNSTERILISED APPLE POMACE MEDIA

Sterilisation of substrates prior to microbial protein production by fermentation is an energy intensive operation and if practiced would add significantly to the costs of the process. For this step to be eliminated, the inoculated micro-organism must be capable of competing with and supplanting the growth of the natural biota of the medium. The ability to do this will depend on the size of the inoculum and its preference for the growth conditions provided and available substrates, ie. $\mu_{inoc} >> \mu_{biota}$.

For this series of growth trials on unsterilised apple pomace the yeasts *Candida utilis* Y15 and *Yarrowia lipolytica* IFO1659 were selected initially. The growth of the normal microflora in an uninoculated medium was monitored in a separate trial and a control of sterilised apple pomace was included (Figure 2). During the course of the fermentations the yeast flora was determined by colony morphology on the plate counts and by microscopic observation of wet mounts. The bacterial population was monitored by Gram staining heat fixed mounts and their microscopic





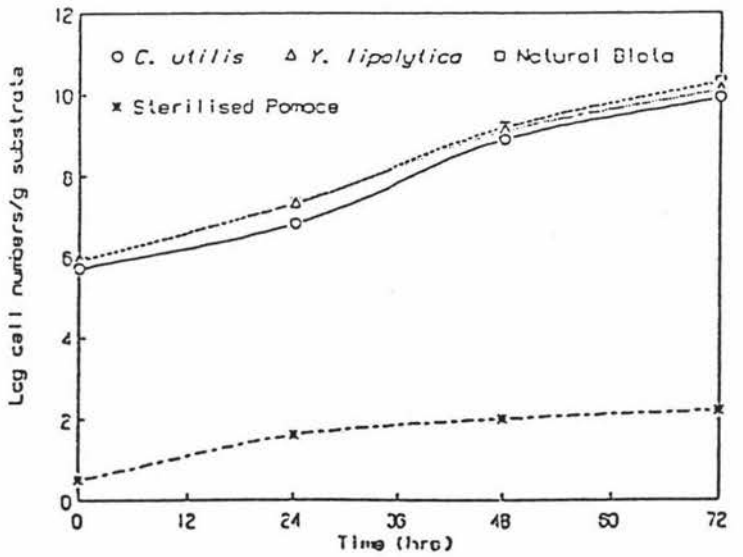


Figure 2. Changes in cell growth, pH and media moisture content during yeast growth on unsterilised apple pomace

appearance at 1000 times magnification was noted. Moisture content, yeast growth and pH were also measured.

1) Bacterial Flora

At the beginning of the growth trials the bacterial flora of the pomace was observed to be a diverse mixture of Gram negative rods, Gram positive rods and Gram positive cocci except for the control (sterilised pomace) where no bacteria were detected. During the propagation of the microflora, however, the Gram positive cocci became predominant until, at 48 hours, these cocci comprised the sole observed bacterial population. The Gram positive cocci formed extensive chains and were present in substantial numbers when the pH of the media reached 3.9 indicating that they were probably members of the genus *Lactococcus*, a common plant micro-organism (Brock, 1979). Only a low but heterogeneous population of bacteria was observed after 72 hours in the control.

2) Yeast Propagation

At the initiation of yeast growth, plate morphology and microscopic observation demonstrated the predominance of *C. utilis* or *Y. lipolytica* populations. At 24 hours both the colony morphology and the microscopic observation indicated that the yeast inocula were being supplanted by a yeast from the natural microflora of the pomace. By 48 hours this unknown apiculate, bipolar budding yeast was predominant and at 72 hours it was the sole component

population observed and detected. Thus the inocula had died out. This unknown yeast was isolated and identified (Section 3.2) as *Kloeckera apiculata* and was the sole yeast observed in the trial using uninoculated, unsterilised pomace. The yeast flora in the sterilised media were small but heterogeneous populations during the growth trials. To reduce any ambiguities in the interpretation of results, sterilised pomace was used in all the remaining yeast propagation trials.

Furthermore, in all trials except the control (sterilised pomace) a similar reduction in pH from 4.5 to 4 (Figure 2) and a minor increase in moisture content (Figure 2) was observed throughout. The pH and moisture content of the control remained relatively constant. The size of the changes in pH and moisture were too small for any conclusions to be drawn due to the heterogeneous nature of the apple pomace and the inherent problems associated with the quantification of these and other data.

3.1.2 GROWTH OF SELECTED YEASTS ON APPLE POMACE

Five yeasts were selected for propagation trials on sterilised apple pomace and the most appropriate yeasts chosen for further experiments. *C. utilis* Y15 and *Saccharomyces cerevisiae* Y10 were selected on the basis of their histories in single cell protein production (Litchfield, 1980). *Y. lipolytica* IFO1659 had been used in the production of a microbial biomass product from apple pomace (Hours *et al.*, 1985).

Schizosaccharomyces pombe H115 was chosen due to its ability to assimilate malic acid, a major acid in apple pomace. *Kloeckera apiculata*, isolated from the natural biota of the pomace, was selected due to its affinity for apple pomace as a fermentation medium. Yeast growth, pH and moisture content were monitored. Yeast growth was determined as a function of yeast cell concentration and moisture content was calculated at the initiation and completion of the trials. All trials were conducted in duplicate.

To limit the number of yeast strains to be tested and thereby reduce the number of subsequent trials to a manageable level, an elimination criterion was required. A rather simple yet quick method of yeast selection was used which had its basis in the maximum cell concentrations reached on unsupplemented sterilised apple pomace.

Figure 3 illustrates the preferences of the selected yeasts for the apple pomace substrate. At 48 hours the plate counts showed that *Kl. apiculata* had the highest cell concentration followed by *C. utilis*, *Sacch. cerevisiae*, *Y. lipolytica* and lastly *Schiz. pombe*.

The fluctuations in pH for each yeast during the fermentations. *Kl. apiculata* growth demonstated the greatest decrease in pH while only *Y. lipolytica* growth was accompanied by a rise in pH.

Small rises in the moisture content of 2% and 3% were observed for *C. utilis* and *Kl. apiculata* respectively (Figure 3). Small reductions in moisture content of 2%

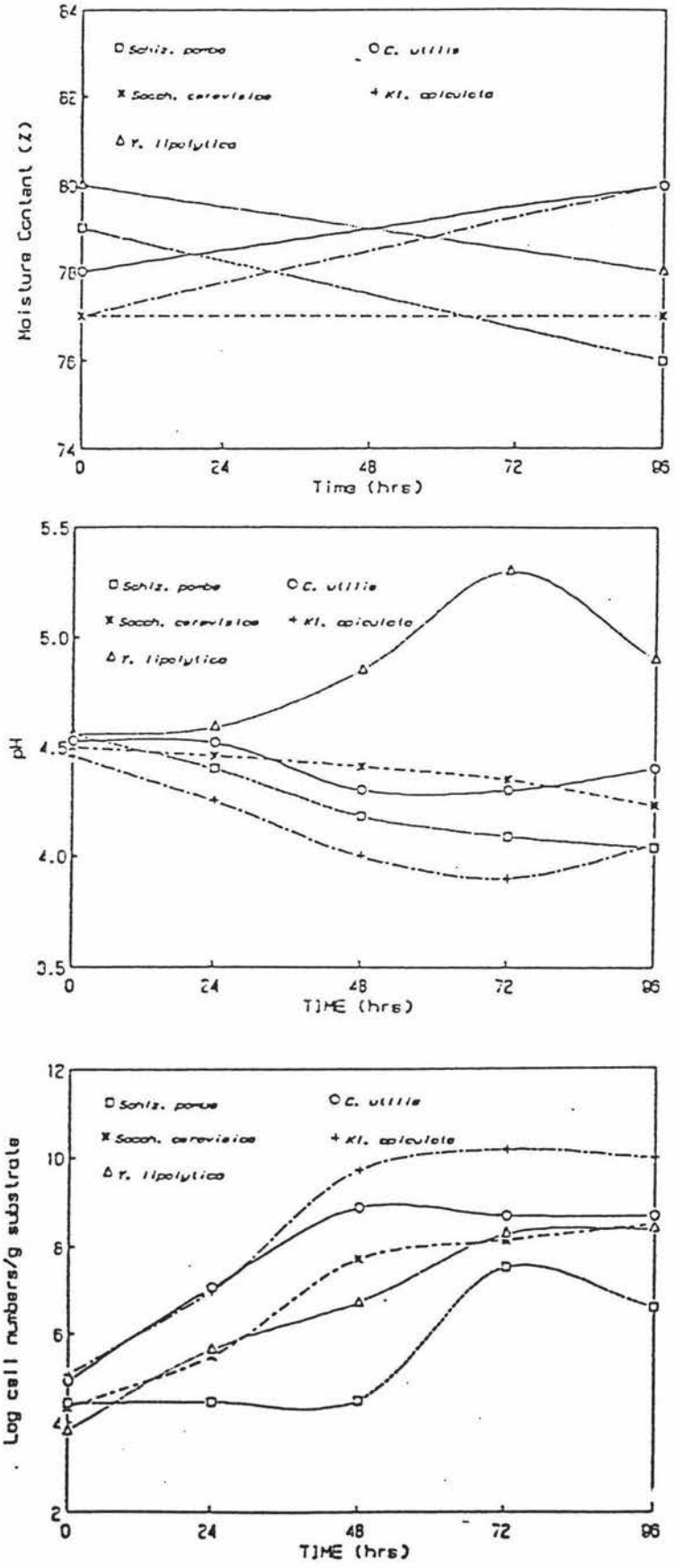


Figure 3. Changes in cell growth, pH and media moisture content during the growth of selected yeasts on sterilised apple pomace

and 3% occurred with *Y. lipolytica* and *Schiz. pombe* while the moisture content during *Sacch. cerevisiae* growth remained constant. However, as the initial moisture contents calculated varied from 76 - 80% it was not possible to determine whether these fluctuations were significant.

The three yeasts *C. utilis*, *Sacch. cerevisiae* and *Kl. apiculata* were chosen for further trials due to their rapid growth on the media and also for the natural diversity that exists between them. *C. utilis* and *Sacch. cerevisiae* are the yeasts traditionally used for the production of microbial proteins (Litchfield, 1980) although they differ with respect to their fermentative capabilities and their range of assimilable carbon sources. *Kl .apiculata* exhibited the greatest affinity for the medium and was also of special interest as no literature was found on its use in microbial protein production. The two remaining yeasts, *Y. lipolytica* and *Schiz. pombe*, were eliminated from further trials as they failed to demonstrate significantly better growth than the other three yeasts. However, this should not totally preclude further investigation into the use of these two yeasts as minor modifications of the pomace medium may have a major beneficial effect on their growth characteristics.

3.1.3 EFFECT OF APPLE POMACE MOISTURE CONTENT ON YEAST GROWTH

The moisture content of the apple pomace (Batch 1) received from the New Zealand Apple and Pear Marketing Board was approximately 80% by weight (Table 1). At this moisture level, pomace is a moist fibrous mass which is prone to forming a pastey material upon physical agitation or processing. After the first 24 hours of solid state fermentation the initial fibrous mass of pomace assumes a characteristically pastey consistency which mostly adheres to the sides of the glass fermentation jars, especially if the medium has been sterilised by autoclaving. Unsterilised pomace also adhered to the sides of the jars but tended to retain its fibrous nature. The remaining pomace conglomerated to form principally large (1 - 2 cm diameter) ball-shaped solids which tumbled freely during rotation of the fermentation vessels.

Trials were initiated to determine the effect of reducing the moisture content of the apple pomace on its consistency and on yeast growth. Apple pomace moisture levels of 80%, 75%, 70%, 65%, 55% and 45% were chosen for fermentation experiments using *C. utilis*, *Kl. apiculata* and *Sacch. cerevisiae*. Yeast growth, pH and moisture content were monitored. All trials were conducted in duplicate. Growth rates (μ) were calculated for each yeast over the first 24 hours of growth using the formula:

$$\mu\ (hr^{-1}) = \frac{\log N_2 - \log N_1}{\text{Time } N_2 - \text{Time } N_1} \times 2.303$$

1) *C. utilis*

The growth of *C. utilis* on apple pomace of 75% and 80% moisture followed nearly identical curves from 48 hours to 96 hours (Figure 4). At a moisture content of 80% a growth rate of $\mu = 0.19\ hr^{-1}$ was calculated for *C. utilis* propagation. Cell numbers at moisture contents of 65% and 70% showed similar values after 48 hours but with cell growth at 70% attaining a higher cell concentration at 96 hours. A lag phase period of growth was clearly observed for 65 - 75% moisture but not at 80% moisture when sampling at 24 hour intervals. At 55% moisture little cell growth was seen and at 45% moisture the growth curve declined until a cell concentration of 0 was obtained at 72 hours, indicating that the cell culture had died.

Reductions in pH were observed for cell growth at moisture levels ranging from 65 - 80% with the lowest pH achieved at 80% moisture (Figure 4).

For the media with initial moisture contents of 70%, 75% and 80% the moisture content of the media remained constant during the solid state fermentations (Figure 4). From 65% moisture down to 45% moisture a progressively greater drying effect was observed over the 72 hours of fermentation.

2) *Kl. apiculata*

Growth curves for *Kl.apiculata* propagation on apple pomace from 65% to 80% moisture were very similar (Figure 5). At 70, 75 and 80% moisture levels a growth

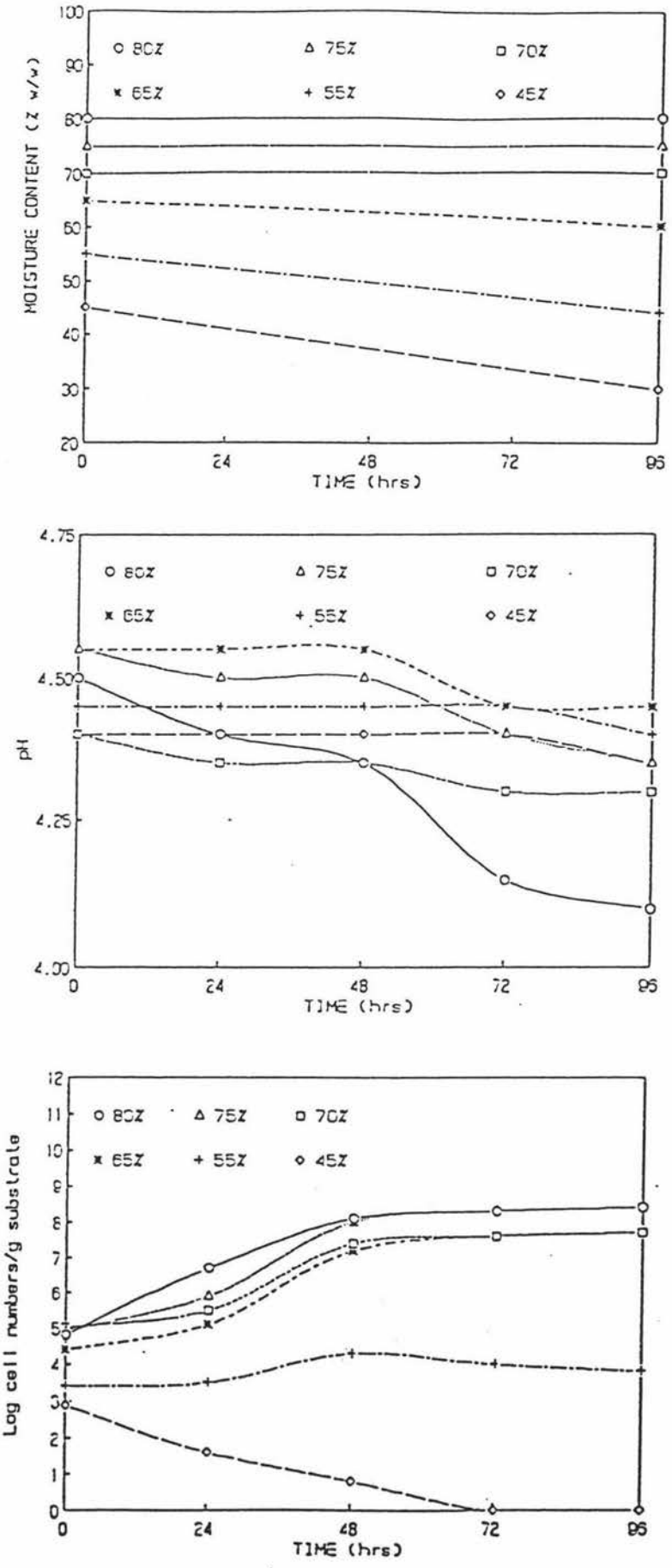


Figure 4. The effect of various pomace moisture contents on cell growth, pH and moisture content during the growth of *C. utilis*

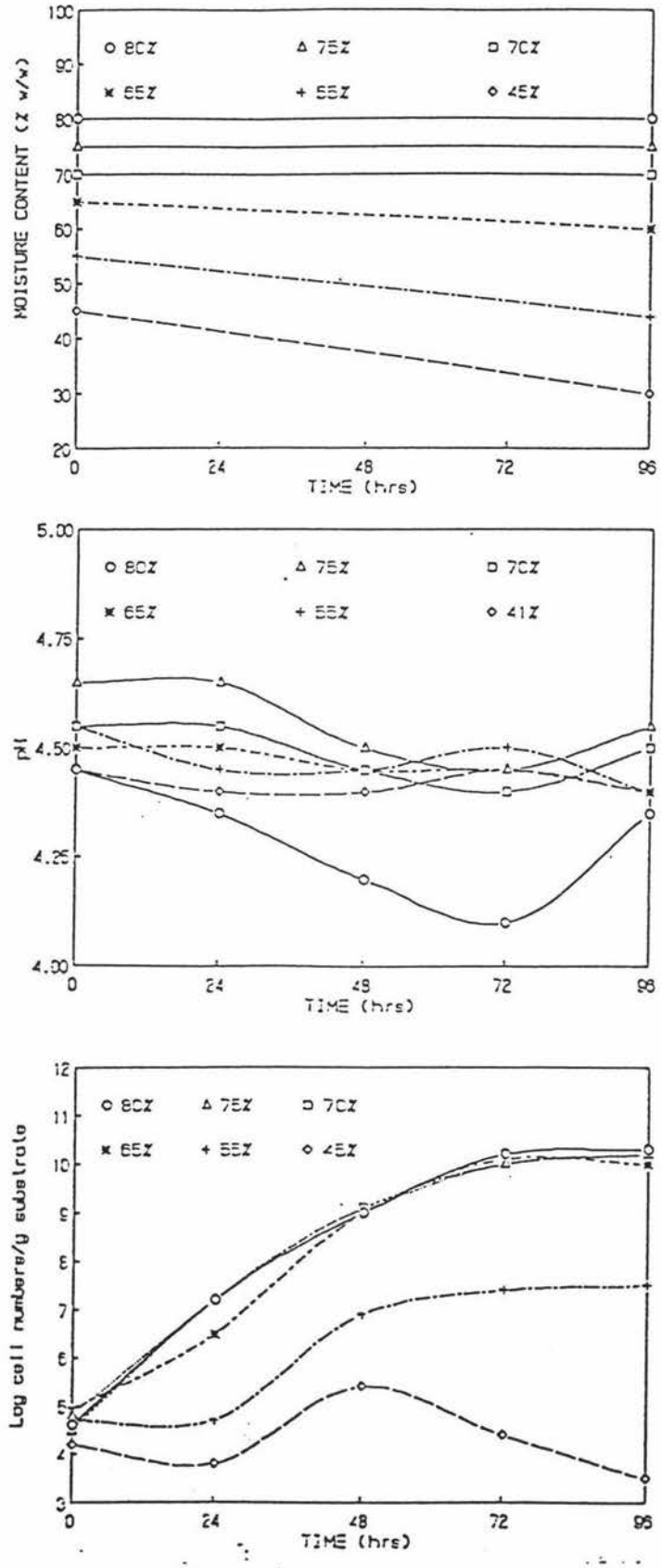


Figure 5. The effect of various pomace moisture contents on cell growth, pH and moisture content during the growth of *M. spiculata*

rate of $\mu = 0.25\ hr^{-1}$ was calculated for *Kl. apiculata*. However, cell concentrations at the 70 - 80% levels were still increasing after 96 hours whereas at 65% a maximum cell concentration was reached before 96 hours.

Monitoring the pH during cell growth revealed a gradual reduction in pH decline as the moisture content of the pomace was reduced (Figure 5). Cell growth at moisture levels of 70%, 75% and 80% showed an increase in the pH after 72 hours indicating the possible assimilation of organic acids during the latter part of the trials.

At the initial moisture contents of 70%, 75% and 80% no change in moisture content was detected after 72 hours of yeast propagation (Figure 5). From 65% moisture down to 45% moisture a progressively greater drying effect was observed.

3) *Sacch. cerevisiae*

Maximum cell concentrations were achieved at 72 hours with 65 - 80% moisture and thereafter decreasing (Figure 6). At 70, 75 and 80% moisture levels a growth rate of $\mu = 0.15\ hr^{-1}$ was calculated for *Sacch. cerevisiae*. At 55% moisture little cell growth was observed and at 45% cell concentration gradually decreased.

Only minor reductions in pH were observed during *Sacch. cereisiae* growth with the greatest decline occurring at 80% moisture (Figure 6).

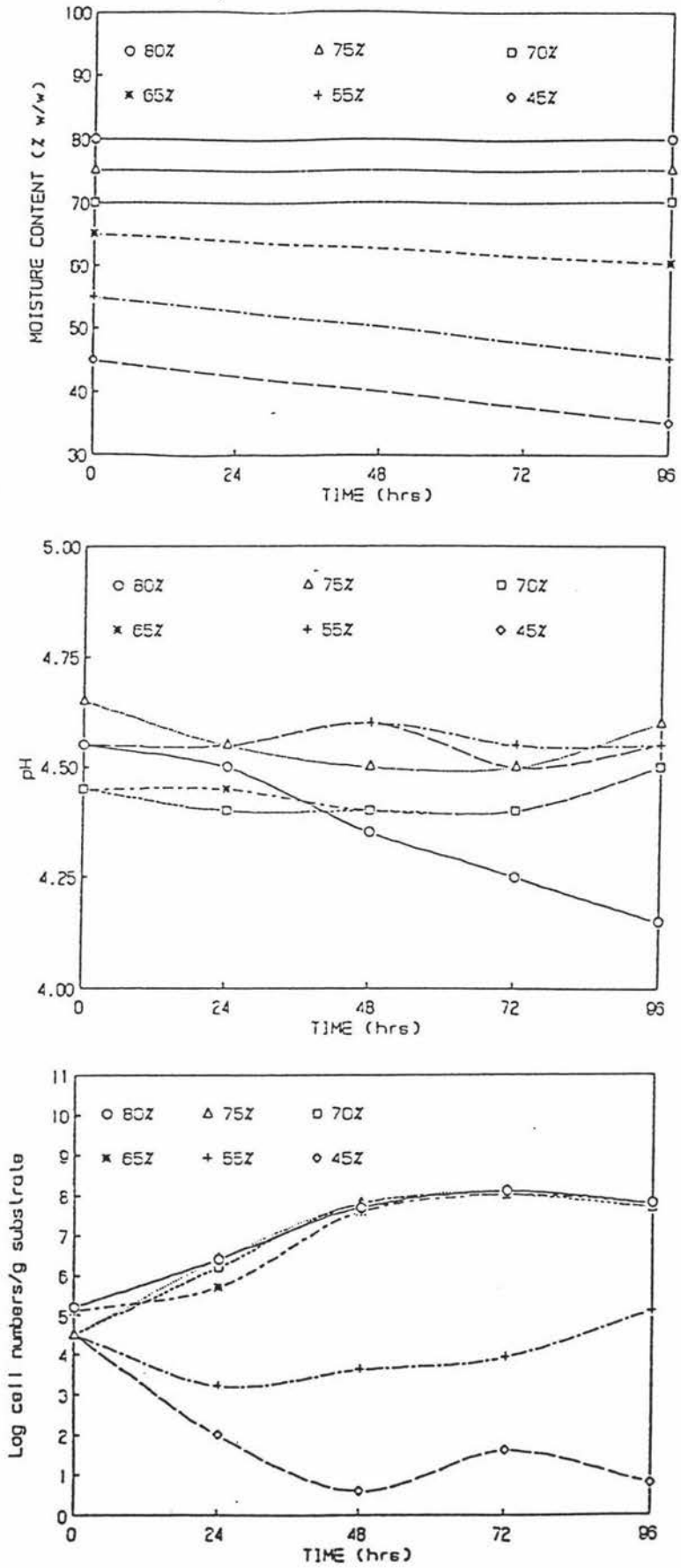


Figure 6. The effect of various pomace moisture contents on the growth, pH and moisture content during the growth of *Sacch. cerevisiae*

The changes in the media moisture contents during the growth of *Sacch. cerevisiae* (Figure 6) were very similar to those obtained with the growth of *C. utilis* and *Kl. apiculata* with more rapid loss of moisture occurring when drier pomace was used.

The consistency of the apple pomace was identical for each yeast at each initial moisture content. From 80% moisture to 70% moisture, the pomace medium in the fermentation vessels was observed to either adhere to the walls or conglomerate to form ball-shaped solids. From 65% moisture down to 45% moisture, progressively less pomace adhered to the walls of the vessels and the occurrence of the ball-shaped solids diminished until, at 45% moisture, the entire medium tumbled freely.

From these trials and in view of the the yeast's growth responses it was ascertained that future work should use a medium with an initial moisture content at or, preferably, above 65%. The yeasts *C. utilis* and *Kl. apiculata* were selected for further experiments due to the higher cell concentrations and growth rates they attained on the pomace media during these trials.

3.1.4 SUPPLEMENTATION WITH NITROGENOUS GROWTH SUBSTRATES

Trials conducted by Hours *et al.* (1985) and Hang (1988) on microbial biomass production from apple pomace indicated that nitrogen addition could significantly improve yeast growth. As a result, various nitrogenous sources were used in this research

to supplement the apple pomace (at 80% moisture) in solid state fermentations with *C. utilis* and *Kl. apiculata*. Urea, ammonium hydroxide and ammonium sulphate were included as nitrogenous supplements in the *Kl. apiculata* growth trials and urea, ammonium hydroxide, ammonium sulphate and ammonium nitrate were used with *C. utilis*. Urea, ammonium hydroxide and ammonium sulphate were selected arbitrarily while ammonium nitrate was chosen because of the ability *C. utilis* has to assimilate nitrate as a nitrogen source. Also included in the trials were apple pomace in which the pH was adjusted with sodium hydroxide as in previous experiments and apple pomace with no pH adjustment. No pH adjustment of the media to which nitogenous compounds were added was conducted other than that caused by the compounds themselves. The quantities of nitrogenous growth substrate added and the actual nitrogen (N) inclusion rate are summarised in Table 3. Yeast growth and pH were monitored and all the trials were conducted in duplicate.

1) *Kl. apiculata*

Kl.apiculata growth (Figure 7) responded best to the addition of ammonium hydroxide (μ = 0.36 hr^{-1}) . Little difference in cell growth rate was observed between the addition of urea, pomace pH adjusted with sodium hydroxide and pomace with no pH adjustment (μ = 0.32 hr^{-1}).

Monitoring of the pH revealed small reductions in pH with the addition of sodium hydroxide, ammonium hydroxide and ammonium sulphate (Figure 8). Only a

Table 3. Quantitiy of nitrogen added for each nitrogenous growth substrate.

Substrate	Nitrogenous Growth Substrate Inclusion Rate	Nitrogen Inclusion Rate (gN/100g)
2 N ammonium hydroxide	1% v/w	0.028
ammonium nitrate	1% w/w	0.35
ammonium sulphate	1% w/w	0.21
urea	1% w/w	0.47

minor decrease in pH was observed using the medium with no pH adjustment while the addition of urea resulted in a large pH increase, possibly signifying an active urease enzyme in this isolate.

2) *C. utilis*

C. utilis grew faster ($\mu = 0.36\ hr^{-1}$) on ammoniumn sulphate compared to the remaining nitrogenous compounds ($\mu = 0.21\ hr^{-1}$) which showed some improvement over the unsupplemented pomace (Figure 9) in terms of the final populations reached.

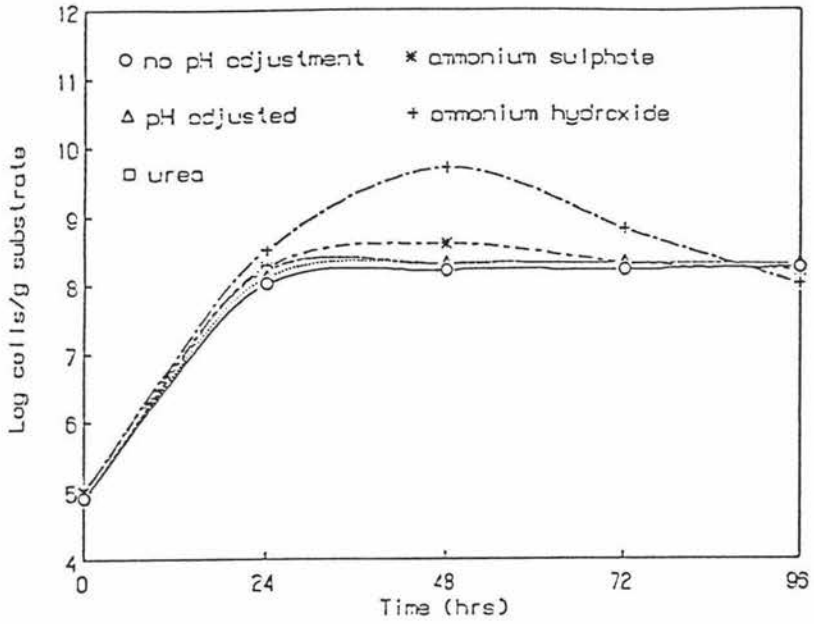


Figure 7. Growth of *Kl. apiculata* on pomace supplemented with various nitrogen sources.

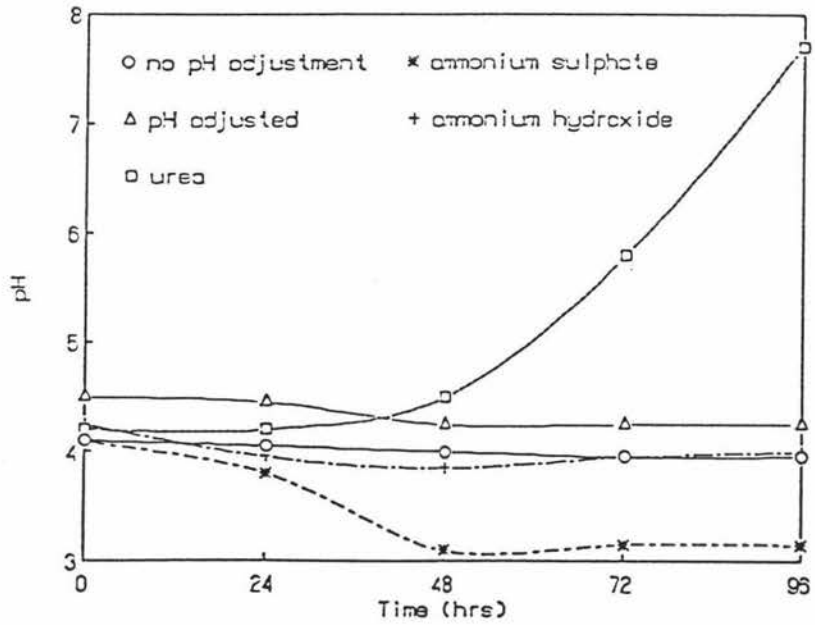


Figure 8. Changes in pH during the growth of *Kl. apiculata* on the supplemented media.

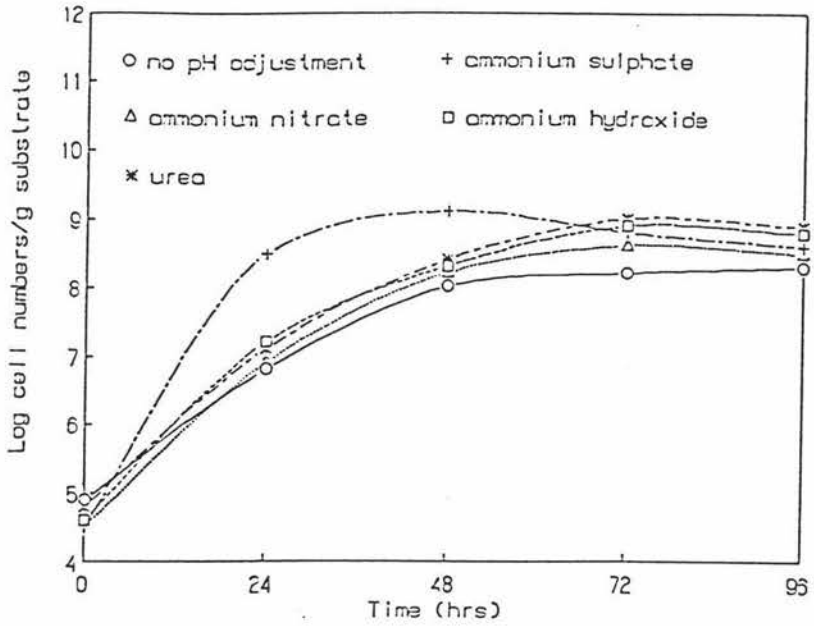


Figure 9. Growth of *C. utilis* on pomace supplemented with various nitrogen sources.

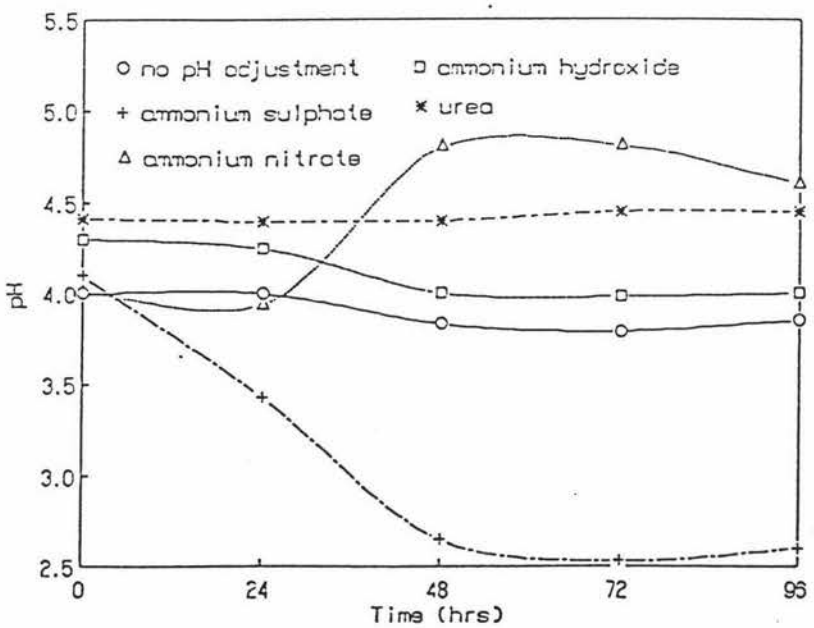


Figure 10. Changes in pH during the growth of *C. utilis* on the supplemented media.

Monitoring of the pH fluctuations showed a large pH decrease from pH 4.1 to pH 2.6 in the medium supplemented with ammonium sulphate (Figure 10). Small reductions in pH were observed in the unsupplemented medium and the medium supplemented with ammonium hydroxide. Growth on the urea supplemented medium showed no pH change while the addition of ammonium nitrate resulted in a pH increase of 0.85 pH units.

3.1.5 PHYSIOLOGICAL ASPECTS OF YEAST GROWTH ON APPLE POMACE

Further trials were conducted to analyse some of the physiological aspects of *Kloeckera apiculata* growth on supplemented apple pomace media. The view adopted was that assessment of these aspects should not only lead to a greater understanding of the growth of this yeast on the pomace media, but it should also give an indication of the nutritional improvements achieved through its propagation. In addition, the efficiency with which the yeast can convert the available substrates to microbial proteins can be ascertained. Cell concentration, pH, crude protein, soluble sugar fractions, ethanol and fatty acids were monitored during the yeast propagation trials. *Kl. apiculata* was grown on pomace supplemented with ammonium hydroxide. Two concentrations of ammonium hydroxide addition were used: one with 1% v/w 2 N ammonium hydroxide (0.028 g N/100 g) and the other with 1% v/w 7.8 N ammonium hydroxide (0.11 g N/100 g).

However, stocks of the apple pomace used in previous

trials had become depleted at this stage. Consequently a new batch (Batch 2) of pomace was obtained from the New Zealand Apple and Pear Marketing Board, Hastings for use in these solid state fermentation trials. This second batch had a visibly different complement of apple varieties to the first batch used. A comparison of the moisture content, pH, soluble sugar fraction and crude protein content are given in Table 4. The second batch was moister, more acidic and had a lower nutrient content than the first batch.

1) *Kl. apiculata*

Figure 11 (1% v/w 2 N ammonium hydroxide) and Figure 14 (1% v/w 7.8 N ammonium hydroxide) show the utilisation characteristics of the soluble glucose, fructose and sucrose fractions present in the pomace during *Kl. apiculata* growth on the two supplemented pomace substrate. The naturally low concentrations of soluble sucrose in the pomace remained constant during the yeast propagation. No preference was shown for the utilisation of fructose or glucose.

Figure 12 (1% v/w 2 N ammonium hydroxide) and Figure 15 (1% v/w 7.8 N ammonium hydroxide) illustrate the changes in apple pomace organic nitrogen content with respect to the utilisation of extractable non-protein nitrogen (NPN) and the total nitrogen content of the system for both supplemented media. The constant total nitrogen values during the course of the trials indicates that the fermentation is a closed system with respect to nitrogen.

Table 4. Comparison of the first and second batches of apple pomace

	Batch 1		Batch 2	
	% Dry Weight	% Wet Weight	% Dry Weight	% Wet Weight
Moisture Content:	-	78-80	-	88-90
Soluble Sugars:				
Glucose	17	3.4	22.7	2.5
Fructose	40	8.0	23.6	2.6
Sucrose	5	1.0	1.8	0.2
Total	62	12.4	48.1	5.3
Protein (N × 6.25):	5	1.0	2.7	0.41
pH		4.1		3.5

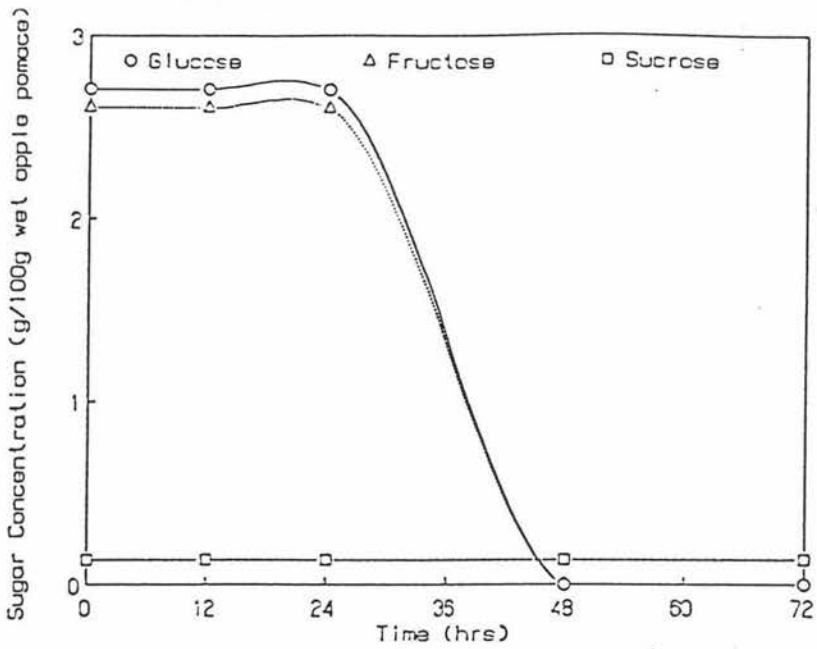


Figure 11. Utilisation of soluble sugars during *Kl. apiculata* growth on pomace supplemented with 1% v/w 2 N ammonium hydroxide.

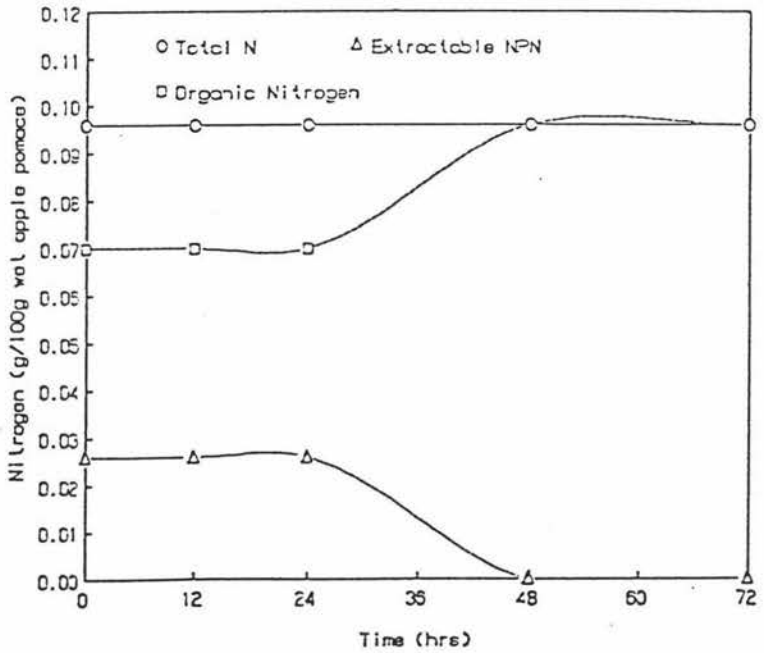


Figure 12. Changes in NPN and organic nitrogen during *Kl. apiculata* growth on pomace supplemented with 1% v/w 2 N ammonium hydroxide.

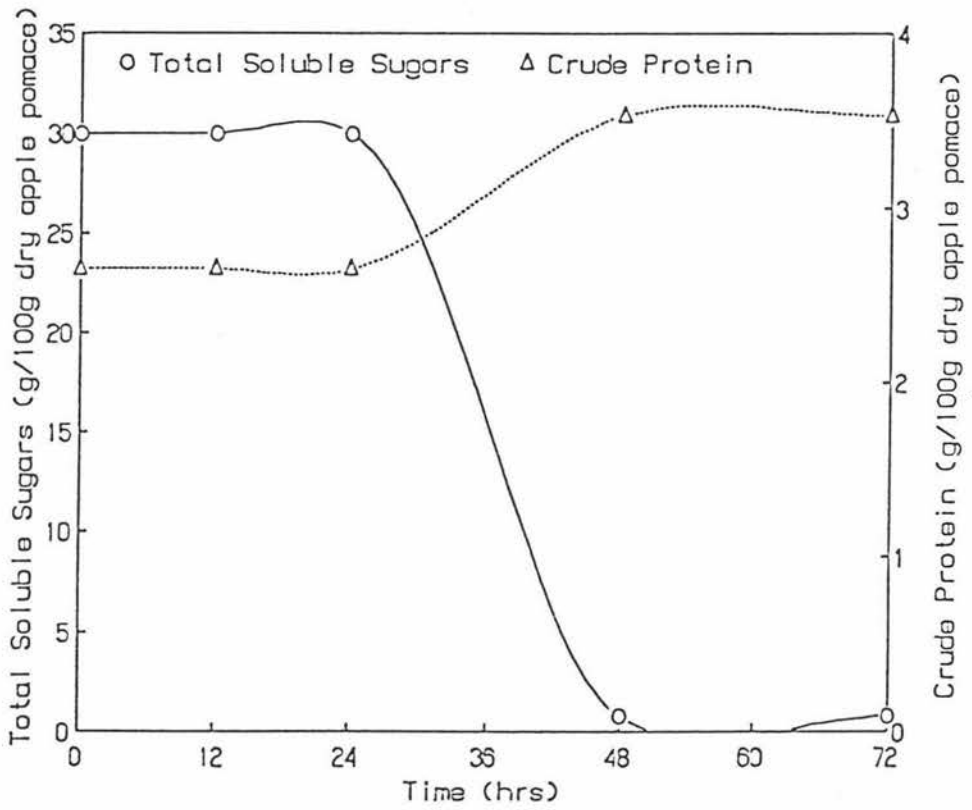


Figure 13. Crude protein production with respect to the utilisation of total soluble sugars during *Kl. apiculata* growth on pomace supplemented with 1% v/w 2 N ammonium hydroxide.

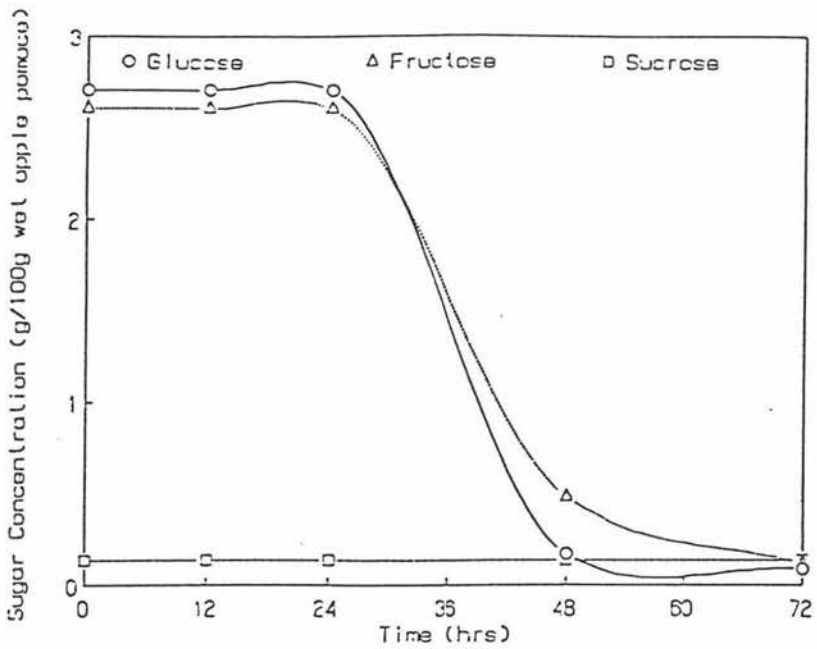


Figure 14. Utilisation of soluble sugars during *Kl. apiculata* growth on pomace supplemented with 2% v/w 7.8 N ammonium hydroxide.

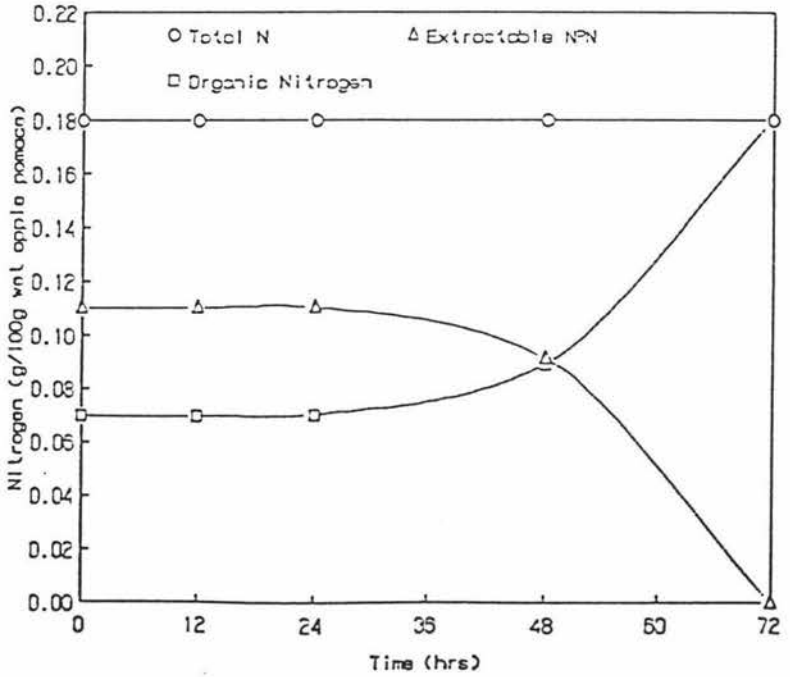


Figure 15. Changes in NPN and organic nitrogen during *Kl. apiculata* growth on pomace supplemented with 1% v/w 7.8 N ammonium hydroxide.

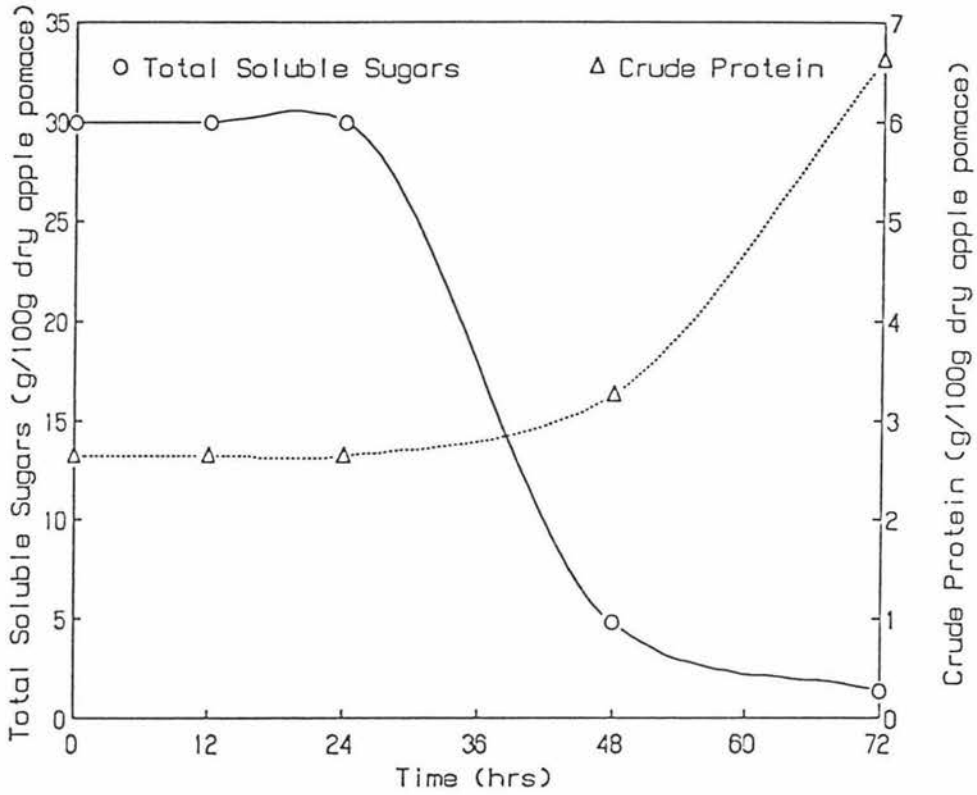


Figure 16. Crude protein production with respect to the utilisation of total soluble sugars during *Kl. apiculata* growth on pomace supplemented with 1% v/w 7.8 N ammonium hydroxide.

Figure 13 (1% v/w 2 N ammonium hydroxide) and Figure 16 (1% v/w 7.8 N ammonium hydroxide) show the increase in crude protein content of the fermenting samples (measured on a wet weight basis) with respect to the utilisation of total soluble sugars.

The maximum crude protein content achieved for *Kl. apiculata* growth on each medium was: 0.55% (measured on a wet weight basis) on a medium supplemented with 1% w/w 2 N ammonium hydroxide after 48 hours; and 1.06% crude protein on pomace supplemented with 1% v/w 7.8 N ammonium hydroxide after 72 hours, ie. increasing ammonium hydroxide addition increased the protein production and also increased the production time.

Figure A1 (1% v/w 2 N ammonium hydroxide) and Figure A2 (1% v/w 7.8 N ammonium hydroxide), both in the Appendix, illustrate the cell growth patterns and pH changes observed for the growth of *Kl. apiculata* on the second batch of pomace media supplemented with the two levels of ammonium hydroxide.

Only very minor concentrations (less than 0.2% w/w) of ethanol and acetic acid were monitored through to completion, and these were already present at the initiation of the growth trials. No propionic, n-butyric or iso-butyric acids were detected.

The yield coefficients for crude protein production (Y_{cps} - crude protein increase/sugars consumed), cellular protein production (Y_{ps} - cellular protein increase/sugars consumed) and biomass production (Y_{xs}

- biomass increase/sugars consumed) were determined for *Kl. apiculata* on its respective media (Table 5).

Crude protein yields were calculated from the dry weight data obtained by Kjeldahl analysis and non-protein nitrogen extraction, eg.

$$Y_{cps} = \frac{CP_2 - CP_1}{S_2 - S_1}$$ where CP = crude protein, S = substrate

The cellular protein yields were determined from the amino acid analysis of the final fermented product for *Kl. apiculata* growth on pomace supplemented with 1%v/w 7.8 N ammonium hydroxide. Biomass yields were calculated from the total cellular protein contents obtained during the amino acid analyses conducted in Section 3.3. These values allow a comparison of the efficiency with which the yeast converts the available substrates to crude protein, cellular protein or biomass.

The increases in crude protein (Figure 13 and Figure 16) were due to the formation of microbial proteins and non-protein nitrogen substances, such as nucleic acids. The final proportion of the total crude protein content attributable to yeast and pomace proteins was about 25:10 (yeast:pomace) for the yeast growth on pomace supplemented with 1% v/w 7.8 N ammonium hydroxide and approximately 13:10 for the yeast growth on pomace supplemented with 1% v/w 2 N ammonium hydroxide. However, there was probably some deamination and decarboxylation of the pomace proteins by the yeast during growth.

Table 5. Yield coefficients for crude protein (Y_{cps}), cellular protein (Y_{ps}) and biomass (Y_{xs}) production for *Kl. apiculata* growth on various supplemented pomaces.

Growth Medium	Y_{cps}	Y_{ps}	Y_{xs}
1% v/w 2 N ammonium hydroxide	0.03	NA	0.07
1% v/w 7.8 N ammonium hydroxide	0.12	0.15	0.28

NA - not available

3.2 AMINO ACID ANALYSIS

Amino acid profiles were generated for the yeasts *Saccharomyces cerevisiae* Y10, *Candida utilis* Y15 and *Kloeckera apiculata* (Table 6) and compared to that of *Torula* . The amino acid profile data for *Torula* was obtained from Callihan and Dunlap (1969). These amino acid profiles were generated to provide a comparison between the three yeast strains, and particularly the *Kl. apiculata* isolated from the apple pomace, with published data for a yeast commonly used for the production of microbial protein. As expected, the profiles were similar for all of the yeasts, although *Kl. apiculata* did have a considerably greater content of serine and lysine while *C. utilis* Y15 had a greater content of glycine.

An amino acid profile of the final, freeze-dried fermented product was also generated (Table 7). The sample used was taken from the fermentation of apple pomace supplemented with 1% v/w 7.8 N ammonium hydroxide using *Kl. apiculata* at the 72 hour time point. The total cellular protein, as calculated from the amino acid data, was 7.14% w/w, which was slightly higher than the total crude protein content of 6.9% w/w determined by Kjeldahl analysis. A comparison of the amino acid profile of the microbially modified pomace with some amino acids in barley and wheat was made (Table 7). The modified pomace was found to have a comparable glycine/serine and lysine content but was lower in all the remaining amino acids compared. The amino acid profiles for barley and wheat were obtained from Snowden (1984).

Table 6. Amino acid profiles of *Sacch. cerevisiae* Y10, *C. utilis* Y15 and *Kl. apiculata*.

Amino Acid	*Sacch. cerevisiae*	*C. utilis*	*Kl. apiculata*	*Torula*
Asp	4.81	4.16	4.72	NA
Thr	2.51	2.40	2.42	2.19
Ser	2.53	2.40	4.72	NA
Glu	5.68	6.89	6.25	NA
Pro	1.80	1.46	1.33	NA
Cys	0.10	0.10	0.10	NA
Gly	2.09	4.73	3.21	NA
Ala	2.72	1.42	1.80	NA
Val	2.69	2.99	2.61	2.41
Met	0.71	0.65	0.65	0.65
Ile	2.26	2.30	2.28	2.75
Leu	3.82	3.65	3.53	3.44
Tyr	1.79	1.80	1.57	1.85
Phe	2.08	2.10	1.95	2.19
His	1.17	0.96	0.98	0.95
Lys	4.01	3.79	4.73	3.66
Arg	2.67	2.04	2.49	NA
Total	43.44	43.84	45.34	NA

The values in Table 6 are expressed as a percentage of cell dry weight.

Table 7. Amino acid profile of freeze-dried, fermented apple pomace.

Amino Acid	Fermented Apple Pomace (% w/w)	Barley (% w/w)	Wheat (% w/w)
Asp	0.72	NA	NA
Thr	0.38	0.48	0.43
Ser	0.39	NA	NA
Gly	0.87	NA	NA
GlySer	1.26	1.15	1.31
Glu	0.87	NA	NA
Pro	0.28	NA	NA
Ala	0.42	NA	NA
Cys	0.08	NA	NA
Met	0.11	NA	NA
MetCys	0.19	0.37	0.42
Val	0.41	0.64	0.62
Ile	0.38	0.44	0.46
Leu	0.59	0.69	0.92
Tyr	0.32	NA	NA
Phe	0.32	NA	NA
TyrPhe	0.64	1.12	1.04
Lys	0.51	0.52	0.44
His	0.17	0.27	0.33
Arg	0.32	0.59	0.70
Total	7.14	10.0	9.0

3.3 ISOLATION AND CHARACTERISATION OF AN UNKNOWN YEAST FROM APPLE POMACE

During the yeast growth trials (Section 3.1.1) a yeast from the natural microflora of the apple pomace was observed to have a particular affinity for the apple pomace media. This yeast was isolated as a pure culture by repetitive streaking on ME agar plates.

Characterisation of the yeast was achieved principally by the methods used in Kreger-van Rij (1984) and Lodder (1970).

1) Morphological Characteristics

Growth in yeast extract-malt extract broth: After 3 days at 25°C the single cells were apiculate and round to oval, (1.5 - 3.0) × (2.5 - 6.0) µm (Figure 19). Asexual reproduction was by bipolar budding. A sediment was present.

Growth on yeast extract-malt extract agar: After 1 month at 30°C the isolated colonies were white, smooth, glossy and with a raised centre.-

Growth on Dalmau Plate and Rivalier and Seydal Slide: True and pseudomycelia were absent. Both aerobic and anaerobic growth occurred.

Growth on sporulation media: Sporulation was absent after 7 days, 14 days and 1 month of growth, at 30°C, on a carrot wedge, gypsum block, Wickerham's malt

extract agar or Mc Clary's acetate agar.

2) Physiological Characteristics

Data for the standard *Kl. apiculata* yeast was obtained from Kreger-van Rij (1984).

Table 8 presents results of fermentation tests and it can be seen that glucose was the only substrate fermented.

Table 8. Fermentation Tests.

Compound	Unknown Yeast	*Kl. apiculata* Standard Description
Glucose	+	+
Maltose	-	-
Sucrose	-	-
Lactose	-	-
Galactose	-	-
Raffinose	-	-

Table 9 lists the data obtained from nitrogenous compound assimilation tests and shows that only ethylamine-hydrogen chloride was utilised.

Table 9. Assimilation of Nitrogenous Compounds.

Compound	Unknown Yeast	*Kl. apiculata* Standard Description
Potassium nitrate	-	-
Potassium nitrite	-	NA
Ethylamine-hydrogen chloride	+	NA

NA = not available

The results arising from the carbon assimialtion trials (Table 10) were identical to those available in literature for *Kl. apiculata*.

Table 10. Assimilation of Carbon Compounds.

Compound	Unknown Yeast	*Kl. apiculata* Standard Description
D-glucose	+	+
D-galactose	-	-
Arbutin	+	+
Sucrose	-	-
Maltose	-	-
Cellobiose	+	+
Trehalose	-	-
Lactose	-	-
Raffinose	-	-
Inulin	-	-
Starch	-	-
D-xylose	-	-
L-rhamnose	-	-
D-arabinose	-	-
D-ribose	-	-
Glycerol	-	-
D-mannitol	-	-
Citric acid	-	-

Table 11 shows that the growth of the unknown yeast on 50% (w/w) glucose-yeast extract agar was the same as data published for *Kl. apiculata*. While no comparative published data was found for the remaining growth trials these may add valuable information to the body of existing knowledge concerning the unknown yeast.

Table 11. Other Growth Characteristics.

Growth Conditions	Unknown Yeast	*Kl. apiculata* Standard Description
50% (w/w) glucose-yeast extract agar	+	+
Vitamin-free medium	-	NA
Urease	+	NA
Actidione agar	+	NA
34°C	+	NA
37°C	-	NA

NA = not available

From the characterisation studies conducted, the yeast isolated from the natural microflora of the apple pomace was identified as *Kloeckera apiculata*. The unknown yeast was found to fit the standard description of *Kloeckera apiculata* as found in Kreger-van Rij (1984).

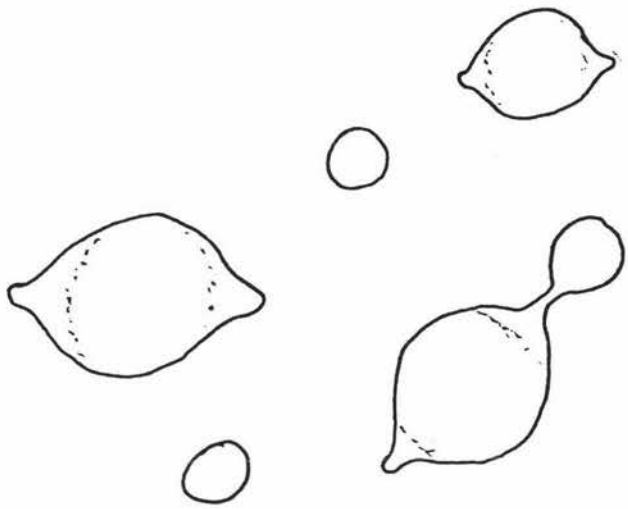


Figure 17. Unknown yeast after 3 days incubation at 25°C in yeast extract-malt extract broth.

CHAPTER 5

DISCUSSION

1) Yeast Growth on Unsterilised Apple Pomace

Trials on the effects of unsterilised media on yeast growth demonstrated that potential difficulties may be encountered particularly if one moves to larger scale operations. Neither *Candida utilis* Y15 nor *Yarrowia lipolytica* IFO1659 were capable of maintaining a dominant growth over the natural microflora of the apple pomace at the inoculum rate and under the growth conditions employed. A yeast from the pomace microflora, isolated and characterised as *Kloeckera apiculata*, was found to supplant the growth of both introduced yeasts.

These results contrast markedly with those obtained by Hours *et al* (1985) where growth of the yeast inoculum was apparently unaffected by the natural microflora. This may have been due to the larger inoculum, the growth conditions or a geographical difference in the types of microflora present on the apple pomace used. However, Hours *et al* (1985) did find that the normal flora in the apple pomace strongly competed with the growth of the fungus *Trichoderma reesei* resulting in a decreased yield. Growth trials conducted by Hang (1988) on unsterilised apple pomace suggested that the *C. utilis* inoculum was unaffected by the natural microflora. However, it is unclear whether the

diversity of the microflora was monitored during yeast propagation in the trials conducted by both Hours *et al.* (1985) and Hang (1988). If not, it is quite possible that the microbial population of the final fermented product may not have been solely due to the inoculum.

2) Growth of Selected Yeasts on Apple Pomace

Kloeckera apiculata demonstrated a considerably greater affinity for the apple pomace medium compared to the other selected yeasts. The growth of *Schizosaccharomyces pombe* H115 was marked by a long lag phase followed by a brief increase then decline in yeast cell concentration. *Candida utilis* Y15, *Saccharomyces cerevisiae* Y10 and *Yarrowia lipolytica* IFO1659 all demonstrated very similar growth patterns.

The growth of all the selected yeasts, except *Y. lipolytica*, was accompanied by a decline in pH. The increase in pH during the growth of *Y. lipolytica* was most likely due to the assimilation of citric acid, a characteristic of *Y. lipolytica* (Kreger-van Rij, 1984).

It would appear that this is the first comparison of yeast growth on unsupplemented apple pomace under solid state fermentation conditions as no comparable information was found during the literature research for this thesis.

3) Effect of Apple Pomace Moisture Content on Yeast Growth

One major reason for the greater biomass yields and growth rates obtained under submerged fermentation conditions, compared to solid state fermentation conditions, is the greater moisture content. A lower moisture level results in greater difficulties in extracting the water necessary for cell growth from the fermentation medium (Brock, 1979). In the case of an apple pomace medium, the availability of water would be predominantly affected by the adsorption of the water to the pomace matrix, although the solute content of the available water may also have some bearing. The effect of apple pomace moisture content on yeast growth was addressed in this study.

The propagation of all three yeasts was not adversely affected until the pomace moisture content was reduced below 65%. Only *Kl. apiculata* grew at a moisture content of 55% and no yeast growth was observed at a moisture content of 45%.

As far as can be ascertained this is the only work to have been conducted on the effects of moisture content on yeast growth in solid state fermentations, although similar results were obtained by Han *et al* (1978) for the growth of a bacterial mixed culture on annual ryegrass straw of different moisture contents.

A common method of improving yeast growth is by increasing the oxygen transfer rate. The limiting step in oxygen transfer is usually intraparticle diffusion

provided that oxygen is freely accessible through interparticle canalisation (Laukevics *et al.*, 1984). The oxygen access is achieved most frequently by periodic mixing. However, this would probably further decrease the porosity of the apple pomace substrate which, at 80% moisture, already tends to assume a pastey form. By reducing the moisture content of the apple pomace, it was found to maintain its characteristic particulate form during the growth trials. This particulate form would be conducive to improved aeration efficiency and probably explains the continued growth of the three yeasts down to a moisture content of 65%.

An alternative approach to increasing the porosity of the medium is the addition of a bulking agent. However, introduction of a bulking agent, such as straw, would be accompanied by a reduction in the concentration of nutrients. With respect to the soluble sugars, this may not necessarily have a detrimental effect upon microbial protein production. Raw apple pomace (Batch 1) has a high soluble sugar concentration of approximately 12%, which may result in some catabolic repression or respiratory inhibition of yeast growth. Any decrease in the soluble sugars could be compensated for by the reduced respiratory inhibition or catabolic repression and by the increased oxygen transfer resulting from the improved porosity of the medium.

4) Supplementation with Nitrogenous Growth Substrates

The growth of *Kl. apiculata* on an apple pomace medium responded best to the addition of ammonium hydroxide. Only minor differences were monitored between yeast growth on pomace supplemented with ammonium sulphate, urea, pomace pH adjusted with sodium hydroxide and pomace with no pH adjustment or supplementation.

The relative effectiveness of the nitrogenous growth substrates was unexpected when the amounts of supplemented nitrogen were considered (Table 3). Although the greatest improvement in the propagation of *Kl. apiculata* was achieved with ammonium hydroxide supplementation, this was in fact accomplished at the lowest nitrogen inclusion rate. This would tend to suggest that the growth of *Kl. apiculata* on an apple pomace medium suffers from only a minor nitrogen limitation.

It was initially suspected that *Kl. apiculata* may lack the enzymes necessary to obtain assimilable nitrogen from urea, but this was not indicated by the changes monitored for pH. The large increase in pH observed with the urea addition suggested that *Kl. apiculata* was able to generate the enzymes required for the reduction of urea to ammonia but that not all the ammonia was assimilated. Literature research indicated that most *Kloeckera* species, including *Kl. lindneri*, lacked the urea amidohydrolyase (E.C. 3.5.1.5) enzyme (Barnett *et al.*, 1983), although no information on urea amidohydrolyase activity specific to *Kl. apiculata* was found. *Kl. lindneri* was considered a

synonym of *Kl. apiculata* by Phaff (1970), but some contention over the validity of this claim exists (Meyer *et al*, 1978). To determine if this *Kl. apiculata* strain did possess the urease enzyme it was propagated on urea agar (Section 3.3). That the test was negative suggests the possibility that flora indigenous to the apple pomace may be responsible for the reduction of urea with the released ammonia becoming available to the yeast.

The largest reduction in pH was monitored for yeast growth on an ammonium sulphate supplemented medium. As this pH decline was not accompanied by the largest increase in cell concentration, it may be inferred that the concomitant addition of sulphate was responsible. The reduction of the pH of the medium to 3 after 48 hours may have also resulted in adverse growth conditions for the propagation of *Kl. apiculata*.

2) *C. utilis*

C. utilis growth benefited most from the addition of ammonium sulphate. While the maximum cell concentration achieved with ammonium sulphate supplementation was similar to those obtained with urea and ammonium hydroxide, the time taken to reach this value was reduced by 24 hours. The addition of ammonium nitrate led to a smaller improvement in yeast growth when compared to the unsupplemented medium.

The propagation of *C. utilis* on a pomace medium supplemented with ammonium sulphate produced the greatest reduction in pH, as it did with *Kl. apiculata* growth. This large decrease in pH may have been due to the improved growth or the result of sulphate addition, as postulated for *Kl. apiculata*. Whether the growth of *C. utilis* was affected at this low pH (pH 2.6) requires investigation. Although yeasts are generally considered to be acidophiles very low pH values can be inhibitory and even toxic (Brock, 1979). By maintaining a constant pH with the controlled addition of a base, the inhibitory effects could be eliminated and the yeast growth consequently improved. Control of the pH can also be utilised as a means of nitrogen supplementation, by using an ammonium hydroxide solution for the maintenance. Research by Hang (1988) demonstrated that the 1% w/w ammonium sulphate supplementation (used also in these trials) was the optimum for the solid state fermentation of apple pomace with *C. utilis*.

An increase in pH was monitored for the growth of *C. utilis* on the ammonium nitrate supplemented medium. This increase implied that the yeast initially assimilated the nitrate as its nitrogen source with the consequent release of the ammonium ions responsible for the pH change. Between 48 hours and 72 hours the pH began to decline indicating a transfer from nitrate assimilation to the assimilation of the dissociated ammonium ions. What benefits were gained from the preferential uptake of nitrate was not clear as this medium was the least effective at improving yeast growth. A speculative reason may be that a

certain concentration of dissociated nitrate ions, which would be released if the ammonium ions were assimilated first, is toxic to the yeast, hence their immediate removal.

No change in pH was detected for *C. utilis* growth on the medium supplemented with urea. *C. utilis* possesses the enzyme urea amidohydrolyase (E.C. 3.5.1.5) necessary for the reduction of urea to ammonium ions (Metzler, 1977). The rate of urea reduction may be controlled such that it is equivalent to the rate of nitrogen assimilation.

The reasons for ammonium sulphate being the preferred nitrogenous growth substrate are varied. The nitrogen inclusion rate resulting from ammonium hydroxide addition is considerably less than the other nitrogen sources and the assimilation of nitrate, from ammonium nitrate, is less effective than ammonium at improving cell growth. With respect to the addition of urea, it is probable that some time is necessary to generate the enzyme pool required for the efficient utilisation of the urea.

As far as can be ascertained, no trials on the effect of various nitrogen sources on yeast propagation under solid state fermentation conditions have been conducted. Hang (1988) used an apple pomace medium supplemented with various amounts of ammonium sulphate to propagate *C. utilis* under solid state conditions. The maximum protein and vitamin increase was achieved with a 1% w/w ammonium sulphate supplementation. Gupta *et al* (1989) studied the addition of various

nitrogenous sources on the production of alcohol from apple pomace by two species of *Saccharomyces*. Although a different end-product was desired, it was found that ammonium phosphate was responsible for the greatest response followed by ammonium sulphate. Gupta *et al.* (1989) suggested that phosphate and sulphate ions, as well as nitrogen, may be stimulatory to the yeasts.

5) Physiological Aspects of Yeast Growth on Apple Pomace

The data contained in Table 4 demonstrates the increase in crude protein yields, cellular protein yields and biomass yields for *Kl. apiculata* growth with increasing rates of ammonium hydroxide addition. However, the time taken to obtain the maximum crude protein content also increased. More research would be required to determine the optimum rate of supplementation as further increases in the protein yield and final protein content and a reduction in the production time may be possible. For any commercial operation of this nature a reduction in processing time would increase its cost effectiveness.

The fact that no ethanol was produced during *Kl. apiculata* propagation on the unsupplemented and supplemented pomace media indicates that the growth was due solely to aerobic respiration. This was rather suprising as it was initially suspected that a possible catabolic repressive or respiratory inhibitive effect may still have been associated with the fairly high soluble sugar concentrations and the pastey consistency of the second batch of apple

pomace, resulting in predominantly anaerobic growth. The absence of low molecular weight fatty acid production was beneficial as it indicates that the metabolism is being directed to other, possibly more profitable, end products. While these volatile fatty acids are an energy source, they are of no use in a fermentation product that would most likely require drying to obtain the final feed supplement.

The protein yield obtained in this trial (Y_{ps} = 0.15) was considerably lower than that achieved by Hours *et al.* (1985) with a submerged fermentation of apple pomace using *Saccharomycopsis* (now *Yarrowia*) *lipolytica* (Y_{ps} = 0.45). The final protein content achieved in this research, measured on a dry weight basis, was also considerably lower; 7.1% compared to the 13% obtained by Hours *et al* (1985). In addition, this value of 7.1% protein was considerably lower than the 15% crude protein obtain by Hang (1988) using *C. utilis* in a solid state fermentation of apple pomace supplemented with 1% w/w ammonium sulphate. However, to put the protein content achieved in this research into perspective, a comparison with results obtained by Rahmat (personal communication) was beneficial. Rahmat (1990-91) conducted a solid state fermentation of apple pomace supplemented with 1% w/w ammonium sulphate using the yeast *C. utilis* Y15, replicating as closely as possible the experiment conducted by Hang (1988). This trial yielded a crude protein content, on a dry weight basis, of 7.5%. That this crude protein figure is also relatively low compared to the results obtained by Hang (1988) suggests that the apple pomaces used differed substantially. The apple pomace

used by Rahmat was from the same second batch (batch 2) used towards the end of the research conducted for this thesis. As can be seen from Table 4 the seasonal variations in the apple pomace due to differing apple varieties and growing conditions are quite large. This may partly account for the low crude protein levels achieved in this research and by Rahmat (1990-91). The fermentation conditions used in this research, and their control, were very similar to the conditions used by Hang (1988) except for the temperature which was not maintained at a constant 25°C.

If the seasonal variations in apple pomace were largely responsible for the reduced crude protein content, one must question the ability and feasibility of producing a consistent fermented feed product during the apple processing season. Should this prove to be impracticable the value of a fermented, pomace feed product would be reduced. More data would be required on the fermentability of and yields from apples collected at different times throughout the season. The data collected in this research may represent the minimum values that might be achieved.

6) Nutritional Evaluation

With the New Zealand livestock industry being based primarily upon pastoral farming practices, an apple pomace microbial biomass product would be limited in its application as a stock feed supplement. It is unlikely that a microbial biomass product would be able to command a reasonable return as a cattle or

sheep feed at present even though it is probably more suitable for ruminants due to its high fibre content of approximately 15%, measured on a dry weight basis (Snowden, 1984). As such, its greatest markets would probably be found with non-ruminant animals that have a formulated feed diet, especially pigs and poultry. Research conducted by Snowden (1984) suggested that an apple pomace feed product would command a higher return if used in the pork industry. Although research on the characteristics and quality of a dried apple pomace feed with pigs has been conducted (Bowden and Berry, 1959) an investigation of the nutritional value of the fermented pomace as a pig feed is still required. None-the-less, the potential may exist for this material's development as an exportable product.

Assuming a final, dried feed product of 10% moisture, generated from the solid state fermentation of apple pomace supplemented with 1% v/w 7.8 N ammonium hydroxide and using the yeast *Kl. apiculata*, a comparison with the constraints placed on diet designs for growing pigs and those for breeding pigs (Snowden, 1988) can be made with respect to the amino acid contents. From these assumptions, and a protein content of 6.5% determined for a final, dried feed product of 10% moisture, Table 10 was constructed. This table suggests a deficiency in all amino acids except glycine and serine for both growing pig and breeding pig diets, and leucine and lysine for breeding pig diets only, although tryptophan values were not available (NA) for the feed product. Methionine is usually the first limiting amino acid and its low content is typical for many microbial

Table 10. Comparison of the estimated amino acid profile of a microbially modified apple pomace feed product with those recommended (Snowden, 1984) for growing pig and breeding pig diets.

Amino Acid	Pomace Feed Product (% w/w)	Growing Pig Constraints (% w/w)	Breeding Pig Constraints (% w/w)
Asp	0.65	NA	NA
Thr	0.34	> 0.56	> 0.4
Ser	0.35		
Gly	0.78		
GlySer	1.13	> 1.0	> 1.0
Glu	0.79	NA	NA
Pro	0.26	NA	NA
Ala	0.38	NA	NA
Val	0.37	> 0.6	> 0.43
Cys	0.07		
Met	0.13	> 0.3	> 0.3
MetCys	0.20	> 0.5	> 0.4
Ile	0.34	> 0.5	> 0.45
Leu	0.53	> 0.9	> 0.24
Tyr	0.29		
Phe	0.29		
TyrPhe	0.58	> 1.0	> 1.0
His	0.15	> 0.36	> 0.24
Lys	0.46	> 0.9	> 0.5
Arg	0.29	> 0.5	> 0.5
Try	NA	> 0.18	> 0.18

protein products (Schacklady and Gatumel, 1973). However, the ultimate measure of the nutritive value of this fermented apple pomace feed product would be its performance in animal feeding studies.

However, it is quite possible that the fibre content of the final feed product will be the limiting factor with respect to its inclusion rate in pig diets.

The fibre content of the apple pomace will be unaffected by solid state fermentation using non-cellulolytic yeasts, but its concentration will probably increase due to inefficiencies in the bioconversion of soluble sugars to microbial proteins. In this study, the soluble sugars accounted for 30% of the pre-fermentation pomace (on a dry weight basis) and the biomass increase accounted for 16% of the post-fermentation pomace. This leaves 14% of unaccounted for fermentation products. Many of these products will be volatile, such as ethanol, esters and volatile fatty acids, or gaseous, such as carbon dioxide, and will be lost from the system during yeast growth or during later processing. As a result, the fibre concentration of apple pomace will increase. With the recommended fibre content on growing pig and breeding pig diets being < 4% (Snowden, 1984) it is possible that the solid state fermented apple pomace product may in fact necessitate its inclusion in reduced amounts as compared to unprocessed dried pomace. As such, a microbially modified pomace feed supplement may be more suitable for incorporation into

a feed for ruminants, although this market would probably be limited in New Zealand for the reasons previously stated.

7) Summarisation

As has been mentioned, a reduction in the time required to obtain the maximum crude protein content is of major benefit if a process of this nature was to be commercialised. Various methods could be employed to further reduce the propagation times and increase protein yields.

Ideally, for the propagation of microbial cells, the yeast inoculum should be prepared on apple pomace supplemented as in the growth medium. This allows the yeast population to generate the enzyme pool necessary for the immediate and efficient utilisation of the substrate prior to inoculation. The subsequent reduction or elimination of the lag phase period of growth would decrease the microbial protein production time. An increased inoculum size may also have a beneficial effect upon yeast growth and negate the need to sterilise the medium.

The important fermentation parameter temperature was not investigated during the course of this research due to time limitations and the unavailability of facilities required for the precise control of fermentation temperatures. All the fermentation trials were conducted in a large pilot plant room with an inefficient temperature control. As a result,

fermentation temperatures varied from 18 - 22°C in winter to 22 - 26°C in summer. These laboratory scale experiments were probably conducted at temperatures below the optimum for maximum yeast growth, especially during the winter season. Further research into the optimisation of the temperatures required for maximum microbial protein production would be beneficial. However, in a large scale solid state fermentation of this nature the greatest temperature associated problem may actually be the removal of the heat generated during the exothermic cell growth (Aidoo *et al.*, 1982).

While a microbial biomass product formed from apple pomace may be used directly as a feed supplement, the high moisture content would make storage impractical as microbial degradation would occur. As a result, supply of the pomace feed supplement would be limited to the juice processing season, which generally runs from February to July (Marks, personal commuication). The distribution area would also be confined to local demands due to the bulky nature of the feed. To improve the storage characteristics of the fermented pomace, drying of the feed to produce a microbially stable product would be required. Not only would this improve storage life but it would also decrease its bulk, reduce transportation costs and increase the area of distribution. As drying is an energy intensive and, therefore, a costly operation research would be necessary to determine the maximum moisture content at which the feed product is microbially stable.

If the fibre content of the fermented pomace is, as speculated, considerably higher than the constraints placed on pig diets, it may be possible to reduce the amounts by using a mixed culture of a cellulolytic fungus and a non-cellulolytic yeast. Mixed cultures have been used previously in the solid state fermentation of cellulosic materials such as ryegrass straw (Han *et al*,. 1976) and wheat straw (Laukevics *et al.*, 1984; Viesturs *et al.*, 19). A mixed culture of *Trichoderma reesei* and *Yarrowia lipolytica* has been used for the submerged batch and fed-batch fermentation of apple pomace (Hours *et al.*, 1985). It is possible that a mixed culture, such as the latter example, may reduce the fibre content of the apple pomace during solid state fermentation while maintaining or improving crude protein production and production time.

CHAPTER 5

CONCLUSIONS

1 Yeast growth on sterilised apple pomace media demonstrated that, at the inoculation rate and under the conditions employed, the growth of *Candida utilis* Y15 and *Yarrowia lipolytica* IFO1659 was supplanted by a yeast from the natural microflora. This yeast was isolated and identified as *Kloeckera apiculata*.

2 Of the yeasts selected, *Kloeckera apiculata* demonstrated the greatest affinity for the apple pomace medium. *Candida utilis* Y15, *Saccharomyces cerevisiae* Y10 and *Yarrowia lipolytica* IFO1659 all exhibited similar growth patterns. The growth of *Schizosaccharomyces pombe* H115 on the apple pomace medium was poor.

3 The growth of *C. utilis*, *Kl. apiculata* and *Sacch. cerevisiae* was unaffected by reductions in apple pomace moisture content down to 65%.

4 Of the nitrogenous growth substrates studied, the growth of *Kl. apiculata* benefited most from the addition of ammonium hydroxide.

5 Of the nitrogenous growth substrates studied, the growth of *C. utilis* responded best to supplementation of the apple pomace medium with ammonium sulphate.

6 The greatest increase in the crude protein content of the apple pomace from approximately 2.7% to 7.2%, on a dry weight basis, was achieved by the solid state fermentation of a pomace medium supplemented with 1% v/w 7.8 N ammonium hydroxide using the *Kl. apiculata* strain isolated in this study.

7 A comparison of the amino acid profile of the microbially modified apple pomace and a recommended profile for growing and breeding pig diets indicates that the feed would be deficient in nearly all the essential amino acids.

Research indicates that the greatest potential market in New Zealand for an apple pomace feed supplement is in the pig industry (Snowden, 1984). However, the microbially modified apple pomace generated in this research would be inadequate as a pig feed on its own although it may find some use as a supplement. Before any further research is conducted, feeding trials of the microbial biomass product formed on growing and breeding pigs would be required to determine its nutritional value.

However, a new process has been proposed for the extraction of apple juice in New Zealand (Marks, personal communication). This new, commercially sensitive method of juice extraction would result in an altered pomace waste stream. If the new process was adopted for commercial use, this research would be of limited value to the New Zealand apple industry and potential markets.

APPENDIX

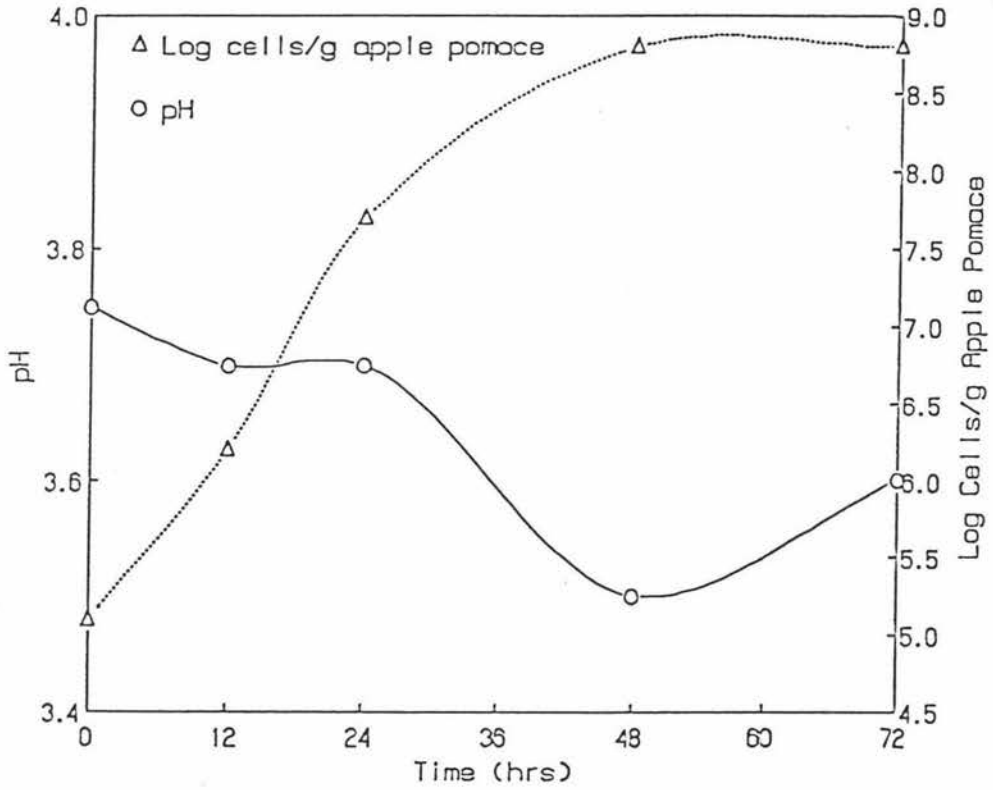


Figure A1. Cell growth and pH changes during *Kl. apiculata* growth on media supplemented with 1% v/w 2 N ammonium hydroxide.

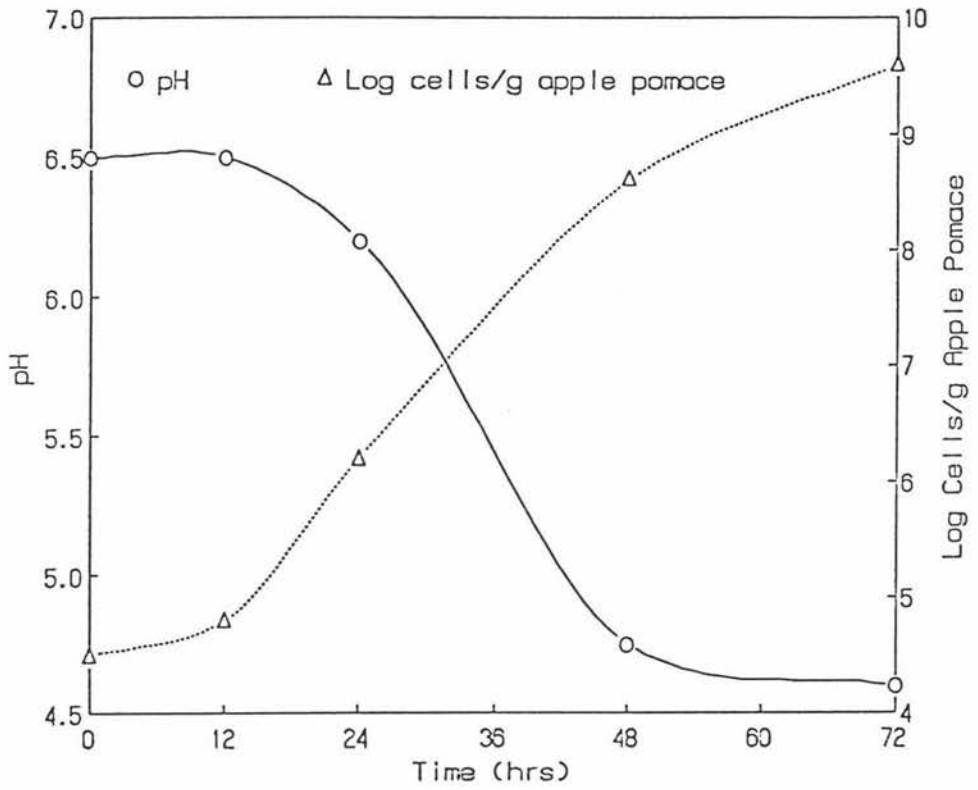


Figure A2. Cell growth and pH changes during *Kl. apiculata* growth on media supplemented with 1% v/w 7.8 N ammonium hydroxide.

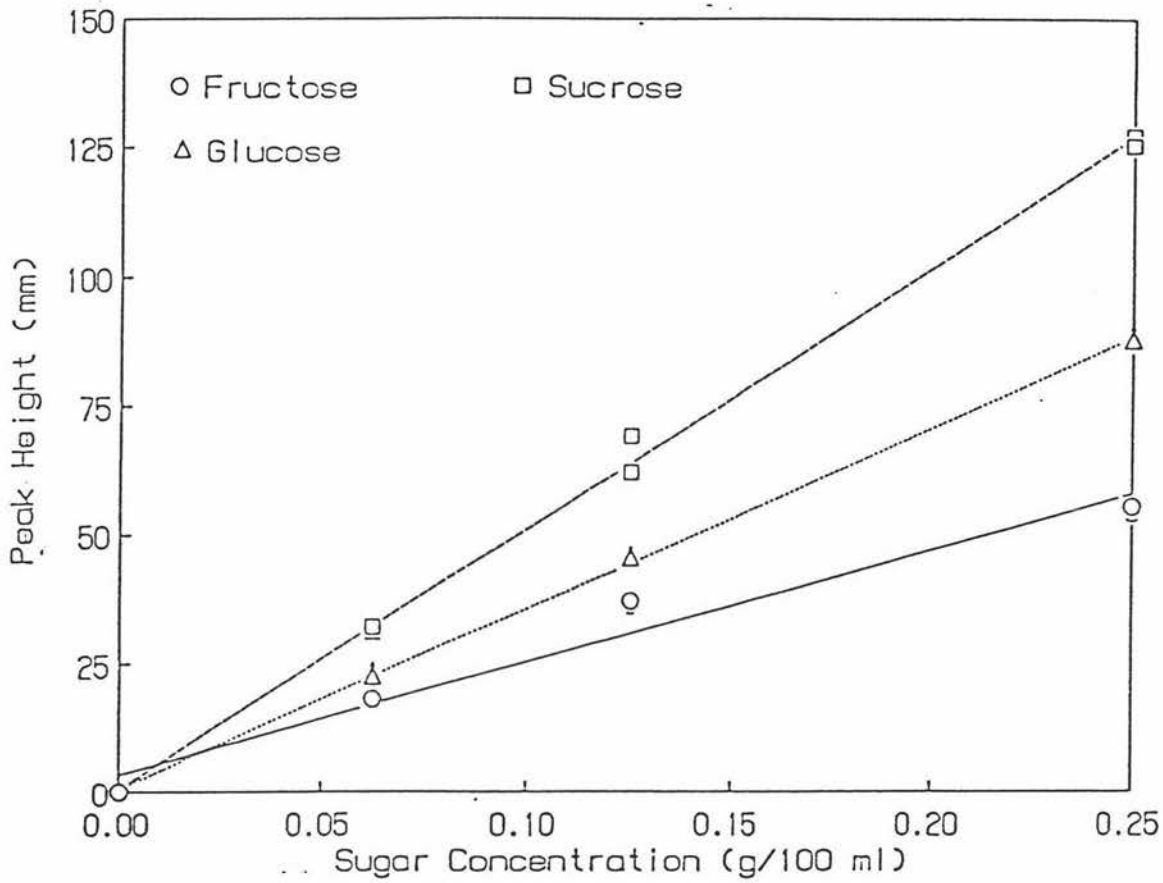


Figure A3. Standard curves for the quantification of soluble glucose, fructose and sucrose by HPLC analysis.

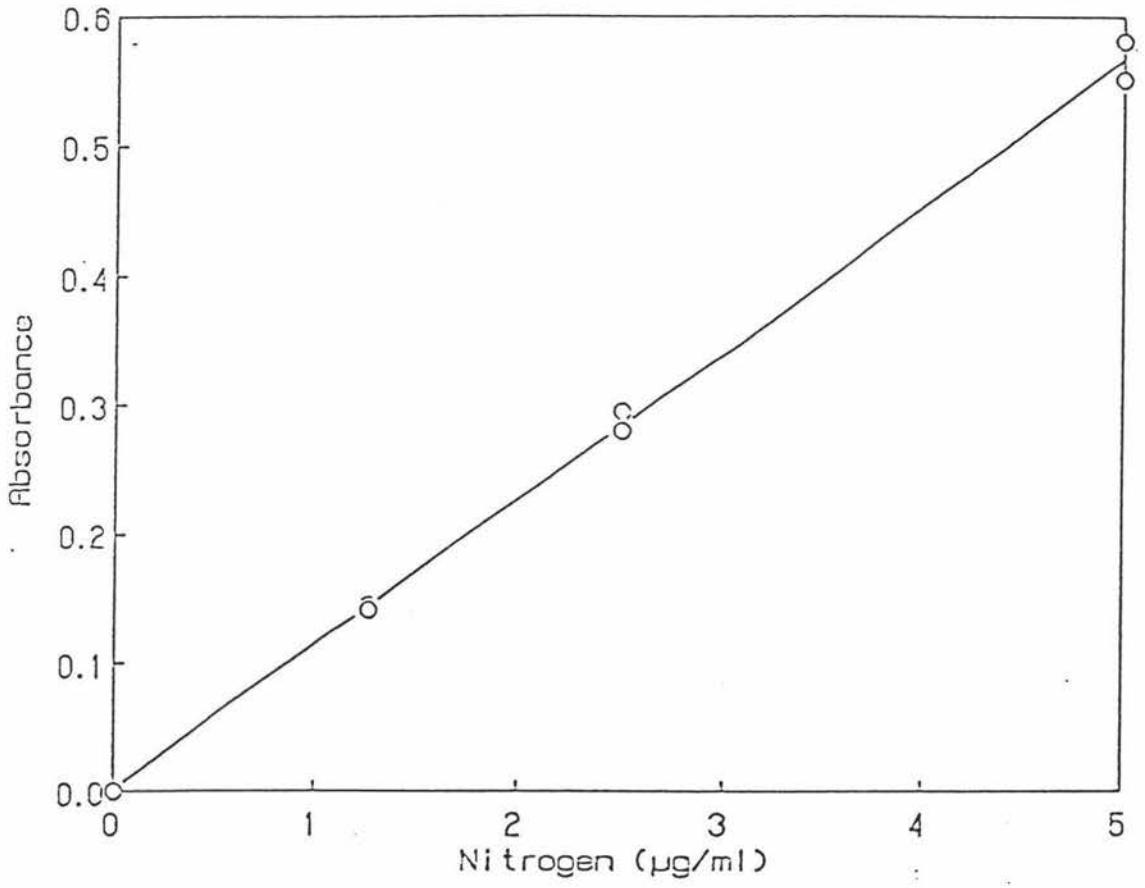


Figure A4. Standard curve for the quantification of nitrogen.

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