T H E

ACETONE BUTANOL ETHANOL

FERMENTATION:

PRELIMINARY STUDIES ON SOME PRACTICAL ASPECTS

by
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a thesis presented in partial fulfilment of
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ABSTRACT

The dilute nature of solvents at the end of fermentation and slow overall rate of fermentation are major economic burdens on a commercial plant producing acetone, butanol, and ethanol. A preliminary feasibility costing of such a plant showed the cost of fermenters represents almost 50% of the total purchased equipment cost, and emphasised the need for improvements in the fermentation.

Experiments were performed in 10-litre and 30-litre pressure vessels, a 1.5-litre vessel at atmospheric pressure, and trial runs in 100 ml bottles.

Good correlations were found for the different fermentation headspace pressures (100 to 250 kPa abs.) and minimum observed pH's (pH 4.2 to pH 4.65) with final butanol yields (0.92 to 11.6 g/l); increases in both parameters correlating with increased butanol concentration. Ethanol was found to be correlated with pressure only, and acetone with neither parameter directly. Other chemical species present in the broth were also correlated with each other. It was found that a tree diagram drawn using the strongest correlations resembled closely the known metabolism of the organism in terms of the metabolic pathways, specification of active forms of the metabolites, and effect of external influences. Use of multiple linear regression in this manner was named The Factor Correlation Method, and is potentially useful for research on metabolism and similar investigation on a much broader basis. Application of this technique showed that the pressure effect was possibly due to more than a single metabolic cause, and further experiments also emphasised the complex nature of the pressure effect.

The experimental work also highlighted the potential hazard of culture degeneration leading to substandard

fermentation yields and eventual nonviability. Discussion on the experimental results and of the literature suggests the phenomenon is due to infection by lysogenic phage rather than spontaneous mutation, and an approximate model based on simultaneous partial differential equations parallels some observed characteristics of the phenomenon.

Other topics include theoretical exercises with laboratory work on the water tolerance of methanol-petrol mixtures, the error associated with cell enumeration using a haemocytometer, and evaluation of growth and solvent production characteristics and some relevant parameters.

ACKNOWLEDGEMENTS

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CONTENTS

LIST OF VARIABLES AND ABBREVIATIONS

Variable	Name	Units
a	a constant	$
a_a	specific area of packing	$m^2\ m^{-3}$
acet	acetone	
A	area	m^2
A_a	active area on the plate	m^2
$[AA]_f$	final total acetate concentration	(g/l)
$[AA^-]_f$	final, calculated, dissociated acetic acid concentration	(g/l)
A.B.E.	acetone butanol ethanol fermentation	-
$[A]_f$	final acetone concentration	(g/l)
A_s	area free for gas flow	m^2
b	a constant	$\$m^3$
b.p.	boiling point	°C
but	butanol	
B	bottoms flow	$mol\ s^{-1}$
$[BA]_f$	final total butyrate concentration	(g/l)
$[BA^-]_f$	final, calculated, dissociated butyric acid concentration	(g/l)
$[B]_f$	final n-butanol concentration	(g/l)
BOD	Biological Oxygen Demand	ppm
c	specific heat	$Jg^{-1}\ K^{-1}$
c′	total cost of fermenters	$
CM	Cooked Meat Medium	
CoA	Coenzyme A	-
COD	Chemical Oxygen Demand	ppm
CW	cooling water	-
d.f.	degrees of freedom	-
d_g	gas density	$kg\ m^{-3}$
d_l	liquid density	$kg\ m^{-3}$

D	diameter	m
D′	distillate flow	mol s^{-1}
DC	direct cost	$
DE	time taken between viral infection and cell burst	hr
Dist	distillation section	-
DPC	direct product cost	$pa
DR	rate of death of active infected cells	t_d^{-1}
e	error	
e_a	human absolute error	cells
equ.	equation	
eth	ethanol	
E	number of cells per square expected	cells
$[E]_f$	final ethanol concentration	(g/l)
f	fractional error	-
f_a^2	human fractional error	-
frac.	fraction	
F_g	fraction of cross sectional area open to gas flow	%
FC	fixed charges	$pa
FCI	fixed capital investment	$
Fd	ferridoxin	
Ferm	fermentation section	-
FFAP	Free Fatty Acid Phase	
FP	feed plate position (numbered from the top of the column).	-
GE	general expenses	$pa
GLC	gas-liquid chromatography	
Glu	d-glucose	
h	liquid hold up as a fraction of the total bed volume	-
h_c	heat of combustion	Jg^{-1}
h_v	heat of vapourisation	Jg^{-1}

$[H^+]_f$	final hydrogen ion concentration	(M)
$[H^+]_m$	minimum hydrogen ion concentration observed	(M)
$[HAA]_f$	final, calculated, undissociated acetic acid concentration	(g/l)
$[HBA]_f$	final, calculated, undissociated butyric acid concentration	(g/l)
HTU	height of a transfer unit	m
H_2O	water	
i	inhibition power constant	-
I	inhibitor concentration	g/l
IC	indirect cost	$
IPC	individual purchase cost of item updated to September 1980	$
k	growth rate estimated from the logistic equation	hr^{-1}
K´	a constant	
K_m	a constant	
K_v	a constant	-
L	length	m
LMTD	log mean temperature difference	K
LN	log mean cell concentration	10^6/ml
m	mass flow rate	kg hr^{-1}
m_f	slope of feed line for a McCabe-Thiele Construction	
mol	mole	
m_r	slope of rectification section operating line	-
MC	manufacturing costs	$pa
MEK	methyl ethyl ketone	
M_i	general term for a property	-
MLR	Multiple Linear Regression	

M_m	general term for a property of a mixture	-
MON	motor octane number	-
Mr	molecular weight	g/mol
MU	growth rate	hr^{-1}
M15	mixture of 15% (v/v) methanol with 85% (v/v) petrol	-
M85	mixture of 85% (v/v) methanol with 15% (v/v) petrol	-
n	number of squares counted	squares
no.	number of operating fermenters (excluding spare).	-
N	cell concentration	10^6/ml
NT	number of trays	-
N.C.P.	National Chemical Products Ltd	-
N_{log}	cell population estimated by the logistic equation	10^6/ml
N_o	cell concentration immediately after inoculation	10^6/ml
NPN	non-proteinaceous nitrogen	
N_t	average stationary cell population	(X 10^6 cells/ml)
N_s	stationary phase cell population	10^6/ml
N_{sm}	cell population smoothed using three point moving averages	10^6/hr
NTU	number of transfer units	-
NV	burst size	$cell^{-1}$
O	observed cell population	10^6/ml
OD	oven dried	
p	pressure	kPa
pa	per annum	
pH_f	final broth pH	

pH_m	minimum observed pH	
PEC	purchased equipment cost	$
P_l	wetted column pressure drop	Pa m^{-1}
PLB	rate of conversion of lysogenic to virial cell	t_d^{-1}
P_o	dry column pressure drop	Pa m^{-1}
Prod	product section	-
PS	success constant	
P_{sm}	product concentration smoothed using three point moving averages	g/l
q	specific rate of production of product	µg/l hr cell
q´	ratio of heat required to vapour in the feed to a distillation column to its heat of vapourisation	-
Q	average WP flow rate (15 m^3 hr^{-1})	m^3 hr^{-1}
Q_l	liquid flow rate	mol s^{-1}
Q_g	gas flow rate	mol s^{-1}
r	ratio of fermenter filling rate to Q.	-
rpm	revs per minute	rev. min^{-1}
R	ratio of fermenter emptying rate to Q.	-
R_m	minimum reflux ratio	-
RON	research octane number	-
R_r	external reflux ratio	-
R´	maximum rate of butanol production	q $l^{-1}hr^{-1}$
RR	$RR = 0.8\ (r^{-1} + R'^{-1})$	-
s	absolute standard error	cells
s & t	shell and tube heat exchanger	-
s.g.	specific gravity	-
std.	standard	-

s^2	variance of cell counts	$cells^2$
S	substrate concentration	g/l
t	time	s
t_d	doubling time for a healthy cell	hr
t_{sm}	time smoothed using three point moving averages	hr
t_t	turnaround time	hr
T	temperature	K
TCI	total capital investment	$
TLV	threshold limit value	ppm
TN	total nitrogen	
U	overall heat transfer coefficient	$kW\ m^{-1}\ K^{-1}$
v	volume of single fermenter	m^3
v´	velocity	$m\ s^{-1}$
V	total fermentation volume	m^3
V_f	flooding velocity	$m\ s^{-1}$
V_o	optimum velocity	$m\ s^{-1}$
V_{tt}	Total tank volume	m^3
V'_t	superficial velocity without downcomers	$m\ s^{-1}$
w	heat flux	W
WC	working capital	$
WP	Sulphuric acid casein whey permeate	-
WSH	Wood Sugar Hydrolysate	
WSR	Wood Sugar Hydrolysate Residue	
$\bar{x}$	arithmetic mean	-
x_i	composition	mol. frac.
y	composition (operating line)	mol. frac.
y*	equilibrium composition	mol. frac.
y_d	distillate composition	mol. frac.
Y	conc. of healthy cells	10^6/ml
YALL	YALL = Y + YY + YL	10^6/ml
YE	yeast extract powder	-
YL	conc. of lysogenic cells	10^6/ml

y_o	bottoms composition	mol. frac.
YY	conc. of infected cells	10^6/ml
YYV	conc. of infected cells a period D before the time of interest	10^6/ml
$Y_{.68}$	68% confidence level envelope	cells
ΔG_o	standard change in free energy	J/mol
$\Delta G'$	change in free energy	J/mol
α	a constant	-
β	a constant	-
μ	growth rate	hr^{-1}
$\int$	integral sign	
ϕ	voidage	-
$\emptyset$	a constant	-

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1. INTRODUCTION

1.1 PRELUDE

Since 1973 there has been a rapid rise in the price of petroleum and derived products. This has led to increasing interest in the use of fermentations for production of various chemicals in the belief that such processes may become economic, as they were prior to the era of cheap oil. One such fermentation process is the now obsolete acetone/butanol/ethanol (A.B.E.) fermentation. Imports of acetone, butanol, and ethanol into New Zealand are shown in Table 1.1.

Historical uses of n-butanol are shown in Fig. 1.1 but the compound has been used in recent times as a cosurfactant/coagent in tertiary oil recovery from existing wells (Compere and Griffith, 1979), and in solvents for nitrocellulose lacquers (Cheremisinoff, 1980).

The greatest incentive to recent research, however, has been for alternative fuels applications. Table 1.2 compares the fuel characteristics of some lower alcohols and acetone with gasoline. As a complete replacement fuel, the chief advantage of butanol over methanol or ethanol is its greater energy per unit mass (50% and 20% respectively). As a fuel extender, butanol has two very important advantages over both methanol and ethanol. First, butanol blends in mixtures with petrol for spark ignition engines (as in the private vehicle fleet) have water tolerance levels greatly superior to those of methanol and ethanol blends. In fact, iso-butanol has been considered as a blending agent for blends of methanol with gasoline to increase their water tolerance (Judd and Graham, 1979). Work done by the present author as a prelude to this thesis, has shown n-butanol to be equally effective (Appendix 1). A blend of 5% t-butanol in automotive fuel was once used

Table 1.1. Imports of Acetone, Butanol, and Ethanol.

Solvent	Acetone	Butanol	Ethanol
Approx. Import Volume[a] (litres)	1,338,283	361,133	4,382,137
Est. ex Factory Price[b] ($/kg)	0.98	0.90	0.60
N.Z. Retail Price[c] ($/kg)	1.22	1.12	0.74
Approx Total Cost of Imports	1,036,099	263,266	2,074,504

a: assume 50% total butanol imports are n-butanol

b: 1978-79 figures

c: based on estimated ex factory

Refs: British Petroleum (1980)

N.Z. Department of Statistics (1981)

Fig. 1.1. Uses for n-Butanol in 1927 (Anon, 1927).

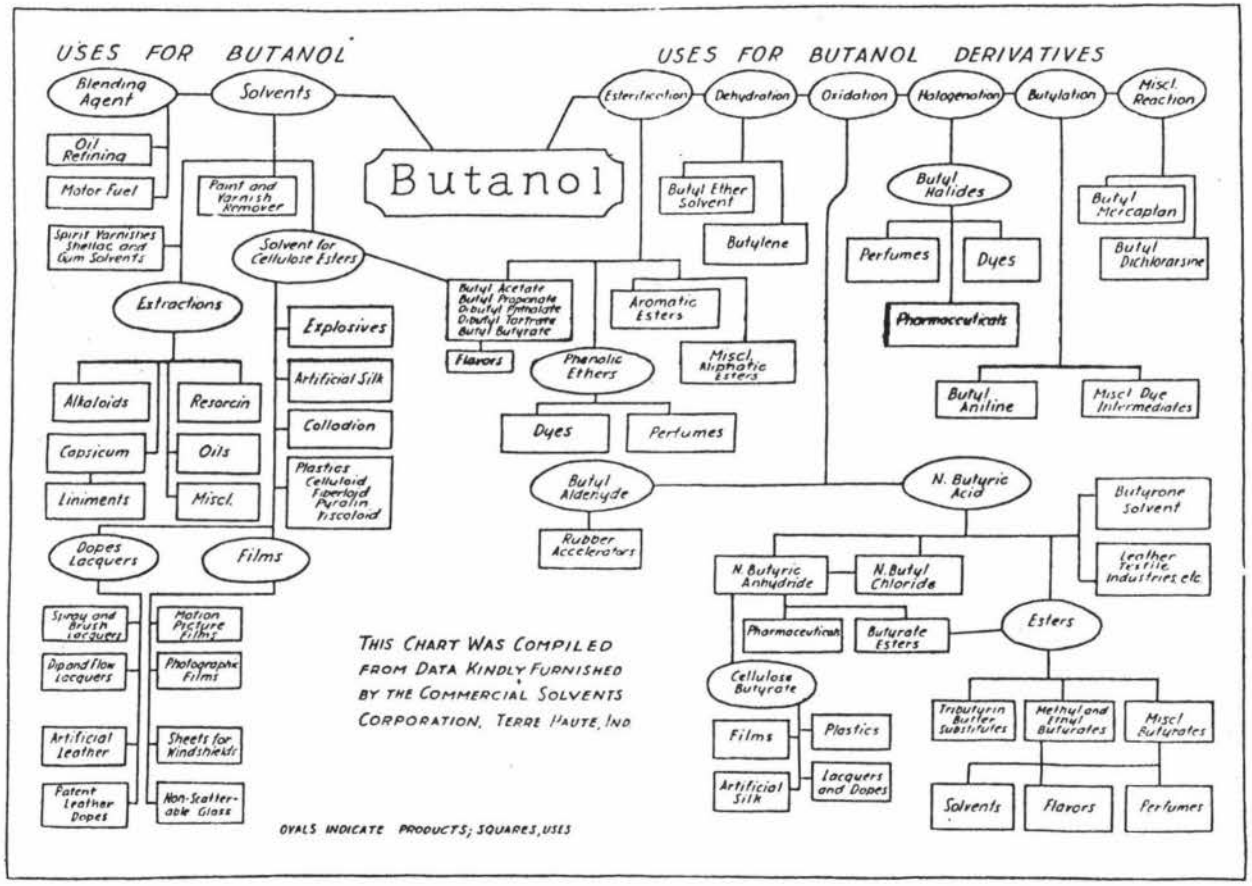


Table 1.2 CHARACTERISTICS OF SOME PURE LIQUIDS

Property[a]	Units	Premium Gasoline	Methanol	Ethanol	n-Butanol	Acetone
Mr	g mol^{-1}	-	32.04	46.07	74.12	58.08
s.g.	-	0.73-0.74	0.793	0.789	0.810	0.792
b.p.	°C	40-200	65	78.4	117	56.5
h_v	Jg^{-1}	350	1,170	879.2	619.7	550.9
h_c	Jg^{-1}	47,100	23,492	29,690	36,049	30,838
St. Ratio[b]	g/g	14.7	6.5	9.0	11.1	9.5
Octane Rating						
RON[c]	-	96	110	100	-	-
MON[d]	-	91	87, 94	94	83	93
TLV	ppm	250-700	200	1,000	100	1,000

a: See List of Variables and Abbreviations for explanation.
b: St. Ratio = Stoichiometric Ratio (air:fuel)
:Molecular Weight of air = 0.79 x 28 + 0.21 x 32
= 28.9 g mol^{-1}
c: Research Octane Number
d: Motor Octane Number

Refs: Judd (1979)
Noon (1981)
Perry and Chilton (1973)
Thompson and Ceckler (1977)
Weast (1977)

under the trade name ARCONOL by the Atlantic Richfield company (Cheremisinoff, 1980). At this level there was no loss in 'driveability'. At higher concentrations (10% and 15%) however, some loss was apparent. ARCONOL also appeared to have good carburettor anti-icing characteristics, octane performance, and water tolerance properties (Cheremisinoff, 1980). The Goodyear Tire and Rubber Co. have a patent on a t-butanol/gasoline/water blend reported to have cold weather properties superior to both methanol and ethanol blends (Cheremisinoff, 1980).

In fact, up to 20% n-butanol can be added to petrol with only very minor engine modifications (Noon, 1981). Miller et al. (1981) state that butanol's low latent heat of vapourisation (h_v) relative to methanol and ethanol would probably avoid cold start problems, its low vapour pressure avoid vapour lock problems, and its longer carbon chain reduce water tolerance problems. For example, a 20% n-butanol mixture does not phase separate with 1% added water whereas a 20% ethanol mixture does (Miller et al., 1981). In addition, the present author has shown in experiments similar to those of Appendix 1 that a blend of 20% ethanol with a laboratory petrol mixture has a much greater water tolerance than similar methanol blends (at either 20% or 85%). This implies that the most stable blend is with butanol, followed by ethanol and then methanol.

The second advantage of n-butanol as a fuel extender is that it can be mixed directly with diesel fuels for use in the compression combustion engines so extensively used in industry and transport. This is practicable to a concentration of 30-40% (Miller et al., 1981; Noon, 1981), whereas both methanol and ethanol are very much more troublesome, requiring perhaps spraying the fuels separately into the cylinders (Cheremisinoff, 1980), or the creation of fine, stable emulsions by chemical means. Diesel mixtures are limited to just 30-40% to prevent engine knocking.

Slight cetane depression is experienced at these concentrations but the problem is minor. The long term effect of the mixture on lubrication was not examined in either publication.

Professor A.L. Titchener has outlined nine major problems with alcohol fuels (McEldowney, 1982), namely, poor cold weather starting characteristics, low calorific values only partially offset by higher engine efficiencies, greater heats of vapourisation, poor engine lubrication, attack of some engine materials, aldehyde emissions, toxicity, poor diesel substitution, and logistics of introduction. Butanol would compare little worse than the lower alcohols on few of these, and is vastly superior in diesel applications which he suggested were the only major technical hurdles.

In addition to traditional uses and these liquid fuel possibilities, resurgence of interest in solvent delignification of wood (originally for pulp production) (Aronovsky and Gortner, 1936) could also give the A.B.E. fermentation a future in forest utilisation (U.S. Dept. Energy, 1979). Aronovsky and Gortner (1936) found aqueous n-butanol solutions to be the best of a range of solvents for the delignification process.

It is for these reasons as well as the savings on imports for industrial use that the present study was initiated. The two organisms Clostridium acetobutylicum and Cl. butylicum were used, as strains of these two organisms have proved to be amongst the best microorganisms for the fermentation. They are both motile, saccharolytic bacteria and are obligate anaerobes. These two organisms will be described in more depth in Sections 1.3 and 2.2.

The choice of raw material remained. Traditionally, either starch or molasses has been used, but neither is particularly cheap or readily available in New Zealand.

Wix and Woodbine (1958) mentioned the use of whey as a substrate for the A.B.E. fermentation. New Zealand dairy companies produce large volumes of whey and whey deproteinated by ultrafiltration (whey permeate). The whey production over a year for a typical dairy factory is shown in Fig. 1.2, and the eighty dairy factories in New Zealand would produce some 7,000 million litres annually. Maddox (1980) has shown that whey permeate is a suitable substrate for the A.B.E. fermentation. Due to recent growth in the New Zealand dairy industry this supply of whey and whey permeate will outgrow the traditional outlets, principally spray irrigation onto pasture and lactose production. Dumping to waste (into streams, waste treatment plants, or the sea) puts a vast load on the receiving system (the BOD of whey is 40-60,000 p.p.m. (Wix and Woodbine, 1958)), and represents a loss of potentially useful material. The surplus whey or whey permeate therefore represents a possible substrate. An attractive feature of using whey permeate as a substrate for the A.B.E. fermentation is its relatively low sugar concentration (ca. 50g/l lactose). This is ideal because product toxicity by n-butanol limits the n-butanol concentration in the spent broth to ca. 12g/l (Hastings, 1978) and total solvents to about 20 g/l, and thereby limits the sugar utilised to ca. 50-60g/l. This, in addition to the presence of cheap readily available whey permeate, made whey permeate an attractive substrate. However, another more abundant substrate would be required for very large volume production. The total production capacity from the whey may be estimated as

$$\begin{aligned} \text{prod.} &= (\text{sugar conc.}) \times (\text{conversion efficiency}) \times (\text{whey volume}) \\ &= 50 \text{ g/l} \times 0.32 \text{ g/g} \times 7 \times 10^{9} \text{ l/yr} \times 10^{-6} \text{ tonne/g} \\ &= 110{,}000 \text{ tonnes/yr.} \end{aligned}$$

where 0.32 g/g is the lactose to solvent conversion efficiency (Section 3.3.3.9). This represents only ca. 7% of the petrol and ca.10% of the diesel supplied to the N.Z.

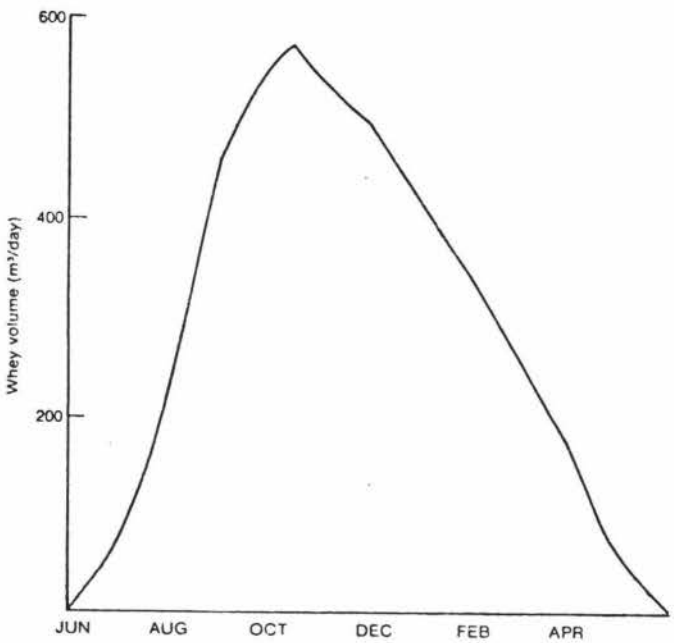


Fig. 1.2. Typical Seasonal Flow of Whey for a New Zealand Dairy Factory (Marshall, 1981).

consumer in the early 1980's. Delivery to the pump was 1.6×10^6 tonnes/yr for petrol and 1×10^6 tonnes/yr for diesel in 1980 and 1981 (N.Z. Dept. Statistics, 1982). So unless this rough calculation is out by a factor of 10, or more it can be seen that there is insufficient whey. The calculation for ethanol fermentation is virtually identical.

Alternative fermentation substrates would have to include both wood hydrolysates and sugar beet. Leonard et al. (1947) showed that wood sugar hydrolysate could be used for the fermentation, although difficulties would be encountered. This possibility is considered further in Chapter 4.

1.2 HISTORY

The history of the A.B.E. fermentation up to the 1950's is well documented by Wynkoop (1943), Prescott and Dunn (1959), and Ross (1961). At one stage it was second in importance only to ethanol production (Spivey, 1978).

The fermentation was first noticed by Pasteur in 1861 (Ross, 1961). In 1911, Strange and Graham Limited (U.K.) employed Fernbach and Schoen of the Pasteur Institute and Perkin and Weizmann of Manchester University to study the butanol fermentation as a source of raw material for possible synthetic rubber production. Weizmann left their employ in 1912 and later isolated an organism capable of producing an acetone yield four times that of previous isolates (Compere and Griffith, 1979). Unfortunately, initial hopes of synthetic rubber production from butanol were not fruitful, and it was not until World War I and the great demand for acetone for explosives and nitrocellulose wing dopes for aeroplanes that the first commercial plants were established. After the war the demand for acetone diminished and many plants were closed (Prescott and Dunn, 1959).

However, soon after World War I, butyl acetate was found to be an excellent solvent for the nitrocellulose lacquers used on cars and so the rapidly growing car industry made the process viable through n-butanol, which had previously been a waste product (Ross, 1961). Fig. 1.1 shows uses for n-butanol in 1927 (Anon, 1927). Production facilities grew rapidly between the wars and new uses for n-butanol were discovered in wood pulping (Aronovsky and Gortner, 1936), urea-formaldehyde resins and coatings, and as a plasticiser or solvent in other plastics (Wynkoop, 1943).

After World War II, when the demand for acetone once again plummetted, the A.B.E. fermentation again decreased in importance. At this time a new synthetic chemicals industry based on ethanol from yeast fermentations began competing in the same market. Developments in the A.B.E. industry resulted in increased efficiency and early competition, but the synthetic chemical industry soon began to dominate. In an effort to survive, previous "waste-products" were marketed (Hastings, 1978). Hydrogen gas was used for methanol manufacture and foodstuffs. Carbon dioxide was compressed and used as dry ice, and vitamin B_{12} and other growth factors from the residual solids made economic the total evaporation of spent medium to protein rich animal food. Finally, however, simultaneous increases in the price of molasses for fermentation and the sudden availability of cheap petroleum as an alternative raw material for the synthetic chemicals industry meant that the A.B.E. process was no longer economic. Only in sugar- or molasses-producing "non-petroleum" countries such as some Eastern European countries, Egypt, and South Africa has the fermentation continued. The history to 1978 is recorded by Hastings (1978).

1.3 THE ORGANISMS

Clostridium acetobutylicum is an anaerobic, spore-forming,

motile, rod-shaped bacterium. Bergey's Manual (Smith and Hobbs, 1974) provides a good description of the species, and describes it as Gram-positive and usually 4 μm x 1 μm. Spivey (1978) describes the species as Gram-positive or Gram-variable depending on culture age.

Storage of the organism to prevent culture deterioration requires specific attention. Repeated subculturing of many butyric acid-forming bacteria is reported to result in serious culture deterioration, leading eventually to complete loss of viability (Kutzenok and Aschner, 1952). Storage of Cl. acetobutylicum on sand or soil as a means of culture maintenance has proved successful (Spivey, 1978). Conversely, heat shocking of the spores soon after inoculation has been frequently reported to result in faster fermentation rates and higher yields (Hastings, 1978; Prescott and Dunn, 1959; Ross, 1962; Spivey, 1978).

Resistance of the organism to the toxic effects of the solvents produced, in particular n-butanol, is of great importance as this is thought to be one of the limiting factors in the final solvent yield. Inhibition occurs at concentrations as low as 10 g/l, and 12 g/l or 13 g/l n-butanol halts the fermentation (Hastings, 1978; Ross, 1961). Efforts to increase the resistance of the organism have met with only slight success (U.S. Dept Energy, 1980). Jerusalemskii seems to have been the only person to increase the n-butanol tolerance significantly (Ross, 1961). He used continuous culture methods over a 200 day period to obtain a culture which could tolerate 25g/l n-butanol from a strain initially tolerating only 8g/l. He also found that the resistance was transferred through further generations.

Clostridium butylicum is very similar to Clostridium acetobutylicum, and perhaps the only major difference which can be ascribed to strain variation is Cl. butylicum's inability to hydrolyse gelatine (Smith and Hobbs, 1974).

1.4 THE PROCESS

Beesch (1952), and Prescott and Dunn (1959) present good reviews of some fermentation processes prior to 1959. Spivey (1978) gives details of an A.B.E. fermentation plant currently in operation. Figs. 1.3, 1.4, and 1.5 show the process flow diagram, distillation system, and the pH, acid, solvent, and gas evolution curves for a typical batch fermentation at the National Chemical Products (N.C.P.) plant in South Africa (Spivey, 1978). Briefly, each pressure fermenter of 90,000-litre working volume (constructed of stainless steel-clad mild steel) and associated piping is left under steam pressure while not in use (for sterilising), flushed with sterile CO_2 (to prevent vacuum formation during cooling and to maintain anaerobic conditions), and filled with medium. The medium (molasses) is diluted to about 60-65g/l fermentable sugar concentration in order that the normal 30% final yield on sugar would result in less than 20g/l total solvent. This is desirable as solvent toxicity would otherwise halt the fermentation prematurely. The medium is sterilised immediately prior to entering the fermenter using steam injection, a plate heat exchanger, and a holding tank to give retention time of 4 minutes at 128°C. The medium is cooled to 34°C and run into the fermenter. Oxygen is stripped from the medium by CO_2 agitation for 20 minutes before and after inoculation. Finally, if required, the pH is adjusted to pH 5.8-6.0 using ammonia. The fermenter head-space pressure is then adjusted using CO_2 to 35 kPa and held for 5 hr., after which it is released to 18 kPa to allow for build up due to gas evolution by the organism. When 35 kPa is again attained, excess gas is drawn off.

Gas production ceases abruptly after 30-34 hours and the fermentation ends. The beer is then sent for distillation. Typically, solvent yields are as shown in Table 1.3. About 50% (wt. basis) of the sugar goes to carbon dioxide, and 2% to hydrogen.

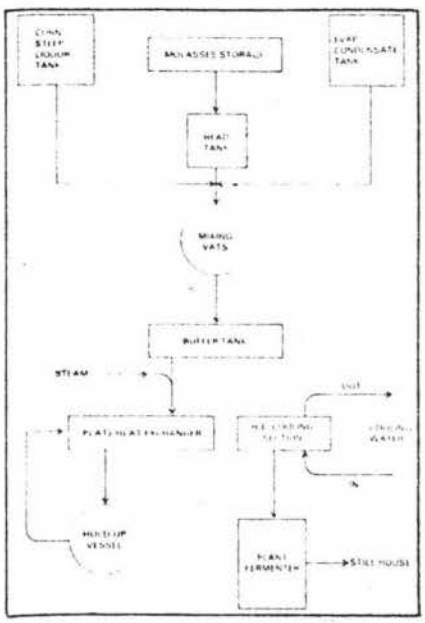


Fig.1.3. Process Flow Diagram.

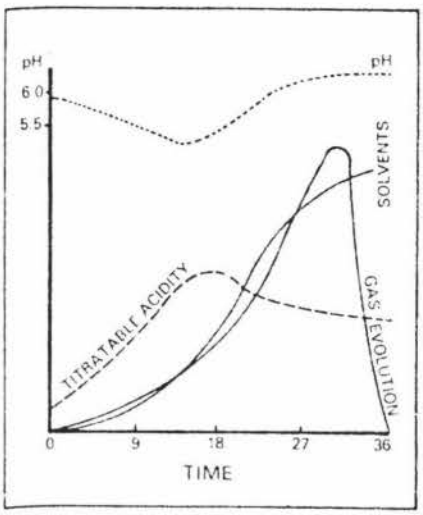


Fig. 1.4. Progress of the Fermentation.

Fig. 1.5. Distillation Plant Flow Diagram.

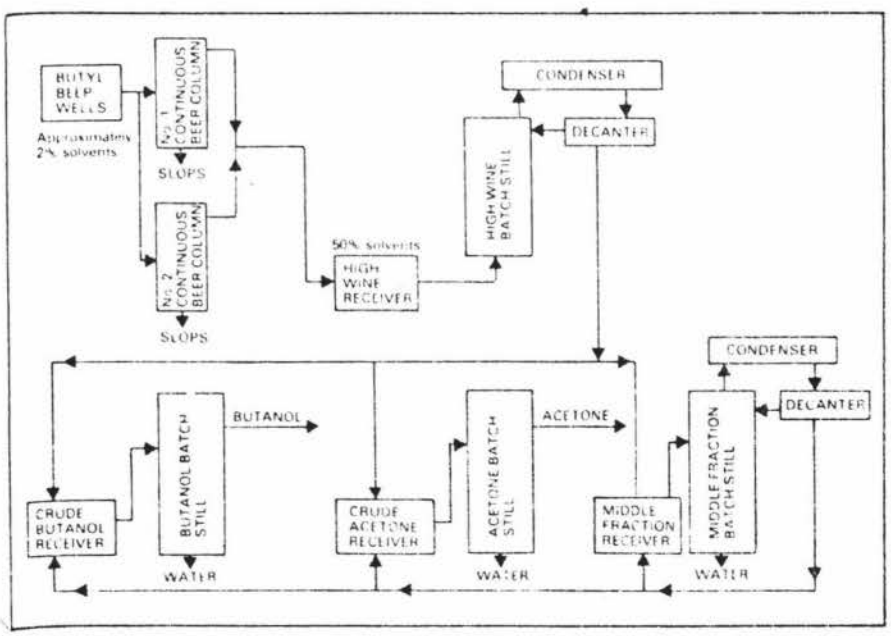


Table 1.3 TYPICAL BROTH CONCENTRATIONS
AT NATIONAL CHEMICAL PRODUCTS (Spivey, 1978)

Species	Concentration (g/l)
Initial Fermentable Sugar	63
Butanol	11
Acetone	6
Ethanol	2

The total "turnaround time" per batch fermenter is 48 hours, and multiple vessels allow continual production.

Continuous fermentation has not yet been attempted on an industrial scale although some workers showed promising results in the laboratory (Ross, 1961).

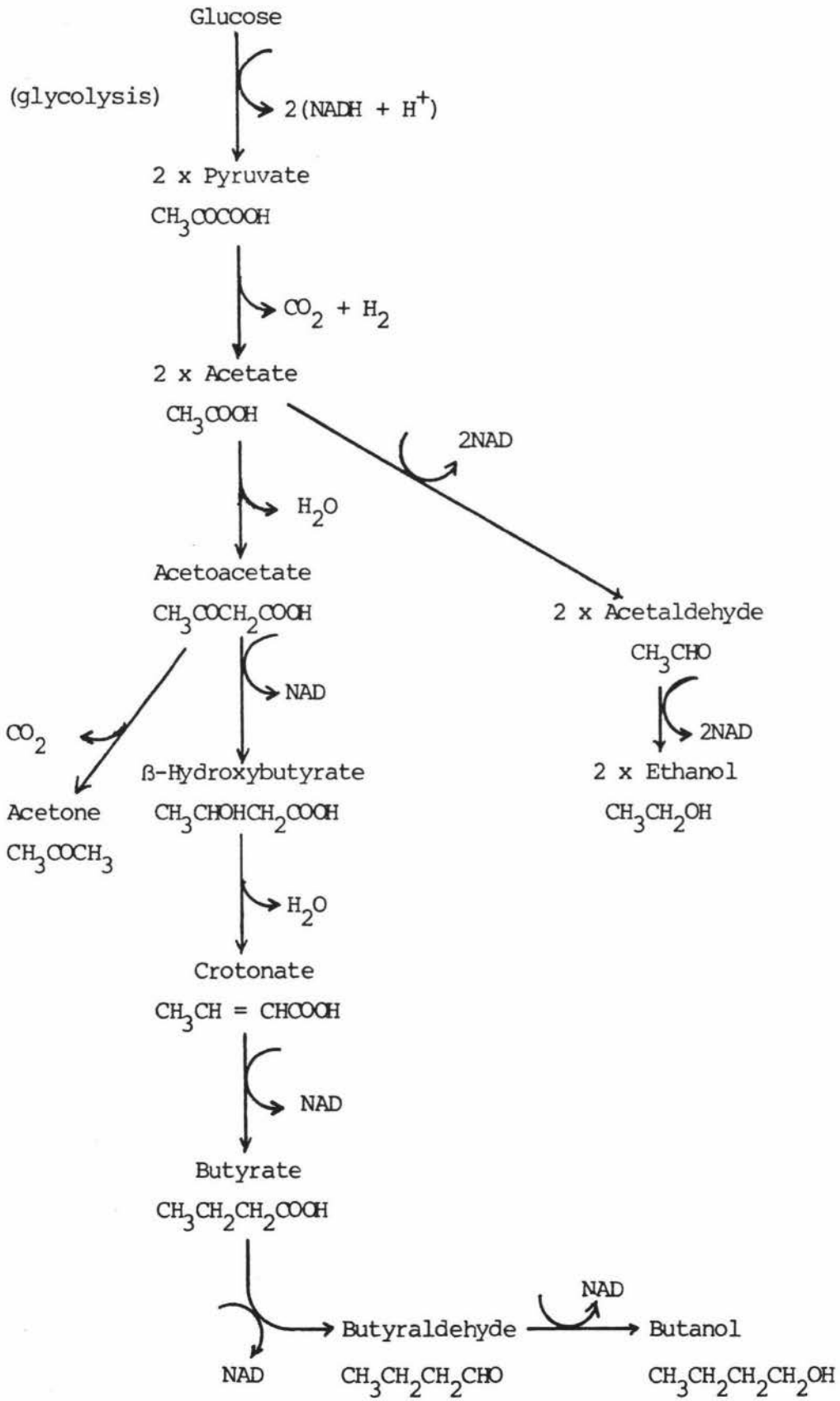
1.5 THE BIOCHEMISTRY

The organism converts sugar to pyruvate via the glycolytic pathway. The pyruvate is then reduced to acetate which can be used for ethanol production and/or condensed to aceto-acetate and used for production of acetone and/or n-butanol.

The basic fermentative pathways are shown in Fig. 1.6.

The biochemistry is outlined in more detail in Section 3.3.3.5 (especially Fig. 3.15).

Figure 1.6 THE METABOLIC PATHWAY TO THE SOLVENTS
(Coenzyme A forms omitted for convenience)



2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Media

Sulphuric acid casein whey permeate (WP) was obtained from the New Zealand Dairy Research Institute (Palmerston North, New Zealand). Its preparation is described by Matthews et al. (1978) and the average seasonal composition by Marshall (1981) (Table 2.1).

Yeast Extract (YE) was purchased from Sigma Chemical Co. Ltd. (St. Louis, Missouri, U.S.A.).

Cooked Meat Medium (CM) was obtained from Difco Laboratories (Detroit, Michigan, U.S.A.).

Wood Sugar Hydrolysate (WSH) fractions (pentose-rich prehydrolysate and hexose-rich hydrolysate), and WSH residue from the bottom of a beer still (stripper) after a yeast fermentation (WSR), were obtained from the Forest Research Institute (Rotorua, New Zealand). The compositions of the two hydrolysates are given in Tables 2.2 and 2.3, and for the residue in Table 2.4 (Mackie, 1982).

For the preparation of the hydrolysate, the Pinus radiata wood feed was presteamed at 148°C for 35 minutes, then cooked at 160°C for 30 minutes in 6.7-litres of 0.5% H_2SO_4 per kg OD (oven dried) feed to produce the prehydrolysate. Further cooking at 185°C for 140 minutes in 20-litres of 0.5% H_2SO_4 per kg OD feed produced the main hydrolysate. The final sugar yield was approximately 48% of OD feed (Mackie, 1982).

2.1.2 Sugars

L-arabinose, D-glucose, and D-xylose were obtained from

Table 2.1 AVERAGE SEASONAL COMPOSITION
OF WHEY PERMEATE (Marshall, 1981)

Characteristic	Concentration (g/l)
Total Solids	56.4
Lactose	46.0
Protein (TN-NPN)	0.3
NPN	0.32
Ash	7.9
PO_4^{3-}	2.0
Ca^{2+}	1.4
SO_4^{2-}	2.8
Mg^{2+}	0.11
Na^{+}	0.50
K^{+}	1.4
Cl^{-}	0.9
pH	4.5 - 4.6

Table 2.2 COMPOSITION OF WOOD SUGAR HYDROLYSATE FRACTIONS
(Mackie, 1982)

	Concentration (g/l)	
	Prehydrolysate (Pentose Rich)	Main Hydrolysate (Hexose Rich)
Total Solids	35	26
Suspended Solids	1.4	3.0
Arabinose	2.5	0
Xylose	5.9	0.9
Mannose	12.0	1.3
Galactose	3.4	0.3
Glucose	6.8	20.0
Total Carbohydrate	ca.32.0	ca.25.5

Table 2.3 COMPOSITION OF COMBINED FRACTIONS
(Mackie, 1982)

Property	Concentration (mg/l)
Acidity	7,675 $CaCO_3$
Sulphate	5,000 (as sulphuric acid)
Metals:	
Mn	1
Fe	16
Ni	40
Cr	18
Cu	0.3
Phenols	0.76
Colour	1,900 (chloroplatinate units)
COD	32,840
BOD	18,000

Table 2.4 WOOD SUGAR HYDROLYSATE RESIDUE COMPOSITION
(Mackie, 1982)

Species	Concentration (g/l)
pH	4.5-5.0
Acidity	1.25 $CaCO_3$ (pH 8.3)
Sulphate	1.5
Nitrogen	0.018 (Kjeldahl)
Phospherous (Total)	0.003
Calcium	1.4
Phenols	0.0005
Metals	(as for combined hydrolysate)
Total Solids	21.9
Suspended Solids	1.6
COD	22
BOD	13
Colour	2,400 (chloroplatinate units)
Xylose	4.3
Arabinose	2.2

B.D.H. Chemicals Ltd (Palmerston North, New Zealand). Lactose, for use in culture media, was purchased from Wyngate (Hawera, N.Z.) and, for use as an analytical standard, from Sigma Chemical Co. Ltd.

2.1.3 Chromatographic Standards

Butan-1-ol, butan-2-ol, ethanol, acetone, and acetic acid (UNIVAR grades) were obtained from Ajax Chemicals (Sydney, Australia). N-butyric acid (ANALAR grade) was purchased from B.D.H. Chemicals Limited.

2.1.4 Gases

Hydrogen and oxygen-free nitrogen were purchased from New Zealand Industrial Gases Ltd., (Palmerston North, New Zealand).

2.1.5 Other Chemicals

Industrial grade ammonia solution was obtained from Andrew Chemical Co. Ltd. (Takapuna, New Zealand), and formalin from Laboratory Supply Pierce (N.Z.) Ltd. (Birkenhead South, New Zealand).

2.2 ORGANISMS

Clostridium acetobutylicum NCIB 2951 was purchased as a freeze dried specimen from the National Collection of Industrial Bacteria, Aberdeen, Scotland.

Clostridium butylicum NRRL B-592 was purchased as a freeze dried specimen from the Northern Regional Research Centre, U.S. Dept. of Agriculture, Peoria, U.S.A.

Both strains were reconstituted into 20 ml CM supplemented with 50g/l D-glucose contained in 25 ml screw-capped bottles. Soon after gassing ceased, the organism was

inoculated into 90 ml CM, supplemented with 50g/l D-glucose, heat shocked at 75°C for one minute, and incubated at 30°C. After sporulation the culture was stored at 7°C. Stock cultures for regular use in inoculum preparation were prepared from this master stock into CM supplemented with 50g/l of either D-glucose (for WSH media experiments) or lactose (for WP experiments).

Cl. acetobutylicum NCIB 2951 was used for all experiments except those described in Section 4.2 and Chapter 5.

Cl. butylicum NRRL B-592 was used for the experiments in Section 4.2 and Chapter 5.

2.3 MEDIUM AND EQUIPMENT STERILISATION

All media of volumes less than 3-litres were sterilised in an autoclave at 120°C for 15 min. Greater volumes were sterilised in the autoclave at 120°C for 20 min.

Gas filters, used to sterilise gases for fermentation, were filled with glass wool and heated in an oven at 160°C for ca. 3 hours.

Electrodes for insertion into fermenters were sterilised in 5% (v/v) formalin solution, and rinsed with sterile distilled water immediately before use.

2.4 INOCULUM PREPARATION

Unless otherwise noted, inocula were prepared by introducing 1 ml of stock culture into 100 ml of CM supplemented with 50g/l lactose. After incubation for two days at 30°C in an anaerobic jar (Gas Pak BBL 70304 for carbon dioxide and hydrogen production) fermentations were inoculated either by pipette, or by pouring directly into the fermenter allowing a minimum of solids to leave the inoculum bottle.

2.5 FERMENTATION EQUIPMENT AND METHODS

2.5.1 100 ml Scale Fermentations

100 ml nominal volume experiments were performed in 120 ml screw-capped "medicine bottles" containing ca. 90 ml of the required medium and adjusted to pH 6.5 with aqueous ammonia (5N) prior to autoclaving. After sterilisation and cooling, 1 ml of inoculum was added and the bottles were incubated at 30°C.

Approximately 8 ml of sample was aseptically withdrawn by pipette as required. Cell numbers and culture pH were determined immediately, and the sample stored at -20°C prior to chemical analysis.

2.5.2 1.5-litre Fermentations

1.5-litre nominal volume fermentations were performed in a Microferm Laboratory Fermenter (New Brunswick Scientific Co. Ltd, New Jersey, U.S.A.) using a 2-litre glass vessel containing 1.5-litre of medium. Continuous measurement and one-way control of pH was performed by a Horizon Model 5997-20 pH Controller (Horizon Ecology Co., Chicago, USA) coupled to an EIL 33 1070 030 toughened glass electrode and an EIL 33 1320 210 laboratory sealed reference electrode. Ammonia (5N) flow, when called by the controller, was stopped every 29 sec. by a cam-timer to prevent gross over-correction. The ammonia was gravity fed from a dropping funnel through a Fluorocarbon Delta model DV2 122A2 solenoid valve (Delta Solenoid Valves, Anaheim, California, USA).

Fig. 2.1a is a photograph of the Microferm unit, with fermenter in place, and recording equipment. Fig. 2.2a is a diagram of the same.

Immediately after sterilisation, the fermenter and contents

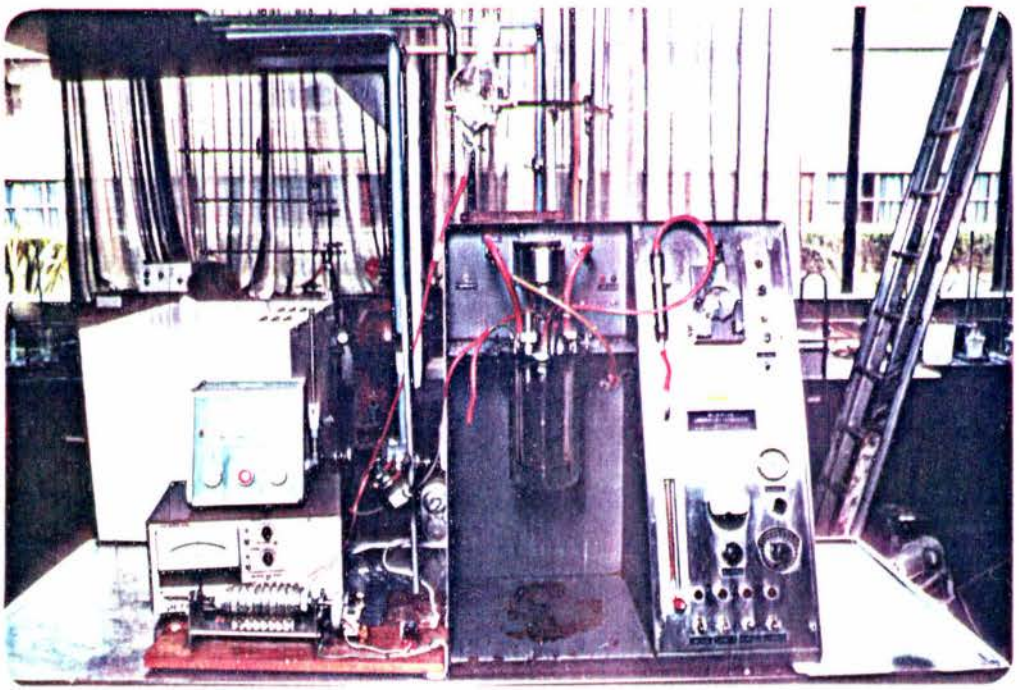


Fig. 2.1a. The 1.5-litre Fermenter in the Microferm.

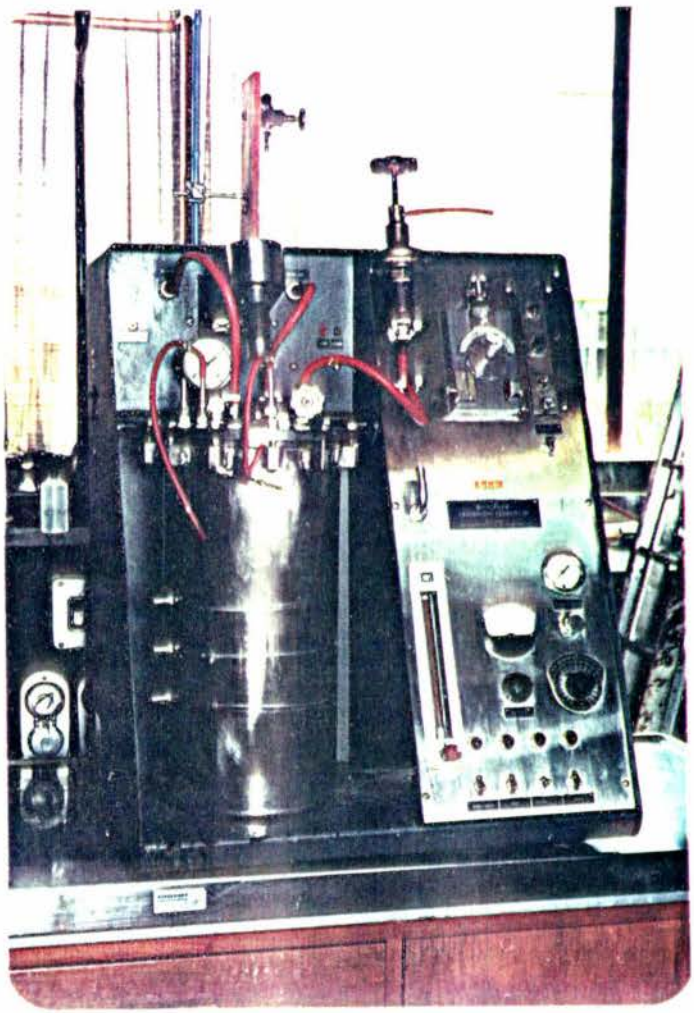


Fig. 2.1b. The 10-litre Fermenter in the Microferm.

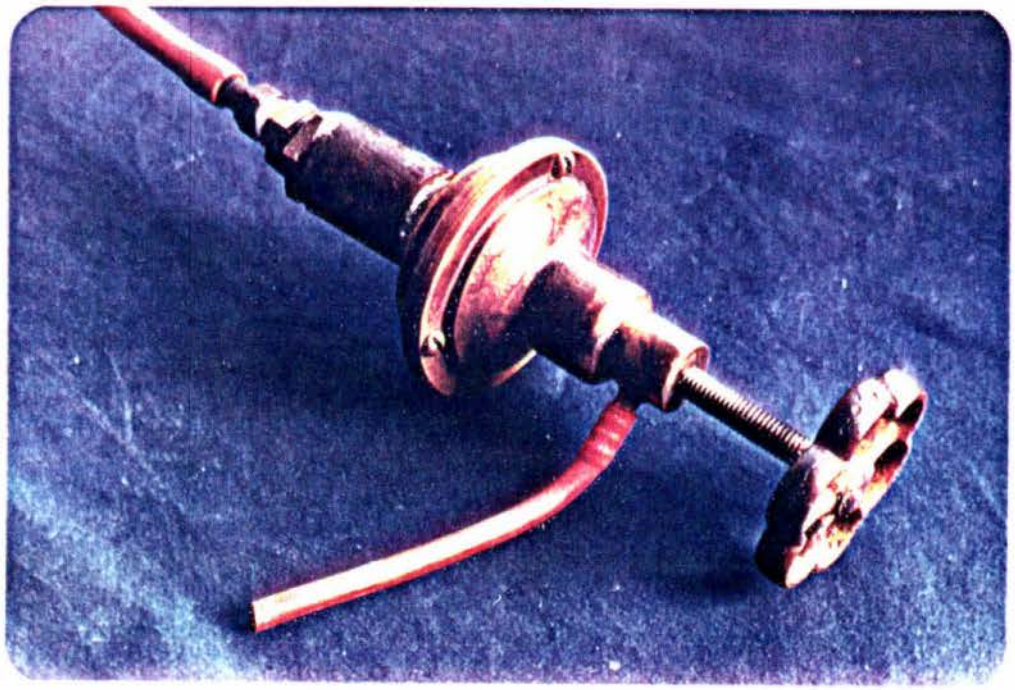
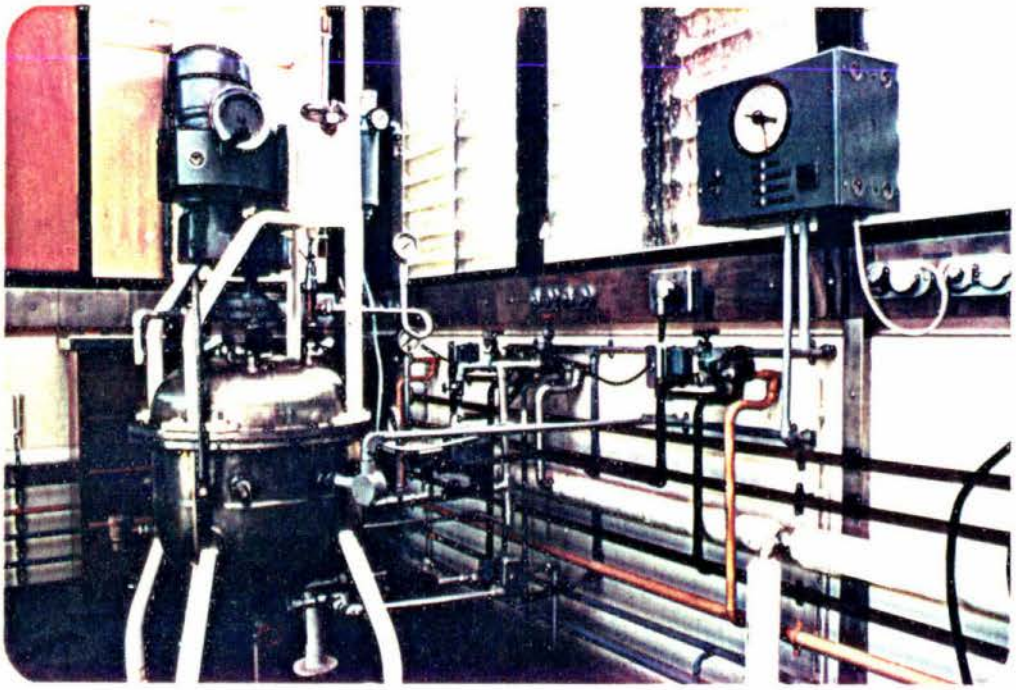
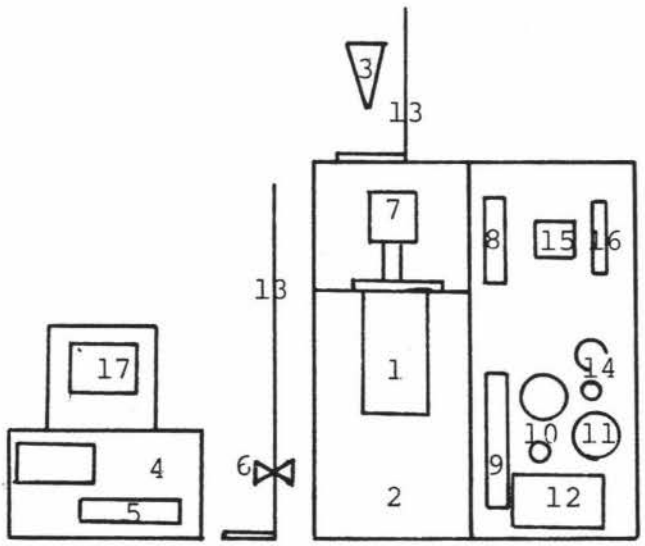


Fig. 2.1c. The Pressure Controller.

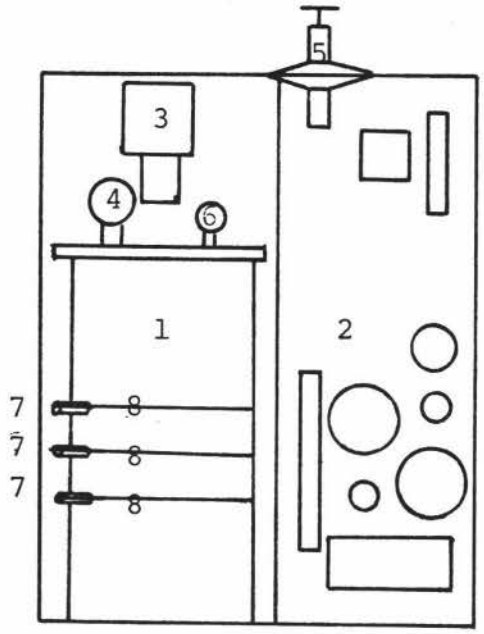
Fig. 2.1d. The 30-litre Fermenter.





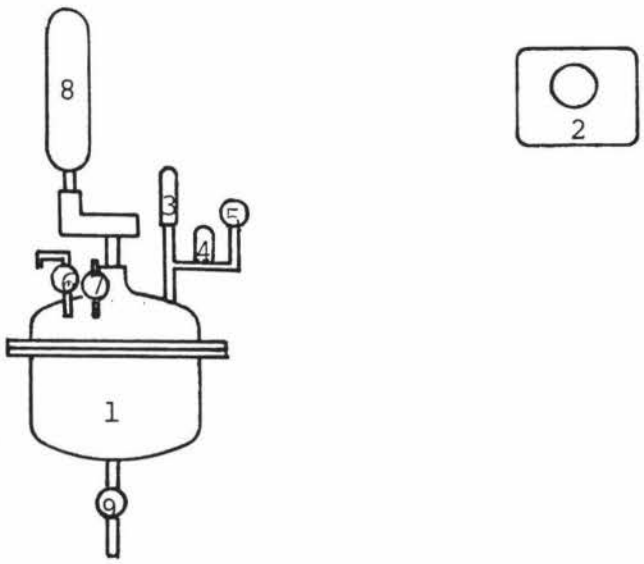
1. 1.5-litre fermentation vessel.
2. Microferm support unit.
3. Dropping funnel for ammonia.
4. pH controller.
5. Cam timer.
6. Solenoid valve for pH control.
7. Agitator drive.
8. Gas filter.
9. Gas flow measurement and control.
10. Agitation control and rev. counter.
11. Temperature control.
12. Main switch, heating, cooling, and agitator switches and indicator lights.
13. Retort stands.
14. Pressure control (not used).
15. Antifoam pump (not used).
16. Antifoam controls (not used).
17. E_c measurement (not used).

Fig 2.2a. Schematic Diagram of 1.5-litre Fermentation Vessel in the Microferm Unit.



1. 17-litre 316 stainless steel pressure fermenter.
2. Microferm unit (see Fig 2.2a.).
3. Drive unit in raised position.
4. Pressure gauge.
5. Pressure control valve.
6. Valve for pressure release.
7. Openings for continuous fermentation (not used).
8. Wire bands (to restrain plugs under pressure).

Fig 2.2b. Schematic Diagram of Microferm Unit and Pressure Fermenter.



1. 30-litre jacketed pressure fermenter.
2. Automatic temperature controller (controls steam,hot water, or cold water as selected).
3. Pressure safety valve.
4. Vacuum relief valve.
5. Pressure gauge.
6. Pressure relief and free-steaming valve.
7. Sampling valve.
8. Motor and variable speed gear box for agitation (used only for sterilisation and cooling).
9. Draining valve.

Fig. 2.2c. Schematic Diagram of 30-litre Industrial Style Fermenter.

were placed onto the Microferm unit, electrodes were inserted, gas flow was started (either sparging or sweeping across the broth surface as required), and temperature control (30°C) and agitation were switched on while the fermenter was still hot (ca. 90°C). The system was then left for ca. 1 hour and allowed to equilibrate to pH 6.5. About 150 ml of inoculum were then poured directly into the fermenter (attempting to retain as much of the inoculum solids in the bottle as possible) and the conditions were set for that particular experiment.

Broth samples were taken as required by blocking off gas outlets and forcing liquid out using gas pressure. About 10 ml were drawn to clear the sample line, and 8-10 ml of sample were taken. A cell count was performed immediately, and the sample was stored at -20°C prior to chemical analysis.

2.5.3 10-litre Fermentations

10-litre nominal volume fermentations were conducted with 10-litres of the required medium in a 17-litre, 316 stainless steel vessel built in the workshop of the Biotechnology Department of Massey University, and capable of holding about 170 kPa. Figs. 2.1(b) and 2.2(b) respectively are a photograph and diagram of the fermenter. The fermenter did not have an agitator. The assembled, vented, fermenter was sterilised by autoclaving, and cooled in cold water maintaining a positive pressure of ca. 50 kPa oxygen-free nitrogen in the headspace to prevent formation of a vacuum and to maintain anaerobic conditions in the fermenter. The fermenter was then placed in the Microferm unit for operation.

Pressure control was achieved using a modified domestic hot water pressure valve vented to atmosphere by bubbling through water. Unfortunately the valve leaked and so gas flow rate measurements were not accurate. The modifica-

tions required to allow adjustment of pressure consisted of providing a tapping for a threaded shaft which pressed onto the rubber diaphragm. Fig. 2.1(c) shows a photograph of the controller.

Samples were taken as required. About 20 ml was drawn to clear the sample lines, followed by ca. 8 ml of sample. If there was insufficient pressure to expel the culture, the sample was drawn into a sealed flask using a slight vacuum.

Cell concentration, pH, and temperature were measured and recorded immediately on sampling, and samples were then stored at -20°C prior to chemical analysis.

2.5.4 30-litre Fermentations

30-litre nominal volume fermentations were conducted in a ca. 50-litre vessel registered for 140 kPa broth pressure and 170 kPa jacket pressure, and manufactured by Burns and Ferrall Limited (Auckland, New Zealand).

Figs. 2.1c and 2.2c respectively are a photograph and a diagram of the fermenter.

Sterilisation was achieved by passing steam through the jacket and controlling the pressure to hold the broth temperature at 120°C for 15 minutes. Cooling was then achieved by passing cold water through the jacket. When the temperature fell below 100°C, oxygen-free nitrogen was introduced to prevent formation of a vacuum and to maintain anaerobic conditions. Subsequent temperature control throughout the fermentation was by means of hot water passing automatically through the jacket when the temperature fell below 30°C. The fermenter was inoculated with 150 ml culture and the gas pressure was allowed to build up to that required.

Sampling and pressure control was as for the 10-litre fermentations.

2.6 ANALYTICAL METHODS

2.6.1 pH Measurement

Routine laboratory pH measurements were performed using a Metrohm Herisau pH-Meter E520 (Switzerland).

2.6.2 Cell Counts

These were performed immediately after sampling using a standard haemocytometer under 400X magnification. Only vegetative cells were counted.

2.6.3 Solvents and Fatty Acids

Solvents and low molecular weight fatty acid concentrations were determined by gas-liquid chromatography (GLC) using a Shimadzu gas chromatograph (GC - 5A). A temperature programming unit (TP - 3A), electrometer (EM - 55), control panel (FVS - 5), basic unit (BAS - 5), and Shimadzu chart recorder were also used. A programmable integrator (Varian CDS 111 (Chromatography Data System) performed the internal standard calculations.

The column (2m x 3mm ID) consisted of 10% FFAP (Free Fatty Acid Phase) on a 100/120 mesh Chromosorb G AWDMCS support.

A nitrogen carrier gas flow rate of 80 ml min^{-1} was used, and the temperature programme held the column at 87°C for 7 minutes, followed by an increase to 180°C at a rate of 20°C min^{-1}. Typical retention times are shown in Table 2.5.

Sample (1 ml) was added to an equal volume of internal standard solution containing 1% (v/v) butan-2-ol and either 20% (v/v) formic acid or 10% (v/v) orthophosphoric acid. The sample was then centrifuged prior to injection (ca. 5 µl).

Table 2.5 TYPICAL GLC RETENTION TIMES

Compound	Retention Time (minutes)
acetone	1.4
ethanol	2.3
butan-2-ol	3.6
butan-1-ol	7.3
acetic Acid	13.4
butyric Acid	15.6

2.6.4 Lactose

A Yellow Springs Sugar Autoanalyser (YSI Model 27) was used for lactose analysis. The samples were centrifuged to remove particulate matter and diluted as appropriate using distilled water prior to analysis.

3. PRODUCTION OF n-BUTANOL FROM WHEY PERMEATE WITH DIFFERENT HEADSPACE PRESSURES

3.1 INTRODUCTION

Maddox (1980) showed sulphuric acid casein whey permeate to be a suitable substrate for the A.B.E. fermentation on a 100 ml scale. The purpose of the work in this chapter was to scale up the fermentation to laboratory scale fermenters and to identify important fermentation variables, in particular the effect of headspace pressure. Spivey (1979) stated that some of the hydrogen gas produced by the organism during fermentation was utilised during the subsequent production of butanol, and that the NCP process was controlled to a minimum pH of pH 5.5. McInerney and Bryant (1982) also mention the effect of the partial pressure of hydrogen, but in relation to methane production. Hence, the effect of headspace pressure and the controlling of pH to a minimum of pH 5.5 on the A.B.E. fermentation were studied. First, the effect of composition of sparging gas and pH control to pH 5.5 on 1.5-litre atmospheric pressure fermentations followed by the effect of headspace gas pressure on a 10-litre scale fermentation was examined. Secondly, the fermentation was attempted in a 30-litre industrial-style pressure fermenter. Due to the work of Maddox (1980), attention was paid almost entirely to the pressure effect and the fermentation itself rather than the effect of substrate, noting only that the permeate with 5 g/l added YE gave healthy fermentation. At no stage was any attempt made to optimise the fermentation with respect to medium, inoculum preparation, or fermentation temperature.

During analysis of the fermentation results it was noticed that the minimum observed pH as well as pressure was well correlated with yield of the butanol. This result was quantified using multiple linear regression. It was then found that use of multiple linear regression to find

correlations between concentrations of species measured at the end of the fermentation structured these metabolites into a system almost identical to the organism's metabolic pathways. This approach, which was named the Factor Correlation Method, appears novel when used to this extent, and could well be useful in future work on metabolic pathway elucidation.

3.2 1.5-litre EXPERIMENTS

3.2.1 Results

The medium used for all fermentations was sulphuric acid casein whey permeate supplemented with 5g/l yeast extract, and adjusted to pH 6.5 using 5N NH_4OH prior to autoclaving. The organism used was Cl. acetobutylicum NCIB 2951. The fermentations were conducted with 1.5-litre of broth as outlined in Section 2.5.2.

Three systems of gas flushing were used in order to vary the level of hydrogen in solution, and pH was either controlled to a minimum of pH 5.5 or not controlled at all. Sparging with oxygen-free nitrogen (200 ml/min) with vigorous agitation (200 rpm) was intended to remove both hydrogen and carbon dioxide from solution and supply the lowest level of dissolved hydrogen (Gas Level A). An intermediate level of hydrogen was provided by sweeping oxygen-free nitrogen (50 ml/min) across the medium surface without agitation, allowing the levels of dissolved hydrogen and CO_2 to be controlled by the composition of the fermentation gases (ca. 1:1 H_2:CO_2 (volume basis)) (Gas Level B). Hydrogen sparging (200 ml/min) with agitation (200 rpm) was used to strip all the CO_2 from solution, but maintain the hydrogen as close to saturation as possible (Gas Level C). This last provided the highest hydrogen concentration.

The results of the fermentations were similar to those shown in Fig. 3.16. (page 83.) for a typical fermentation. It shows the typical fall in pH as acids and cells are produced, followed by a rise in pH and temporary "dip" in the acid levels as solvent production begins. The order in which the experiments were done along with the final butanol and cell concentrations are given in Table 3.1.

Table 3.1 YIELD DATA FOR 1.5-LITRE EXPERIMENTS

pH Control	Species	Units	Gas Levels		
			A	B	C
minimum pH 5.5	Butanol	g/l	-	2.4	0.4,0.7**
	Log_{10}(cells)	log cells	-	8.38	9.5,9.1**
	Chronological Order	-	-	2	4,5**
no control	Butanol	g/l	3.4	5.3	5.3
	Log_{10}(cells)	log cells	8.9	8.2	8.8
	Order	-	6	1	3

*Gas Level A :sparge with oxygen-free nitrogen (200 ml min^{-1})
:agitate (200 rpm)

Gas Level B :sweep oxygen-free nitrogen through headspace (50ml min^{-1})
:no agitation
Gas Level C :sparge with hydrogen (200 ml min^{-1})
:agitate (200 rpm)
** One set of duplicates was run.

3.2.2 Discussion

Consideration of Table 3.1 clearly shows that controlling pH to not less than pH 5.5 reduces the final yield of butanol compared to fermentations without pH control.

It is quite possible that controlling the pH to a lower value would show an improvement (Andersch et al., 1982), but since encouraging yields were obtained without any pH control it was decided to perform all subsequent fermentations at the "natural" pH.

In the case of Gas Level B with pH control the broth pH fluctuated greatly. This fluctuation was caused by lack of mixing in the fermentation broth, and it is likely that some regions in the broth had a pH below pH 5.5. This possibility may well account for the higher yield of butanol in this run (Andersch et al., 1982).

The three runs without pH control suggest that hydrogen does have an effect on the final yield of butanol. Gas Level A showed the lowest butanol yield, while the runs at Gas Levels B and C returned the highest yields. However, if the concentration of dissolved hydrogen were to effect butanol yields, it would be expected that Gas Level C show a yield superior to B. It is possible that the lack of CO_2 at Gas Level C caused this effect. This possibility is discussed further in Section 3.3.3.11 in reference to a 10-litre run with reduced carbon dioxide concentrations, and in Section 7.2. Alternatively, experimental error may be masking a difference. The exact cause could not be conclusively determined. The possibility that an overall time trend produced the variation in butanol yield was discounted due to high yields in both the first and last runs, with the lowest yields ocurring in between (refer Table 3.1).

A possible mechanism by which dissolved hydrogen could

effect the fermentation is via the equilibrium

$$NADH + H^+ \rightleftharpoons NAD^+ + H_2$$

(McInerney and Bryant, 1981) where higher levels of H_2 would act in accordance with Le Chatelier's principle to supply the bacteria with a better supply of NADH. This NADH would then be available for oxidation by the reduction reactions, such as alcohol production.

It was decided that the most convenient method of increasing the concentration of hydrogen in solution would be to trap the fermentation gases (H_2 and CO_2) in the vessel headspace, so that the headspace pressure would build up. This would then cause an increased concentration of hydrogen in solution. There seemed to be no advantage in attempting to create an atmosphere of pure hydrogen and so the natural fermentation gases were used. Thus, a sealed fermentation pressure vessel was constructed of 316 stainless steel for use in subsequent experiments. Pressure control was achieved using a domestic hot water supply pressure relief valve modified to allow variable pressure control.

It is interesting to compare the results of Table 3.1 with those of Maddox (1980) who achieved butanol yields in excess of 10 g/l. His work was done on a 100 ml scale in screw-capped bottles which allowed the headspace pressure to increase during fermentation. It might therefore be postulated that increased headspace pressure caused an increase in the concentration of dissolved hydrogen, and was responsible for the higher butanol yields. Mention of a possible effect of CO_2 is made later in Section 3.3.3.11.

It was thought that the effect of the H^+ ion concentration (i.e. pH), which appears also in the NAD/NADH equilibrium, would be more important in its effect on enzyme and other cellular activity than on the NADH level, the former swamping any possible effect of the last.

3.3 10-litre SCALE EXPERIMENTS

3.3.1 Results

Whey permeate (10-litres) supplemented with 5g/l YE was fermented in a 10-litre stainless steel pressure vessel with temperature controlled to 30°C. The inoculum was 150 ml of Cl. acetobutylicum NCIB 2951 in CM supplemented with 50 g/l lactose. The maximum headspace pressure allowed to accumulate during the fermentation was the only variable altered, with the exception of Run I which had a pressure of 101 kPa pure H_2 from inoculation.

Figs. 3.1 - 3.10 describe the course of the fermentations. The pH after autoclaving started near pH 5.5 and dropped to between pH 4.2 and pH 4.7 as acids and cells were produced. Once solvent production began, the pH rose slightly in spite of an overall increase in acid levels. Only in Run I did the pH show a significant rise after inoculation. Data for times greater than 100 hr. are drawn at 100 hr.

Table 3.2 describes typical microscopical observations of the organism during the fermentations. These observations showed no real variation between different fermentations.

Table 3.3 summarises for each fermentation the maximum pressure (p), the average stationary phase cell concentration (N_s), final butyric acid ($[BA]_f$), acetic acid ($[AA]_f$), butanol ($[B]_f$), acetone ($[A]_f$), and ethanol ($[E]_f$) concentrations, and also the final pH (pH_f) and minimum observed pH (pH_m) for these runs.

Table 3.4 shows variables calculated directly from the experimental values of Table 3.3 and used in the discussion section. Table 3.5 shows some results predicted by correlations of the data in Tables 3.3 and 3.4, and is included here as it gives the quantitative

cont. p. 55/

Fig. 3.1. Fermentation Results for Run A.

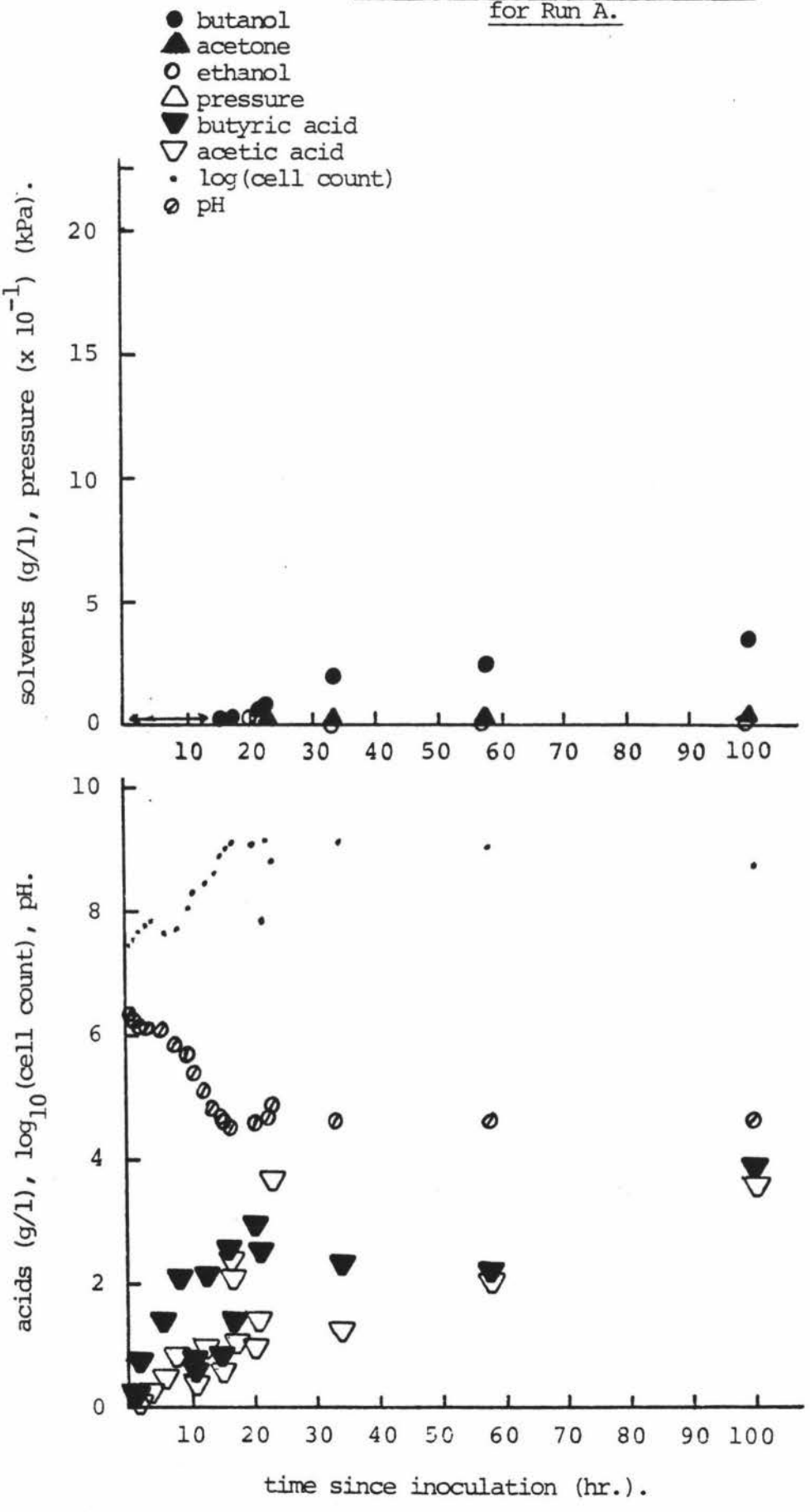


Fig. 3.2. Fermentation Results for Run B.

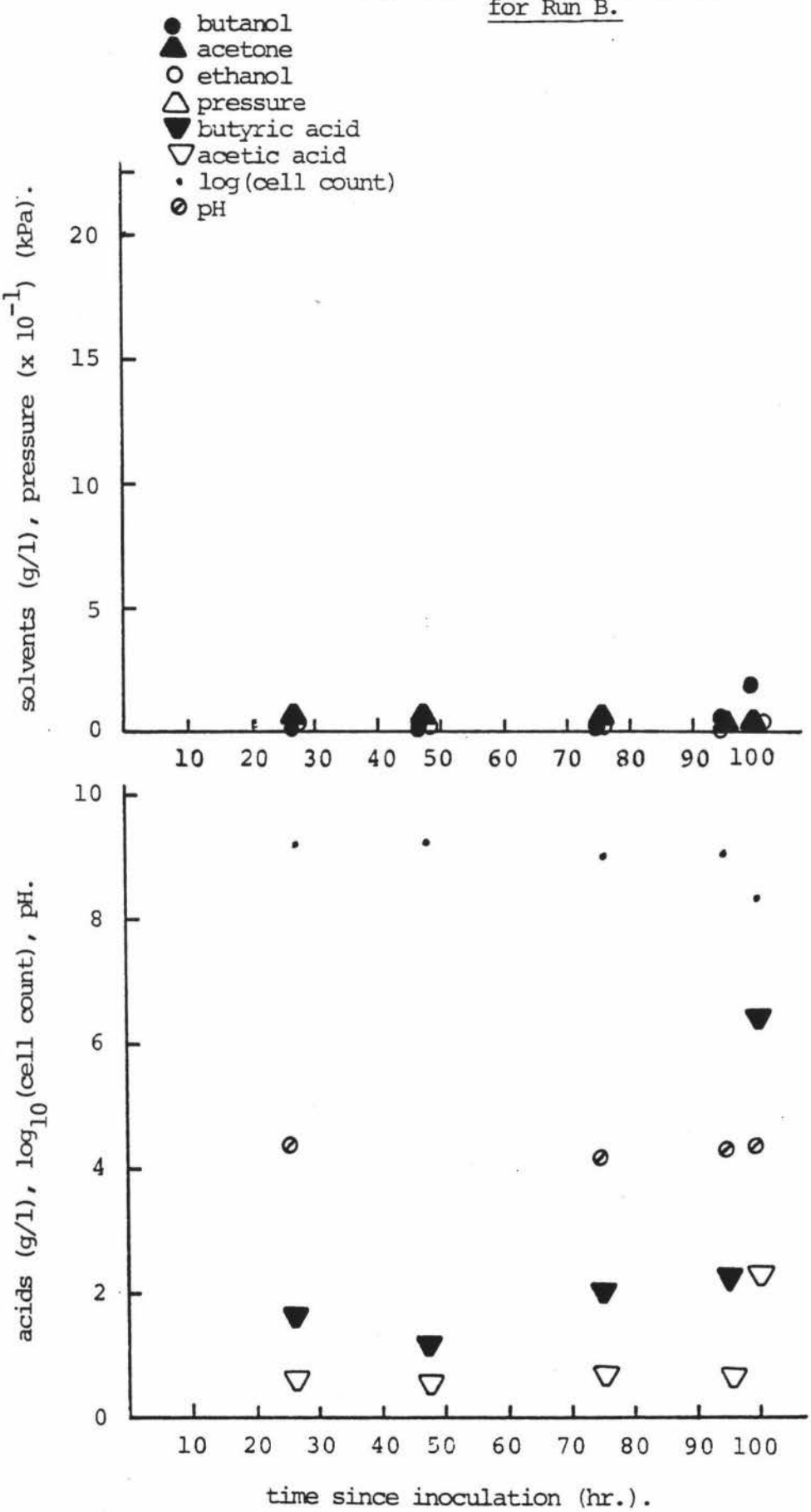


Fig. 3.3. Fermentation Results for Run C.

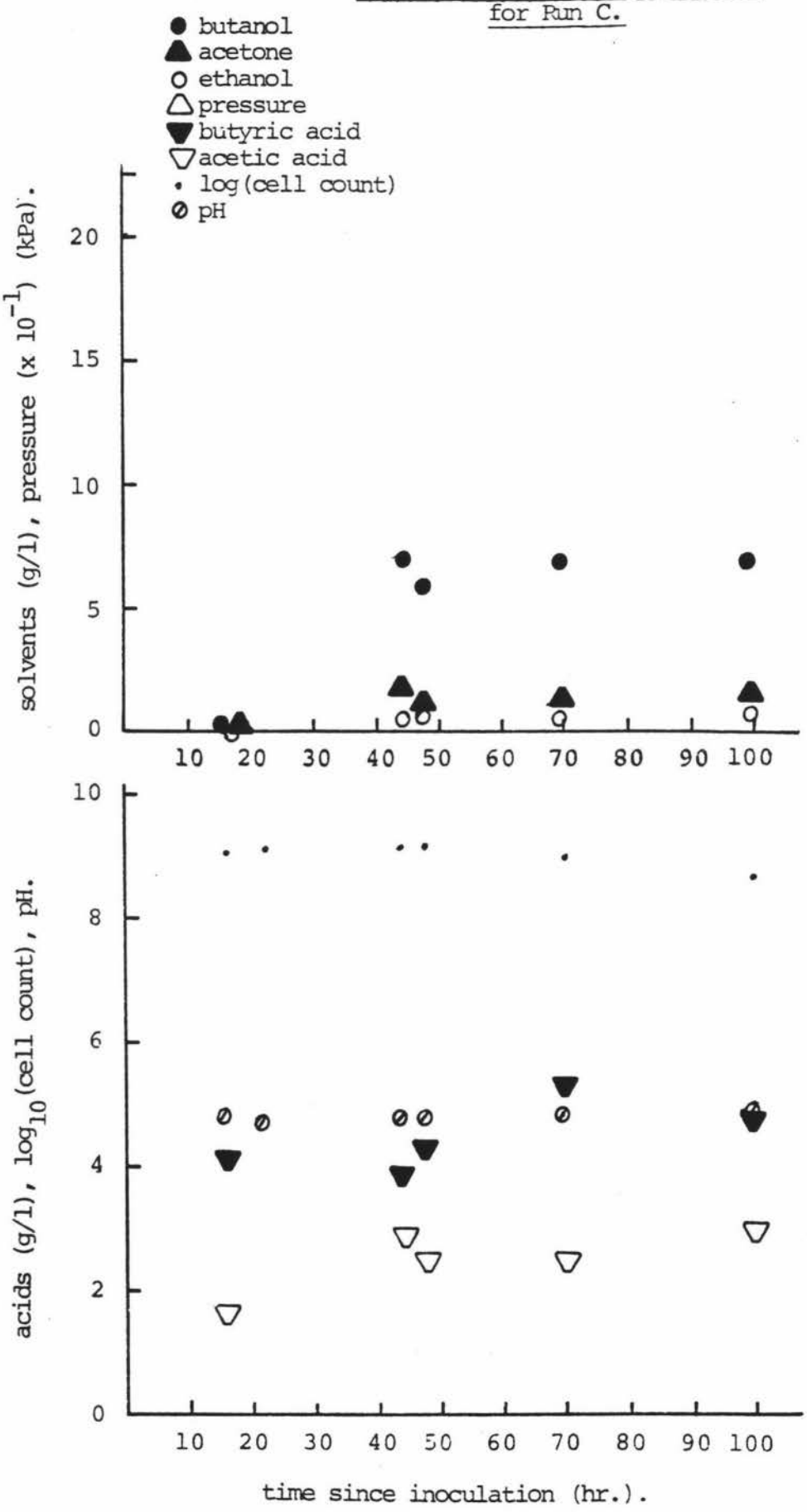


Fig. 3.4. Fermentation Results for Run D.

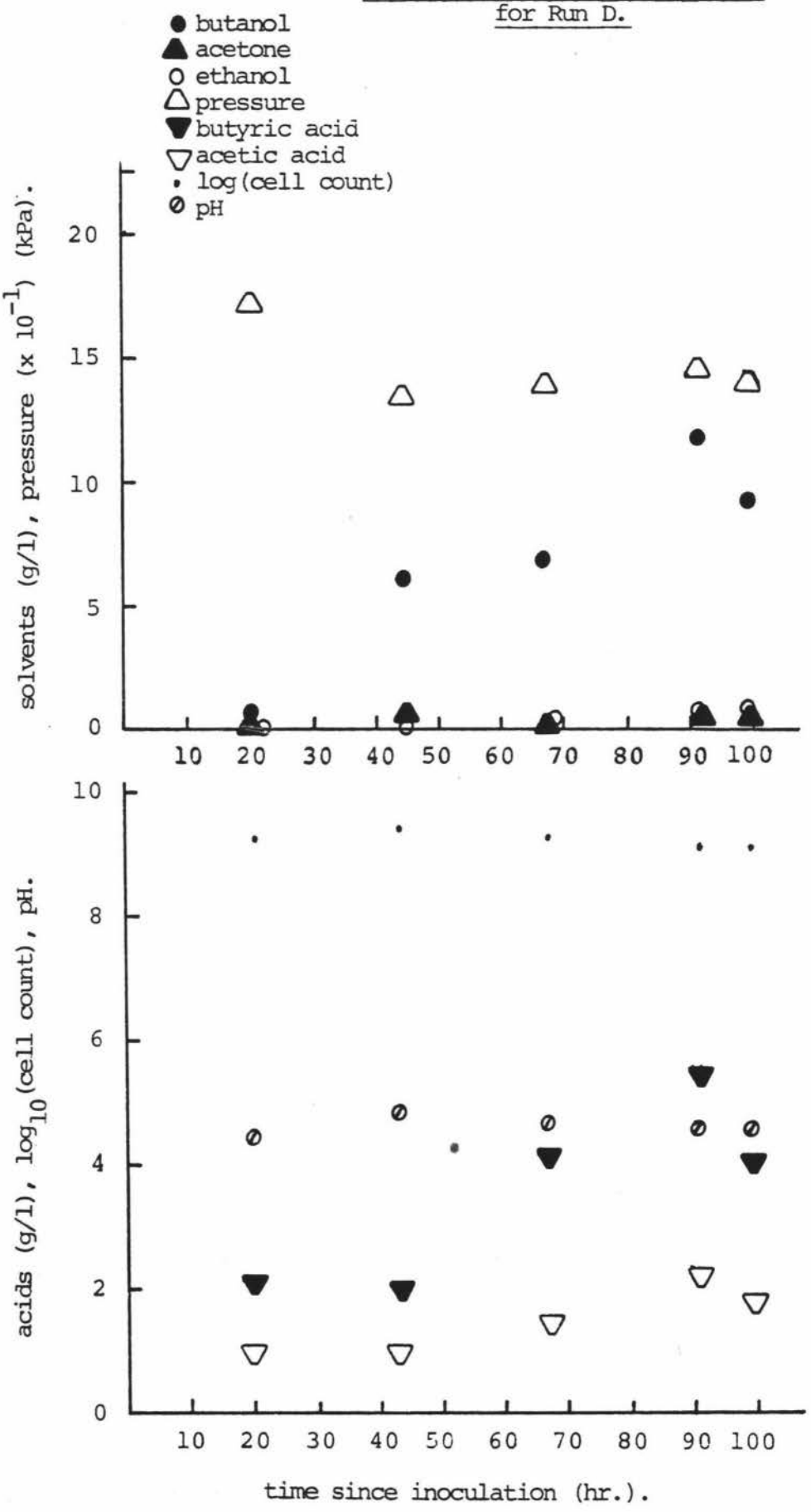


Fig. 3.5. Fermentation Results for Run E.

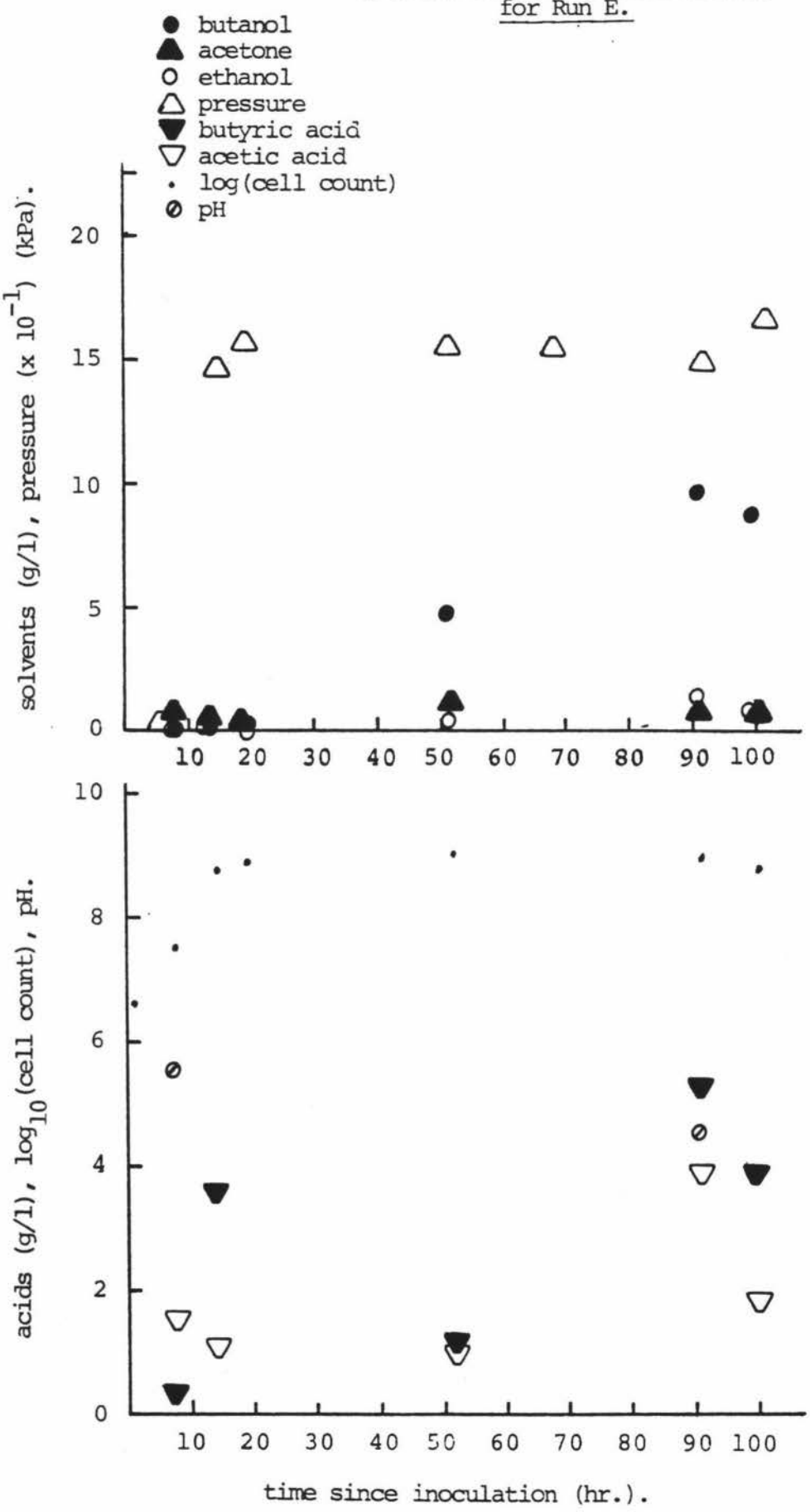


Fig. 3.6. Fermentation Results for Run F.

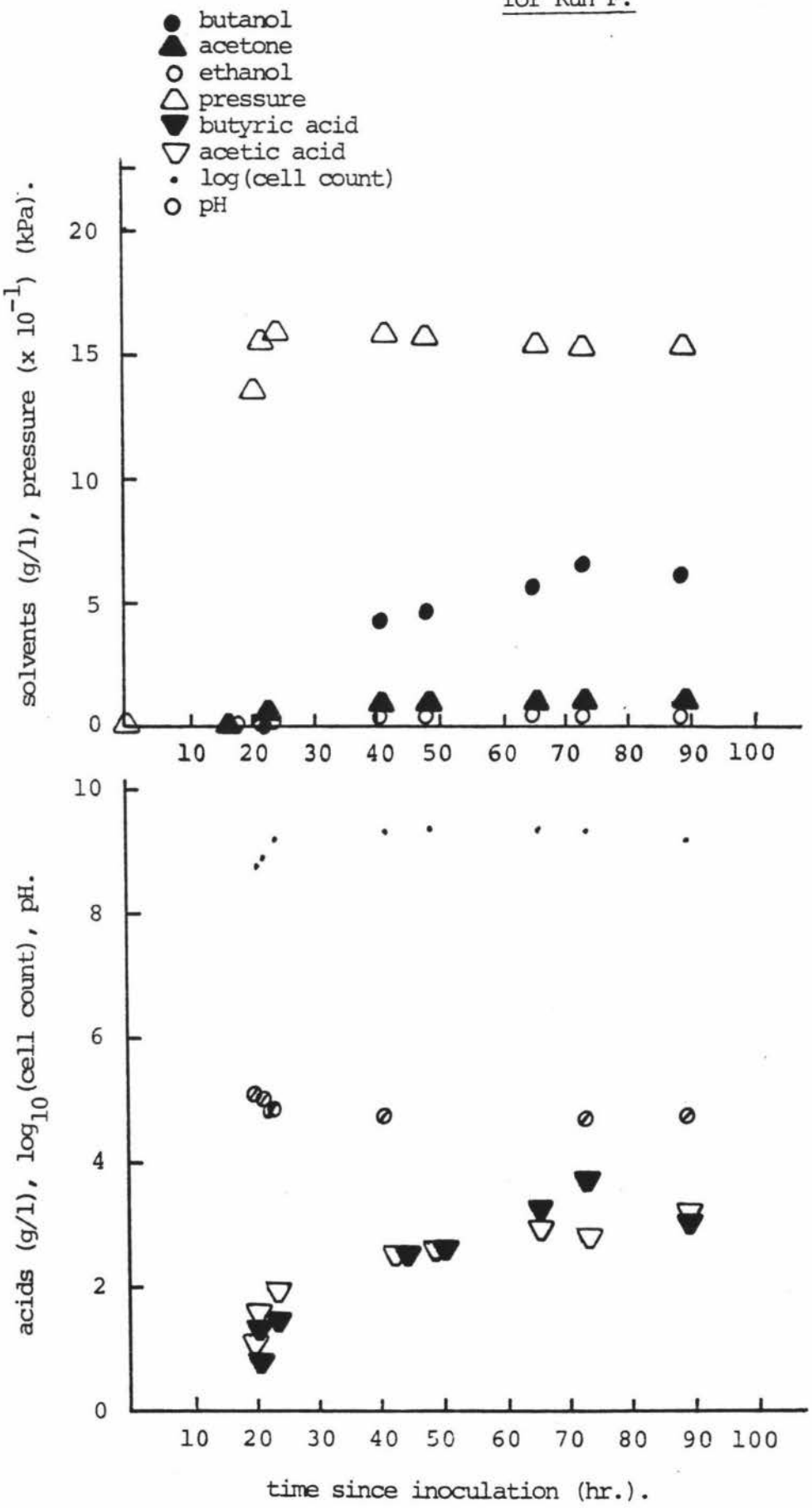


Fig. 3.7. Fermentation Results for Run G.

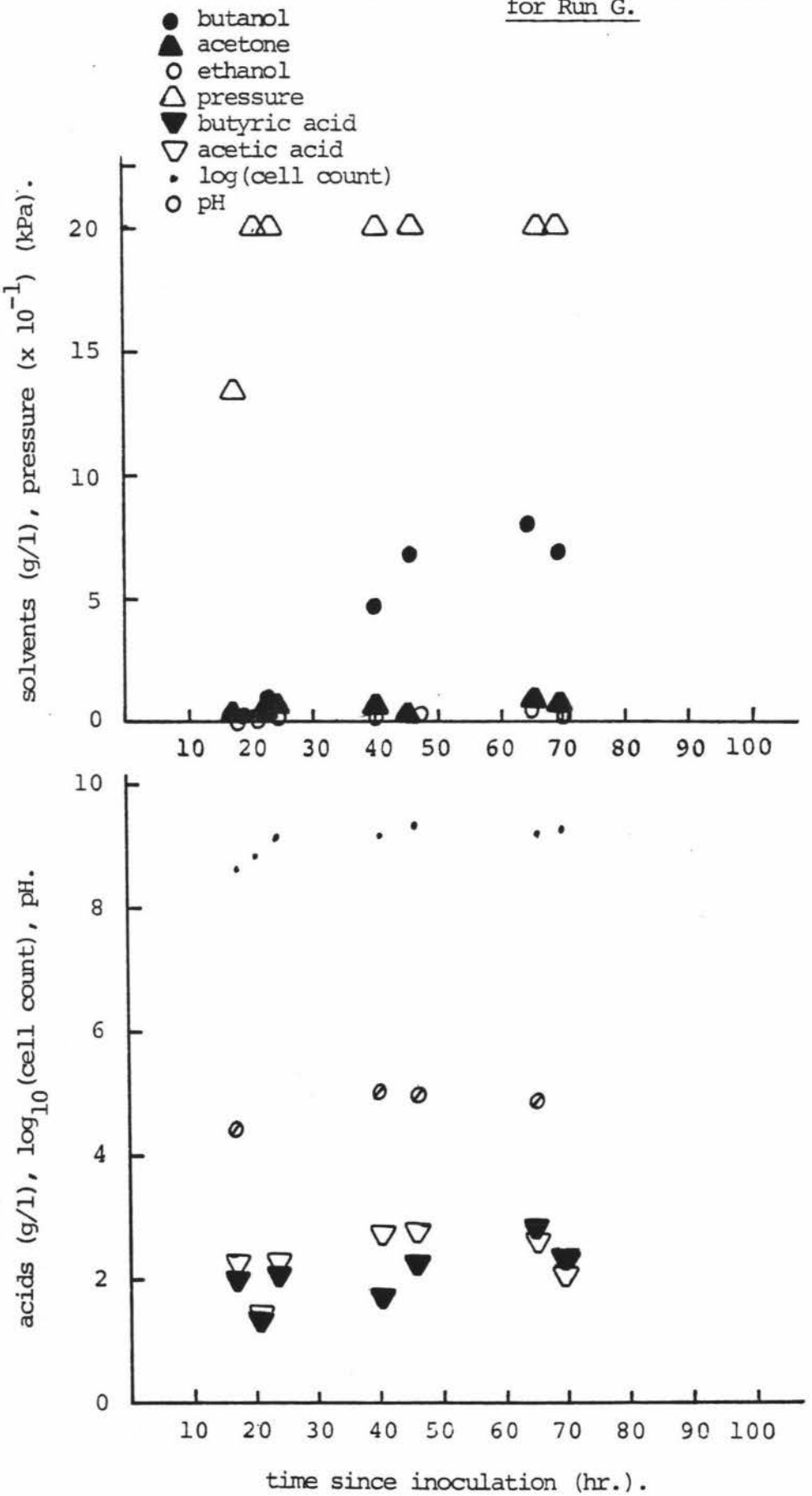


Fig. 3.8. Fermentation Results for Run H.

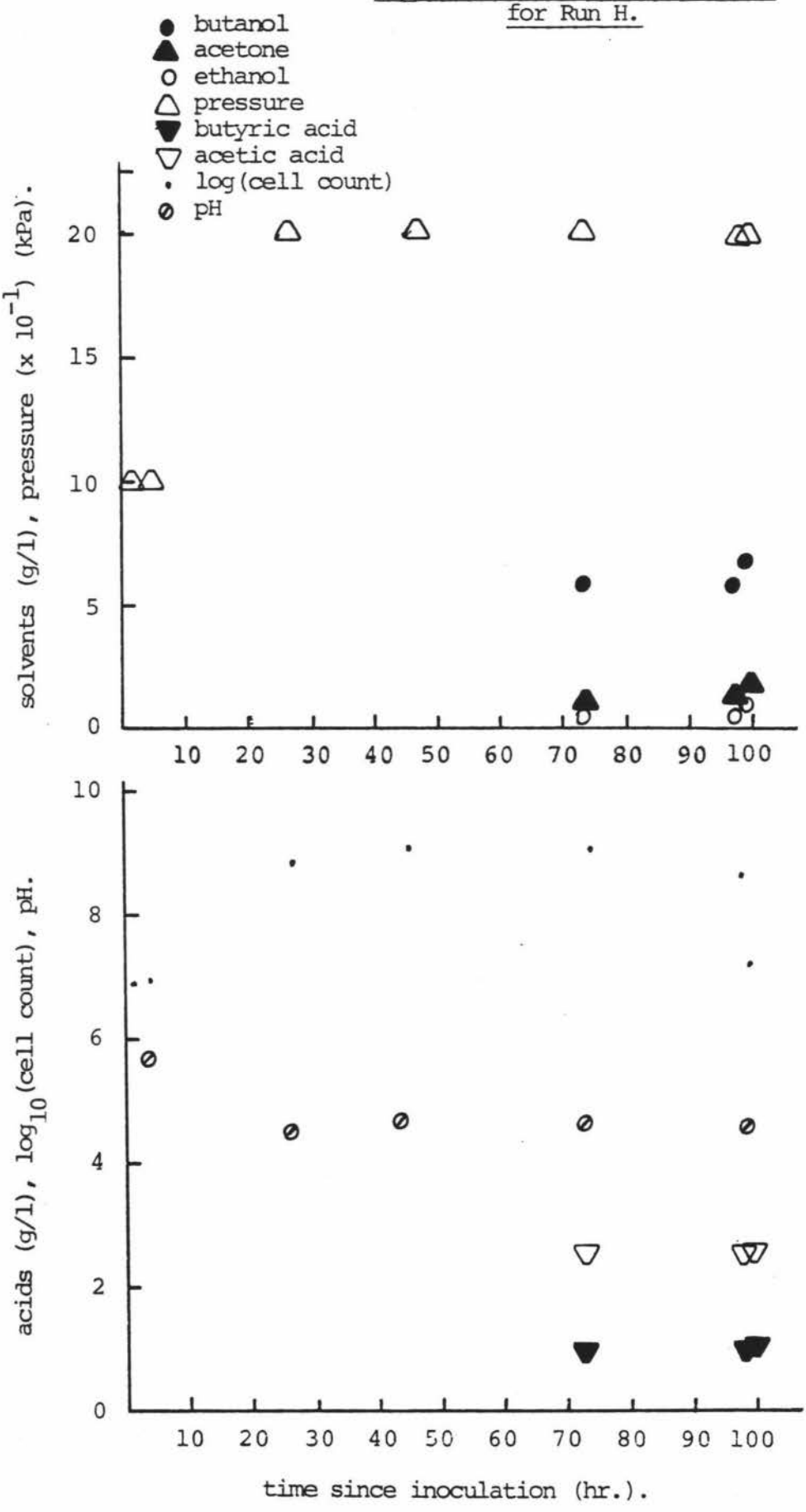


Fig. 3.9. Fermentation Results for Run I.

note: This fermentation had 101kPa pure H_2 applied at inoc.

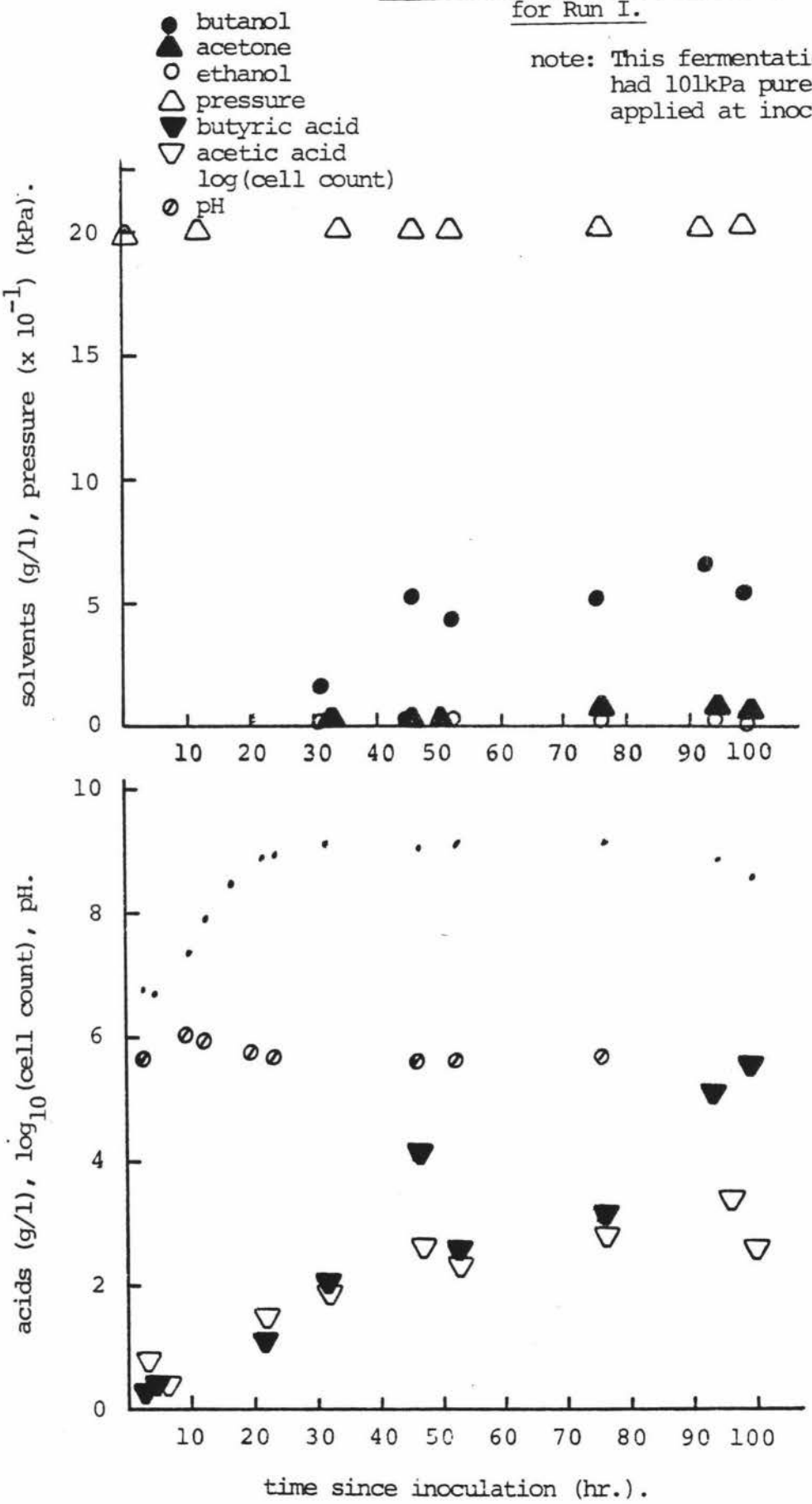


Fig. 3.10. Fermentation Results for Run J.

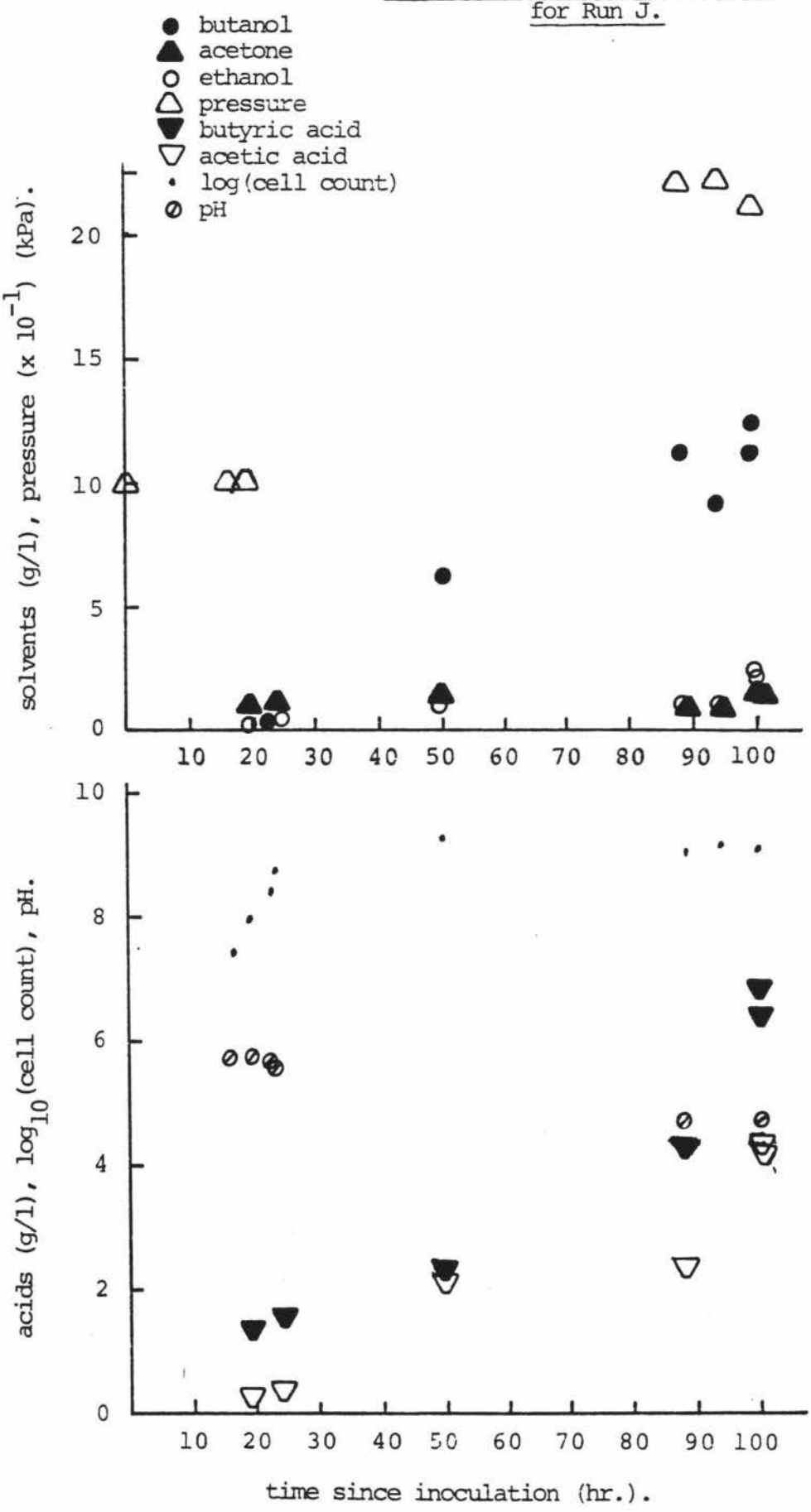


Table 3.2 SUMMARY OF TYPICAL MICROSCOPICAL OBSERVATION OF Cl. ACETOBUTYLICUM NCIB 2951 DURING FERMENTATIONS

Time (hrs)	Length (μm)	Motility
0		
0.25	1.5 - 2	some slightly motile
1.5	0.25 - 0.5	15-20% slightly motile
4	2 - 4*	brownian motion
	ca. 0.1*	extremely motile (ca. 5-10% population)
8	1.5 - 2.5	most motile
10	1.5 - 2.5	all well motile
17	1 - 1.5	all well motile
20	1 - 1.5	less motility
23	1 - 1.5	stationary, wiggling
33	1 - 1.5	almost non-motile
55	1 - 2	non-motile
130	1 - 3	non-motile

* There were two distinctly different populations present. One large and non-motile, the other very small and extremely motile.

Table 3.3 SUMMARY OF 10-LITRE RUNS

Run	p	pH_m	pH_f	N_s	$[B]_f$	$[A]_f$	$[E]_f$	$[BA]_f$	$[AA]_f$
A	101	4.5	4.63	1093	3.40	0.50	0.46	2.41	2.10
B	101	4.2	4.35	1068	0.92	0.27	0.28	-	-
C	101	4.6	4.85	1373	6.75	1.34	0.55	5.21	3.25
D	140	4.6	4.6	1977	10.5	0.45	0.82	4.82	2.62
E	157	-	4.6	994	9.40	0.67	1.11	4.24	2.89
F	160	4.65	4.65	1875	6.15	0.93	0.46	3.37	2.73
G	201	4.45	4.85	1685	7.40	0.67	0.51	2.89	2.73
H	201	4.5	4.65	1110	8.20	1.95	1.05	1.16	2.31
I	207	4.6	4.65	1183	4.60	0.47	0.27	3.66	3.15
J	250	4.65	4.75	1317	11.6	0.99	1.80	5.98	3.57
s.e.	-	-	-	26%	48%	61%	65%	40%	16%

Table 3.4 SUMMARY OF CALCULATED VARIABLES FOR 10-LITRE RUNS

Run	p	$[H^+]_m$ ($\times 10^{-5}$)	$[H^+]_f$ ($\times 10^{-5}$)	log N_s	$[HBA]_f$	$[BA^-]_f$	$[HAA]_f$	$[AA^-]_f$	μ	R' ($\times 10^{-9}$)
A	101	3.16	2.37	9.0386	1.47	0.93	1.20	0.90	0.33	0.091
B	101	6.31	4.47	9.0284	-	-	-	-	-	0.037
C	101	2.51	1.41	9.1377	2.53	2.67	1.44	1.81	-	0.233
D	140	2.51	2.51	9.2960	3.02	1.79	1.53	1.09	-	0.101
E	157	-	2.51	8.9972	2.65	1.57	1.69	1.20	0.28	0.121
F	160	2.24	2.24	9.2730	2.02	1.35	1.52	1.21	0.33	0.101
G	202	3.55	1.41	9.2266	1.40	1.48	1.21	1.52	0.16	0.142
H	202	3.16	2.24	9.0453	0.69	0.46	1.28	1.02	0.18	0.135
I	208	2.51	2.24	9.0730	2.19	1.47	1.75	1.40	0.25	-
J	250	2.23	1.78	9.1196	3.24	2.72	1.78	1.78	0.42	0.137
s.e.	-	-	-	1.2%	39%	46%	15%	25%	33%	43%

Table 3.5 SOME ESTIMATED VALUES
(From Correlations in Table 3.7)

RUN	$[B]_f$ [a]	$[B]_f$ [b]	$[HBA]_f$ [a]	$[HBA]_f$ [b]	$[HAA]_f$ [a]
A	4.8	4.3	1.22	1.19	1.19
B[c]	-	-	-	-	-
C	6.6	5.7	2.06	2.02	1.43
D	8.7	7.3	2.39	2.21	1.48
E[d]	8.6	-	2.93	-	-
F	7.6	8.7	2.35	2.64	1.60
G	7.8	7.4	1.26	1.19	1.19
H	6.5	8.3	1.51	1.70	1.33
I[e]	9.3	10.0	3.15	2.54	1.57
J	12.5	12.3	3.26	3.11	1.74
s.e.e.[f]	15%	23%	23%	27%	5%

Notes:

a estimated from measured data

b estimated from estimated data

c concentrations for Run B could not be estimated as no experimental acid concentrations were available

d concentrations for Run E could not be estimated as no reliable pH_m was available

e Run I had different experimental conditions, the effects of which are emphasised by these estimates

f the standard error of the estimated variable compared with the measured or calculated variable (Tables 2.3a or c) divided by the mean variable and expressed as a percentage (s.e.e.)

(contd. from p.40)

result of the text and correlations of Section 3.3 (see especially Table 3.17). Table 3.5 may be compared directly with Tables 3.3 and 3.4.

Some acid data for Run B were not calculated as acid levels rose so severely late in the fermentation. N_s was evaluated over the apparent stationary phase.

3.3.2 Analysis of Data

3.3.2.1 Method of Data Analysis

In order that any relationships be clearly established, the data of Table 3.3 were correlated using multiple linear regression on the Massey University PRIME 750 computer (PRIME Computer Inc., U.S.A.). The software package used was the Minitab package developed by Pennsylvania State University (1981). The use of quadratic terms for curvature in the relationships was tried, but the term was never significant.

Relationships were established by trying all combinations of variables possible, retaining only those variables where the correlation showed a high t-statistic. For final correlations, only variables significant at greater than the 90% confidence level were retained. Automatic selection of significant variables by the computer software proved unreliable because the measure of significance of a variable depended strongly on the variables present in the correlation due to the non-orthogonality of the data. Careful consideration of anomalous correlations containing different combinations of the same variables clarified problems as shown in section 3.3.3.2.

The use of multiple linear regression on the final broth chemical concentrations in this fashion was named the Factor Correlation Method.

3.3.2.2 Solvent Relationships with Pressure and Minimum pH

Fig. 3.11 shows the relationship between solvent yields and p, while Fig. 3.12 shows the relationship between the solvent yields and pH_m. The results of a multiple linear regression fitting the model

$$[B]_f = a + b\ (\text{pressure}) + c\ (pH_m)$$

are given in Table 3.6.

Results of a similar treatment of data for acetone, ethanol, and total solvents are shown in Table 3.7. The equations fitted were

$$[A]_f = d + e(p) + f\ (pH_m)$$
$$[E]_f = g + h(p) + i\ (pH_m)$$
$$[T]_f = j + k(p) + l\ (pH_m)$$

over the ranges

$$0 \text{ kPa} < p < 250 \text{ kPa} \quad : < = \text{"less than or equal to"}$$
$$4.2 < pH_m < 4.65$$

These results, summarised by Table 3.8, show the effect of p and pH_m on the different solvents. $[T]_f$ is of course very strongly influenced by $[B]_f$, and so their regression coefficients and levels of significance will also be closely related. This can be seen in the tables. The regressions were performed on the data for the eight runs A, B, C, D, F, G, H, and J. Run E was not used as no reliable pH_m value was available, and Run I was not used as the fermentation conditions were different.

Table 3.8 shows acetone is affected by neither pressure nor pH_m, hence the correlation for acetone should be written:

contd. p. 62/

Fig. 3.11. Solvent Concentration vs. Pressure.

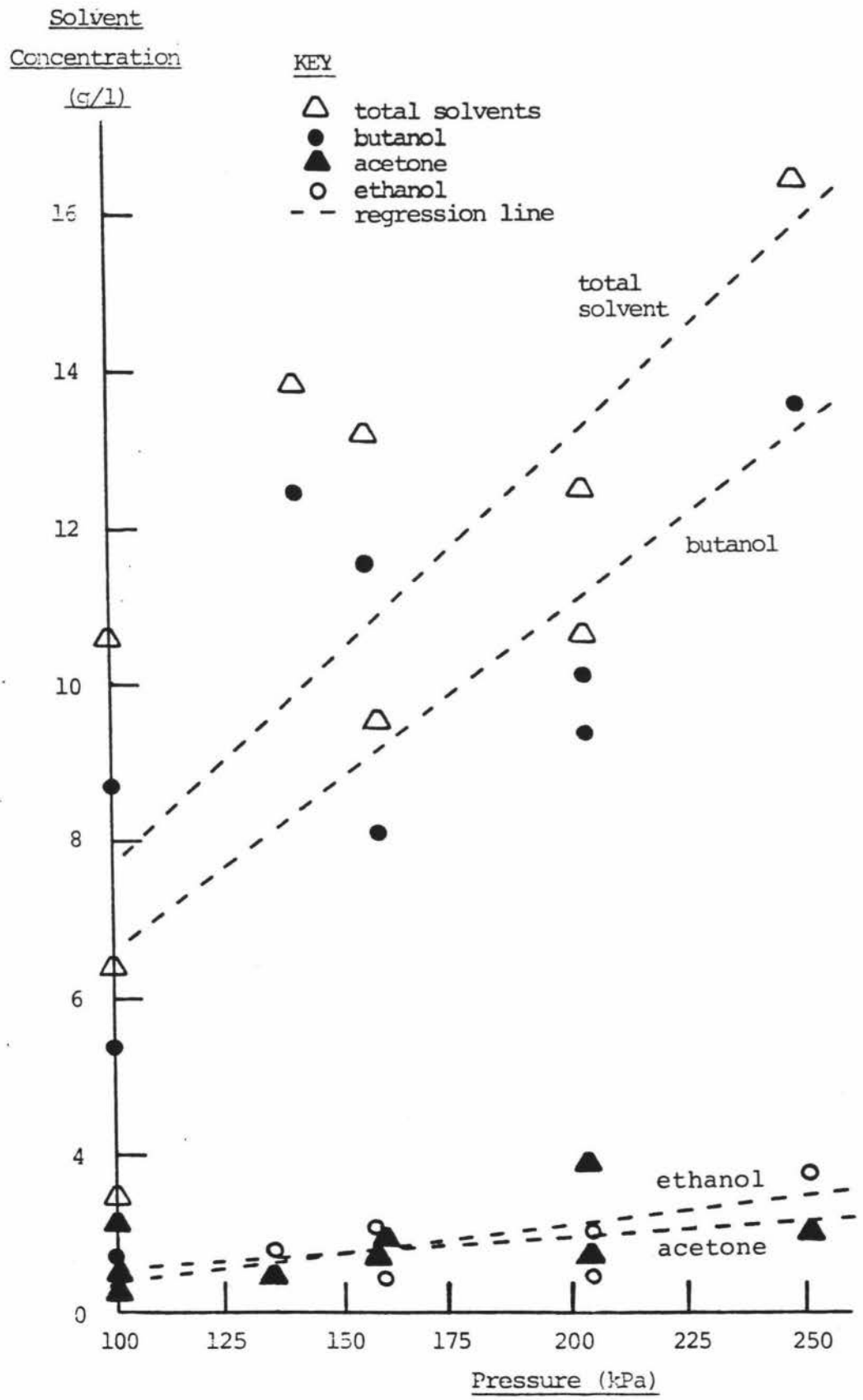


Fig.3.12. Solvent Concentration vs. Minimum pH.

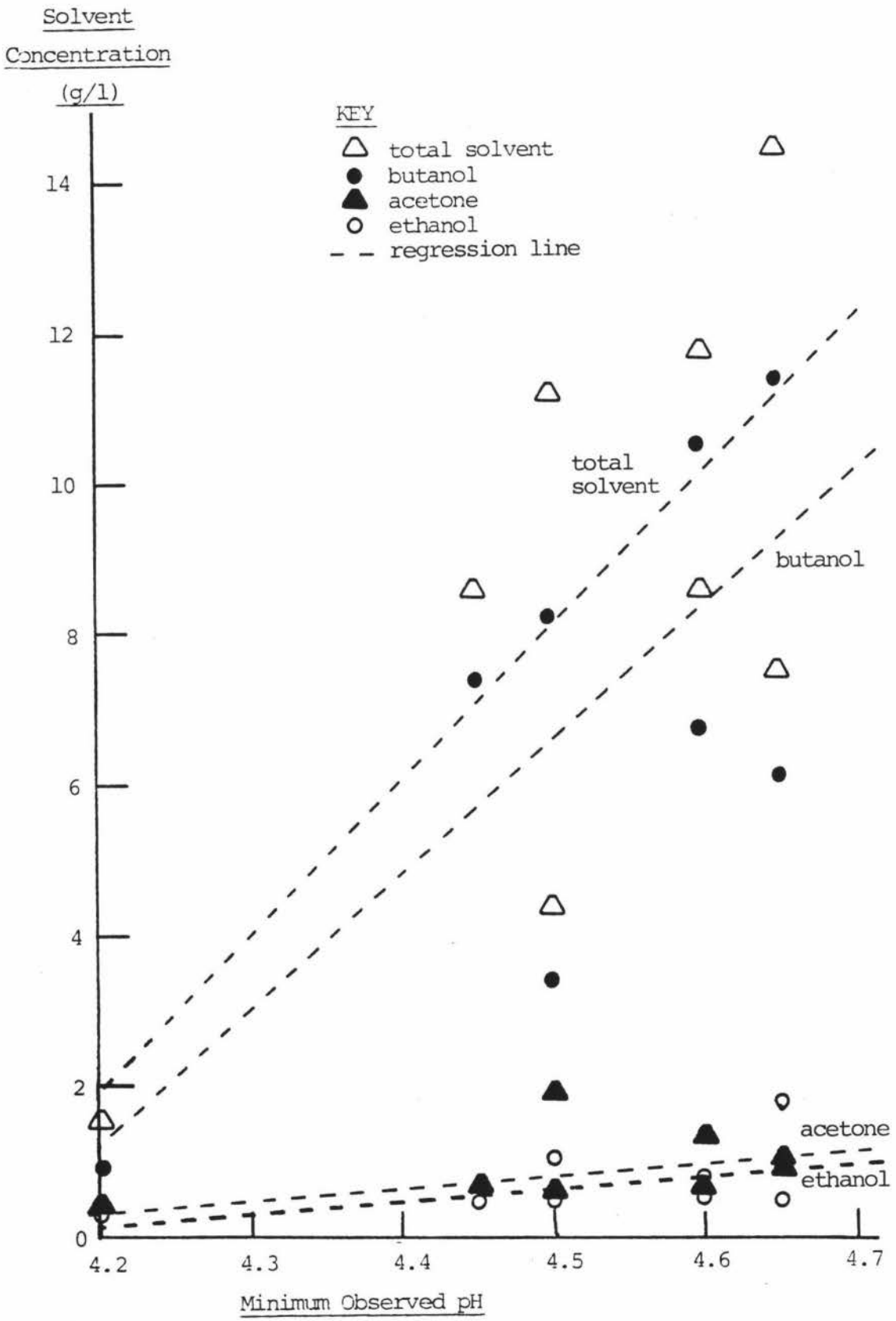


Table 3.6 RESULTS OF MULTIPLE LINEAR REGRESSION (MLR) OF FINAL BUTANOL CONCENTRATION (B_f) WITH HEADSPACE PRESSURE (p) AND MINIMUM BROTH pH (pH_m)

Variable*	Value	t-ratio**
a	-57.28	-2.51
b	0.0314	2.35
c	13.09	2.52

*a = intercept term
b = pressure coefficient
c = pH_m coefficient

**Confidence limits of t for 5 degrees of freedom are:

$t_{.90} = 2.015$
$t_{.95} = 2.571$

Table 3.7 RESULTS OF MULTIPLE LINEAR REGRESSION ON FINAL ACETONE CONCENTRATION (A_f), FINAL ETHANOL CONCENTRATION (E_f), AND FINAL TOTAL SOLVENT CONCENTRATION (T_f).

Solvent	Variable	Value	t-ratio*
acetone	d	- 4.18	-0.61
($[A]_f$)	e	0.00281	0.69
	f	1.022	0.65
ethanol	g	- 3.884	-0.97
($[E]_f$)	h	0.00591	2.51
	i	0.8164	0.90
total solvent	j	-65.34	-2.60
($[T]_f$)	k	0.04013	2.72
	l	14.93	2.61

* $t_{.90} = 2.015$
$t_{.95} = 2.571$

Note: all correlations have 5 degrees of freedom.

Table 3.8 SUMMARY OF SOLVENT RELATIONSHIPS WITH HEADSPACE PRESSURE AND MINIMUM BROTH pH

Solvent	Pressure	pH_m
n-butanol	*	*
acetone	x	x
ethanol	*	x
total	**	**

x probably unrelated

* related at 90% confidence level or better

** related at 95% confidence level or better

(contd. from p. 56)

$$[A]_f = 0.86 \text{ g/l} \pm 60\%$$

for these runs.

Table 3.8 also shows that ethanol is affected only by p and not by pH_m, and so a new correlation for $[E]_f$ may be made

$$[E]_f = 0.00684\ p - 0.2908$$

i.e., g = -0.2908, h = 0.00684, i = 0 (cf Table 3.7).

with r = 0.757 (7 d.f)
and $r_{98\%}$ = 0.750 (7 d.f) (Eaton Tables, 1974)

(where r is Pearson's Product Moment Correlation Coefficient). Run E has been included in this correlation as pH_m data is not required.

The butanol concentration is given by

$$[B]_f = -57.3 + 0.03142\ p + 13.1\ pH_m$$

and total solvent by

$$[T]_f = [B]_f + [A]_f + [E]_f$$
$$= -65.34 + 0.04013\ p + 14.93\ pH_m$$

(obtained by summing the above relationships).

3.3.2.3 Solvent Relationships with Acids, Pressure, Final pH and Minimum pH

In order that a more complete understanding of the concentration relationships could be constructed, each variable was regressed with all other variables in Table 3.3. It was then found that certain variables other than p and pH_m explained the observed variation in solvent yields better.

For example, the variation in $[B]_f$ is better explained by variation in $[BA]_f$ and p rather than pH_m and p. The overall picture developed initially using experimental data was as shown in Fig. 3.13. However, the final pH (pH_f) seemed to have an effect, but at worse than the 90% confidence level. In an effort to further understand the effect of pH_f, pH_f was combined with the experimentally measured values of $[BA]_f$ and $[AA]_f$ to calculate four new variables; $[HBA]_f$, $[BA^-]_f$, $[HAA]_f$, and $[AA^-]_f$. It was assumed that the H^+ ion was the only cation of importance, and that the following relationships held.

$$K_{BA} = \frac{[H^+]_f [BA^-]_f}{[HBA]_f} \quad -(1)$$

$$[BA]_f = [BA^-]_f + [HBA]_f \quad -(2)$$

$$K_{AA} = \frac{[H^+]_f [AA^-]_f}{[HAA]_f} \quad -(3)$$

$$[AA]_f = [AA^-]_f + [HAA]_f \quad -(4)$$

$$pH_f = -\log_{10}[H^+]_f$$

where K_{BA} = dissociation constant for butyric acid in water

$= 1.484 \times 10^{-5}$ M

$= 1.484 \times 10^{-5} \, Mr_{HBA}/(Mr_{BA} - Mr_{H^+})$

$= 1.484 \times 10^{-5} \times 88.10/(87.1 \times 1.0)$

$= 1.501 \times 10^{-5}$ g/l

K_{AA} = dissociation constant for acetic acid in water

$= 1.750 \times 10^{-5}$ M

$= 1.780 \times 10^{-5}$ g/l

Mr = relative molecular weight (g/mol).

The temperature was 30°C. The results of these calcul-

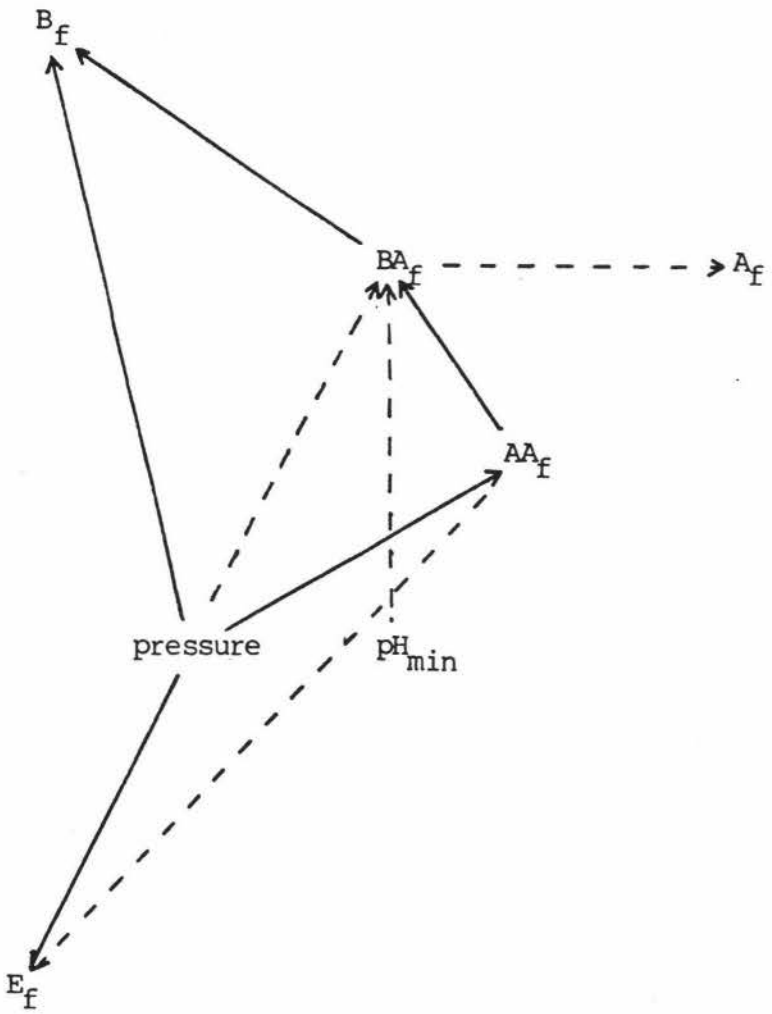


Fig 3.13 POSSIBLE CONCENTRATION INTERRELATIONSHIPS

——— Probable relationships

– – – Possible relationships

ations can be seen in Table 3.4. The regression procedure was then repeated. The resulting correlations using the undissociated acid concentration instead of total acid concentrations showed improved correlations, and it was interesting to notice that in regressions where undissociated and dissociated forms were present together, the dissociated form frequently dropped out but the undissociated form remained. Tables 3.9 - 3.16 show some important correlations for $[B]_f$, $[HBA]_f$, $[HAA]_f$, $[E]_f$, $[A]_f$, N_s, and R'. The specific growth rate, μ, was calculated for the exponential growth phase from a graph of ln N versus time. The maximum specific rate of butanol production, R', was estimated as the maximum observed rate of production divided by the stationary phase cell population (N_s). These tables give some of the trial and error correlations attempted before arriving at a correlation containing only significant variables. The t-statistic in conjunction with the degrees of freedom gives a measure of the degree of importance of the corresponding variable. As an approximate rule of thumb, any variable with a t-statistic in excess of 2.0 is likely to be significant at greater than the 90% confidence level for any degree of freedom (df) except df = 1.

3.3.2.4 Summary of Correlations

A summary of the final correlations selected from Tables 3.9 - 3.16 is given in Table 3.17, and accurate confidence levels of the t-statistic at different degrees of freedom in Table 3.18. Fig. 3.14 shows these relationships diagrammatically. In brief, then, it seems that the stationary cell population is related only to the two alcohol concentrations, increasing with butanol and decreasing with ethanol. In particular the stationary cell population seems best related to the ratio of the alcohols. Ethanol is related to pressure and undissociated acetic acid concentration, while butanol is related to pressure and undissociated butyric acid. The undissociated butyric acid concentration is in turn related to the undissociated

contd. p. 79/

Table 3.9 CORRELATIONS FOR FINAL BUTANOL CONCENTRATION (B_f)

Related Variable	Coefficient	t-Statistic	Degrees of Freedom
const	6.574	1.93	5
P	0.0311	2.29	
$[H^+]_m$	-144573	-2.47	
const	-0.388	-0.15	5
P	0.0303	2.48	
$[BA]_f$	0.876	2.16	
const	-3.58	-0.33	2
P	0.0317	2.00	
$[H^+]_m$	70904	0.25	
$[HBA]_f$	3.385	0.77	
$[BA]_f$	-0.787	-0.37	
const	-1.023	-0.42	4
P	0.0317	2.76	
$[HBA]_f$	2.399	1.95	
$[BA^-]_f$	-0.873	-0.62	
const	-2.278	-0.38	4
P	0.0289	1.98	
$[HBA]_f$	1.426	0.92	
$[HAA]_f$	1.630	0.24	
const	-0.958	-0.42	5
P	0.0310	2.89	
$[HBA]_f$	1.762	2.75	
no const	-	-	
P	0.0275	4.32	6
$[HBA]_f$	1.605	3.32	

Table 3.10 CORRELATIONS FOR THE UNDISSOCIATED BUTYRIC ACID CONCENTRATION (HBA)

Related Variable	Coefficient	t-Statistic	Degrees of Freedom
const	2.287	0.66	3
P	-0.00264	-0.48	
$[H^+]_m$	-84456	-1.19	
$[AA]_f$	0.9224	1.18	
const	-18.03	-3.71	3
P	-0.0150	-4.08	
$[H^+]_m$	255846	3.10	
$[HAA]_f$	10.889	4.96	
const	5.8409	3.27	4
P	0.0004714	0.09	
$[H^+]_m$	-139693	-2.49	
const	-2.0283	-0.32	4
$[H^+]_m$	-20832	-0.20	
$[HAA]_f$	3.272	1.27	
const	-2.662	-2.13	5
P	-0.004724	-1.32	
$[HAA]_f$	3.834	4.32	
const	-2.907	-2.22	6
$[HAA]_f$	3.4566	3.88	

Table 3.11 CORRELATIONS FOR THE UNDISSOCIATED ACETIC ACID CONCENTRATION ($[HAA]_f$) AND TOTAL ACETIC ACID ($[AA]_f$)

Related Variable	Coefficient	t-Statistic	Degrees of Freedom
*const	5.853	10.79	3
P	0.00210	1.84	
$[H^+]_m$	-69727	-5.63	
$[H^+]_f$	-76378	-5.32	
**const	2.253	9.24	3
P	0.001383	2.71	
$[H^+]_m$	-36622	-6.59	
$[H^+]_f$	-2302	-0.36	
**const	2.192	14.14	4
P	0.00142	3.22	
$[H^+]_m$	-36325	-7.48	

* correlation for $[AA]_f$

** correlation for $[HAA]_f$

Table 3.12 CORRELATIONS FOR THE FINAL ACETONE CONCENTRATION ($[A]_f$)

Related Variable	Coefficient	t-Statistic	Degrees of Freedom
const	0.5071	1.10	6
$[B]_f$	0.02373	0.24	
$[E]_f$	0.2387	0.34	
const	0.888	0.88	2
P	0.002631	0.66	
$[H^+]_m$	-13010	-0.76	
const	0.523	0.23	3
$[H^+]_m$	-45469	-0.86	
$[BA]_f$	-0.5274	-2.18	
$[AA]_f$	1.326	1.87	
const	-1.098	-0.90	5
$[BA]_f$	-0.4331	-2.31	
$[AA]_f$	1.321	2.09	
const	-	-	6
$[BA]_f$	-0.3228	-2.30	
$[AA]_f$	0.7813	3.87	
const	-0.244	-0.16	4
$[H^+]_f$	-44770	-1.18	
$[HBA]_f$	-0.7684	-2.12	
$[HAA]_f$	2.566	1.70	
const	-0.624	-0.39	5
$[HBA]_f$	-0.6652	-1.82	
$[HAA]_f$	2.044	1.36	
const	0.8633	(std. error = ± 60%)	8

Table 3.13 CORRELATIONS FOR THE FINAL ETHANOL CONCENTRATION ($[E]_f$)

Related Variable	Coefficient	t-Statistic	Degrees of Freedom
const	0.0371	0.06	5
P	0.006	2.49	
$[H^+]_m$	-7699	-0.74	
const	-6.718	-1.23	3
P	0.001606	0.39	
$[H^+]_m$	88474	0.96	
$[HAA]_f$	3.378	1.37	
const	-1.662	-1.54	4
P	0.00467	1.77	
$[HBA]_f$	-0.018	-0.06	
$[HAA]_f$	1.2164	1.01	
const	-8.432	-2.94	4
$[H^+]_m$	118094	2.51	
$[HAA]_f$	4.194	3.63	
const	-0.2744	-0.76	7
P	0.00667	3.07	
const	1.2987	2.87	6
$[H^+]_m$	-17365	-1.32	
const	-1.371	-1.50	6
$[HAA]_f$	1.521	2.45	
const	-1.6142	-2.31	5
P	0.00476	2.34	
$[HAA]_f$	1.147	2.31	

Table 3.14 CORRELATIONS FOR THE STATIONARY PHASE CELL POPULATION (N_S)

Related Variable	Coefficient	t-Statistic	Degrees of Freedom
**const	9.1360	54.56	3
$[B]_f$	0.04109	2.64	
$[A]_f$	-0.06876	-1.39	
$[E]_f$	-0.22830	-2.66	
$[H^+]_m$	-1314	-0.45	
**const	8.977	91.50	5
P	0.000835	0.97	
$[B]_f$	0.03896	2.55	
$[E]_f$	-0.3310	-2.77	
**const	9.0447	132.04	6
$[B]_f$	0.04225	2.85	
$[E]_f$	-0.2782	-2.64	
*const	$1{,}112 \times 10^6$	5.06	6
$[B]_f$	142×10^6	2.98	
$[E]_f$	-942×10^6	-2.78	
**const	8.9	130.91	7
$[B]_f/[E]_f$	0.02388	3.60	
*const	6.38×10^8	2.80	7
$[B]_f/[E]_f$	7.82×10^7	3.52	

* N_S used for correlation.

** log N_S used for correlation.

Table 3.15 CORRELATIONS INCLUDING THE SPECIFIC GROWTH RATE (μ)

Variable	Related Variable	Coefficient	t-Statistic	Degrees of Freedom
μ	const	0.3912	1.09	2
	$[HBA]_f$	0.0622	1.05	
	$[H^+]_m$	-7562	-0.80	
$[BA]_f$	const	-0.1118	-0.07	4
	μ	12.188	2.14	
$[HBA]_f$	const	-2.892	-1.30	3
	R'	18.84	1.33	
	μ	8.856	2.97	
B_f	const	6.304	1.52	4
	μ	4.90	0.35	
μ	const	0.2855	1.62	4
	P	-1.22×10^{-5}	-0.01	
$[HBA]_f$	const	-0.1280	-0.15	4
	μ	7.209	2.43	
$[HBA]_f$	const	-2.599	-2.31	3
	μ	2.862	1.11	
	$[HAA]_f$	2.5584	2.55	
μ	const	0.7181	3.99	3
	$[H^+]_m$	-15124	-2.45	
μ	const	-4.071	-2.23	3
	pH_m	0.9571	2.39	

Table 3.16 CORRELATIONS INCLUDING
THE SPECIFIC RATE OF BUTANOL PRODUCTION (R′)

Variable	Related Variable	Coefficient	t-Statistic	Degrees of Freedom
$[A]_f$	const	0.1228	0.32	7
	R′	6.061	2.05	
$[AA^-]_f$	const	0.5374	1.86	6
	R′	5.868	2.82	
pH_f	const	4.35411	59.20	7
	R′	2.4900	4.46	
R′	const	-0.7001	-0.83	4
	$[A]_f$	0.03109	1.37	
	$[AA^-]_f$	0.05035	0.86	
	pH_f	0.1570	0.81	
R′	const	-1.0818	-3.38	6
	pH_f	0.2532	3.60	
	$[A]_f$	0.0284	1.38	
R′	const	-0.8722	0.96	5
	$[AA^-]_f$	0.0461	0.73	
	pH_f	0.2010	0.97	
R′	const	-0.01639	-0.37	5
	$[A]_f$	0.03380	1.55	
	$[AA^-]_f$	0.0892	2.84	
B_f	const	3.667	1.28	7
	R′	28.48	1.31	

contd./

Table 3.16 (contd) CORRELATIONS INCLUDING THE SPECIFIC RATE OF BUTANOL PRODUCTION (R′)

Variable	Related Variable	Coefficient	t-Statistic	Degrees of Freedom
$[HBA]_f$	const	1.338	5.29	6
	R′	0.0067	0.00	
R′	const	0.08298	5.73	3
	μ	-0.08579	-2.69	
	p	2.475×10^{-4}	5.75	
$[H^+]_f$	const	4.06×10^{-5}	8.22	7
	R′	-1.42×10^{-4}	-3.79	
$[BA]_f$	const	-4.810	-3.68	3
	μ	5.962	2.10	
	$[AA]_f$	2.337	4.32	
$[HBA]_f$	const	0.593	2.95	5
	R′	-4.816	-3.46	
	$[BA]_f$	0.57851	14.84	
$[HBA]_f$	const	1.009	0.89	2
	R′	-7.341	-0.98	
	μ	-0.665	-0.29	
	$[BA]_f$	0.5936	4.73	
B_f	const	-13.217	-2.55	3
	R′	133.75	4.08	
	μ	16.531	2.36	

Table 3.17 CORRELATION SUMMARY TABLE

Dependent Variable	Related Variable	Coefficient	t-statistic	d.f.
$\log N_s$	const	8.9	130.91	7
	$[B]_f/[E]_f$	0.02388	3.60	
N_s	const	$1{,}112 \times 10^6$	5.06	6
	$[B]_f$	142×10^6	2.98	
	$[E]_f$	-942×10^5	-2.78	
$[B]_f$	const	-	-	6
	p	0.0275	4.32	
	$[HBA]_f$	1.605	3.32	
$[A]_f$	const	$0.86 \pm 60\%$*	(9 values)	
$[A]_f$	const	-	-	6
	$[BA]_f$	-0.323	-2.30	
	$[AA]_f$	0.781	3.87	
$[E]_f$	const	-1.6142	-2.31	5
	p	0.004759	2.34	
	$[HAA]_f$	1.147	2.31	
$[HBA]_f$	const	-2.907	-2.22	6
	$[HAA]_f$	3.457	3.88	
$[HAA]_f$	const	2.192	14.14	4
	p	0.00142	3.22	
	$[H^+]_m$	-36327	-7.48	

contd./

* standard error (approx. one standard deviation)

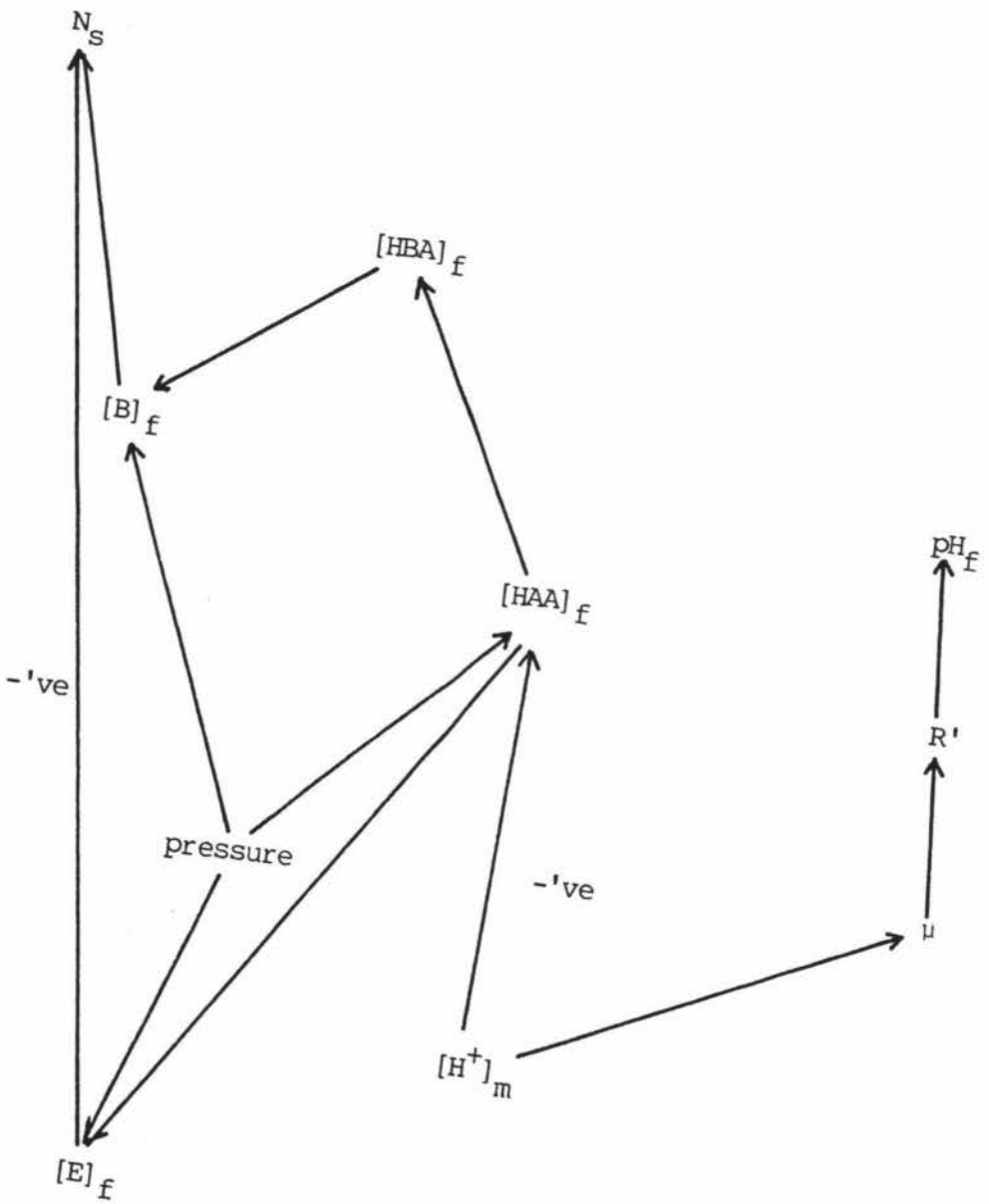
Table 3.17 CORRELATION SUMMARY TABLE (contd)

Dependent Variable	Related Variable	Coefficient	t-statistic	d.f.
pH_f	const	4.35411	59.20	7
	R′	2.4900	4.46	
R′	const	0.08298	5.73	3
	μ	-0.08579	-2.69	
	p	2.475×10^{-4}	5.75	
μ	const	0.7181	3.99	3
	$[H^+]_m$	-15124	-2.45	

Table 3.18 LEVELS OF SIGNIFICANCE FOR THE t-STATISTIC
(QUINN, 1974)

d.f.	Level of Probability		
	90%	95%	99%
1	6.314	12.71	63.66
2	2.920	4.303	9.925
3	2.353	3.182	5.841
4	2.132	2.776	4.604
5	2.015	2.571	4.032
6	1.943	2.447	3.707
7	1.895	2.365	3.499
8	1.860	2.306	3.355
9	1.833	2.262	3.250
10	1.812	2.228	3.169

Fig 3.14 POSSIBLE CONCENTRATION INTERRELATIONSHIPS*



*The independent variables were pressure and $[H^+]_m$.

acetic acid concentration and probably not to the pressure or minimum pH (refer sect. 3.3.3.2). Undissociated acetic acid is in turn related to pressure and minimum pH. Acetone, however, appears best related to the total acid concentrations (as opposed to undissociated acid concentrations), increasing with butyric acid and decreasing with acetic acid.

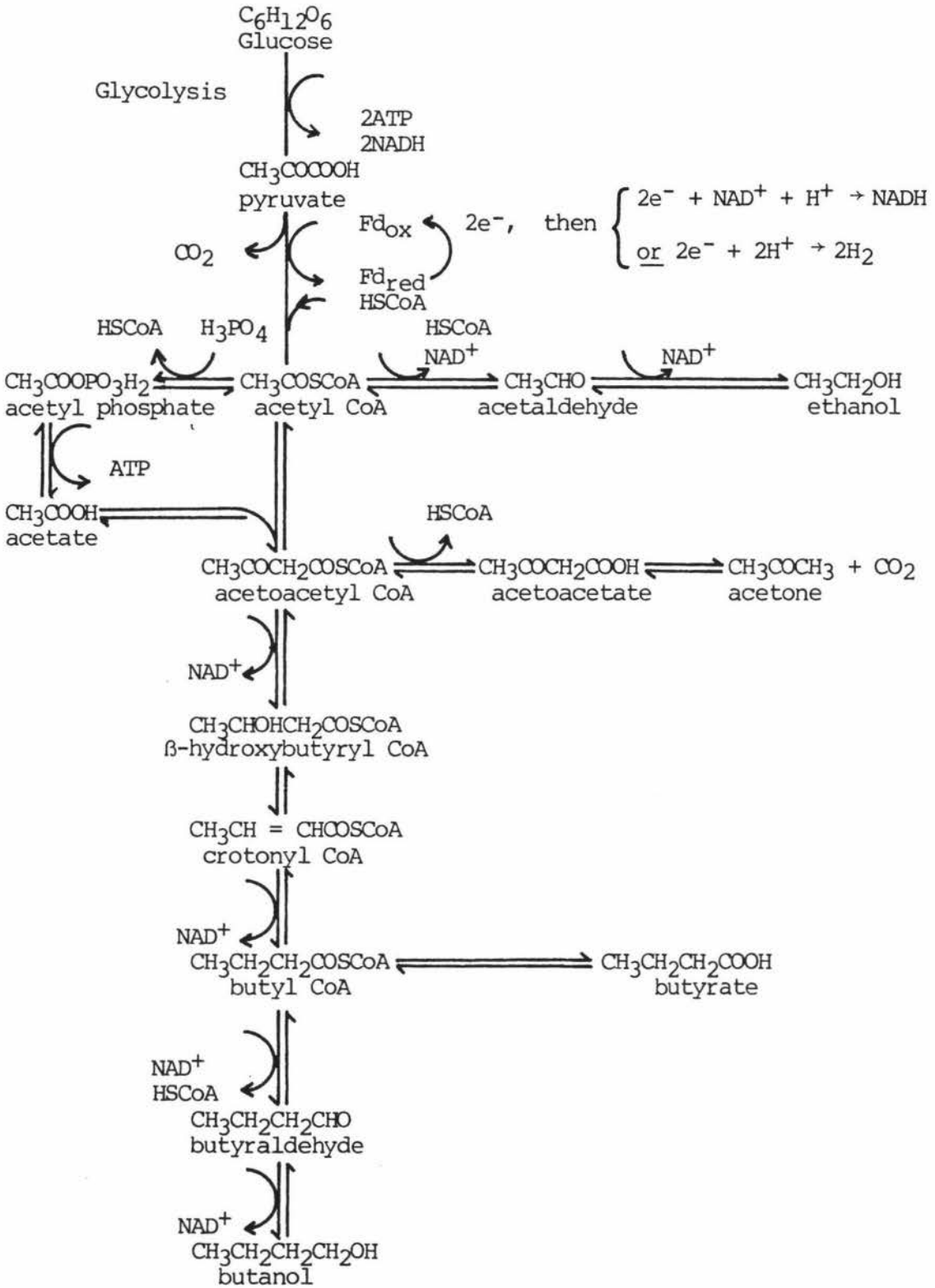
It appears that pH_f is dependent on R', which is dependent on μ and p, and μ is dependent on pH_m. These last correlations can be seen in Tables 3.15 and 3.16.

3.3.3 Discussion of Correlation Results

3.3.3.1 Overview

Confidence in the general validity of the relationships presented in Table 3.17 and Fig. 3.14 can be gained by their comparison with the basic metabolic pathways of the organism. Fig. 3.15 shows the metabolism in much more detail than presented earlier (Fig. 1.6) (Rhodes and Fletcher, 1966; Ross, 1962; Thauer et al., 1977). Butanol is made from butyl coenzyme A (butyl CoA) which in turn is made from acetyl CoA. Ethanol and acetone are made from acetyl CoA also.

Hence, not only do the t-statistics in Tables 3.17 and 3.18 suggest that the correlations are strong (with almost all correlations at 95% or 99% confidence levels), but the results resemble the known biochemistry to a large extent (Fig. 3.15). The only correlations showing less than 95% confidence are those for acetone and ethanol (at the 90% confidence level). This may be ascribed in part perhaps to the low concentrations of these solvents in the broth relative to butanol.



Also: $NADH + H^+ \rightleftharpoons NAD^+ + 2H$

$2H \rightarrow H_2$

and Fd is ferredoxin

Fig 3.15 DETAILED BIOCHEMISTRY

Discussion of each correlation in Table 3.17 and Fig. 3.14 with its suggested metabolic cause follows in the next few sections.

3.3.3.2 Anomalies in the Correlations

It is quite noticeable that when $[HAA]_f$ is included in a correlation, then both P and $[H^+]_m$ also appear to be important. In the case of $[HBA]_f$ correlations (Table 3.10) neither p nor pH_m show great significance unless both are present. In the case of $[E]_f$ correlations (Table 3.13) the opposite is true - in a correlation including $[HAA]_f$, either p or $[H^+]_m$ will give a significant correlation, but <u>not</u> if both are present together.

The source of these perplexities most probably lies in the very strong correlation of $[HAA]_f$ with both p and $[H^+]_m$ (Table 3.11). The final correlations for $[E]_f$ and $[HBA]_f$ (Table 3.17) were made on the basis of correlations involving only p and $[H^+]_m$ <u>without</u> $[HAA]_f$ in the correlation. Such correlations for $[HBA]_f$ suggest $[H^+]_m$ is the more important of the two, however it drops out when $[HAA]_f$ is used and so neither p nor $[H^+]_m$ was finally included (Tables 3.10 and 3.17). When $[E]_f$ is considered in this manner, p appears as the more important variable and is still significant when $[HAA]_f$ is included in the correlation. It is not surprising from a metabolic view point that $[H^+]_m$ has no importance in the $[E]_f$ correlation as ethanol reaches its maximum concentration before pH_m occurs (Tables 3.13 and 3.17).

The correlations for R' also show some results which are difficult to understand, but this also appears due to the nature of the data.

Further discussion of these results in metabolic terms can be found later.

Another possible cause of a misleading correlation is the effect of coincidence due to random causes alone. Of the 16 variables which appear significant, it is quite possible one is due only to random coincidence. If the confidence level of each are all multiplied together, then the result will be to give an overestimate of the probability that none are due to random coincidence, W, alone:

$$
\begin{aligned}
W = \; & 0.99 \times 0.95 \times 0.95 \times 0.99 \times 0.95 \times 0.9 \\
& \times 0.99 \times 0.9 \times 0.9 \times 0.99 \times 0.95 \times 0.99 \\
& \times 0.99 \times 0.9 \times 0.95 \times 0.9 \\
= \; & 0.99^{6} \times 0.95^{5} \times 0.9^{5} \\
= \; & 0.941 \times 0.774 \times 0.59 \\
= \; & 0.43
\end{aligned}
$$

(from Tables 3.17 and 3.18). This means that there is only a 43% chance that <u>all</u> the correlations in Table 3.17 are a true representation of real effects. Hence, while confidence in any particular correlation may be strong, there is a better than even chance that one of the "related" variables identified is in fact unrelated.

3.3.3.3 The Typical Fermentation

Fig. 3.16 shows a typical tracing against time expected for an experiment run near one atmosphere gauge pressure.

Cell count readings during the first five hours after inoculation usually show a rise then a fall (data for Run A is given in Table 3.19). This effect is due to settling of the cells in the fermenter past the sample point and perhaps to a short lag period. No attempt at agitation was made at any stage during fermentation until after final sampling. If Table 3.2 is considered in conjunction with Fig. 3.16, then the first five hours may be considered a period when the very small cells (ca. 0.1 μm) observed after 4 hours are multiplying to become a

Fig. 3.16. A Typical Fermentation at 1 atm. (ga.) Pressure.

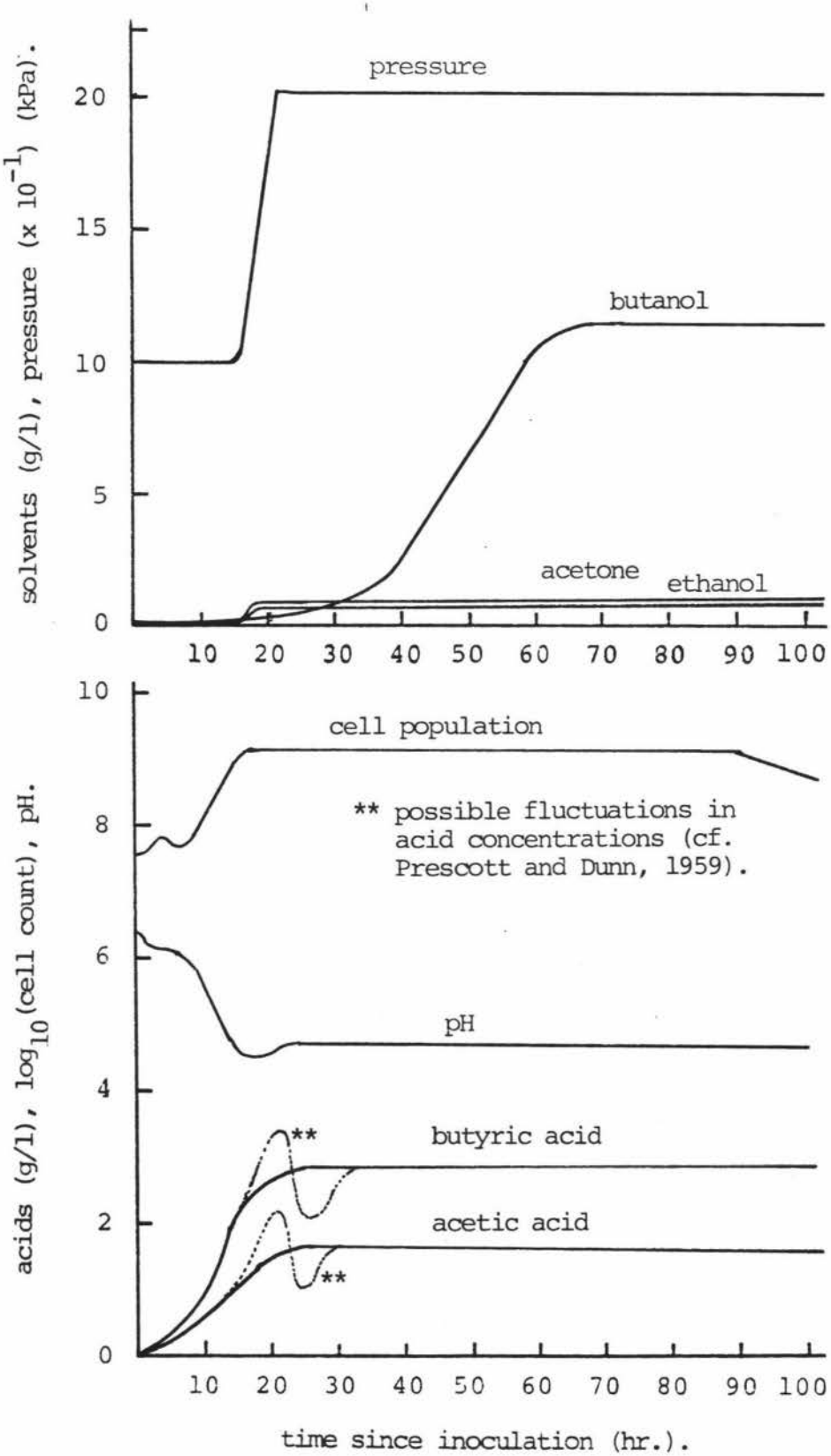


Table 3.19 CELL POPULATION DATA FOR RUN A

Time (hrs)	Cell Count (10^6 cells/ml)
0.42	29
1.25	45
2.5	48
3.8	60
5.2	45
7.7	59
9.2	108
9.9	195
10.5	210
12.0	407
13.6	480
14.6	767
15.3	900
15.8	973
16.5	1,287
19.5	1,113
20.4	873
21.3	1,320
22.5	697
33.5	1,330
57.8	1,033
130.3	560
	(ag.) 713*

*The 130 hour sample was agitated and on recounting was found to increase about 30%.

significant proportion of the population, finally becoming more numerous than the large (ca. 1-2 μm) almost non-motile cells. For this reason, and the lack of agitation (which would be required to make the broth homogeneous and the sample representative), data taken in the first five hours should be used only with great care. Once motility started and gassing began (as indicated by a rise in pressure after about 5 hours), the broth was assumed to be well mixed and the samples representative.

Throughout the exponential growth phase (ca. 5 - ca. 25 hrs) the cells were motile and about 1-2 μm in length. Early in this phase the acid concentration rose rapidly and the pH dropped. When solvent production began (usually all three solvents almost simultaneously), both of the acid concentrations appeared to drop. This dip is not particularly noticeable in Figs. 3.1 - 3.10 due to the low density of sampling in this region. As solvent production began to slow the acid concentrations increased quickly and continue to rise to a new maximum. Both acetone and ethanol appeared to reach their maximum concentrations early in the stationary phase and before pH_m is reached. However, n-butanol continued to be produced until late in the stationary phase.

The decline phase is reflected only by a decreasing cell count and not by any other parameter.

3.3.3.4 Fluctuations in the Stationary Phase Cell Population

An interesting peculiarity of the stationary cell population is the large fluctuations observed after the transition region between the exponential and stationary phases. This can be seen clearly in data from Run A (Table 3.19; Fig. A3.2) although it is often masked in a graph of ln N vs. t (e.g. Fig. 3.1). The exponential phase seemed to start after about 5 hrs and continued for about 11 hours.

After 16 hours (between 16 hours and some time less than 130 hours) the stationary phase was reached, the cell population was seen to oscillate, and finally the decline phase began.

For Run A, the standard deviation of cell numbers in the stationary phase is far in excess of the expected error (7 values; mean of cell number 1,093 x 10^6 cells/ml; standard deviation of 243 x 10^6 cells/ml or 22% of the mean). Appendix 2 shows that the expected error is about 6% (Fig. A2.2). The cell concentration was about 70 cells/square after a 10 fold dilution.

This large fluctuation was unexpected. It was frequently seen to occur after the exponential to stationary transition and may lead to variation of up to a factor of 2 in the total cell count. Workers at Colorado State University (U.S. Department of Energy , 1980) have reported very much more savage variation in their viable cell concentration estimates, and also found that optical density and cell dry weights could increase while their viable cell counts were decreasing. Barber et al. (1979) and Webster et al. (1981) also report a similar phenomenon shortly after commencement of the stationary phase. The fact that pH and other chemical species' concentrations did not similarly fluctuate suggests strongly that the phenomenon is due to some peculiarity in cell behaviour.

A possible explanation for the strange behaviour based on phage infection is discussed in Chapter 5 and in Appendix 4. Various other explanations have also been suggested (Bacon, 1982). If the synchronous growth had resulted (as could possibly result from phenomena seen in Section 3.3.3.3) then the fluctuations might be caused by very rapid autolysis (as in some strains of Bacillus subtilis) followed by growth of a younger generation of cells. Since the period of fluctuation is about the same as the doubling time for the bacteria (3-4 hours) (Table 3.10,

Fig. A3.2) then this is possible. It may also be that lysis of some of the older cells releases reuseable material, but again synchronous growth would be required. Experiments with controlled rates of growth using antibiotics or continuous fermentations may lead to a better understanding of the phenomena.

3.3.3.5 Cell and Solvent Correlation

The average concentration of cells in the stationary phase is well correlated with the two alcohols' concentrations at the end of the fermentation (Table 3.17 and Fig. 3.14). The probable metabolic cause lies in the production of ATP for continued growth by production of acetate (Fig. 3.15) (Rhodes and Fletcher, 1975). Production of acetone, butanol, acetic acid, and butyric acid involves the production of ATP by acetoacetyl CoA formation from acetate (formed from acetyl phosphate) and acetyl CoA. This is the more energetically favourable path for consumption of the acetyl groups, leaving ethanol production as an energetically wasteful side reaction even though it does supply some reductive capacity. Reduction of the NADH to NAD for continued glycolysis may also be achieved by production of butyrate or butanol, while acetone and acetic acid production reduce no NADH. The most metabolically efficient products are therefore butyric acid and butanol, supplying both ATP and reductive power, with butanol being the more reduced compound absorbing two electrons more than butyric acid. Butyric acid production is favoured over acetone or just acetate production due to the need to reduce NADH for continued metabolism.

Hence cell production is favoured by conditions favouring production of acetone, butyric acid, and in particular butanol with consequent ATP generation. Ethanol production generates no ATP. The correlations highlight the relationships with butanol and ethanol in particular, suggesting that reductive capacity may also be important.

3.3.3.6 Solvent and Acid Correlations

At first glance the correlations appear to match the metabolic pathway well. However, as all known chemical concentrations measured correspond to extracellular concentrations, some link between extracellular and intracellular concentrations is required. Bailey and Ollis (1977) make mention of the high resistance of cell membranes to charged chemical groups, while allowing small neutral species relative ease of passage into and out of the cell. An equilibrium, based on the concentration of neutral species alone, is set up across the cell membrane. This allows the cell to readily lose such toxic compounds as acetone, ethanol, and butanol while retaining a high internal salt concentration relative to the surrounding solution. This type of behaviour could explain apparent differentiation in the correlations between undissociated and dissociated acid groups as calculated from the total acid concentration and broth pH. Thauer et al. (1977) outline the excretion of lactic acid by a carrier-mediated process, and liken acetic acid excretion to this. The mechanism is described as independent of metabolic energies and dependent on the undissociated acid forms only. It appears then that there may be a direct relationship between internal and external concentrations of the uncharged species measured. That is, for example, that the external concentration of undissociated acetic acid is directly related to the undissociated acetic acid concentration internally. In the case of passive membrane transport this assumption is well justified, and may also be quite sound for active transport depending on the mechanism. The same assumption is made for butyric acid.

This same effect could also be considered important in that it would also allow rapid flux of dissolved hydrogen while allowing a pH gradient. The results of the correlations appear to support this model, and uncharged species' concentrations external to the cell can be considered

analogous with their internal uncharged concentrations.

In light of the above, then, and the correlation results (Table 3.17), it appears that high levels of butyric acid relate almost directly to high levels of butanol, and high levels of acetic acid relate to both butyric acid and ethanol levels. These results are understandable for systems related by equilibrium reactions frequently seen in cellular metabolism, and resemble well a metabolic pathway where acetic acid is an intermediate for butyric acid production which in turn is an intermediate (in combination with coenzyme A) in butanol production.

3.3.3.7 The Effect of Pressure

If it is assumed that pressure acts through hydrogen (rather than carbon dioxide) as immediately suggested by the 1.5-litre experiments, and that the mechanism is via the equilibrium

$$NADH + H^+ \rightleftharpoons NAD^+ + H_2$$

then it could be assumed that the pressure would effect any reaction utilising NAD or NADH. The source of H_2, H^+, NAD, or NADH would not be important - only the overall level of NADH (and also of H^+ and NAD) is important. McInerney and Bryant (1981) mention this theme in the field of methane production in waste treatment. If the partial pressure (analagous to concentration in this case) of hydrogen is very low (as it is with healthy methanogen utilisation of hydrogen), then the fermentative bacteria ferment pyruvate to H_2, CO_2, and acetate. If the partial pressure of hydrogen is allowed to increase (as occurs for example during overloading of a waste treatment plant) then the fermentative bacteria regenerate their NAD by using H to form reduced compounds such as lactate, propionate, butyrate, and ethanol. In the case of Cl. acetobutylicum it appears that a higher concentration of H

results in production of butanol also. The overall concentration of H, then, effects the products of fermentation, and it is hypothesised that pressure acts via the equilibrium of hydrogen and NAD with NADH.

McInerney and Bryant (1981) suggest that the order of production of reduced products with increasing concentration of H is paralleled by the ΔG_o values for production of the product from pyruvate (refer Table 3.20). McInerney and Bryant further say that $\Delta G'$ values including the partial pressure of hydrogen give a better indication:

$$\Delta G' = \Delta G'_o + 1.36 \log ([\text{products}]/[\text{reactants}])$$

and gives graphs like Fig. 3.17 (McInerney and Bryant, 1981). This equation gives some means of predicting when production of an electron sink compound would become thermodynamically unfavourable and stop. Fig. 3.17 shows $\Delta G'$ for degradation of propionate, butyrate, and ethanol to hydrogen and acetic acid, and for production of methane from H_2 and CO_2. A similar graph could no doubt be drawn for fermentation of hydrogen and acetate to products such as acetone, ethanol, and butanol, and a cut off partial pressure found for their formation. Fig. 3.17 shows that the thermodynamic cut-off partial pressure of hydrogen for ethanol production from acetic acid is at a partial pressure of hydrogen of 10 kPa, and for butyric acid is 100 times lower at 0.1 kPa. Hence ethanol appears well correlated with pressure since it is relatively close to its cut-off level, while butyric acid shows only weak correlation (Table 3.10).

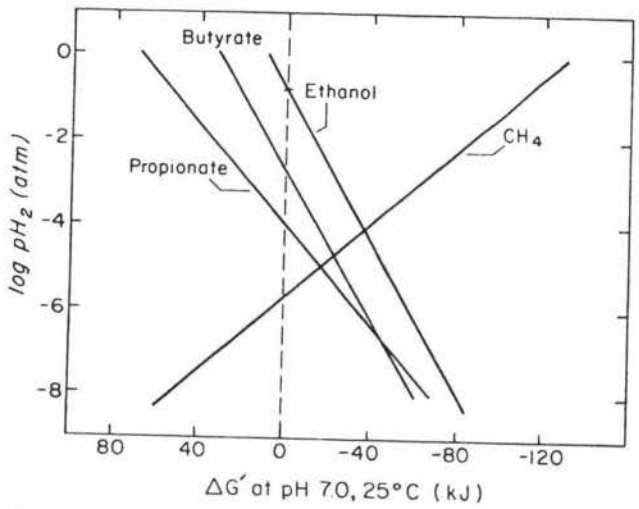
Thauer et al. (1977) elaborate on the mechanism of hydrogen release. The hydrogen liberated to ferredoxin (Fd) at the oxidation of pyruvate to acetyl CoA may be converted to hydrogen stored as NADH and subsequently used in any other reaction utilising NADH, or alternatively the hydrogen may be liberated to form molecular hydrogen. The

Table 3.20 ΔG_o VALUES FOR PRODUCTION OF VARIOUS CHEMICALS FROM PYRUVATE*

Product	ΔG_o (kJ mol^{-1})**
pyruvate	0
acetone	-16.3
ethanol	-38.9
acetate	-47.3
butyrate	-77.4
butanol	-159.0

* McInerney and Bryant, 1981 Thauer et al., 1977.

** Data is only approximate due to difficulty in determination. These values differ by up to 20% in the two references, but the relative positions in the table do not alter.



Effect of the partial partial pressure of H_2 (pH_2) on the change-in-free-energy ($\Delta G'$) for the reactions involving ethanol, propionate, and butyrate degradation (Equations 27, 32, and 28, respectively; Table 2) with H_2 and acetate production and methane formation (Equation 4, Table 2) from H_2 and CO_2. The calculations were based on the assumption that the concentrations of ethanol, acetate, propionate, and butyrate were 1 m*M* each and that of bicarbonate was 50 m*M* and that the partial pressure of CH_4 was 0.5 atm using the equation, $\Delta G' = \Delta G^{\circ'} + 1.36 \log([\text{products}]/[\text{reactants}])$.[44] (From McInerney, M. J. and Bryant, M. P., *Biomass Conversion Processes for Energy and Fuels*, Sofer, S. and Zaborsky, O., Eds., Plenum Press, New York, 1980, in press. With permission.)

Fig. 3.17. The Effect of Hydrogen Concentration on the Change in Free Energy of Several Common Microbial Products (McInerney and Bryant, 1982).

rate of production of molecular hydrogen is governed by the rate of hydrogen conversion from FdH to NADH. This conversion is governed by the ratio of acetyl CoA to CoA since NADH:ferredoxin oxidoreductase requires acetyl CoA as an obligatory allosteric activator, and CoA is competitively antagonistic. In the present case where the partial pressure of hydrogen has been artificially raised by increasing the headspace pressure, the rate of this conversion is increased but will still be regulated by the ratio acetyl CoA:CoA. It might be thought then that acetic acid is related to butyric acid in the correlations (Table 3.17) through this controlling ratio and its effect on NADH levels instead of by virtue of its position in the metabolic pathway. However if this were the case then it would be expected that acetic acid would be more strongly correlated with the other reduced compounds such as ethanol and butanol in addition to their correlation with pressure.

This mechanism based on dissolved hydrogen concentration may well explain the effect of pressure on solvent concentration, but stops short of an adequate reason for the correlation of associated acetic acid ($[HAA]_f$) with pressure. It would seem that a possible explanation for this may be the increased dissolved carbon dioxide concentration on the hydrogen ion concentration (i.e. pH) by the relationship

$$H_2O + CO_2 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-}$$

Correlations of p with pH_f suggest that pressure does not directly affect the final pH. In a separate set of experiments similar to those of Chapter 4 but not presented in this thesis, it was found that the broth pH did rise between samples after pressure release by removal of the cap of the 100 ml bottle and a period of fermentation with a loose cap. This could possibly be explained on the basis of dissolved CO_2. It was not possible however to say whether the effect had any relationship with acetic

acid levels in that case, and since Table 3.11 shows a correlation for $[AA]_f$ and an identical correlation for $[HAA]_f$ (which should have the pH effect removed) it does not seem likely that the correlation of acetic acid (as either AA or HAA) with pressure is due to a pH-equilibrium effect mediated by dissolved CO_2.

It is distinctly possible, however, that CO_2 is reduced to acetate as in many other Clostridia (Thauer et al., 1977). This may be done by fixing both CO_2 and H_2

$$2CO_2 + 4H_2 \rightarrow CH_3 COOH + 2H_2O$$

as in the case of Cl. aceticum, or without hydrogen fixation as in the case of Cl. thermoaceticum, possibly by the anaplerotic reaction:

$$\text{pyruvate} + CO_2 \rightarrow \text{oxaloacetate}$$

(Pelczar et al., 1977). This method may be used if the organism lacks hydrogenase with which to fix hydrogen. In this case the electron donors are unknown, but it is thought that the carboxyl group of the acetate comes from the carboxyl group of pyruvate rather than CO_2 (Thauer et al., 1977).

Whether or not hydrogen is fixed by the organism is unimportant, as either way CO_2 fixation is a possible explanation for the pressure effect on the acetic acid level.

Further evidence that CO_2 fixation may occur can be found in Section 3.3.3.11.

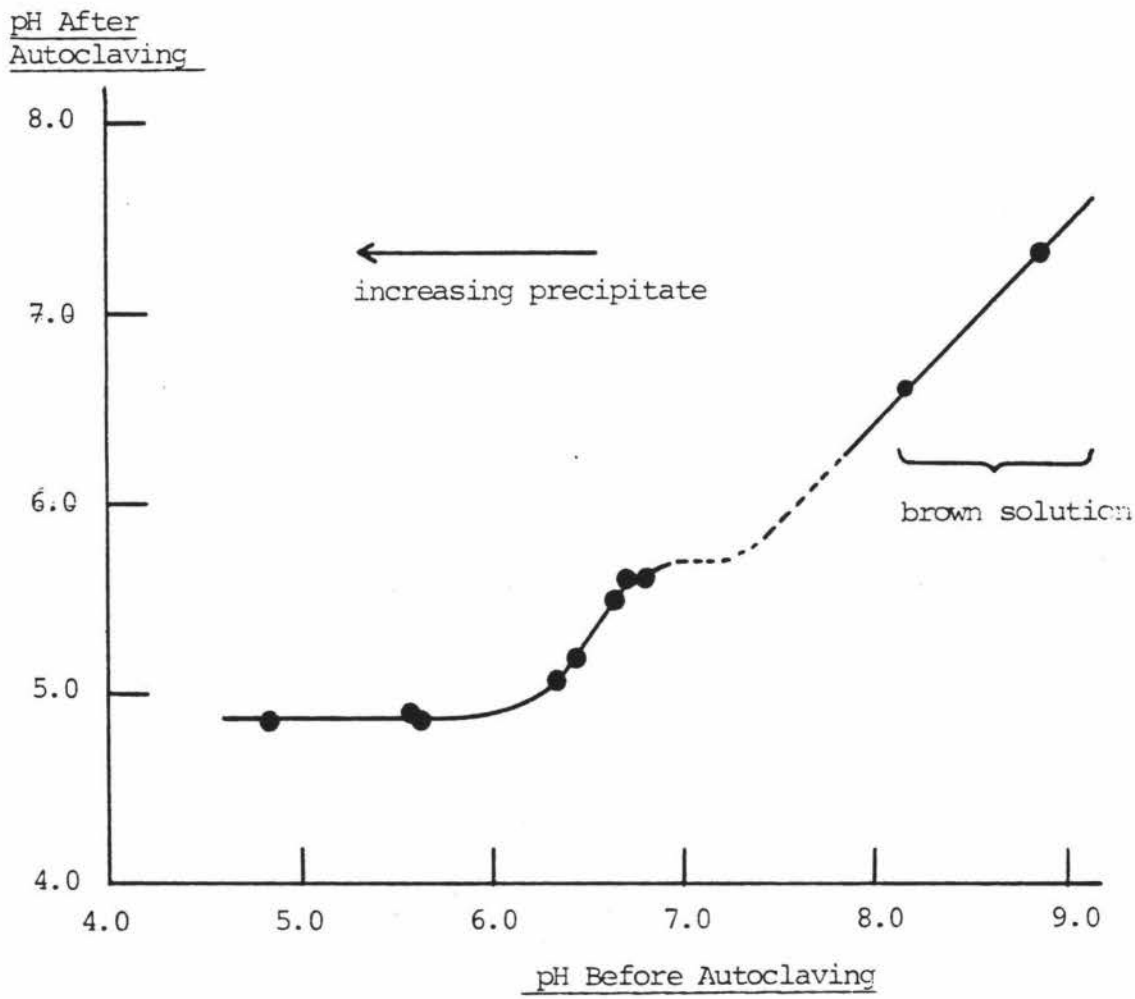
3.3.3.8 The Effect of Minimum pH

The source of the variation in pH_m is uncertain. The possibility that variation in initial pH caused the

resultant pH_m variation was investigated, but there was no correlation in the experimental values between initial pH and pH_m. However, there was no attempt made to measure the levels or fluctuations in buffering capacity (due perhaps to pH adjustment procedure) before or after autoclaving, and since the pH during fermentation will depend not only on the initial pH but also on the buffering capacity of the medium, then anything leading to alterations in the buffering capacity of the whey permeate would cause variation in pH_m. Fig. 3.18 shows the results of a small experiment measuring the pH before and after autoclaving. Each point represents the pH change on autoclaving in a 100 ml beaker of whey permeate, pH adjusted with ammonia prior to autoclaving. This experiment shows the effect of autoclaving on pH before inoculation. It shows at least one region of buffering. If the buffering were the result of irreversible or partially irreversible chemical reactions, then pH fluctuations during media preparation may well have an effect on the overall buffering capacity of the fermentation broth. This in turn would effect the pH during fermentation including the minimum observed pH (pH_m). This is the probable cause of variation in the minimum pH.

The effect of pH_m experienced by the fermentation on $[HAA]_f$ levels and μ is possibly due to some form of cell damage. Whether the damage is irreversible or reversible is unclear, however it does seem to produce a long term effect in its effect on the final broth acetic acid levels ($[AA]_f$) thereby suggesting the effect is in fact durable. pH_m does not seem to affect the final total cell population, although it may conceivably affect the viable cell count. In aerobic cells, increased acid levels due to lactate production in the absence of oxygen cause a drop in pH which leads to leakage of lysozymes into the cytoplasm resulting in degradation of the intercellular constituents (Robbins and Cotran, 1979). This may be a possible mechanism for pH damage in the

Fig. 3.18. pH Before and After Autoclaving*
(Whey Permeate with 5 g/l YE)



* sample size = 100 ml.
autoclaved in pyrex beaker with tin-foil covering
-200 kPa(abs.), 15 min.
samples left to cool before analysis.

anaerobic cell also.

Perhaps a more feasible mechanism for the effect of pH_m is that it characterises the pH curve for a large portion of the fermentation (even though it does not seem to be directly related to pH_f). The intracellular pH of the organism follows the medium pH to within a few pH units (Thauer et al., 1977) and would therefore be correspondingly effected. Hence if pH_m does characterise the pH curve for a large portion of the fermentation then it is not surprising that it has an effect. Why it seems to affect acetic acid principally is not understood, but may suggest the effect acts somewhere in acetate production or in possible CO_2 fixation (Section 3.3.3.7).

It should be noted that the effect of pH_m in the correlations is not reliant solely on the extreme value of pH_m 4.2 in Run B. Except in the case of the correlations for R' (Section 3.3.2.4), the removal of Run B data and recalculation of the correlations gives very similar results to those presented based on all the data in Tables 3.3 and 3.4, hence R' is not considered directly correlated with pH_m.

3.3.3.9 Specific Rates for Growth and Butanol Production

Fig. 3.14 shows the suggested relationships for the rate data. $[H^+]_m$ appears to be the only independent variable which is correlated with μ. The standard deviation about the regression line was estimated at $\pm$ 0.07363 which is 26% of the mean value for μ. (The uncorrelated standard deviation of the μ values is $\pm$0.0989, or $\pm$ 35%.) The remaining scatter about the regression line is probably entirely due to the error in estimation of μ.

The two variables which correlate with R' are μ and p. The fact that neither appears significant on its own is almost certainly due to the nature of the data. The variation not accounted for in a regression against only one variable

appears to swamp any reduction in variance explained by the single variable. There appears to be no relationship between μ and p. However, while it is not surprising that R' be positively correlated to p, R' was not expected to be negatively correlated with μ. The standard deviation about the regression line was $\pm$ 0.00705 ($\pm$6% of the average of R' (0.12217)) which is a marked improvement over $\pm$ 0.0525 ($\pm$43%) for the uncorrelated R'.

Correlations for μ and R', which are both evaluated at periods during the fermentation, against variables evaluated after the fermentation had finished, might also be made. It appears that pH_f is strongly correlated with R'. Since R' might be interpreted as a rate of acid utilisation as well as a rate of alcohol production, then it would appear possible that this correlation does reflect a metabolic effect. Other correlations are more troublesome. While it appears that $[HBA]_f$ is related to μ, μ becomes less important when $[HAA]_f$ is included into the correlation.

From the correlations given, it appears that $[B]_f$ is not related to either μ or R' individually, but again if both variables are included a strong correlation results. Attempts to include $[HBA]_f$ and p into correlations with μ and R' result in groups of correlations which are difficult to interpret and which suffer from insufficient degrees of freedom for confident conclusions. This apparent variation in significance was typical of many correlations of data evaluated during fermentation with data evaluated at the end of the fermentation, and so such correlations were not pursued further for this thesis.

This section has shown several correlations for the rates of growth and butanol production. However it has also shown the inability of linear correlations to deal with all situations, and has pointed to the need for more powerful statistical techniques. Further development of the Factor Correlation Method will have to consider the use of

these more powerful methods. Further study of the method will also need to consider the relationship of variables determined at differing times during fermentation.

3.3.3.10 Carbohydrate Usage and Fermentation Efficiency

Lactose measurements were taken for all samples using the autoanalyser. However some source of interference was evident in the readings. Levels of butyric acid and other species known to interfere were below those suggested by the equipment suppliers; however some source of interference was obvious from a non-monotonic decrease in the lactose levels. For these reasons the lactose analyses are not presented. The interference seemed to be linked to the levels of acetic and butyric acid, but the actual active interfering species was unknown. Further pursuit of accurate lactose levels, while desirable, was not followed as these data were not required to accomplish the aim studying the effect of pressure on solvent yields.

From the literature it appears that good carbohydrate usage can be achieved. If it is assumed that almost all of the carbohydrate is consumed, and the data from Run J is used (total solvent = 11.6 + 0.99 + 1.8 = 14.4 g/l) with an assumed initial concentration of about 45 g/l lactose (refer Table 2.1), then:

$$\text{efficiency} = (14.4 \text{ g/l})/(45 \text{ g/l}) \times 100$$
$$= 32\%$$

which is close to values from the literature (e.g. Leonard et al., 1947; Noon, 1981)

3.3.3.11 Run I

Run I was conducted differently from the other runs. Unlike the other runs which created their own headspace pressures by entrapment of the fermentation gases, Run I

had 200 kPa (absolute) of oxygen-free hydrogen applied immediately after inoculation. As can be seen in Table 3.3, the solvent concentrations were unexpectedly low. Instead of being directly comparable with Run H (with approximately the same pressure and minimum pH), the butanol yield was about half that of Run H (see also Table 3.5), and the other two solvents about one quarter those of Run H. This result was unexpected on the basis of the hydrogen mechanism (Section 3.3.3.7), and points to how little is really known of the mechanisms involved.

One possible explanation of the phenomenon is the lack of carbon dioxide. Even when gas production is proceeding the headspace carbon dioxide concentration and corresponding dissolved concentration in Run I would not be expected to rise as quickly as in the other fermentations because the headspace already had about twice as many moles of hydrogen to dilute in Run I as there was nitrogen in the other fermentations. Also the pressure release mechanism would have been actuated soon after production started for Run I and therefore much earlier than the other runs. This would have caused more fermentation gas (including CO_2) to be lost early in the fermentation. Due to the fairly low level of broth agitation it is hard to assess the strength of the effect of headspace gas concentrations on the broth concentrations. However, since the only difference in Run I was the headspace concentration, it must be assumed that this is the basic cause. The possibility of some effect of headspace mediated through CO_2 rather than mediated through hydrogen was suggested earlier in Section 3.3.3.7 with reference a possible effect of pressure on acetic acid levels mediated by CO_2. This effect could conceivably be linked with the results of Run I, and might also explain the low solvent yields seen in the 1.5-litre experiments (Section 3.2).

Spivey (1978) mentions that NCP (South Africa) set a pressure of only 35 kPa at inoculation, after which the

pressure may be reduced to 18 kPa before increasing naturally to 35 kPa again and gas draw off is started and pressure is controlled to 35 kPa. Spivey (1978) does not specifically state that the initial pressurisation is done with CO_2, but he does state that initial agitation, oxygen stripping, and a slight positive pressure to prevent contamination and for cooling purposes is applied using CO_2. NCP may use CO_2 because it is a convenient gas being a by-product of the fermentation and in preference to a mixture of CO_2 with H_2 to reduce the risk of explosion or to recover more H_2, but they might perhaps have found that pure CO_2 gave better results. The discussion earlier on the possible effect of CO_2 on acetic acid levels (Section 3.3.3.7) may be relevant to Run I also. The fact that CO_2 levels in Run I would be expected to be lower than the other runs suggests that CO_2 does play a major part in the fermentation. However, the acid levels in Run I (and in particular acetic acid) are not significantly different from those of the other runs (Tables 3.3, 3.4 and 3.5) and it is only the concentrations of all three solvents which appear surprisingly low. (Butanol comparisons can be made with Tables 3.3 and 3.5, and the reader can verify for himself the comparison for the other two solvents from Table 3.3 with predictions from Table 3.17.) It should be noted that the early headspace gas concentrations affect the concentrations of solvents which do not appear in significant quantities until after the growth phase appears to have finished, but seems to leave the acid levels unaffected. The fact that pH rose initially in contrast to the other runs is evidence of some early effect. It may be that the lack of CO_2 in solution, the levels of which would be increasing slower than in the other fermentations, is the cause of this effect and that the change in the broth pH (to include amongst the most alkaline pH experienced by any of the fermentations) is the basic cause of low solvent yields in the final fermented liquor.

An alternative explanation for the low butanol yield of Run I is that the timing of application of the increased pressure is critical. This might be reasonable if considering a delayed increase in pressure, but that the increase could come too early is hard to imagine. The fact that Spivey (1978) applied 35 kPa pressure from inoculation would also suggest that this is not the case.

At present the exact cause is unknown. The fact remains, however, that Run I did not produce the solvent yields expected. It may be that this run, which was not repeated, is a "wild" run and not at all typical. This can only be answered by further experimentation. In spite of this possibility, it would seem that the conditions of fermentation for Run I are not conducive to high solvent yields, perhaps for reasons connected with the lack of CO_2, perhaps connected with the effect of pressure on acetic acid (refer Section 3.3.3.7), and perhaps connected with the low solvent yields in the 1.5-litre experiments (Section 3.2). This last possibility appears quite real, but the lack of knowledge about the effects observed prevents further discussion.

3.3.4 Discussion of Factor Correlation Method

Application of multiple linear regression to give an indication of the biochemical pathway and the effect of external influences with possible point(s) of action, appears to be novel. Possibly the reason the method can distinguish into a possible cause and effect structure (such as a metabolic pathway) is that the regressions act to find which variables <u>best</u> explain the scatter seen. If then we say that

$$Y = f\ (B,\ others_1)$$
$$B = f\ (A,\ others_2)$$

where Y, B and A are known, and "others" represents

unmeasured effects (including random error), then regressions for Y including B alone would explain the variation in Y better than A alone. This is because the scatter remaining unexplained after regression is due only to $others_1$ in the first instance, and due to $others_2$ as well as $others_1$ in the second. Hence B would provide a better correlation for Y than A, and would appear with the higher t-statistic in a regression equation. While this is only a simple and qualitative explanation, it gives insight for a basic understanding of the method. Confidence in the method is given by the results themselves rather than a detailed proof of the method.

It is worth noting that, while the method can identify many variables which are correlated, unless all major variables are determined then predictions from correlations will not necessarily be good. Table 3.5 shows some estimates made from the correlations in this chapter. Table 3.5 may be compared with values from Tables 3.3 and 3.4. Even in the case of butanol concentrations estimated using estimates from other correlations the agreement is quite good. The estimates of $[HAA]_f$ are extremely good, with the standard error of the estimate of 5.0% of the mean $[HAA]_f$ measured. This degree of accuracy is quite exceptional as the measured variables themselves would have an associated error equal to or greater than this. The error in the other estimates is somewhat greater and in the order of 15%-27% (Table 3.5), but still represent a large reduction in the original variation of between 39% and 48% (Tables 3.3 and 3.4). Due to the large reduction in variance it might appear that the major variables have been identified, although it is quite possible the 15%-27% represents some unknown variables beyond experimental uncertainties.

Further development of the Factor Correlation Method will require analysis of variance techniques more sophisticated than used here, but such development is beyond the scope of this work.

3.4 30-litre Scale Experiments

3.4.1 Results

30-litres of medium as used in Section 3.3.1 were used for these fermentations. The inoculum also was exactly the same, and was not increased in size.

The runs follow a very similar pattern to Fig. 3.18 (Section 3.3.1) and will not be reproduced here.

The only noticeable microscopical difference in the cells to those for the 10-litre and 1.5-litre fermentations was in cell definition. The cells grown in the 30-litre industrial style fermenter in both runs AA and BB were much easier to see under a microscope than those grown in the 10-litre fermenter. In addition, the broth both during fermention and in the final beer was whiter and contained more suspended solids. Table 3.21 summarises the other results for the 30-litre fermentations.

The only known differences between the 10-litre and 30-litre experiments were the fermentation vessels, and the relative inoculum size. (The percentage inoculum for the 30-litre fermentations were a third that for the 10-litre fermentations.)

3.4.2 Discussion

Run AA was noticeably atypical of all previous runs. It was very slow to commence and never showed healthy gassing. The results for Run AA were discarded from further discussion.

Run BB showed healthy fermentation. Comparison of Table 3.21 with Table 3.3 shows that the 30-litre vessel was able to produce solvent yields comparable with the 10-litre fermenter. Prediction of $[B]_f$ using the correlation in

Table 3.21 SUMMARY OF 30-LITRE RUNS

Run	AA [a] [b]	BB [b]	units
P	0	160	kPa
pH_m	-	4.25	pH
pH_f	-	4.35	pH
N_s	1130[c]	1942	x 10^6 cells/ml
$[B]_f$	0.22[c]	4.77	g/l
$[A]_f$	0.51[c]	1.09	g/l
$[E]_f$	0.19	0.34	g/l
$[BA]_f$	0.84	4.99	g/l
$[AA]_f$	0.87	2.99	g/l
R	-	0.13[c]	g/(l hr)
	-	0.38[c]	hr^{-1}

a Run AA was very slow and appeared atypical. Very few samples were taken.

b Run AA was conducted at atmospheric pressure, and Run BB at 160 kPa (abs.).

c Large error associated with these data.

Table 3.6 for the 10-litre fermentations gives the estimated $[B]_f$ as 3.4 g/l (p = 160 kPa, pH_m = 4.25) compared with $[B]_f$ = 4.8 g/l in the 30-litre broth. $[E]_f$ = 0.34 g/l for the 30-litre fermentation may be compared directly with $[E]_f$ = 0.46 g/l (Run F, Table 3.3) as p is the only significant variable (Table 3.8), and $[A]_f$ = 1.09 g/l compared with $[A]_f$ = 0.723 (Tables 3.17 and 3.21). The differences in solvent yields may not be significant after error due to analytical errors and error about the regression lines is considered (Section 3.3.4), but they are encouraging as the 30-litre acetone and butanol concentrations are both slightly higher than might be expected; the butanol concentration perhaps significantly higher.

The microscopical difference in cell definition between the 10-litre and 30-litre fermentations was observed in both 30-litre fermentations, and also a third 30-litre run (not mentioned elsewhere) during which the temperature control failed. The difference is therefore definite and probably due to vessel size or geometry, or related effect. It is unlikely to be due to inoculum size as the 1.5-litre fermentations (Section 3.2) did not show a similar difference. The direct cause is uncertain.

The difference in broth appearance is possibly due to the greater agitation due to large fermentation volume, since the degree of agitation does not stay constant with increasing fermentation volume. This is most easily understood by realising that the actual volume of gas produced in the fermenter is proportional to broth volume, whereas the vertical speed of the gas flow (based on cross-sectional area) is proportional only to volume to the power of two-thirds. Hence the vertical flow rate will increase disproportionately with increasing volume (all else, such as a medium and geometry, being constant). In the case of the 30-litre fermenter then, the greater degree of agitation might cause more of the sediment to be kept

in suspension. The sediment is thought to be a mixture of precipitated protein and inorganic salts (principally calcium salts). While this is a possible answer, there is no direct proof. The different broth appearance might alternatively be due to a metabolic cause, perhaps related to the greater visual definition of the individual cells.

4. A.B.E. FERMENTATION OF WOOD SUGAR HYDROLYSATES AND PENTOSE SUGARS

4.1 Introduction

The possible use of wood hydrolysates as a fermentation substrate for large scale solvent/fuel production by the A.B.E. fermentation was mentioned in the Prelude (Section 1.1).

The work of Leonard *et al.* (1947) showed that the fermentation of wood hydrolysates by A.B.E. bacteria is difficult, requiring pretreatment of the solution. They added malt sprouts, $(NH_4)_2HPO_4$, $CaCO_3$, and a trace of reduced iron, and inoculated at 8% of the fermentation volume. Their experiments showed that inhibition resulted in long lag periods and slow sugar utilisation, and was partially due to furfural and other substances formed during the acid hydrolysis, and which were easily removed by steam stripping. Dilution of the medium gave better fermentations as did treatment with decolourising carbon. Metal ions did not seem to be a problem but for some other reason an alkaline pretreatment helped. They stated that the addition of reducing agents such as reduced iron or sodium sulphite helped and that the alkaline pretreatment may act similarly. Beesch (1952) found that Cu^{2+} (ca. 2-5 ppm), and Hg^{2+} and Sb^{3+} (much higher levels) were the only metals to significantly inhibit the fermentation. In summary, Leonard *et al.* (1947) found that inhibitor removal by distillation, followed by pH adjustment to pH 6.5 with lime, was simplest, and 24% to 38% yields were obtained (solvent/sugar).

It was decided therefore to do some brief work on this substrate. The effluent from the first beer still after a yeast fermentation of the treated hydrolysate (known as wood sugar residue (WSR)) as well as fresh (untreated (WS)) hydrolysates were examined. Fermentation of pentose sugars was studied simultaneously with the still bottoms (WSR).

4.2 WSR and Pentose Sugar Fermentations

4.2.1 Results

These trials were performed on a 100 ml scale using Cl. butylicum NRRL B-592 on WSR as outlined in Table 4. All bottles had their caps screwed down tightly to cause pressure build up, in spite of the risk of bottle explosion. Unfortunately the cap of bottle 5 leaked badly, and hence no pressure build up occurred in this bottle (as otherwise evidenced by a slight hiss when the cap was loosened for sampling).

Table 4 gives a summary of the fermentation results. Samples were taken only once a day and analysed for approximate concentrations to give approximate comparisons only (i.e. the maximum error in any particular concentration is probably about ± 40%). The values in Table 4 were for a sample taken 13 days after inoculation. The acid concentrations are typically higher than those of the 7 day sample, but solvent concentrations and other species' concentrations were about the same. All fermentation variables (except those for Bottle 1) followed the shape of the typical fermentation curves described by Fig. 3.15, but the time scale should be expanded by a factor of 2 (i.e. the rate of fermentation here was about half the rate of the 10-litre fermentations on WP), or by a factor of 6 for Bottle 3 which fermented much more slowly.

Bottle 1, containing WSR alone (Bottle 1), showed no observable growth until a period of three weeks had passed. Sampling had stopped after 13 days, but the bottles were left to continue incubation for observation.

Bottle 3, supplemented only with glucose with no YE, showed slow fermentation but returned the highest solvent yields of all the bottles.

Table 4. SUMMARY OF WSR FERMENTATIONS *

Bottle	Supplements	pH_m	pH_f	N_s	$[B]_f$	$[A]_f$	$[E]_f$	$[BA]_f$	$[AA]_f$	eff.**
1	none	7.9	-	16	0.6	0.4	0.9	0.2	0.7	-
2	5 g/l Y.E.	5.4	6.7	40	0.2	0.3	0.6	1.8	0.7	-
3	50 g/l Glucose	5.8	6.6	95	9.4	4.3	1.0	0.6	0.5	26%
4	50 g/l Glucose + 5 g/l Y.E.	5.6	6.3	191	4.3	1.1	0.6	1.5	1.2	9%
5	20 g/l D-Xylose + 5 g/l Y.E.	5.5	5.65	200	3.7	0.4	0.5	3.7	0.3	16%
6	20 g/l L-Arabinose + 5 g/l Y.E.	5.5	-	214	1.5	0.1	0.5	4.5	0.6	3%

Notes: * Sampling was infrequent and so these data should be used for approximate comparison only.

** eff. = fermentation efficiency (g solvent/ g sugar utilised).

Bottle 2 fermented only weakly, but all other bottles showed strong gassing with active fermentation.

In all cases the fermented medium was a turbid light amber-brown colour with a black scum coating the surface froth. The inoculated broth had been a slightly turbid dark brown before fermentation.

4.2.2 Discussion

Due to the strong fermentation in the fully supplemented bottles, it appears that WRS does not contain any particularly potent toxic compounds. However, it is apparent from the faster rates of fermentation and higher final yields that supplementation with both YE (probably as a nitrogen source) and carbohydrate is necessary to obtain good solvent yields.

The presence of solvent in Bottles 1 and 2 is due principally to carry over of solvent from the inoculum. This was estimated as 1.5 g/l total solvent, and was subtracted from the total solvent yields in the other bottles to estimate fermentation efficiency. The fermentation efficiency showed considerable variation. Bottle 3, supplemented with 50 g/l glucose only, showed the highest yield of 26%. However it was also the slowest fermentation of all the supplemented media. Supplementation with both YE and 50 g/l glucose provided a much faster fermentation and more cell growth, however the fermentation efficiency dropped to only 9% (Bottle 4, Table 4). If indeed YE was acting as a nitrogen source, then it would appear that WSR has insufficient nitrogen for good growth and fast fermentation. This apparent negative correlation of rate of fermentation and activity with solvent yield may well be connected with the negative correlation of solvent production rate and growth rate seen in Chapter 3 (Table 3.17), but the cause is uncertain.

The two pentose supplemented fermentations showed good activity, however L-arabinose showed poor efficiency (3%) even when compared with that of D-xylose (16%). The reason for this large difference is unknown. Possibly the lower headspace pressure due to the leaking cap of Bottle 5 is the cause. It is more likely that the cause is due to the substrate as suggested by Compere and Griffith (1979).

Compere and Griffith (1979) performed a more complete study of several organisms on chemically defined media, including the fermentation of L-arabinose, D-xylose, and glucose at different concentrations by Clostridium butylicum NRRL B592. They found that both the fermentation efficiency and final solvent ratios depended on both the substrate and substrate concentration, and could vary immensely; a result which is reflected in Table 4. Compere and Griffith (1979) also commented on this particular organism's surprising ability to ferment D-xylose. The present work performed on supplemented wood sugar hydrolysate closely parallels their work, and leads on to the next section of work on the attempted fermentation of untreated wood sugar hydrolysate.

In summary, it appears that WSR contains no potently toxic compounds but is deficient in nitrogen, and that Clostridium butylicum NRRL B592 can readily grow on pentose sugars. The solvent yields and ratios are however dependent on both substrate and substrate concentration. This variation with different strains of the same organism has been used to follow market trends in the past (Hastings, 1978) and is therefore of commercial interest.

4.3 WSH Fractions Experiments

4.3.1 Results

Two 100 ml bottles each of the prehydrolysate and main

hydrolysate supplemented with 5 g/l YE (as in Section 2.5.1) were set up and inoculated with Cl. acetobutylicum. After pH adjustment the solutions both took on a transparent dark brown colour. Growth occurred in one of the two prehydrolysate bottles, but not at all in any of the other three bottles. Growth was accompanied by strong gassing and lightening of the broth to a turbid light amber-brown with accompanying surface scum similar to the transformation seen in Section 4.2. The cell concentration when growth ceased was about 10^9 cells/ml (i.e. comparable with the populations seen in Chapter 3).

Attempts to grow the organism in the 1.5-litre vessel on prehydrolysate supplemented with 5 g/l YE (two attempts) and main hydrolysate also supplemented with 5 g/l YE (one attempt) all failed. No growth was observable to the eye in any of the three attempts.

No pretreatment of any of the hydrolysates was performed other than pH adjustment with aqueous ammonia and autoclaving prior to inoculation.

4.3.2 Discussion

These results show the difficulty associated with attempts to ferment untreated hydrolysate. The fact that one culture did grow suggests that it is possible to develop an inoculum which can grow on the untreated hydrolysate. Leonard et al. (1947) reached a similar result.

Further, it seems that the inhibitory characteristic in the solution is removed by yeast fermentation and distillation since the beer still residue (WSR) fermented in Section 4.2 showed much less (if any) inhibition. Again this is in keeping with Leonard et al. (1947) who found that some volatile components were inhibitory but that these could be removed by various pretreatments, including steam stripping.

5. DECLINING PERFORMANCE IN THE A.B.E. FERMENTATION

5.1 Introduction

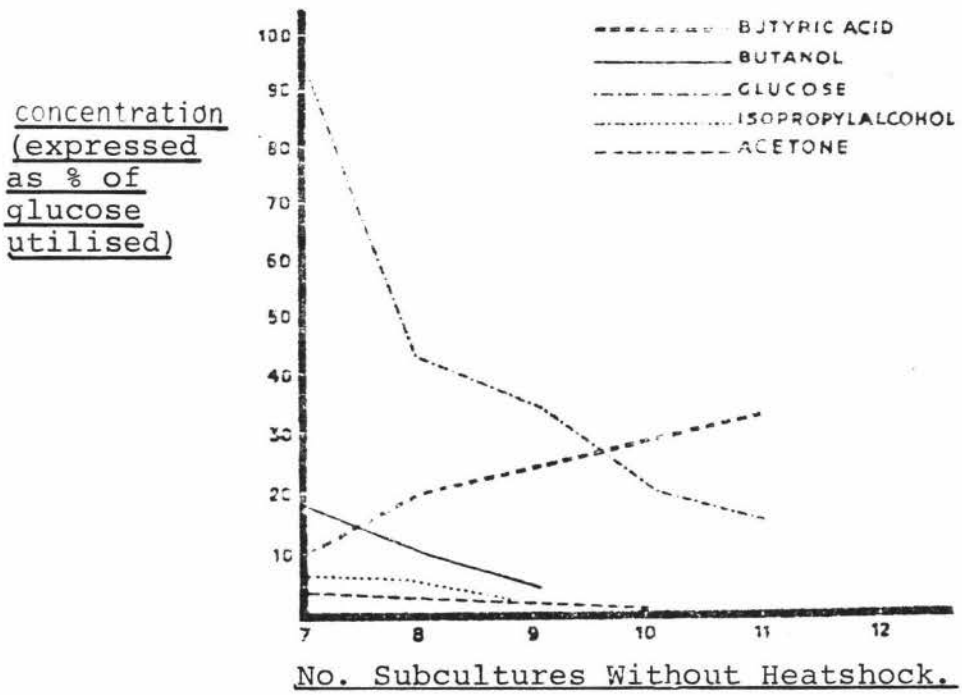
As with all fermentations intended to produce a desired result, contamination by unwanted cellular organisms or viruses, genetic variation, disturbances in physical and chemical conditions, or bad production procedure (such as might affect inoculation) can drastically affect the A.B.E. fermentation. However, assuming that nutritive conditions are met, the system is suitably anaerobic, and hygiene has been maintained to a "reasonable" level, there may still be problems.

Kutzenok and Aschner (1952) made a detailed study of degeneration in a strain of Clostridium butylicum after sequential serial transfer. They observed in each fermentation after transfer, lower use of sugar, lower solvent production, and a relative rise in butyric acid concentration expressed as percentage of glucose utilised. Fig. 5.1 (Kutzenok and Aschner, 1952) summarises their fermentation results. The most striking characteristics were loss of potential for solvent production and less vigorous fermentation. They concluded that individual cells were either degenerate or normal and that the declining culture characteristics were due to an increase in the proportion of degenerate cells in the population, rather than gradual deterioration of all cells simultaneously.

Ogata and Hongo (1979) describe similar fermentation symptoms in Clostridia species' (including Cl. acetobutylicum and Cl. saccharoperbutylicum) fermentations after phage attack - typically a slowing of the process or decreased productivity from a light contamination, or obvious cell lysis from heavy contamination.

They do not mention the effect of serial transfer from contaminated cultures, only saying that stocks known to

Fig. 5.1 Results of Kutzenok and Aschner (1952).



contain lysogens should not be used.

Another factor which seems to affect culture performance is heatshocking of the inoculum (Hastings, 1978). This is said to produce a healthier fermentation and to promote solvent production.

The following series of experiments shows the effect on degeneration during serial transfer of heatshocking at each transfer, and of the fermentation headspace pressure. No attempt was made to determine the cause of degeneration.

5.2 Results

Clostridium butylicum NRRL B-592 was used for this experiment. The culture underwent six passages of serial transfer in 100 ml "medicine bottles" containing sulphuric acid whey permeate supplemented with 5 g/l YE. After each sequential inoculation the freshly inoculated broth was heatshocked for 1.5 min at ca. 75°C, and the remainder of the inoculum used as inoculum for a 10-litre pressure fermentation similar to those outlined in Chapter 3. The headspace pressure for the 10-litre fermentation was controlled to a predetermined level and the final broth concentrations recorded. Subsequent transfers were made some days after gassing had ceased.

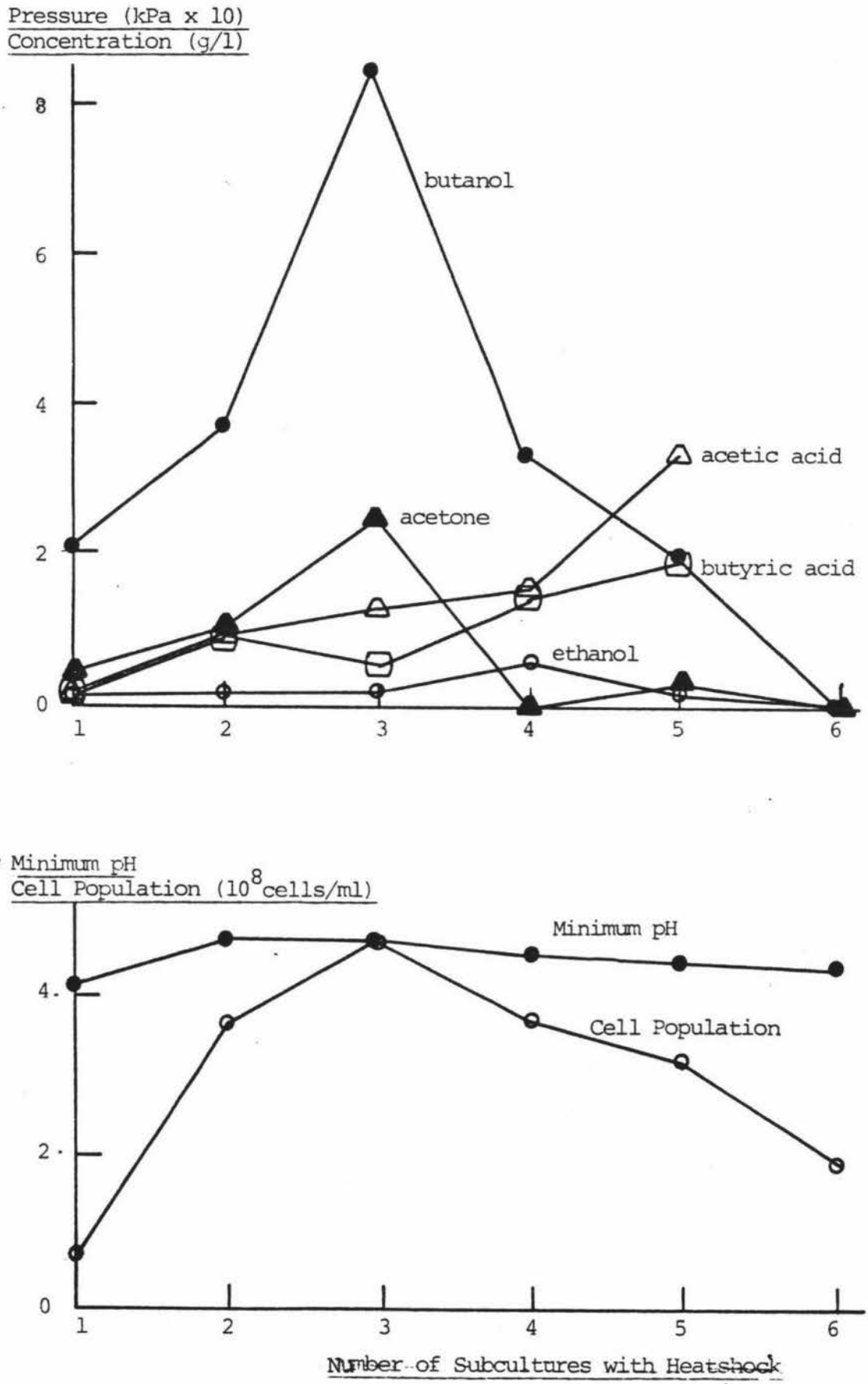
Results at first were encouraging with all culture characteristics (including minimum pH) improving (Fig. 5.2). However, after the third transfer degeneration was rapid, and by the 7th transfer fermentation had almost ceased. Degeneration was marked by decreasing cell numbers and solvent production, and increasing acid levels with associated decreasing pH.

The pressures (absolute) used were 70 kPa, 100 kPa, 65 kPa, 20 kPa, 0 kPa, and 95 kPa respectively for each

Table 5.1 RESULTS OF 10-LITRE FERMENTATIONS ON SERIAL TRANSFER WITH HEAT-SHOCKING

Transfer:	1	2	3	4	5	6	Units
N_s	76	380	490	380	331	195	$10^6 \frac{\text{cells}}{\text{ml}}$
$[B]_f$	2.1	3.7	8.5	3.34	1.99	negl	gl^{-1}
$[E]_f$	0.09	0.15	0.15	0.56	0.11	"	gl^{-1}
$[A]_f$	0.43	1.05	2.5	0	0.31	"	gl^{-1}
pH_m	4.3	4.9	4.85	4.7	4.6	4.55	pH
$[BA]_f$	0.2	0.9	0.5	1.4	1.9	-	gl^{-1}
$[AA]_f$	0.2	1.0	1.3	1.5	3.3	-	gl^{-1}

Fig.5.2. Declining Performance with Subculturing.



successive subculture, but they did not appear to significantly affect the fermentation results.

5.3 DISCUSSION

The characteristics of degeneration observed in this experiment were very similar to those described by Kutzenok and Aschner (1952) as a result of presumed genetic deterioration on serial transfer, and described by Ogata and Hongo (1979) due to phage infection. Initial comparison of the experimental technique and results in this chapter which those of Kutzenok and Aschner (1952) suggests that the degeneration seen was due to similar phenomena and that heatshocking provided some promise of counteraction. However, there was some evidence from cell count data (e.g. Table 5.2) that population fluctuations similar to those seen in Chapter 3 were also occurring in these fermentations although sampling was less frequent and gave a less detailed profile. Fluctuations were in fact seen in most of the fermentations. In addition, there seemed to be an unusual rise in cell population after about 150 hours. For these reasons, the literature describing phage attack on the fermentation was examined for a possible alternative solution and is discussed further in Section A4.1 of Appendix 4. It would appear that the possibility of involvement of phage or other lytic agents cannot be ruled out. It is difficult to comment further other than to stress the need to avoid degeneration in commercial plant by preparing all inocula from a standard stock as in Chapter 3, rather than by serial transfer which is sometimes used in brewing (Hastings, 1978). Stocks of different strains are kept to prevent successive failure due to phage attack (Ogata and Hongo, 1979; Ross, 1961). The fact that continuous fermentation of this type of organism has been successful on a laboratory scale (Ross, 1961) may lead to the conclusion that the degeneration is due to some phenomenon other than adverse spontaneous genetic mutation.

Table 5.2 CELL COUNT DATA IN 10-LITRE FERMENTATION AFTER THE FIRST SERIAL TRANSFER

Time (hrs)	Concentration (million cells/ml)
5.3	25
7.4	23
9.6	19
13	16
21	21
40	54
44	60
48	76
53	58
58	71
65	71
88	88
96	87
167	167
192	131
233	187

6. PRELIMINARY DESIGN AND COSTING OF AN A.B.E. FERMENTATION PLANT

6.1 Plant Design Parameters

General The design is very similar to that published by the author at CHEMECA '81 (Maddox et al., 1981) with some modifications. The main modification being that the spent broth solvent concentrations used were based on the experimental results of Chapter 3 rather than data reported in the literature. The design is a preliminary costing exercise and is based on existing technology with a feed of sulphuric acid casein whey permeate.

Quality The feed is not assumed to be sterile even though it will come from a neighbouring ultrafiltration plant. This is because existing dairy plants are not able to provide reliably sterile permeate. Intermediate storage, if any, might also increase the contamination risk. The temperature is about 50°C, and the composition is given in Table 2.1.

Capacity The average permeate flow rate is assumed to be 300 m^3 day^{-1} (12.5 m^3 $hr.^{-1}$). The plant should be designed with 20% excess capacity (360 m^3 day^{-1}, 15 m^3 $hr.^{-1}$) (Peters and Timmerhaus, 1980).

Location The plant is assumed to be located near the dairy factory supplying the feedstock.

Utilities The dairy factory is assumed to have sufficient surplus capacity to supply utilities (Jebson, 1981).

Operating Time The plant is expected to run 24 hours per day and 270 days per year. The running time in practice will be governed by the flow from the dairy plant which will in turn be governed by the dairy season (Fig. 1.2).

Materials The plant should be constructed of 306 or 316 stainless steel, except for the first distillation column (the beer still) which should be made of Cu/Ni alloy, and off-line and product storage vessels which may be of plain carbon steel construction.

Fermentation The fermentation was assumed to yield 14.4 g/l solvents in the ratios 15:3:1 n-butanol:acetone:ethanol after 65 hr. The solvent ratio chosen is that achieved in the work performed by the author in a 30-litre fermenter (Section 3.4), but due to the low efficiency the figure used for the total solvent yield was that for Run J of Chapter 3 (Table 3.3). The fermentation time used was also from Run J. The solvent ratios and the figure for total solvents yield used are both similar to those reported in the literature, although the time for fermentation is worse. The work in Chapter 4 and by Compere and Griffith (1979) show the large variation in solvent ratios and yields possible when different substrates, conditions, and organisms are used.

Beesch (1952) states that a 48 hr. fermentation on molasses could be expected to give ratios of 15:6.6:0.4, although the average ratios in his survey of 13 fermentations not producing isopropanol was 15:6:0.6. Spivey (1978) summarises a molasses fermentation which took 30-34 hr. to give a yield of 15:8.2:2.7, and Ross (1961) a fermentation of 36-48 hr.

Data for fermentations on whey and whey permeate is sparse. Maddox (1980) gives ratios of 10:1:1 for a fermentation using the same organism on sulphuric acid whey permeate, but his fermentation was on 100 ml scale and rates low. The work presented in Chapter 3 of this thesis also showed an average ratio of 9:1:1 (Table 3.3). Wix and Woodbine (1958) mention an industrial fermentation on an unspecified whey which took 12 to 48 hr, but give no data on solvent yield.

A total solvent yield of 15 g/l and butanol concentration of 12 g/l is quite reasonable on the basis of published data (Ross, 1961; Spivey, 1978) due to toxicity.

The fermentation values were assumed reasonable for initial costing purposes. However the result could very well be far from a practical optimum as no effort was made to optimise the fermentation in Chapter 3, and even the scarce data present in the literature for whey suggests the fermentation can perform very much better than assumed.

6.2 Plant Layout

The layout adopted is shown in Fig. 6.1. Continuous steam sterilising is provided by a length of piping for the required holding time, a regenerative heat exchanger to recover heat, a steam booster to raise the temperature for sterilising, and a water cooler to control the temperature to about 30°C for fermentation. The sterilising system is put in front of the fermenters but after nitrogen addition (such as Yeast Extract). pH adjustment with sterile ammonia (due to its strong basic pH) is performed after cooling prior to the filling of the fermenter.

Multiple batch pressure fermenters were used for the fermentation. Batch fermentation will be used as continuous fermentation is not yet proven commercially. The ability to hold pressure was specified in order to better attain high solvent yields (Chapter 3). The ability to withstand pressure will also be useful for steam sterilising of the process equipment, necessary to help prevent contamination both by unwanted micro-organisms and phage. Multiple fermenters were chosen to reduce the individual fermenter volume to a reasonable size, to supply a continual flow to the distillation unit, to allow continual draw off of whey from the dairy factory, and capitalise on the advantages of multiple vessels (such as convenience, flexibility, turndown, and superior economics when fermentations go "off").

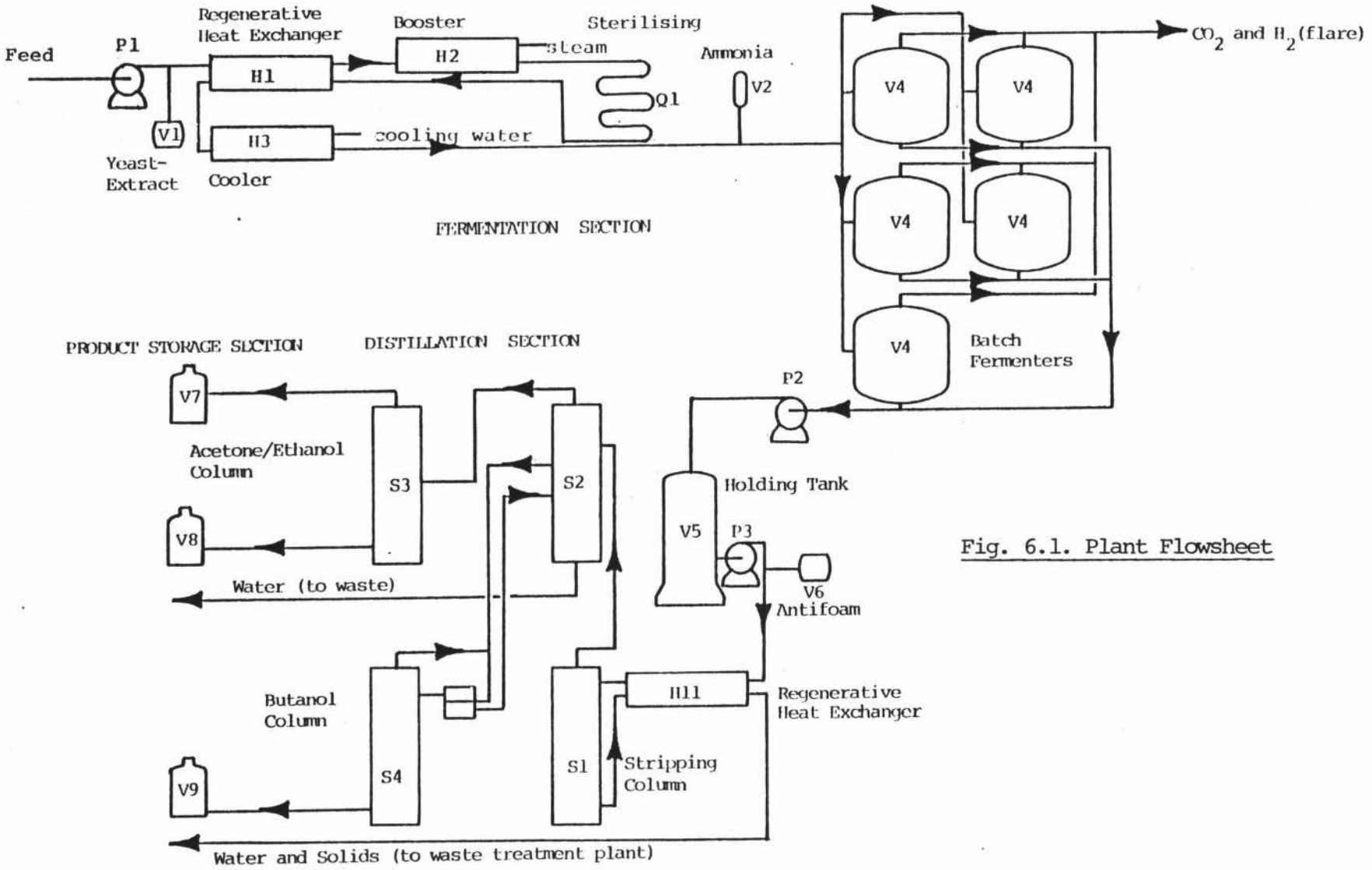


Fig. 6.1. Plant Flowsheet

A vessel after the fermenters helps to smooth the rate of flow to the distillation plant by supplying evening out and final "polishing" of the spent broth (beer) concentrations as well as flow rate buffering. Finally before the distillation section antifoam is added.

Distillation was chosen as the means of solvent separation as it is the only proven means for solvent separation available at present. Separation systems such as liquid/liquid extraction, solid/liquid extraction, and membrane preconcentration and separation are still not commercially proven (U.S. Department of Energy, 1980). The distillation system adopted was that traditionally used (Othmer, 1936), consisting of a beer stripping column and three much smaller columns producing solvent to specification.

Finally, the solvents produced are stored in small product tanks prior to cartage away from site by tanker (rail, boat, or road - unspecified).

6.3 Fermentation Section

6.3.1 Yeast Extract Addition (V1)

Two 3.5 m^3 tanks would be suitable. The tanks should be fitted with agitators to provide mixing for dissolution, and both process water and liquor to provide make-up. Draw off to the liquor stream would be made by metered pump.

It is envisaged that each tank be rinsed out and made up once each per shift, giving a residence time of 8 hours each (use 9 hours for design). The tank solution should be about 100 g/l, and flow metered to give a process stream concentration of about 5 g/l yeast extract. (This is the concentration used in our experiments. It has been shown elsewhere (for example; U.S.Department of Energy, 1980) that 4 g/l yeast extract with 1 g/l $(NH_4)_3PO_4$ is also

suitable. Investigation as to a better, cheaper, alternative nitrogen source at a later date is recommended.)

$$\text{flow of YE} = 5\ g\ l^{-1} \times 15\ m^3\ hr^{-1} / 100\ g\ l^{-1}$$
$$= 0.75\ m^3\ hr^{-1}$$

V_{tt} = total tank volume for 9 hr. residence time
$= 0.75\ m^3\ hr^{-1} \times 9\ hr$
$= 6.75\ m^3$

Use 2 X 3.5 m^3 tanks.

Two tanks were chosen in case contamination became a problem, and in order that staggered filling times would allow continuous flow to the process.

The tanks will be made of mild steel, and the metering pump rated at 0.75 $m^3\ hr^{-1}$ (12.5 l min^{-1}).

6.3.2 Sterilisation Holding Section (Q1)

The sterilisation conditions govern the heat exchanger duties, and should therefore be specified first. The sterilisation conditions decided on were t = 37 sec and T = 130°C (Cleland, 1980; Aiba et al., 1965).

Liquor at 130°C would be fed into a length of stainless steel pipe to provide a plug flow steriliser. In order that turbulent flow is present in the pipe (to prevent uneven cooling of liquor near the walls), a design fluid velocity of 1.2 m s^{-1}(4 ft s^{-1}) should be used (Peters and Timmerhaus, 1980).

Q = liquor flow
$= 15\ m^3\ hr^{-1}$

A = required pipe cross-sectional area
= Q/(velocity)
= 15 m^3 hr^{-1}/(1.2 m s^{-1} x 60^2 s hr^{-1})
= 0.0035 m^2

D = pipe diameter
= $(4A/\pi)^{0.5}$
= 0.0668 m (2.6 ins)

use 2.5 inch (0.0635 m) pipe.

v′= actual pipe velocity

$$= \frac{15\ m^3\ hr^{-1} \times 4}{\pi \times (0.0635\ m)^2 \times 60^2\ s\ hr^{-1}}$$

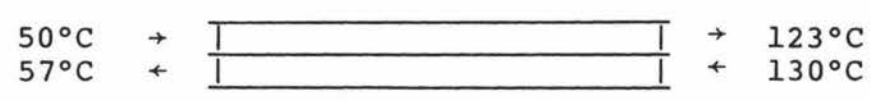
= 1.3 ms^{-1}

and L = length pipe required
= v t
= 1.3 ms^{-1} x 37 s
= 48.1 m

hence use 50 m of 2.5 in (0.064 m) pipe.

6.3.3 Regenerative Heat Exchanger (H1)

This exchanger will be a plate-and-frame type heat exchanger to get good heat recovery and for easy cleaning. Schematically the layout is:



The feed liquor is assumed to be hot from the ultrafiltration plant (50°C), and pure countercurrent flow will be assumed with a temperature difference of 7°C. The following method is suitable for approximate sizing and costing.

$$w = mc\ (T_2 - T_1)$$
$$= (15 \times 1000) \times 4.19 \times (130 - 57)/60^2$$
$$= 1.3 \times 10^3\ kJs^{-1}$$
$$= 1.3\ MW$$

: m = flow rate (kg s^{-1})
: c = specific heat (kJ $kg^{-1}K^{-1}$)
: T = temperature (°C or °K)
: w = energy flow (Js^{-1} or W)

LMTD = log mean temperature difference
= 7°C (°K) (pure countercurrent flow)

U = heat transfer coefficient
= 1.2 kW $m^{-2}\ K^{-1}$
(N.Z. Institution of Engineers, 1980(a))

so A = area of heat exchanger
= w/(U LMTD)
= 1 x 10^6 J s^{-1}/ (1.2 x 10^3 J s^{-1} m^{-2} K^{-1} x 7°K)
= 120 m^2

The second heat exchanger (H2), used to bring the process liquor up to sterilising temperature (130°C), will be a shell and tube steam condenser with energy flow 119 kW, LMTD = 38°C, U = 2.8 kW m^{-2} $°C^{-1}$ (Chemical Engineers' Handbook, 1973), area 1.1m^2, and 200 l hr^{-1} steam.

The third heat exchanger (H3) will be a shell and tube heat exchanger with cooling water on the shell side. The cooling water rise assumed was 10°C and energy flow of 393 kW, LMTD = 11.5°C, U = 1.2 kW m^{-2} $°C^{-1}$ (N.Z. Institution of Engineers, 1980(a)), A = 30 m^2, and 34 m^3hr^{-1} water.

6.3.4 Fermenters (V4)

The fermenters should be stainless steel-clad pressure

vessels. No agitation is required, except for initial gas sparging (with CO_2 or CO_2/H_2 mixture) to strip out oxygen and supply some initial turbulence. No air supply is required as the fermentation is entirely anaerobic.

One spare fermenter will be specified for backup purposes. This may not be required in practice if the risk of fermenter breakdown is considered acceptable. The plant will be operating at peak flow for only a few months each year (depending on the whey supply) and the consequences of a breakdown during this period must be considered. The cost saving in this decision is considered later.

t_t = turnaround time
= cleaning time + sterilisation + filling
+ time for fermentation + emptying
= $3 + 0.5 + 0.8\ v/(rQ) + 65 + 0.8\ v/RQ$
= $68.5 + 0.8\ v\ (r^{-1} + R^{-1})/Q$: units = hours

where v is the volume of a single fermenter. (Assume fermenters operate 80% full.) A cleaning and sterilising time of 3.5 hr. was thought to be reasonable. In the equation r is the ratio of vessel filling rate to the average process flow rate ($Q = 15\ m^3\ hr^{-1}$), and R the ratio of the emptying rate to Q.

now V = total fermentation volume
= $Q\ t_t$ (in the limiting case)
= $n\ v$

hence $nv = 68.5\ Q + 0.8\ v\ (r^{-1} + R^{-1})$
$v = (68.5Q)/(n - 0.8\ r^{-1} - 0.8\ R^{-1})$

and c' = total fermenter cost
= $a\ (n + 1)\ v^{0.56}$: a = constant
= $a\ (n + 1)((68.5Q)/(n - 0.8\ r^{-1} - 0.8\ R^{-1}))^{0.56}$

where n is the number of operating fermenters (excluding

the spare), and c′ is the cost. The ratio c′/a may be considered as a factor proportional to cost. The cost power factor of 0.56 on the size term (v) is the common "six-tenths power rule" for cost adjustment with object size applied to stainless steel pressure reactors, and is typical of small pressure vessels and unpressurised vessels of size similar to the fermenters.

$$c'/b = \text{cost factor}$$
$$= (n + 1)(n - 0.8\,(r^{-1} - R^{-1}))^{-0.56}$$
$$= (n + 1)(n - RR)^{-0.56}$$

: RR = Rate Factor

$$= 0.8(r^{-1} + R^{-1})$$

: b = 68.5 a Q

To find the minimum cost, the expression is differentiated

$$\frac{d\,(c'/b)}{dn} = (n - RR)^{-0.56} + (n + 1)(-0.56)(n - RR)^{-1.56}$$
$$= (n - RR)^{-0.56}\,(1 - 0.56\,(n + 1)/(n - RR))$$
$$= 0 \text{ (at cost minimum)}$$

This assumes that RR is fixed and will only vary with negligible variation in total cost. The minimum cost therefore occurs at

$$0 = 1 - 0.56\,(n + 1)/(n - RR)$$
$$(n - RR) = 0.56\,(n + 1)$$
$$RR = 0.44n - 0.56$$

or $$n = (RR + 0.56)/0.44$$

If then r is set to 1 (i.e. fermenters filled at $15m^3\ hr.^{-1}$)

$$n = (RR + 0.56)/0.44$$
$$= (1.36 + 0.8/R)/0.44$$

then $$R = 0.8\,(0.44n - 1.36)^{-1}$$

Table 6.1 shows values of the emptying rate (R) and the cost ratio (c/b) for different numbers of fermenters with and without a spare. The approximation assumes that varying R and RR incurs negligible cost alterations. It is true that these variations will be significantly less than the fermenter cost.

The negative values of R show that it is impossible to fill the vessels with r = 1 with less than 4 operating fermenters. Conceptually this is obvious in the case of a single fermenter. When filling of the one fermenter is complete, there is no other fermenter to fill while the first is fermenting so filling must stop. This makes it impossible to maintain an average filling rate equal to the average flow (i.e. r = 1). For this reason 4 operating fermenters are specified for r = 1.

If r is increased and the filling rate is greater than $Q = 15\ m^3\ hr^{-1}$, then the Rate Factor RR is used for comparisons. Values of RR are included in Table 6.1, but discussion of the possible advantages allowed by varying r will be left until Discussion (Section 6.8). The decision of allowing r to vary from unity will in itself entail some added expense in extra equipment - i.e. an unpressurised feed tank.

The values for c′/b, R, and RR are the optimum for the particular number of fermenters, and depend only on the number of fermenters and not each other.

The design is then to have n = 4 + 1 = 5 pressure fermenters each of volume (v)

$$v = t_t \times Q/n$$
$$= (68.5\ hr. \times 15\ m^3/hr. + v\ (0.8\ (1 + 2^{-1})))/4$$
$$4v = 1027.5 + 1.2\ v$$

Table 6.1 COST OF FERMENTERS FOR DIFFERENT NUMBERS OF FERMENTERS

Number of Operating Fermenters n	Filling R (r = 1)	Rate Factor RR (r varying)	Cost Ratio c′/b (with spare)	Cost Ratio c′/b (without spare)
1	-0.87	-0.12	1.88	0.94
2	-1.67	0.32	2.24	1.50
3	-20.0	0.76	2.55	1.91
4	2.00	1.20	2.81	2.25
5	0.95	1.64	3.04	2.54
6	0.63	2.08	3.26	2.79
7	0.47	2.52	3.45	3.02

Note: These ratios are all dimensionless.

$$v = 1027.5/(4 - 1.2)$$
$$= 367\ m^3$$

when operating 80% full with one spare fermenter and with an emptying rate of only 2.00 times the average flow, Q.

The turnaround time may then be calculated as:

$$t^t = 68.5\ hr. + 0.8 \times 367\ m^3\ (1 + 2^{-1})/15\ m^3\ hr.^{-1}$$
$$= 97.9\ hours$$

The turnaround time will be shortened by increasing R, reducing the emptying rate, and thereby allowing some down-time for each fermenter. While $367\ m^3/(2 \times 15\ m^3\ hr.^{-1})$ = 12.2 hr. seems a long time allowed to empty a fermenter it is caused by the necessity to have more than three fermenters, and to allow r to equal 1. The long emptying time has the advantage of aiding supply of an even flow of liquor to the distillation plant.

6.3.5 Pumps

Sizing of pumps for costing estimates is based on the hydraulic head required (F). Pumps P1 and P3 were assumed to have to pump against a 10 m head with a capacity of 300 l min^{-1} (430 $m^3\ day^{-1}$) to provide capacity surplus to normal operational requirements. F is calculated as the product of capacity and head. A common spare is used.

Pump P2, which empties the fermenters, was given over twice the required capacity to allow the fermenters to empty in less than 9 hours. At such times as only one of the pumps is used (i.e. the spare is not used), the emptying time will be $367\ m^3/0.7\ m^3\ min^{-1}$ = 524 min or 8.7 hr.

6.3.6 Other Equipment

The process pH adjustment will be made using ammonia (V2) to lessen dependence on yeast extract.

The distillation buffer tank (V5) was sized as slightly bigger than 40% of a single fermenter, and is not a pressure vessel. Agitation should be supplied to prevent settling of cells. Cells will be fed into the first still (beer stripper) and removed as bottoms.

Tables 6.2, 6.3, and 6.4 give a summary of the fermentation section equipment.

6.4 Distillation

6.4.1 Overview of the Distillation Section

The layout of the distillation system used was taken from the literature (similar to Othmer, 1936) and is the subject of several patents (e.g. U.K. Patent 419170, 1934; U.K. Patent 633706, 1949). It is shown schematically in Fig. 6.1. The layout incorporates the use of a phase separator (P) to break the azeotrope in the azeotropic distillation.

The distillation plant represents only a relatively small fraction of the total capital cost and the beer still represents the largest proportion of the distillation plant capital cost, hence only rough sizing of the distillation plant is required. Nevertheless, some areas have had a little more design attention paid to detail as an aid to future design when greater precision is required. Columns S1, S3, and S4 may readily be approximated by binary mixtures with good accuracy, however Column S2 is much more difficult. All four of the components, acetone, ethanol, butanol, and water are represented by high concentrations at some point in Column S2, and the mixture

Table 6.2 SUMMARY OF VESSELS FOR FERMENTATION SECTION

Vessel	Description	Number	Size Parameter
Q1	steriliser section	48.1 meters	2.5 in (0.0635 m)
V1	Yeast Extract stock tank	2	3.5 m^3
V2	ammonia bottle	1	5 m^3 day^{-1} (1.5MPa)
V4	fermenter (pressure)	5	376 m^3
V5	buffer (agitated)	1	300 m^3
V6	antifoam tank	1	40 m^3

Table 6.3 SUMMARY OF HEAT EXCHANGERS FOR FERMENTATION SECTION

H.E.	Area (m^2)	Type	Comments
H1	120	plate	regenerative
H2	1.1	s & t*	superheater (condenser)
H3	30	s & t	cooler (cooling water)

* s & t = shell and tube (cross flow)

Table 6.4 SUMMARY OF PUMPS FOR FERMENTATION SECTION

Pump	Number*	Capacity (l/min)	Head** (kPa)	F*** ($\times 10^3$ m^3 Pa min^{-1})
P1	1	300	100	30
P2	1 + 1	700	100	70
P3	1 + 1	300	100	30

* one spare each (common spare P1 and P3)

** each pump required to raise liquid to height of about 10 m

*** cost data for pumps are usually based on an F factor defined as the product of the capacity and the head.

forms highly non-ideal systems including a two phase region due to the presence of butanol and water in suitable concentrations. In order to provide preliminary design data on the column, the column will be approximated by two columns (S2A and S2B), each of which will be treated as independent columns. The approximations will be dealt with when appropriate. The hydraulic sizing (Section 6.4) has been done for three different tower internals (turbo-grid, plate, and packed columns) for comparison of the three types. In the final specification equipment manufacturers' data should be considered for final selection and design.

6.4.2 Mole Balances

The overall strategy for solution of the system mole balances was to first calculate the distillate for S1, then the distillate and part of the overall bottoms flow for S2, then determine concentrations around S4, and finally column S3. Calculations for other streams were made as required. All balances will be conducted on the basis of molar flow rates (mol s^{-1}). Concentrations are evaluated only as a final step for diagrammatic summary.

The method of analysis was to set up all assumptions and constraints as a system of simultaneous linear equations. These equations are then solved. The equations were solved using a modified TI-58 calculator hard-wired to solve up to a system of 8 simultaneous linear equations. Each set of equations includes component mole balances with other equations formulated as appropriate, and are listed below or in the appropriate table. Examination of the method of formulation will show that this approach is capable of handling quite complex specifications after linearisation. In order that roundoff errors are minimised and consistency maintained throughout the balances, values were recorded to between 4 and 8 decimal places. This accuracy is included for numerical consistency.

The feed to the distillation section was defined as 14.4 g/l total solvent in the ration 15:3:1 butanol: acetone:ethanol.

The mole balance calculations are summarised in Figs. 6.2 - 6.5. The beer still (S1) calculations were performed as follows:

Constraints
(i) mass balance requirements.
(ii) 90% recovery of n-butanol specified.
(iii) negligible loss of acetone and ethanol to bottoms
(iv) overheads are approximately at components' azeotropic concentration. The alcohol concentrations were estimated by assuming each solvent was in the same ratio to water as it was in its binary azeotrope.

Feed

The feed and concentrations to the first still are:

flow rate = 360 m^3 day^{-1} (20% excess capacity)
= 231.4815 mol s^{-1}

butanol = 0.0028051 mol fraction
acetone = 0.0006561 mol fraction
ethanol = 0.0003008 mol fraction
water = 0.9962380 mol fraction

The pronumerals are (where P1T is the label used for the product stream from the top of column S1, and P1B the product stream from the bottom of column S1):

(a) = molar flow rate of distillate stream (P1T)
(b) = molar flow rate of butanol in P1T
(c) = molar flow rate of acetone in P1T

contd. p. 143/

Fig 6.2 MOLE BALANCE DATA AROUND S1

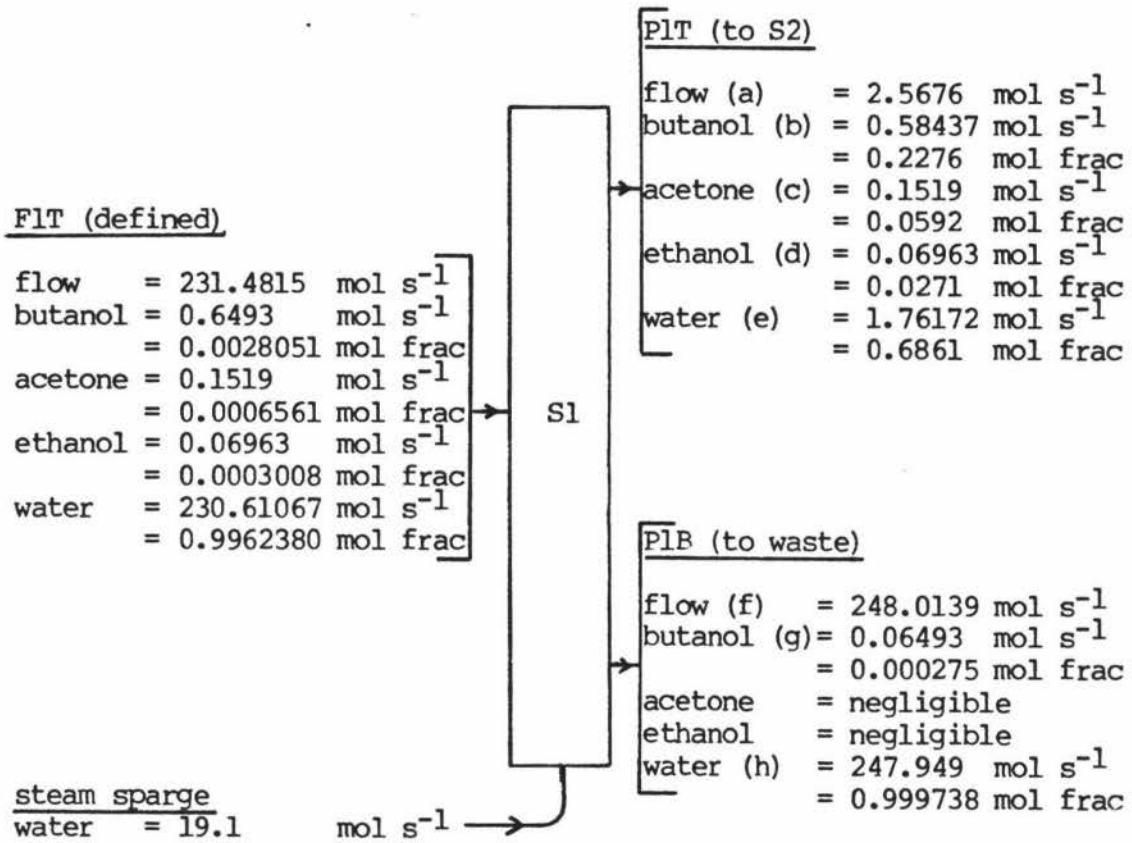


Fig 6.3 MOLE BALANCE RESULTS FOR S2

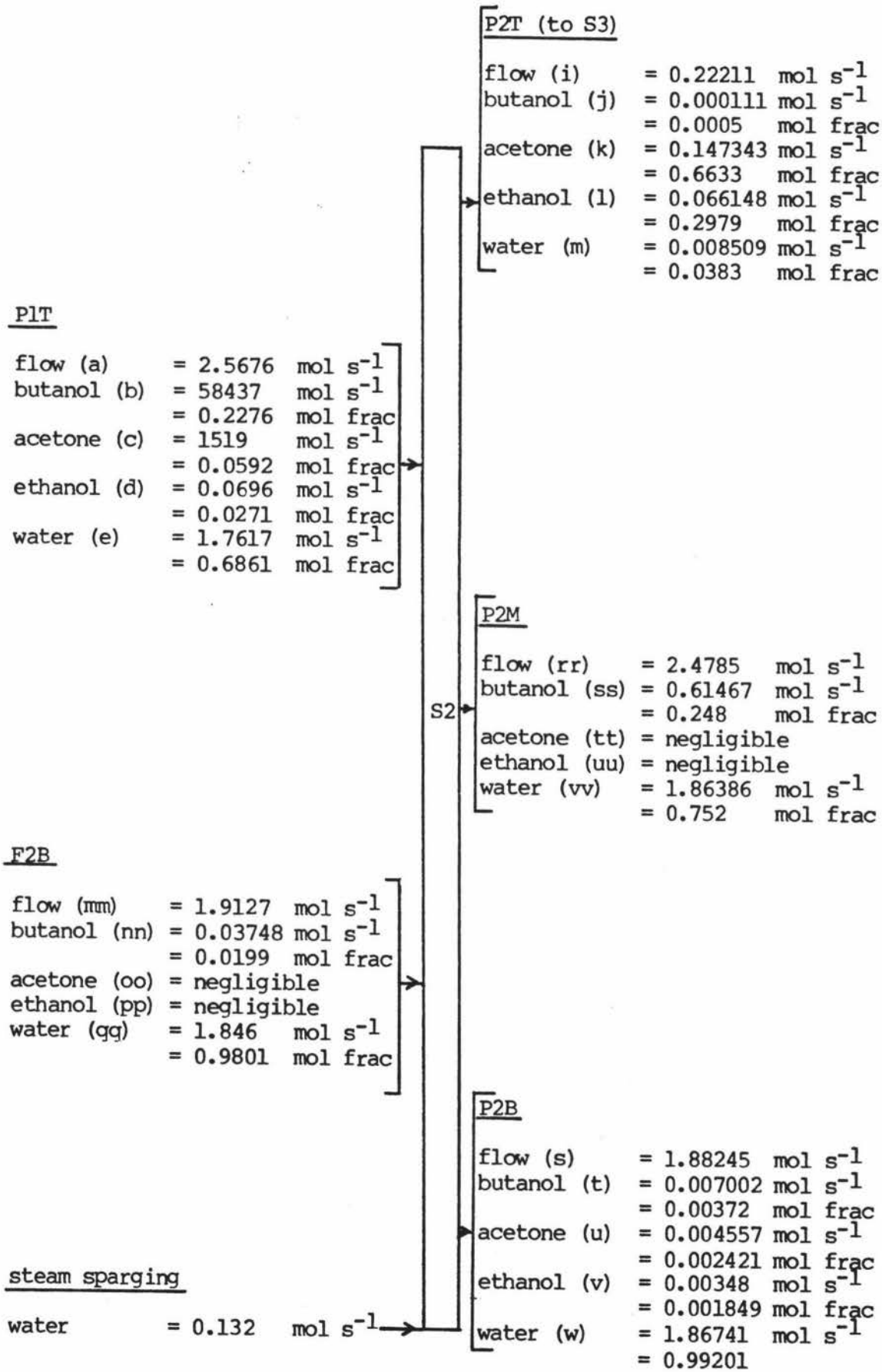


Fig 6.4 MOLE BALANCE RESULTS FOR S3

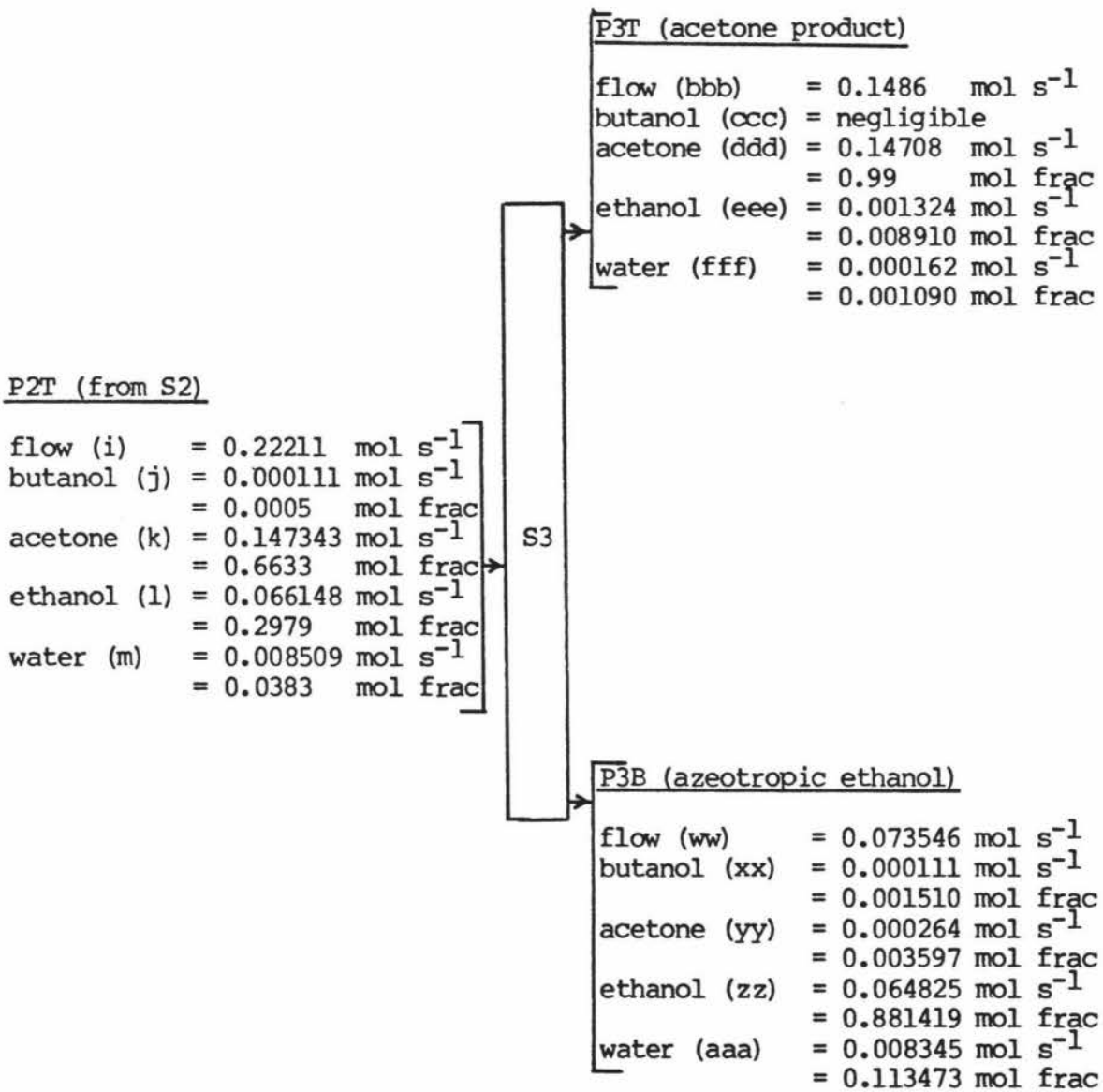
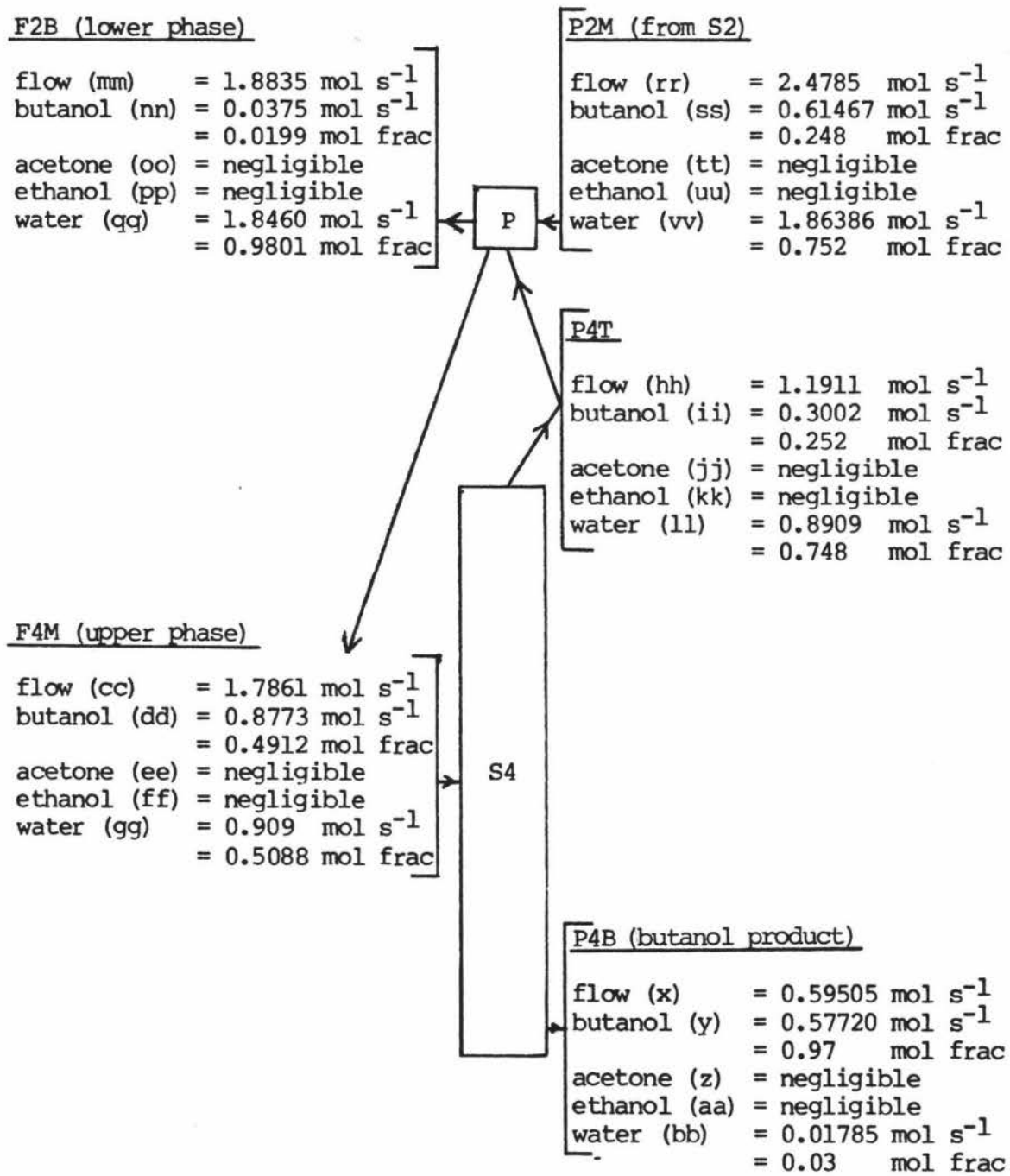


Fig. 6.5 MOLE BALANCE RESULTS FOR THE PHASE SEPARATOR (P) AND S4



(contd. from p. 138)

(d) = molar flow rate of ethanol in P1T
(e) = molar flow rate of water in P1T
(f) = molar flow rate of bottoms stream (P1B)
(g) = molar flow rate of butanol in P1B
(h) = molar flow rate of water in P1B

as shown in Fig. 6.2.

The equations used to determine the unknowns were:

i) Mass balances for butanol, acetone, and ethanol, and an overall mass balance (equations 1 to 4).

ii) Recovery statement (i.e. butanol flow in overheads is 90% of butanol flow in the feed so the ratio of overheads to bottoms is 9 to 1).

$$(b) - 9\,(g) = 0 \qquad \text{(equation 5)}$$

iii) Azeotrope Statement (assume binary ratios)

$$\text{butanol:water} = 0.25:0.75 = 1:3$$

$$\text{ethanol:water} = 0.89:0.11 = 1:0.1236$$

and so adding together to give the total water flow

$$(e) = 3(b) + 0.1236\,(d)$$

$$3(b) + 0.1236\,(d) - (e) = 0$$

(equation 6).

iv) Summation Statement for stream P1B

$$(f) = (g) + (h)$$
$$(f) - (g) - (h) = 0 \quad \text{(equation 7)}.$$

(i.e. the total stream flow must equal the sum of all its components).

Written in matrix form these equations become:

P1T			P1B				F1T*	Balance**
b	c	d	e	f	g	h	(feed)	
1	1	1	1	0	1	1	231.4815	overall
1	0	0	0	0	1	0	0.6493	butanol
0	1	0	0	0	0	0	0.1519	acetone
0	0	1	0	0	0	0	0.06963	ethanol
1	0	0	0	0	-9	0	0	equ 5
3	0	0.1236	-1	0	0	0	0	equ 6
0	0	0	0	1	-1	-1	0	P 1 B

* All equations are solved for unknowns on the basis of mole flow rates, and only converted to mole fractions for diagrammatic representation.

e.g. 0.0028051 x 231.4815 = 0.64932 mol s^{-1}
(butanol flow rate).

** overall = overall mole balance (equation 1)
butanol = butanol mole balance (equation 2)
acetone = acetone mole balance (equation 3)
ethanol = ethanol mole balance (equation 4)
equ 3 = equation 5
equ 4 = equation 6
P1B = summation statement for stream P1B (equation 7)

Solving this system of equations gives:

$b = 0.58437 \text{ mol } s^{-1}$
$c = 0.1519 \text{ mol } s^{-1}$
$d = 0.06963 \text{ mol } s^{-1}$
$e = 1.76172 \text{ mol } s^{-1}$
$f = 228.9139 \text{ mol } s^{-1}$
$g = 0.06493 \text{ mol } s^{-1}$
$h = 228.8490 \text{ mol } s^{-1}$

and the determinant of the matrix was 10. Solving for (a) gives:

$$a = b + c + d + e$$
$$= 2.5676 \text{ mol } s^{-1}$$

Water introduced to the column by steam sparging is added onto the bottoms flow. This is because the water in the distillate streams of S1 and S2 is entirely dependent on the alcohol concentrations in these streams. All other water becomes part of the bottoms. This result is not entirely accurate of a real distillation column, and is forced by the method of calculation. For this reason 19.1 mol s^{-1} water is added to e to account for steam sparging. This quantity of steam is calculated later (Section 6.4.3.1). The bottoms flows then become:

$f = 228.9139 + 19.1 = 248.0139 \text{ mol } s^{-1}$
$g = 0.06493 \text{ mol } s^{-1} = 0.000262 \text{ mol frac}$
$h = (228.8490 + 19.1) \text{ mol } s^{-1} = 0.999738 \text{ mol frac}$

The same adjustment is made for column S2 which has a sparging rate of 0.132 mol s^{-1}. The sparging rate in both cases was assumed equal to the vapour flow at the base of the columns (Tables 6.14 and 6.15).

The results and calculations for the remaining distillation columns are summarised in Figs. 6.2 - 6.5 and Tables 6.5 -

6.10 respectively. All pronumerals used in Tables 6.5 - 6.10 are defined in Figs. 6.2 - 6.5 and 6.6. Column S2 is split up into two columns (S2A and S2B) connected by an imaginary stream (P2AB) which represents the overall mole flow downwards at the point of the imaginary split (Fig. 6.6). Further description of the approximation is provided by Tables 6.6 and 6.7 and (Fig. 6.6). Splitting column S2 into two and use of the imaginary stream greatly simplifies calculations.

6.4.3 Number of Theoretical Stages

Distillation columns may be sized either by the number of theoretical plates required, or by the number of transfer units (NTU) required. The transfer unit is the design parameter used in connection with a continuous tower filling (e.g. packings), and is conceptually equivalent to the ideal plate used in plate tower sizing. For sizing of plate towers the McCabe-Thiele method may be used. This method is restricted to use with ideal mixtures of similar compounds, although for rough sizing and costing this constraint is often relaxed, as it is here. For sizing of packed towers, the McCabe-Thiele method for plate towers may be used and a HETP correlation used as an efficiency and height conversion term, or the NTU method used and a HTU (height of a transfer unit) correlation used for efficiency and conversion to height.

The choice in methods depends on the case in hand, and is often dictated by the availability of a suitable HETP or HTU correlation for the chosen packing.

6.4.3.1 McCabe-Thiele Constructions

It was assumed that the McCabe-Thiele method of distillation column analysis of the non-ideal multicomponent mixtures present would be sufficiently accurate for our purposes.

contd. p. 154/

Table 6.5 MOLE BALANCE FOR S1

Assumption

The feed conditions are specified.

Constraints

1) four mass balances.
2) azeotropic composition in overheads (refer text).
3) negligible loss of acetone and ethanol to bottoms.
4) 90% n-butanol recovery.

Feed

Refer to the text.

Equations

	P1T			P1B			F1T	Balance
b	c	d	e	f	g	h	(feed)	
1	1	1	1	0	1	1	231.485	overall
1	0	0	0	0	1	0	0.6493	butanol
0	1	0	0	0	0	0	0.1519	acetone
0	0	1	0	0	0	0	0.06963	ethanol
1	0	0	0	0	-9	0	0	equ 5
3	0	0.1236	-1	0	0	0	0	equ 6
0	0	0	0	1	-1	-1	0	P1B

Determinant = 10

$b = 0.58437 \text{ mol s}^{-1}$
$c = 0.1519 \text{ mol s}^{-1}$
$d = 0.06963 \text{ mol s}^{-1}$
$e = 1.76172 \text{ mol s}^{-1}$
$f = 228.9139 \text{ mol s}^{-1}$
$g = 0.06493 \text{ mol s}^{-1}$
$h = 228.8490 \text{ mol s}^{-1}$

and $a = b + c + d + e$
$= 2.5676 \text{ mol s}^{-1}$

(refer Fig.6.3,p.139)

Table 6.6 MOLE BALANCE FOR TOP OF S2

(IMAGINARY COLUMN S2A, FIG 6.3, FIG 6.6 (PP. 140 & 149))

Assumption

In order to render column S2 soluble, column S2 was approximated to two stills, one on top of each other, with an imaginary stream (P2AB) connecting the two.

Constraints

1) four mass balances.
2) azeotropic composition in overheads (as for column S1).
3) specify 5% ethanol lost in bottoms and 3% acetone lost in bottoms.
4) specify butanol concentration in overheads at 0.0005 mol. frac. The equations are not particularly sensitive to this , even an error of a factor of 10 produces relatively slight alterations.

Equations

P 2 T				P 2 A B				P 1 T	Balance
j	k	l	m	o	p	q	r		
1	1	1	1	1	1	1	1	2.5676	overall
0	1	0	0	0	1	0	0	0.1519	acetone
0	0	1	0	0	0	1	0	0.06963	ethanol
0	0	0	1	0	0	0	1	1.76172	water
3	0	0.1236	-1	0	0	0	0	0	azeotropes
0	1	0	0	0	-32.3	0	0	0	acetone loss
0	0	1	0	0	0	-19	0	0	ethanol loss
0.9995	-0.0005	-0.0005	-0.0005	0	0	0	0	0	butanol spec.

Determinant = 665.3

j = 0.000111 mol s^{-1} = butanol in S2 distillate
k = 0.147343 mol s^{-1} = acetone in S2 distillate
l = 0.0661485 mol s^{-1} = ethanol in S2 distillate
m = 0.0085091 mol s^{-1} = water in S2 distillate
o = 0.5842389 mol s^{-1} = butanol in P2AB
p = 0.004557 mol s^{-1} = acetone in P2AB
q = 0.0034815 mol s^{-1} = ethanol in P2AB
r = 1.753211 mol s^{-1} = water in P2AB

and i = 0.22211 mol s^{-1} (refer Figs. 6.3 and 6.6 (pp. 140 & 149))
n = 2.3455 mol s^{-1} (refer Fig. 6.6

Fig 6.6 APPROXIMATION FOR COLUMN S2 MOLE BALANCES

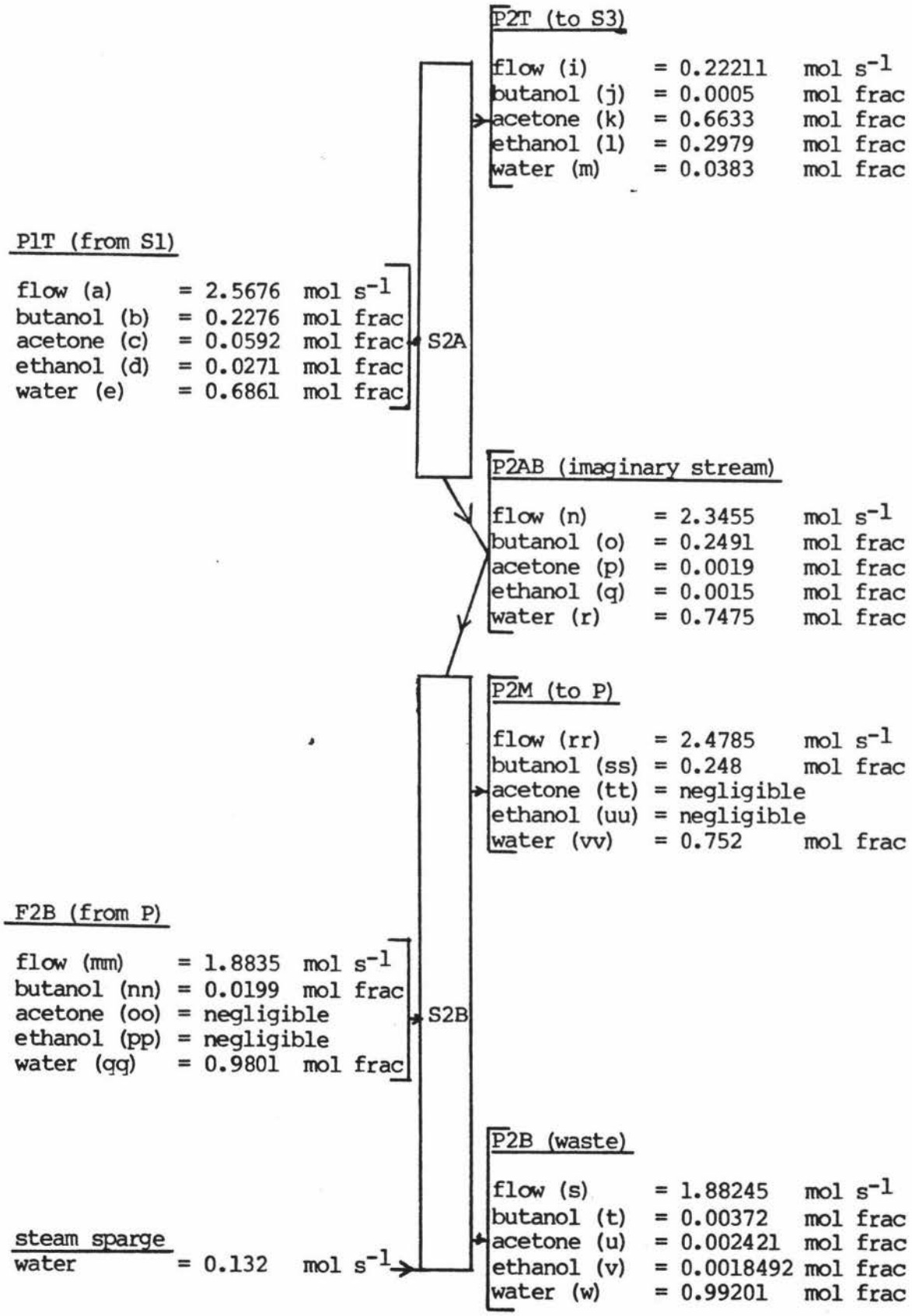


Table 6.7 MOLE BALANCE OVER COLUMNS S2 AND S4

Assumption

1) That acetone and ethanol flows in bottoms from S2 (equivalent to the imaginary stream P2AB) are known.
2) negligible acetone or ethanol escape in the bottoms from S4 (Stream P4B) (assumed due to the high temperature of P4B), and the concentration of acetone and ethanol in streams into and out of P will remain at a low enough level to be ignored. (This assumption cannot of course be fully justified if P2B has non-zero concentrations of these, but as a working assumption it will be accepted.)
3) Specify the butanol concentration in P2B as 0.004.

Constraint

Product butanol is at 0.97 mol frac (industrial grade).

Equations

P2AB = imaginary stream
= (bottoms from S4) + (bottoms from S2)
= P4B + P2B
= P1T - P2T (where P1T and P2T are known).

x	s	P2AB	Balance
1	1	2.3455	Overall
0.97	0.004	0.5842	Butanol

Determinant = -0.966
x = 0.59505 mol s^{-1}
s = 1.75045 mol s^{-1}

and t = 0.004 x 1.75045
= 0.007002 mol s^{-1}

w = (P2B - t - u - v + sparging rate)/(P2B + sparging rate)
= 0.9914 (expressed as a mole fraction).

where p = acetone loss in P2AB
= 0.004557 mol s^{-1}

q = ethanol loss in P2AB
= 0.0034815 mol s^{-1}

also y = 0.97 x 0.59505 = 0.5771985 mol s^{-1}

bb = 0.0178515 mol s^{-1}

t = 0.004 x 1.81854 = 0.007274 mol s^{-1}

:x = bottoms flow from S4
:s = bottoms flow from S2
:t = butanol flow in bottoms from S2
:w = water flow in bottoms from S2
:p = acetone loss
:sparging rate = 0.132 mol s^{-1}
:q = ethanol loss
:u = acetone loss
= p (Table 6.6)
:v = ethanol loss
= q (Table 6.6)

(Refer to Figs. 6.3, 6.5, and 6.6 (pp. 140, 142, 149))

Table 6.8 MOLE BALANCES OVER COLUMN S3
(ref. p. 141)

Assumptions

1) Negligible butanol is present in the feed and its concentration is:
   butanol = 0.0005 mol. frac.
   (refer Table 6.6). All this butanol appears as bottoms (Stream P3B).
2) The water assumed associated with the ethanol and butanol azeotropes will be assumed to remain with them. The system may now be treated as a ternary system. In addition, since all the butanol is assumed to appear in the bottoms, the system may be approximated to a binary system.

Constraints

1) P3B meets industrial grade specifications at about 94.5% (w/w) ethanol.
2) P3T meets industrial grade specifications with 0.99 mol. frac. acetone.

Equations

The grade constraint for ethanol may be rewritten as:

$$0.945 = \frac{46.07\,zz}{46.07zz + 74.12xx + 58.08yy + 18aaa}$$

or $0 = 27.6431xx + 21.6610yy + 6.7131aaa - zz$

bbb	eee	fff	ww	xx	yy	zz	aaa	P2T	Balance
1	0	0	10	0	0	0	0	0.22211	overall
0	0	0	0	1	0	0	0	0.000111	butanol
0.99	0	0	0	0	1	0	0	0.147343	acetone
0	1	0	0	0	0	1	0	0.066148	ethanol
0	0	0	0	3	0	0.1236	-1	0	azeotrope
0.01	-1	-1	0	0	0	0	0	0	P3T
0	0	0	1	-1	-1	-1	-1	0	P3B
0	0	0	0	27.6	21.66	-1	6.71	0	ethanol sp.

Determinant = -24.0932

bbb = 0.148564 mol s^{-1} = distillate from S3(P3T)
eee = 0.001324 mol s^{-1} = 0.008910 mol frac = ethanol in P3T
fff = 0.00016193 mol s^{-1} = 0.001090 mol frac = water in P3T
ww = 0.073546 mol s^{-1} = bottoms from S3(P3B)
xx = 0.0001111 mol s^{-1} = 0.0015100 mol frac = butanol in P3B
yy = 0.0002645 mol s^{-1} = 0.0035972 mol frac = acetone in P3B
zz = 0.0648248 mol s^{-1} = 0.8814192 mol frac = ethanol in P3B
aaa = 0.0083455 mol s^{-1} = 0.1134735 mol frac = water in P3B

Table 6.9 MOLE BALANCE FOR BUTANOL STILL (S4)

Assumptions

1) Flows and concentrations of acetone and ethanol are negligible (refer also Table 6.7).
2) The vapour flow is approaching the azeotropic composition
   i.e. let butanol concentration in the overheads be 0.252 mol. frac.
3) The separator delivers clean phases at concentrations very similar to those for a binary butanol/water system.
   i.e. butanol concentration in the feed (upper layer) is 0.4912.

Equations

cc	-hh	P4B	Balance
1	-1	0.59505	Overall
0.4912	-0.252	0.5771985	Butanol

Determinant = 0.2392

cc = 1.7861 mol s^{-1} :cc = flow of upper phase from P

hh = 1.1911 mol s^{-1} :hh = distillate flow from S4

so ii = 0.252 x 1.1911
= 0.30016 mol s^{-1}

ll = 0.890939 mol s^{-1}

dd = 0.4912 x 1.7861
= 0.87735 mol s^{-1}

gg = 0.90879 mol s^{-1}

(Refer Fig. 6.5, p. 142)

Table 6.10 MOLE BALANCE OVER COLUMN S4 AND THE PHASE SEPARATOR (P)

Assumptions

1) P2M, the middle drawoff stream from S2, is at approximately azeotropic composition.
   i.e. butanol concentration = 0.248
2) Acetone and ethanol concentrations are negligible (refer Table 6.7, p. 150)
3) The butanol concentration from the bottom of the phase separator is given as 0.0199 mol. frac.
4) The butanol concentration in the product stream (P4B) is 0.97 mol. frac.

Equations

mm	-rr	-P4B	Balance
1	-1	-0.59505	Overall
0.0199	-0.248	-0.5771985	Butanol

Determinant = -0.2281

mm = 1.8835 mol s^{-1} : mm = flow of lower phase from P

rr = 2.4785 mol s^{-1} : rr = flow from S2 to P

so nn = 0.0199 x 1.8835
= 0.03748 mol s^{-1}

qq = 1.84602 mol s^{-1}

ss = 0.248 x 2.4785
= 0.61467 mol s^{-1}

vv = 1.86386 mol s^{-1}

(Refer Fig. 6.5, p. 142)

(contd. from p. 146)

The concentrations, binary approximations, and calculation for all the towers are given in Table 6.11. The beer still (S1) calculations for the number of theoretical plates and reflux ratios are outlined below.

The concentrations of the feed, overheads, and the bottoms around S1 are shown in Table 6.11. The system will be assumed to suitably approximated by a butanol/water binary mixture since butanol is generally the least volatile solvent. Fig. 6.8 shows the relevant portion of the vapour-liquid equilibrium diagram for this binary along with the McCabe-Thiele construction.

The feed is heated to 55°C due to heat exchange with the bottoms, and live steam is sparged directly into the base of the column to drive the distillation. This avoids reboiler fouling problems due to the high concentrations suspended and dissolved solids concentrations in the feed liquor. The heat exchanger will be sized for full recovery of heat (equivalent to feed heating to 93°C). In addition the exchanger will be specified as a plate heat exchanger which can be departmentalised, allowing both cleaning and flexibility.

Due to the dilute solvent concentrations in the feed, the relevant thermodynamic properties of the feed will be approximated to those of water. Hence:

h_v = heat of vapourisation of feed
= 40,600 J mol^{-1}

c = specific heat of feed
= 75.4 J mol^{-1} K^{-1}

h_f = energy to raise feed to boiling point
= c (T_2 - T_1)
= 75.4 J mol^{-1} K^{-1} (100°C - 55°C)
= 3393 J mol^{-1}

Table 6.11. SUMMARY OF McCABE-THIELE CALCULATIONS

Column	Binary Approximation	Feed Conc.	Top Conc.	Bottoms Conc.	R_m	R_r	m_r	m_s	NS	FP (m)	H [d]
S1[a]	butanol/water	0.003	0.25	0.0003	-	-	0	11.6	6 + 0	1	7.5
S2A[b]	ethanol/water	0.029	0.86	0.0015	3.58	5.37	0.84	5.44	16 + 0	14	8
S2B	butanol/water	0.02	0.25	0.004	0.0232	0.035	0.0336	14.25	4 + 1	3	2
S3[c]	acetone/ ethanol	0.66	0.99	0.004	2	3	0.75	1.22	37 + 1	31	18.5
S4	butanol/water	0.49	0.25	0.97	0.0346	0.052	0.0493	1.47	5 + 1	2	2.5

Notes: a) Calculations for S1 are given in the text.

b) 1) Column S2 was split into two imaginary columns as in Fig. 6.7 for McCabe-Thiele.
2) The middle draw-off is near the azeotrope.
3) The "bottoms" from S2A is considered as the reflux for S2B.
4) Feed enters at the bubble-point.

c) The number of plates could be reduced by increasing the reflux ratio, or by relaxing the product specifications. The cost alteration involved will not be significant for this costing.

d) Calculated as in Sect. 6.4.5 using a 60% plate efficiency.

Key: R_m = minimun reflux ratio

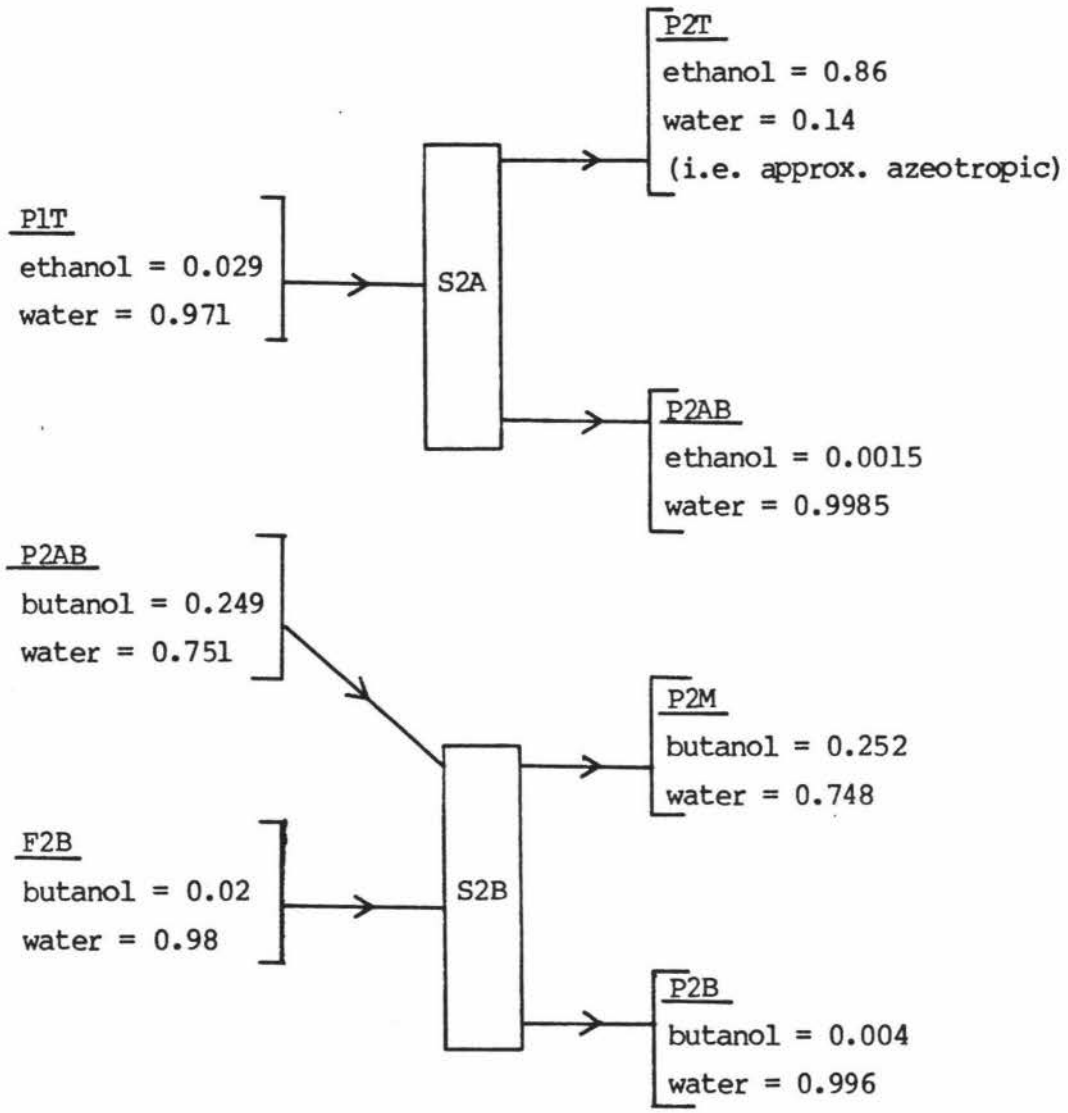
R_r = operating reflux ratio*

m_r = slope of rectification section operating line $= R_r/(R_r + 1)$

NS = number of theoretical stages

FP = feed plate position (numbered from the top).

Fig. 6.7 APPROXIMATION FOR S2 FOR McCABE-THIELE CALCULATIONS

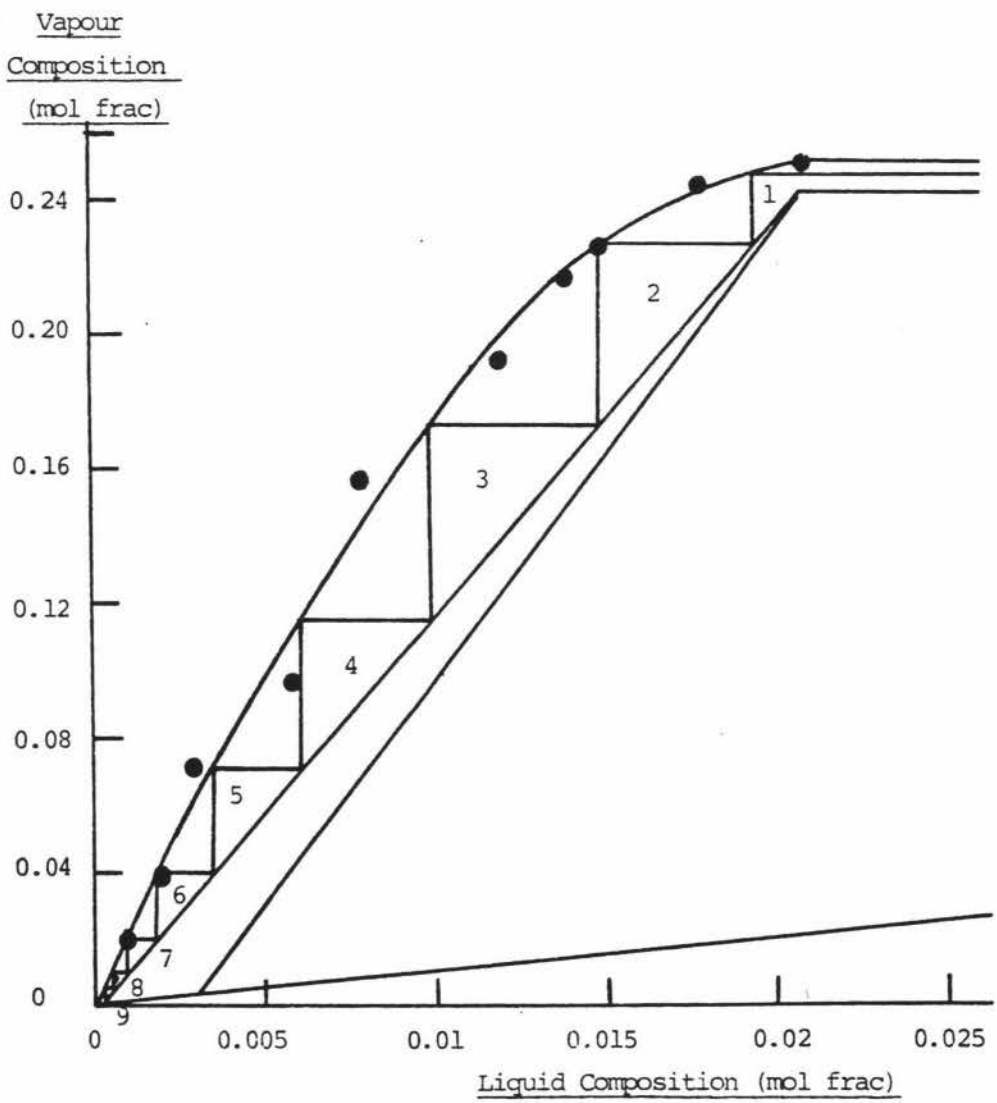


Assumptions:

1) acetone behaves like ethanol in S2A, but remains predominantly in the gas phase.
2) butanol behaves much like water in S2A
3) acetone and ethanol have negligible effect below the middle draw off (i.e. in S2B), and may be considered as part of the butanol fraction.

NOTE: These assumptions parallel simplification of multi-component mixtures of ideal components by specification of key components. The two approximations for P2AB differ due to the choice of different key components for S2A and S2B.

Fig. 6.8. McCabe-Thiele Construction for S1



q' = (heat required to vapourise the feed)/h_v
= (3,393 + 40,600)/(40,600)
= 1.084

and m_f = slope of the feed line for a McCabe-Thiele Construction
= $q'/(q'-1)$
= 1.084/0.084
= 13

Values of R_m, R, M_1, N_t, FP, and H estimated using the McCabe-Thiele constructions may be found in Table 6.11. The hydraulic calculations will be outlined shortly. Data from the McCabe-Thiele constructions are also used in the hydraulic calculations for the column internals.

The approximation for Column S2 for sizing using the McCabe-Thiele Method is shown in Fig. 6.7. Ethanol was used to represent the distillate compounds from S2A as it is less volatile than acetone. Due to temperature considerations, acetone is likely to remain mainly in the vapour phase. Water was used to represent the bottoms components. Due to the low temperature of the distillate (ca. 78°C) it was decided that water (b.p. = 100°C) rather than butanol (b.p. = 117°C) would be the better choice as the bottoms component. Since the distillate from S1 would be near an azeotropic composition, the separation in S2A is conceptually a separation of acetone and ethanol (with some water) from a butanol-water azeotrope. Unfortunately suitable three component data was not found, and so the pseudo-binary approximation of ethanol against the butanol-water azeotrope could not be made.

The lower imaginary column (S2B) was approximated by a butanol-water mixture with its distillate at the azeotropic composition. The imaginary stream (S2AB) was assumed also to be at the butanol-water azeotrope and

therefore could be treated as a reflux.

For columns which have an external reflux (i.e. S2, S3, and S4), a quantity m_r can be evaluated. m_r is the slope of the rectification section operating line in the McCabe-Thiele diagram and is equivalent to two important ratios

$$m_r = R_r/(R_r + 1)$$
$$\text{and} \quad m_r = Q_{lr}/Q_{gr}$$

where R_r is the external reflux ratio (ratio of the distillate flow to the reflux flow), Q_l is an internal molar flow rate of liquid down the column, and Q_g is an internal gas flow up the column. The subscript r denotes quantities determined for the rectification section. A third equation of importance is the difference of Q_g and Q_l, which equals the overall flow rate (D). The two internal hydraulic flow rates may be found for the rectification section by solving two simultaneous equations:

$$m_r = Q_{lr}/Q_{gr}$$

$$\text{or} \quad Q_{lr} - m_r Q_{gr} = 0 \qquad \text{equation (1)}$$

$$\text{and} \quad Q_{gr} - Q_{lr} = D' \qquad \text{equation (2)}$$

where D is the distillate (or overheads) flow for calculations. Two similar equations are used for the stripping section calculations:

$$m_s = Q_{ls}/Q_{gs}$$

$$0 = Q_{ls} - m_s Q_{gs} \qquad \text{equation (3)}$$

$$\text{and} \quad B = Q_{ls} - Q_{gs} \qquad \text{equation (4)}$$

where the s subscript denotes the stripping section.

Distillate and bottoms flow rates for each column are in Figs. 6.2 - 6.5. These equations along with the McCabe-Thiele construction and the distillate and bottoms flows give the internal flow rates required for the column hydraulic design.

The same format for simultaneous equations as used in the last section will be used to summarise the calculations. It is unnecessary to perform the calculations using matrices. The two equations for rectification above then become:

Q_{lr}	Q_{gr}	Answer	Equation
1	$-m_r$	0	(1)
-1	1	D	(2)

Since S1 is a stripping column, no external reflux is required and the feed is put onto the top tray. This corresponds to a rectification section operating line of slope zero ($m_r = 0$) and there is no rectification section. Hence only stripping section flows can be calculated.

The operating line for the stripping section of S1 was drawn to prevent the column operating with a two phase liquid. In this case the format is:

Q_{ls}	Q_{gs}	Answer	Equation
1	$-m_s$	0	(3)
1	-1	B	(4)

Substituting in values for the beer stripping Column S1:

Q_{ls}	Q_{gs}	Answer	Equation
1	-11.6	0	(3)
1	-1	228.9166	(4)

Q_{ls} = 250 mol s^{-1} : Determinant = 10.6
Q_{gs} = 21.6 mol s^{-1}

These internal fluid flows are required later for the hydraulic design of the columns (Section 6.4.4), and Q_{gs} for estimation of plant steam and cooling water consumption (Section 6.7).

In summary then, column S1 will require 6 theoretical stages. The feed will be introduced onto the top plate. No reboiler will be used but live steam will be sparged directly into the base of the column.

The McCabe-Thiele calculations are summarised in Table 6.11 the flow rate calculations are summarised along with the other hydraulic calculations in Tables 6.14 - 6.17.

These approximations are assumed to be suitably accurate for preliminary sizing and costing, but in more detailed design multicomponent calculations might be preferable. This problem are mentioned later (Section 6.4.5).

6.4.3.2 Number of Transfer Units

Packed columns may alternatively be sized using a unit known as a transfer unit. The number of transfer units (NTU) may be approximately calculated using

$$NTU = \int_{y_o}^{y_d} dy/(y^* - y)$$

where NTU = number of transfer units.

y_d = distillate composition

y_o = bottoms composition

y^* = equilibrium composition

y = actual composition (on the operating line).

The vapour-liquid equilibrium diagram has its feed and operating lines drawn up as for the McCabe-Thiele Method, but a transformation of the area between the equilibrium and operating lines (represented by values of y^* and y respectively) is integrated instead of drawing the number of theoretical plates required. This integral then gives the number of transfer units required. Strictly speaking NTU should be estimated separately for different sections but this degree of accuracy is unnecessary.

Calculations for column S3 are outlined here and in Fig. 6.9 and Table 6.12, while Table 6.13 gives a summary of the calculations for all the columns, including the heights which will be referred to later.

Once the equilibrium diagram and operating lines have been drawn as for the McCabe-Thiele construction (Fig. 6.9), a table of y, y^*, and $(y^* - y)^{-1}$ is drawn up (Table 6.12) and the integral evaluated. The integral is most easily evaluated by evaluating the table for equally spaced values of y and evaluating the integral using Simpson's Approximation. Greater accuracy may be obtained by evaluating the integral over two or three concentration ranges. The integral for S3 was evaluated for three separate concentration ranges as shown in Table 6.12. The total integral is then:

$$\begin{aligned} \text{Integral} &= \text{range 1} + \text{range 2} + \text{range 3} \\ &= 7.94 + (1.15 + 1.50) + 39.02 \\ &= 49.6 \\ &= 50 \text{ transfer units.} \end{aligned}$$

Fig. 6.9 The Vapour-Liquid Equilibrium Diagram for Acetone and Ethanol showing Regions of Integration for Packing Calculations for Column S3.

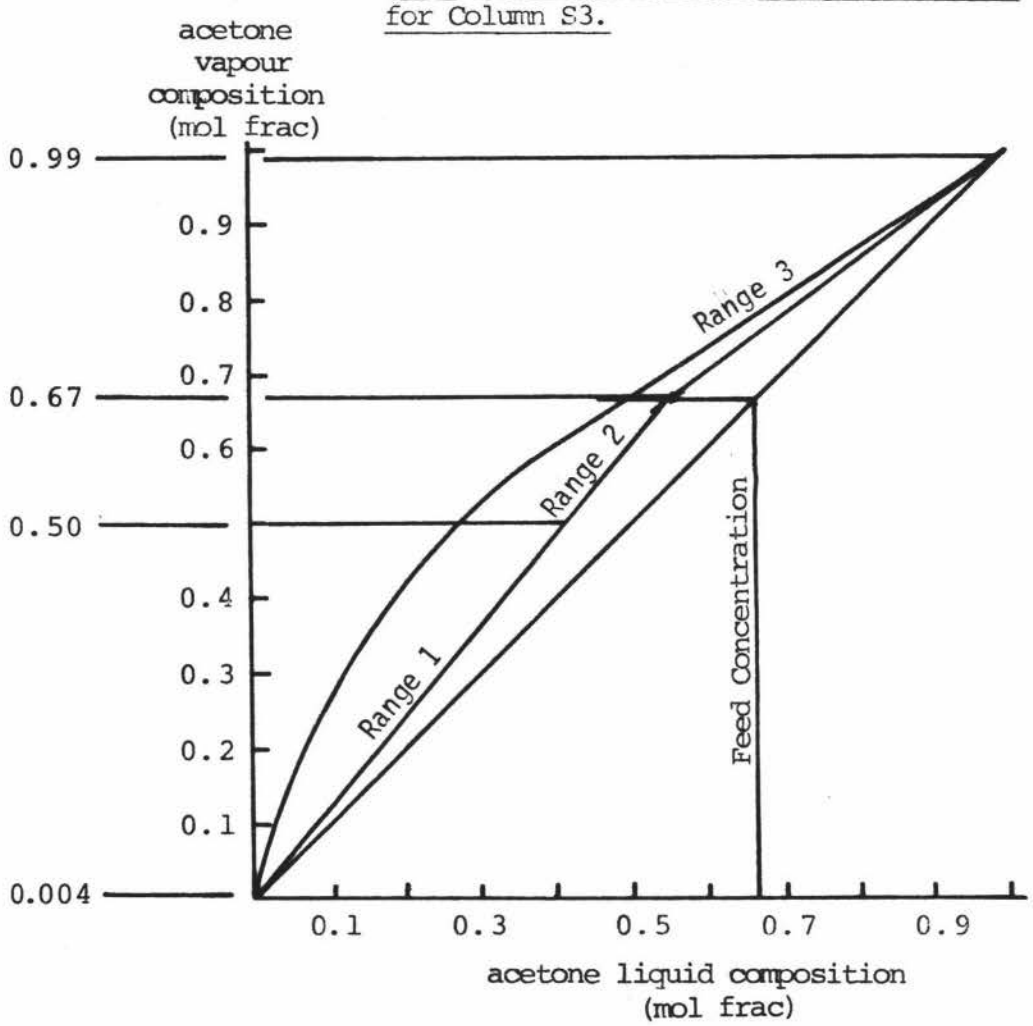


Table 6.12 SUMMARY OF INTEGRAL CALCULATIONS
FOR NTU CALCULATIONS FOR COLUMN S3

Range	y (mol. frac.)	y* (mol. frac.)	$(y^* - y)^{-1}$
1	0.004	0.012	125
	0.1	0.23	7.69
	0.2	0.375	5.71
	0.3	0.475	5.71
	0.4	0.55	6.67
	0.5	0.615	8.70
2	0.5	0.615	8.70
	0.6	0.67	14.29
	0.67	0.705	28.57
3	0.67	0.705	28.57
	0.734	0.76	38.46
	0.798	0.82	45.45
	0.862	0.88	55.56
	0.926	0.932	166.67
	0.99	0.994	250.00

Table 6.13 SUMMARY OF COLUMN HEIGHTS

Column	N_t**	Unit Type	Efficiency	Height (m)
S1*	9	plate	60%	9
S2	38	transfer units	HTU = D***	9.3
S3	50	transfer units	HTU = D***	8
S4	22	transfer units	HTU = D***	6

* 0.6m (2 ft) tray spacing.

** N_t = number of theoretical units (tray or transfer units).

*** HTU = D is a very rough approximation suitable for initial costing calculations only. (refer Section 6.4.5).

where the central range (range 2) was evaluated as two trapezia (i.e. by linear joining of the points followed by area estimation), and the two larger integrals (ranges 1 and 3) were evaluated using Simpson's Approximation. Simpson's Approximation is a second order numerical integration procedure which, effectively, fits a quadratic equation to successive groups of three points for area estimation (Chemical Engineers' Handbook, 1973).

6.4.4 Hydraulic Design and Column Diameter

Each column was designed for sieve plates and for packing, and Column S1 was also designed for Turbogrid plates. Example calculations for each are given below, and the data and summary calculations are given in Tables 6.14 - 6.17. Flow rates were calculated in section 6.4.3, and converted to volume flow rates using an approximate density. The molecular weight (Mr), density (d), and surface tension (t) were all calculated by the simple approximation

$$M_m = \Sigma x_i M$$

where M is the property of concern and x_i the mole fraction of i.

6.4.4.1 Column S1 Design for Turbogrid and Sieve Plates.

Tables 6.14 - 6.17 give a summary of the properties and calculations.

Due to the high solids content of the feed to the beer still (S1) and the high liquor flow rates, the first tower should in fact be designed for turbogrid plates or sieve plates. The calculations for Column S1 are outlined here.

The design correlation for turbogrid plates comes from Földer (1960) and assumes typical parameters:

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Table 6.14 SUMMARY OF HYDRAULIC CALCULATIONS FOR COLUMN S1

Quantity	Rectification	Stripping	Units
liquid flow (Q_l)	0	250	mol s^{-1}
	0	4.50	l s^{-1}
gas flow (Q_g)	2.5676	21.6	mol s^{-1}
	77.11	660	l s^{-1}
molecular wt.	33.9	18.0	g mol^{-1}
temperature	93	100	°C
pressure	1	1	atm
liquid density	0.8766	1	sg
gas density	-	0.000588	sg
liquid viscosity	48	58.8	dynes/cm

worst conditions = stripping section

Quantity	Turbogrid	Sieve Plate	Packing	Units
gas velocity	19.8	3.1	-	m s^{-1}
cross sect. area	0.029	0.188	-	m^2
diameter	0.19	0.49	-	m

Table 6.15 SUMMARY OF HYDRAULIC CALCULATIONS FOR COLUMN S2A AND S2B

Summary of Data for S2A

Quantity	Rectification	Stripping	Units
liquid flow (Q_l)	1.1661	2.87	mol s^{-1}
	0.0780	-	l s^{-1}
gas flow (Q_g)	1.3882	0.53	mol s^{-1}
	37.828	-	l s^{-1}
molecular wt.	53.0	32.1	g mol^{-1}
temperature	59	93	°C
pressure	1	1	atm
liquid density	0.7939	0.89	sg
gas density	1.95	-	kg m^{-3}

Summary of Data for S2B

Quantity	Rectification	Stripping	Units
liquid flow (Q_l)	0.00463	1.8826	mol s^{-1}
	-	0.0347	l s^{-1}
gas flow (Q_g)	0.1377	0.132	mol s^{-1}
	4.132	0.405	l s^{-1}
molecular wt.	32.1	18.4	g mol^{-1}
temperature	93	100	°C
pressure	1	1	atm
liquid density	0.89	0.99	sg

contd./

Table 6.15 (contd)

The worst conditions were taken as the rectification section of column S2A. In fact, due to the very large differences in flow rates between the two sections, the final design may require two different diameters at the top and at the bottom of the tower.

Quantity	Sieve Plate	Packing	Units
gas velocity	1.22	-	$m\ s^{-1}$
cross sect. area	0.031	0.045	m^2
diameter	0.199	0.239	m

Table 6.16 SUMMARY OF HYDRAULIC CALCULATIONS FOR COLUMN S3

Quantity	Rectification	Stripping	Units
liquid flow (Q_l)	0.4458	0.0478	mol s^{-1}
	0.03264	-	l s^{-1}
gas flow (Q_g)	0.5944	0.3343	mol s^{-1}
	16.088	9.6	l s^{-1}
molecular wt.	57.9	43	g mol^{-1}
temperature	56	78	°C
pressure	1	1	atm
liquid density	0.792	0.80	spec. grav.
gas density	2.14	-	kg m^{-3}

worst conditions = rectification section

Quantity	Sieve Plate	Packing	Units
gas velocity	1.17	-	ms^{-1}
cross sect. area	0.0138	0.0198	m^2
diameter	0.133	0.159	m

Section 6.17 SUMMARY OF HYDRAULIC CALCULATIONS FOR COLUMN S4

Quantity	Rectification	Stripping	Units
liquid flow (Q_l)	0.0618	1.8611	mol s^{-1}
	2.21×10^{-3}	0.1636	l s^{-1}
gas flow (Q_g)	1.253	1.266	mol s^{-1}
	37.6	40.43	l s^{-1}
molecular wt.	32.1	72.4	g mol^{-1}
temperature	93	117	°C
pressure	1	1	atm
liquid density	0.8896	0.8114	sg
gas density	-	2.26	kg m^{-3}

worst conditions = stripping section

Quantity	Sieve Plate	Packing	Units
gas velocity	1.1	-	m s^{-1}
cross sect. area	0.037	0.056	m^2
diameter	0.217	0.268	m

(contd. from p. 166)

(a) F_g = fraction of cross sectional area free
= 20%

(b) V_o = optimum loading velocity
= 80% of flooding velocity (V_f)

and the design equation:

$$V_f = 6.9\ F_g\ (Q_l/Q_g)^{-0.34}\ (d_l/d_g)^{0.5} \quad : Mr_l = Mr_g$$

= actual gas velocity in column.

where d is the fluid density.

so $v' = 0.8 \times 6.9 \times 0.2 \times (250.0/21.6)^{-0.34} (1/0.000588)^{0.5}$

$= 19.8\ ms^{-1}$

A = cross sectional area of column
$= Q_g/V_o$
$= 584 \times 10^{-3}\ m^3\ s^{-1}/19.8\ ms^{-1}$
$= 0.029\ m^2$

D = column diameter
$= (4A/\pi)^{0.5}$
$= 0.19$ m.

The method of design for a sieve plate tower comes from Peters and Timmerhaus (1980) and assumes the typical parameters:

(a) 24 in (0.61 m) tray spacing
(b) weir height = 12.5% of tray spacing
= 0.038 m
(c) ideal gas laws hold
(d) foaming is controlled by antifoam agent
(e) A_s = free area of holes
$= 0.1\ A_a$
A_a = active plate area
(f) downcomers are 5% of total cross-sectional area.

The correlation used is:

$$(Q_l/Q_g)(d_g/d_l)^{0.5} = (250.0/21.6)(0.588/1000)^{0.5}$$
$$= 0.28$$

and from the graph for the proportionality constant K_v (Peters and Timmerhaus, 1980, Perry and Chilton, 1973)

$$K_v = 0.21$$

so V'_t = superficial velocity without downcomers

$$= K_v (d_l - d_g)^{0.5} d_g^{-0.5} (\sigma/20)^{0.2}$$
$$= 0.21(1000 - 0.588)^{0.5} 0.588^{-0.5} (58.9/20)^{0.2}$$
$$= 10.7 \text{ ft s}^{-1}$$: where σ = surface tension

v_t = true superficial velocity

$$= 10.7 \times 0.95$$
$$= 10.2 \text{ ft s}^{-1}$$
$$= 3.1 \text{ ms}^{-1}$$

A = tower cross sectional area

$$= 584 \times 10^{-3} \text{ m}^3 \text{ s}^{-1}/3.1 \text{ ms}^{-1}$$
$$= 0.188 \text{ m}^2$$

giving

D = diameter of tower

$$= (4A/\pi)^{0.5}$$
$$= 0.49 \text{ m}$$

Other towers used an 18" tray spacing for tray designs.

6.4.4.2 Column S2 Design as a Packed Tower

The top of column S2A was chosen as the worst case as it had the highest gas velocity and relatively high liquid velocity (refer Table 6.15).

Fig. 6.10. Graph for Determination of K_v.
(from Peters and Timmerhaus, 1980)

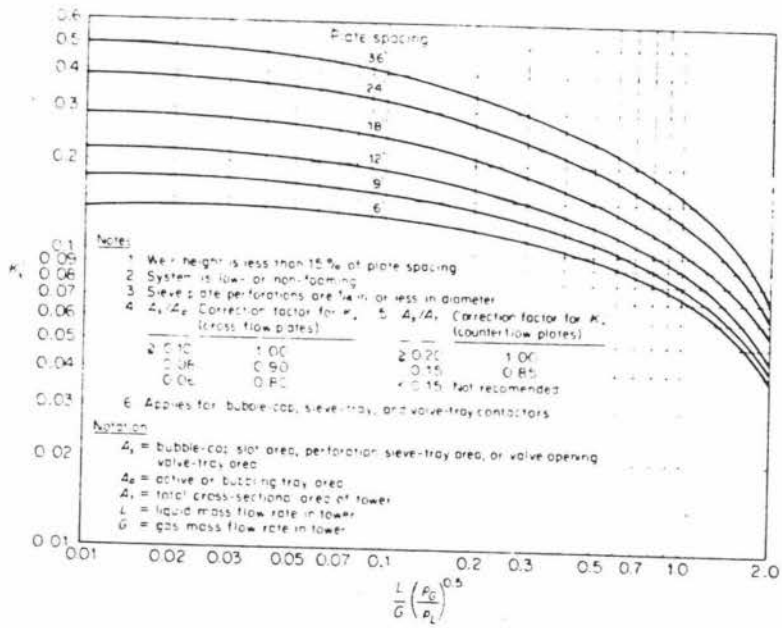

Chart for estimating values of K_v (± 10%) [J. R. Fair, *Petro/Chem. Eng.*, 33(10): 45 (1961). *With permission.*]

The packed tower method of design for 5/8 inch (16mm) polypropylene Pall Rings is taken from Bremer and Kalis (1978) who give typical values which will be used for these calculations:

(a) packing = 5/8 inch polypropylene Pall Rings
(b) voidage ($\not{\varepsilon}$) = 0.87
(c) specific surface area, a = 341 m^2/m^3
(d) $\emptyset$ = 0.8 ($\emptyset$ = a constant)
(e) ß = 0.485 (ß = a constant)

The correlations presented by Bremer and Kalis (1978) are:

$$P_l/P_o = (1 - h/(2\ ß^{5/3}\ \emptyset\ \not{\varepsilon}))^{-5}$$

$$P_o = (0.29\ Q_{vg}^2\ a\ d_g)/(\emptyset^2\ A^2\ \not{\varepsilon}^3)$$

and $$h = 0.34a\ (\mu_l/d_l)^{2/3}\ Re^{2/3}$$

where P_l = wetted column pressure drop (Pa m^{-1})
P_o = dry column pressure drop (Pa m^{-1})
h = liquid holdup as a fraction of the total volume of the bed.
$\emptyset$, ß = constants
$\not{\varepsilon}$ = voidage
d_g = gas density (kg m^{-3})
d_l = liquid density (kg m^{-3})
A = tower cross sectional area (m^2)
a_a = specific area of packing (m^2/m^3)
μ_l = liquid viscosity

These three equations can easily be rearranged to give an expression with A the only unknown:

$$\frac{(P_l\ \emptyset^2\ A^2\ \not{\varepsilon}^3)^{-1/5}}{(0.29\ Q_g^2\ a_a d_g)^{-1/5}} = 1 - \frac{(0.34\ a_a^{1/3}\ Q_l^{2/3})}{(2ß^{5/3}\ A^{2/3}\ \emptyset\ \not{\varepsilon})}$$

which can be solved for A using the method of bisection between the limits (0.01, 100).

Substituting in values for the top of S2 (i.e. for the imaginary column S2A)

$$\frac{(500\times0.8^2\times A^2\times0.87^3)^{-1/5}}{(0.29\times0.0378^2\times341\times1.946)^{-1/5}} = 1 - \frac{0.34\times341^{1/3}(7.8\times10^{-5})^{2/3}}{(2\times0.485^{5/3}A0.8\times0.87)^{2/3}}$$

$$765.22\ A^2 = (1 - 1.3922\times10\ \ A^{-2/3})^{-5}$$

$$765.22\ A^2\ (1 - 1.039\times10^{-2}\ A^{-2/3})^5 - 1 = 0$$

$$A = 0.0448\ m^2$$

$$\text{so}\quad D = (4A/\pi)^{-0.5}$$
$$= 0.239\ m$$

At this point the diameter should be checked to ensure it is greater than 8 times the packing diameter (to help avoid excessive channelling). Since D is greater than 8 x 16 mm = 128 mm (0.128m), D is acceptable. Tables 6.14 - 6.17 give a summary of calculations.

6.4.5 Efficiencies and Tower Height

Typical efficiencies can be obtained from the literature (Peters and Timmerhaus, 1980; Perry and Chilton, 1973), and are typically about 85-90% for the overall column efficiency of a sieve plate tower, and 85-90% for a bubble-cap if operated at appropriate conditions. The number of actual plates multiplied by the tray spacing, with added height for end arrangements, gives an overall height.

The efficiency of packed towers is calculated differently. If the height of packing required has been calculated as

the number of transfer units required (NTU), then the efficiency term is incorporated in the height of the transfer unit (HTU). A very rough approximation suitable only for initial costing exercises is to assume that HTU and D are approximately equal. While this is not particularly sound theoretically, it gives a rough costing approximation (Spedding, 1979). Manufacturer's data for a specific packing, will provide more accurate calculation.

Many papers and books give some alternative, quick, easy to use correlations. However, these correlations are often limited to specific packing types and are limited in number but should be used where possible. For mathematical, more general, possibly more accurate methods of efficiency calculation, the calculation of liquid and gas side heat-transfer coefficients may be used either over the whole column, or at several critical points in the column. These methods are outlined in many texts (Foust et al., (1980); McCabe and Smith, (1976); Peters and Timmerhaus, (1980); Perry and Chilton, (1973); Treybal, 1968)). Experimental data can often be found in the CRC Handbook (1977) or Perry and Chilton (1973). Correlations for data prediction for extrapolation to different conditions, and for estimation of mixture properties can be found in Perry and Chilton (1973, Chapter 3) for viscosity, density, surface tension, and diffusion coefficients. McCabe and Smith (1976), and Cussler (1976) (for multicomponent mixtures) also give data on diffusion and diffusivities. Other texts such as Treybal (1968), and in particular Reid et al. (1977) may be consulted to supply additional data and correlations.

In this design, a plate efficiency of only 60% was assumed for tower S1 due to the extremely non-ideal nature of the system (although in this case the non-ideality actually seems to be of benefit) and because erring on the conservative side would reduce overall solvent losses significantly. For packed tower designs (i.e. all towers in the

present design except S1) the rough approximation of HTU = D was used. In further design for both types of tower internals (especially for packed tower design) equipment suppliers should be consulted. Table 6.13 gives a summary of the height calculations.

6.5 Post Distillation

The post distillation section (product storage) has been sized to provide about one month's storage capacity with a minimum emptying rate of 30 m^3 hr^{-1} for each solvent. Example calculations for each piece of equipment have already been given, and a summary of these calculations can be found later in Section 6.6 (Table 6.20).

6.6 Equipment Summary and Costing

Tables 6.18 - 6.21 give summaries of equipment specifications and costings for the three sections; fermentation, distillation, and product storage. A very brief description of each item of equipment is given, along with the sizing characteristic used for costing. Initial individual purchase costs (IPC) are from the New Zealand Institution of Engineers (1980(b)) and Peters and Timmerhaus (1980) and were updated to September 1980 costs (IPC) using the New Zealand Ministry of Works plant index, and adjusted from mild steel to stainless steel by multiplying by 1.7 (New Zealand Institution of Engineers, 1980(b)). In the US a lower factor is used for stainless steel (Peters and Timmerhaus, 1980) but the NZ domestic market is different, and should be treated differently. The costs for the distillation columns (Peters and Timmerhaus, 1980) include all components normally associated with columns. The total IPC is called the purchased equipment cost (PEC), and the direct cost (DC) of each article was calculated using factors for different cost components (New Zealand Institution of Engineers, 1980). These components are listed and the total costs for each section itemised in

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Table 6.18 SUMMARY OF FERMENTATION SECTION EQUIPMENT AND COSTS ($)

Item	Descrip.	Size	Units	IPC	DC
P1	Centrif	30	MPa l min^{-1}	2,260	8,864
V1(two)	Yeast Extract Mixers	3.5	m^3	6,400	9,000
H1	Plate, Regen	170	m^2	66,500	90,996
H2	S & T, Steam	1.1	m^2	4,100	11,280
Q1	Pipe, Ster	48	m	-	-
H3	S & T	17	m^2	17,500	32,025
V2	pH bottle	700	m^3d^{-1}	-	-
V4(five)	Fermenter	367	m^3	426,500	748,020
P2	Empty Fermenter	70	MPa l min^{-1}	2,954	11,586
V5	Smoothing	300	m^3	54,000	76,896
V6	Antifoam	40	m^3	11,000	23,452
			Totals	591,214	1,012,119 (PEC)
			Services & Yard (50% PEC)		295,607
			Land (6% PEC)		35,473
			FERMENTATION TOTAL		1,343,199

Table 6.19 SUMMARY OF DISTILLATION SECTION EQUIPMENT AND COSTS ($)

Item	Descrip.	Size	Units	IPC	DC
H11	Plate HE	185	m^2	68,300	92,205
P3(=P1)	Centrif	30	MPa 1 min^{-1}	1,130	4,432
S1	Beer, Turbogrid	7.1 x 12	(DiaxHeight) ins x ft	8,700	26,709
S2A	Packed	9.4 x 144	(DiaxHeight) ins x ft	40,300	91,884
S2B	Packed	9.4 x 98.4	(DiaxHeight) ins x ft	27,600	62,928
S3	Packed	6.3 x 105	(DiaxHeight) ins x ft	20,000	45,000
S4	Packed	10.6 x 17.4	(DiaxHeight) ins x ft	7,750	23,793
			TOTALS	173,780 (PEC)	347,551
			Services & Yard (50% PEC)		86,890
			Land (6% PEC)		10,427
			DISTILLATION TOTAL		$444,868

Table 6.20 SUMMARY OF PRODUCT STORAGE SECTION EQUIPMENT AND COSTS ($)

Item	Description	Size	Units	IPC	DC
V7	Acet. St.	30	m^3	11,230	19,203
V8	Ethanol St.	11	m^3	6,740	14,154
V9	Butanol St.	145	m^3	26,200	37,728
PE	Centrif Pumps	200	MPa 1 min^{-1}	3,290	12,367
			TOTALS	47,458 (PEC)	83,452
			Services + Yard (50% PEC)		23,729
			Land (6% PEC)		2,847
			PRODUCT TOTAL		$110,028

Table 6.21 SUMMARY OF COMBINED TOTAL COSTS ($)

PEC (Ferm + Dist + Prod)	=	812,452
DC (Ferm + Dist + Prod)	=	1,443,122
Services + Yard (50% PEC)	=	406,226
Land (6% PEC)	=	48,747
TOTAL DIRECT COST	=	$1,898,095

(contd. from p. 178)

the next section.

6.7 Overall Cost Breakdown and Product Cost

The final cost estimates are summarised in Tables 6.22 - 6.24. Table 6.22 summarises the equipment costs from Tables 6.18 - 6.21, Table 6.23 the running costs, and Table 6.24 the Breakeven-price calculation. The running cost estimations will now be described further.

Raw materials were estimated to cost no more than $100,000. This can be achieved by assuming whey permeate comes from the dairy factory at approximately zero cost and returned to the dairy factory's waste-treatment facilities with greatly reduced BOD. This allows treatment of the whey between ultrafiltration and any nutrient supplementation a cost of about $\$1.10/m^3$ whey permeate based on a 300 day year. Yeast Extract addition at the rate of 5 g/l (450 tonnes/yr) must then cost less than about $0.20/kg (Brown et al., 1982).

Manning levels were estimated for 24 hour operation with three shifts on per day, and one shift off. Each shift would consist of three people on an average wage equivalent to $15,000 p.a. (ca. $7.80 per hour). Salaries and wages for other staff are estimated as outlined by Peters and Timmerhaus (1980) on the basis of the FCI (maintenance and repairs), Operating Labour (laboratory charges), or TPC (administration, distribution, research and development).

Utility costs were estimated on the basis of estimated consumption calculated during equipment sizing. Steam was estimated from the figures for Q_{gs} of the stripping sections for the four columns (Tables 6.14 - 6.17). The Stripping Column (S1) used over 90% of this steam, hence the properties for all were taken as those of water. Also, since steam can be sparged directly into columns S1

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Table 6.22 CAPITAL INVESTMENT COSTS*

COST	ITEM	FERM ($1000)	DIST ($1000)	PD ($1000)	TOTAL ($1000)	TOTAL (% TDC)	COMMENTS
DC	Equipment	591.21	173.78	47.46	812.45	42.8	PEC
	Installation	46.44	33.80	2.73	82.97	4.4	
	Piping	101.73	42.92	16.45	161.10	8.5	
	Instruments	86.75	32.78	5.69	125.22	6.6	
	Electrical	57.70	17.94	3.20	78.84	4.2	
	Civil	65.73	14.79	6.59	87.11	4.6	
	Buildings	29.49	23.48	1.33	54.30	2.9	
	Lagging	33.07	8.06	negl	41.13	2.2	
	Services, Yard	295.61	86.89	23.73	406.23	21.4	(50% PEC)
	Land	35.47	10.43	2.85	48.75	2.6	(6% PEC)
	TOTALS	1,343.20	444.87	110.03	1,898.10	100.0%	TDC
IC	Engineering & Supervision	268.64	88.97	22.01	379.62		(20% TDC)
	Construction expenses and contractors	268.64	88.97	22.01	379.62		(20% TDC)
	Contingency	134.32	44.49	11.00	189.81		(10% TDC)
	I.C.	671.60	222.44	55.02	949.05		TIC
FCI		2,014.80	667.31	165.05	2,847.15		(DC + IC)
WC		223.87	74.15	18.34	316.35		(10% TCI)
TCI		2,238.67	741.46	183.39	3,163.50		TCI

* for explaination of abbreviations, see List of Variables and Abbrevs (p.x)

Table 6.23 TOTAL PRODUCT COST*

Cost	Item	Amount ($1000)	Comments
DPC	Raw Materials	100.00	(low cost substrate)
	Operating Labour	180.00	(4 shift, 3 people)
	Utilities**	100.00	(refer Section 6.7)
	Maintenance & Repairs	142.36	(5% FCI)
	Operating Supplies	19.93	(14% Maintenance)
	Laboratory charges	60.00	(30% Operating Labour)
	Direct Production Cost	602.29	DPC
FC	Depreciation	189.81	(linear FCI for 15 yrs)
	Insurance and Taxes	142.36	(5% FCI)
	Overheads	108.00	(60% operating labour)
	Fixed charges	440.17	FC
MC		1,042.46	MC, (DPC + FC)
	Administration	101.91	(6% TPC)
	Distribution	152.87	(9% TPC)
	Research & Devel	84.93	(5% TPC)
	Financing	316.35	(10% TCI)
	General Expenses	656.06	GE
	Total Production Cost	1,698.52	TPC

* These costs are based on a full year, and would be little altered for a reduced year (assuming staff is paid all year round).

** steam, 0.64 kg s^{-1}, $85,000 pa (gas).
electricity, 12.6 kW, $2,000 pa.
cooling water, 780,000 m^3 yr^{-1}, $15,000 pa (river water).

Table 6.24 BREAKEVEN COST

Solvent	Production (l/year) (270 d/year)	Production (l/year) (330 d/year)	Comments
Butanol	1.08×10^6	1.32×10^6	
Acetone	0.22×10^6	0.27×10^6	
Ethanol	0.08×10^6	0.10×10^6	
Total*	1.38×10^6	1.69×10^6	TP
Cost** ($/l)	1.28	1.04	cost $\frac{TPC}{TP}$
($/kg)	1.57	1.28	

* Assuming no income from by-products (H_2, CO_2, vitamins, stock feed).

** Assuming all products at same price. This cost is the ex factory Breakeven Price.

and S2 an overall column efficiency of 95% was assumed. The mass flow of steam required is:

$$Q_s = (19.1 + 0.132 + 0.3343 + 1.266)/0.95 \text{ mol } s^{-1}$$
$$= 21.93 \text{ mol } s^{-1}$$
$$= 0.39 \text{ kg } s^{-1}$$

with a boiler duty of (allowing generation efficiency of 75%):

$$q_s = 21.9 \text{ mol } s^{-1} \times 40.6 \text{ kJ mol}^{-1}/0.75$$
$$= 1,186 \text{ kW}$$

The cost basis used was \$3.09 per GJ (Palmerston North City Corp, 1981) assuming gas was available to raise the steam. On an annual basis (270 days) then:

$$\text{cost} = (q_s \times 60^2 \times 24 \times 270) \times (\text{price of gas})$$
$$= 1,186 \times 10^3 \text{ J } s^{-1} \times 2.33 \times 10^7 \text{ s } yr^{-1} \times 3.09 \times 10^{-9} \$/J$$
$$= \$85,000 \text{ per annum}$$

Electricity usage was estimated from the pumping data in Tables 6.18 - 6.21. The size statistic, F, is the hydraulic power required per pump. This figure, if expressed in units of MPa l min^{-1}, may be multiplied by $10^3/60 = 16.7$ to give power in Watts. Hence the required pump power (q_p) at an average efficiency of 90% is

$$q_p = (30 + 70 + 30) \times 16.7/0.90$$
$$= 2.4 \text{ kW}$$

The product pumps were neglected as they only run occasionally. The cost of the electric power to the pumps was based on a rate of \$0.140 per kW hr (Palmerston North City Corporation, 1981) discounted by multiplying by 1.15^{-2} to give \$0.1059 per kW hr. At this rate q_p would cost

$$\text{cost} = q_p \times 24 \text{ hr.} \times 270 \text{ days} \times (\text{electricity price})$$
$$= 2.4 \times 24 \times 270 \times 0.1059$$
$$= \$1,647 \text{ per annum}$$

which is ca. 2% of the gas cost, hence even a large error in the estimation of this utility cost would have comparatively little effect. For the purposes of the costing the plant will be assumed to consume about twice the pumping power requirement and incur an electricity cost of about \$3,000 pa.

Cooling water usage was based on the distillation steam duty and a design temperature increase of 10°C. The columns were assumed to be approximately adiabatic, and feeds near their bubble points. This will provide an overestimate of the required cooling water, allowing some margin for error. Hence

$$q = mc\ (T_1 - T_2)$$
$$\text{or } m = q/(c\ (T_1 - T_2))$$
$$= 889 \times 10^3 \text{ J s}^{-1}/(4.2 \times 10^3 \text{ J kg}^{-1} \times 10°C)$$
$$= 21 \text{ kg s}^{-1}$$
$$= 500,000 \text{ m}^3 \text{ yr}^{-1}$$

The cost basis used was from Peters and Timmerhaus (1980) at \$30 per 1000 m^3 for river water. Thus, the cost is

$$\text{cost} = (\text{volume used}) \times (\text{cost of cooling water})$$
$$= 500,000 \text{ m}^3 \text{ yr}^{-1} \times \$30/1000 \text{ m}^3$$
$$= \$15,000 \text{ per annum}$$

Hence the total calculated cost for steam, electric power, and cooling water is about \$85,000 + \$3,000 + \$15,000 = \$103,000. A figure of \$100,000 pa. is used.

Other costs were estimated as suggested by Peters and Timmerhaus (1980).

Table 6.24 shows the estimated production cost. The average solvent cost was estimated at $1.51/kg for a 270 day year ($1.23/kg for a 330 day year), but it was assumed the plant by-products would have no value.

6.8 Discussion

The present costing may be considered a Study Estimate and as such has an expected accuracy of about ±30% (Perry and Chilton, 1973; Peters and Timmerhaus, 1980). As both references point out, however, the actual estimate envelope is better described as -20% to +40% as costs are more likely to overrun than underrun, expecially where inflation has an insidious balloony effect. This study is dated as September 1980. In an industrial situation, the cost of producing such an estimate for a $3 million plant is in the order of $2,000-$6,000 (Perry and Chilton, 1973; Peters and Timmerhaus, 1980).

Comparison of the estimated solvent cost of $1.57/kg (Table 6.24) against the estimated "ex-factory" price of $0.90/kg for butanol already on the market (Table 1.1) suggests that the present proposal is uneconomic (as at September 1980). Extending the production season to 330 d/yr (by use of some other substrate) may be able to bring the cost down as far as $1.28/kg.

In order that the proposal become economic the plant size should be increased as pointed out earlier (Maddox et al., 1981). If the present plant were increased to twice the capacity (600 m^3 day^{-1}) then the solvent cost would drop dramatically. Major plant items are of such a size that no duplication would be required for this increase. An increase of this magnitude would result in the total capital investment of approximately $4.6 million and an annual production cost of approximately $2.6 million. This would cause the solvent price to be $1.18/kg for a 270 day season and $0.96/kg for a 330 day season. This larger

plant may therefore become feasible if selling to the New Zealand market on a 330 day/year basis, especially if the by-products are saleable and the solvent prices can be reduced by 5-10% as discussed shortly. Some of the larger dairy plants in NZ could support a plant of this size (cf. Fig. 1.2). However, the present NZ domestic market for butanol is smaller than the estimated production from a 300 m^3 day^{-1} plant, and so may not be large enough to absorb the output of a 600 m^3 day^{-1} plant (Table 6.24 and Table 1.1). If domestic consumption were to expand through addition into domestic petrol or diesel fuels then the price of the fuels would have to rise slightly. Even at the petrol price 18 months later in early 1982 ($0.68/l = $0.93/kg) there would be some increase. Use as a blending agent in lower alcohol fuel blends would show a smaller rise due to the cost of the lower alcohol and reduced butanol concentrations.

If NZ were to become a net exporter of the solvents the picture would be different. The quoted world price of n-butanol in November 1980 was $0.58/l or $0.72/kg (f.o.b. New York) and as such NZ would not be competitive.

Further increase in plant size would result in better economics (Maddox et al., 1981), but the scale-up would be subject to substrate supply. Duplication of fermenters would of course be necessary, but only if whey flow rate exceeds 3,000-4,000 m^3/day would the beer still require duplication. These would be the only equipment requiring duplication.

Analysis of the costing shows some room for cost savings. If biotechnological research into the fermentation realised improvements early enough to be incorporated into the plant design, then cost reductions could be made as summarised in Tables 6.25 and 6.26. These two tables show the effect of increased rates of fermentation and reductions of fermenter cost. The rate of fermentation

Table 6.25 REDUCTION IN FERMENTER COST WITH TIME FOR FERMENTATION

Fermentation Time (hr)	Reduction from 65 hr (%)	Fermenter Volume (m^3)	Reduction in Fermenter Cost (%)
65	0	367	0
60	8	340	4
55	15	313	8
48	26	276	15
43	34	249	19
38	42	222	24
34	48	201	29
24	63	147	40

Table 6.26 REDUCTION IN BREAKEVEN PRICE WITH FERMENTER COST

Reduction in Fermenter Cost (%)	Fermenter Cost ($)	FCI ($)	TPC ($)	Reduction in Breakeven Price (%)
0	748	2847	1699	0
5	711	2760	1667	2
10	673	2672	1636	4
15	636	2585	1605	6
20	598	2497	1574	7
30	524	2322	1512	11
40	449	2147	1449	15
50	374	1972	1387	18
60	299	1797	1325	22

could be increased at least to that of NCP (Spivey, 1978) (30-34 hr. fermentation) resulting in about an 11% reduction in the Breakeven Price. One possibility for increasing the rate of solvent production is to cause production to begin earlier. Spivey (1977) seems to have an organism capable of beginning solvent production soon after inoculation (Fig. 1.4) - a marked contrast to the fermentations of Chapter 3 (Figs. 3.1 - 3.10, and 3.16). Gottschal and Morris (1981(b)) show that high levels of butyric acid can also help to induce early production of butanol. Increasing the butanol tolerance by say 10% would allow a reduction of 10% in the required fermenter volume, but partially offset a larger time for the fermentation. This improvement might result in something like a 4% reduction in the Breakeven Price.

Biotechnological research resulting in increased fermentation rates and yields after plant design could also result in handsome returns. A 10% increase in the final yield, or the rate of fermentation would result in an increase of approximately the same percentage of the final solvent yield and plant income (assuming good marketing). A large laboratory staff will be required in any case to maintain a smoothly running plant due to problems such as those seen in Chapter 5 as well as perform process research.

Reduction of the number or size of fermenters could result in significant benefits. Fermenters make up 60% of the total purchased equipment cost (Tables 6.18 and 6.21) and so any reduction in this could result in significant savings. If the filling rate (r) were increased, then the fermenters could be reduced to two 443 m^3 fermenters and (2.81 - 2.24)/2.81 = 20% in fermenter costs saved (Table 6.1), which would cause a reduction of 6% in the breakeven cost (Table 6.26). This saving would be partially offset by the extra cost of pumps and another tank. If no spare fermenter was required the filling rate increased then the fermenter costs could be reduced by (2.81-1.50)/2.81 = 47%

with an 11% reduction in the total production cost. The cost involved in increasing the filling rate was not included. This decision requires an alteration in the initial specification and a deeper study of costs and of the risks involved, and finally would be a decision for the plant management.

Fermenters designed as horizontal units might save up to 40% of their direct cost (New Zealand Institution of Engineers, 1980(b)) resulting in about a 15% reduction in solvent costs without incurring extra costs. This is an obvious design modification to be included in future designs if this saving can actually be realised. Further examination of this possibility is obviously warranted.

Another possible cost saving arrangement is to have the spare fermenter operating as the smoothing vessel immediately before distillation. However, at such times as the spare fermenter is required, flow to the distillation system would be less steady with resultant problems.

Alternatively research into the separation technique would be useful. The beer-still incurs about 66% of the utility cost, or 4% of the total production cost. This is due in part to the dilute nature of the beer solvents, and therefore again biotechnological research could help reduce costs. Alternatively improved separation techniques could be used. At present however only distillation is a proven technique.

Thus it can be seen that plant and market size, fermenter costs, and the microbiology/biotechnology aspects of the plant are the three major factors effecting the plant economics.

Unfortunately the substrate must be low cost. If another, higher cost substrate were used to extend the season to 330 days, then this cost would be spread over the entire

year's production. An increase product cost by \$0.04/kg would allow about \$68,000 pa for suitably treated substrate. If an average of 200 m^3/day were used for 60 day/year (the rest being whey permeate), then an approximate price of \$5.67/m^3 could be paid. Brown et al., 1978 give the price of sugar beet as \$20/tonne. If sugar beet were about 200 g/l fermentable carbohydrate, then a broth of concentration 50 g/l would cost about \$5/m^3, and may therefore be a useful substrate. Some extra equipment may be required to allow this substrate to be used, but the extra cost should not be great.

In summary, it appears that a plant of 300m^3 day^{-1} capacity would be uneconomic, however a plant of 600m^3 day^{-1} capacity (principally whey permeate) operating for 330 day per year could be economic, especially if fermenter costs can be reduced as suggested.

7. GENERAL DISCUSSION AND CONCLUSIONS

The decision to concentrate efforts on improvement of fermentation characteristics as an aid to ensuring overall process viability (Chapter 3) was well justified by the costing exercise (Chapter 6), and the problems outlined by Chapter 5.

The costing was based on the unoptimised results of Chapter 3 and showed the enormous cost contribution of the fermentation vessels. While the costing showed a plant of 300 m^3 day^{-1} whey permeate consumption would have little chance of economic success, a plant of double this capacity could be successful, especially with the improvements suggested, including extension of the operating year to a full year if a suitable substrate can be found. Unfortunately the work of Chapter 4 precludes the immediate use of raw untreated wood sugar hydrolysates. The economic reliance of the plant on the fermentation also demands that the fermentation be reliable. The length and variation in the overall fermentation time observed in Chapter 3, and the work in Chapter 5, point therefore to the need for continued microbiological research. Indirect evidence implicated possible involvement of lysogenic phage, in which case careful application of historically proven procedures should be successful. This problem must be overcome if optimum plant performance, as opposed to mere viability, is to be attained. The effect of minimum pH is likely to be less of a problem as it appears readily controllable.

The use of the Factor Correlation Method in outlining the possible metabolic reactions of an organism, along with important chemical species and the effect of factors external to the microorganism, appears novel and is perhaps the most fundamentally interesting result of the thesis. This approach may well be useful in guiding research in many fields other than in microbial metabolism

as it provides a simple method to investigate both obvious and subtle effects in even the most complex of systems (such as a microbial cell). It was developed in the present case to determine which of those basic fermentation parameters investigated most affected the final solvent yields, and the possible mechanisms for their action. As a result the minimum observed pH and the headspace pressure, perhaps as a result of both hydrogen and carbon dioxide, were discovered to be two important fermentation variables positively correlated with butanol yields. Ethanol was found to be correlated only with the headspace pressure, and acetone to neither of these variables. The principles behind the Factor Correlation Method were also applied to a much simpler situation in Appendix 2. Appendix 2 uses linear regression to suggest the major sources of variation for cell enumeration by direct visual counting of cells in a haemocytometer. The results were clear and well defined. The variation in cell counts from square to square was attributable to random variation plus a fractional error term to account for human counting error (in this case 10%).

It would appear, therefore, that the specific aim of the work to show that pressure positively effected the final butanol yield was achieved. Additional information on the economics of a commercially sized plant, the fermentation itself, and on methods of experimental results analysis was also obtained.

APPENDIX ONE: WATER TOLERANCE OF METHANOL/PETROL BLENDS WITH ADDED N-BUTANOL

A1.1 Introduction and Method

In order to assess the characteristics of n-butanol as a blending agent for mixtures of methanol in petrol, titrations of distilled water into a defined base blend of alcohol, benzene, and iso-octane were performed. The experiment provides a means of comparison of n-butanol's blending ability with the results published by Judd and Graham (1979).

A mixture of 3:14 (volume basis) benzene:iso-octane was used as the standard "petrol" mixture (Judd and Graham, 1979). This was then added to methanol as shown in Table A1.1 to produce the base blends M15, and M85. The base blends were intended to behave in the same manner as the methanol/petrol blends suggested for possible use in New Zealand in the near future. The base blend was then mixed with the blending agent to be tested to give a test blend with a final concentration of 7.4% (v/v) blending agent.

Fifty millilitres of test blend were then titrated with distilled water at 15°C until phase separation was first observed either as an opaque cloudiness or as two distinct, clear, separated phases. For accurate water tolerances, titrations were done in a temperature controlled water bath at various temperatures near 15°C while agitating vigorously, and the variation with temperature then considered using linear regression. Both methyl ethyl ketone (MEK) and n-butanol were used as blending agents. The MEK was used to provide a means of direct comparison with the results of Judd and Graham (1979).

Estimation of the water droplet size from the titration pipette was necessary due to the small volumes of water involved. This was achieved in a separate experiment by

Table A1.1 BASE BLEND MIXTURES

Blend	Alcohol	Alcohol Conc.	"Petrol" Mix*
M15	Methanol	15	85
M85	Methanol	85	15

"Petrol" Mix = 3:14 benzene:iso-octane

taking a pipette reading after each drop when emptying the pipette from full. This was done twice with the same pipette and pipette filler as used for the titrations.

A1.2 Results

Table A1.2 shows the results of the two water-droplet size experiments. Drop size was calculated as the difference in readings before and after each drop. In all there were 21 readings, giving a drop size of (0.0369 ± 25%) ml (error for two standard deviations, or approx. 95% confidence level).

Fig. A1.1 shows the titration results for M15, giving water tolerance as % of original blend volume against temperature and corrected for water droplet size. The correction was made by subtracting half the drop size (ie: 0.5 x 0.037ml = 0.018ml) from the volume of water used in the titration. Linear regression was then used to estimate the water tolerance at 15°C. Table A1.3 summarises the titration results. The results for M85 blends are for duplicate experiments at 15°C without regression, and are not therefore as accurate as the M15 results which used linear regression to provide an estimate of the water tolerance at 15°C.

A1.3 Discussion and Conclusions

The results for M15 with and without MEK compare favourably with those of Judd and Graham (1979). Water present in the n-butanol prior to titration was checked but found to be low in comparison to the amount added. It is obvious that a marked improvement in water tolerance is obtained with 7.4% (v/v) n-butanol; 15 times and 2.2 times the tolerances for M15 and M85 respectively. The only additives with the same effect or better on M15 in Judd and Graham's (1979) table are hexanol, piperidine, and 2, 4 xylenol. The tolerance for an M15 mixture blended with

Table A1.2 WATER-DROPLET SIZE RESULTS

Trial 1		Trial 2	
Reading (ml)	Drop Volume (ml)	Reading (ml)	Drop Volume (ml)
0		0	
0.04	0.04	0.04	0.04
0.08	0.04	0.075	0.035
0.11	0.03	0.12	0.045
0.15	0.04	0.155	0.035
0.175	0.025	0.19	0.035
0.21	0.035	0.23	0.04
0.25	0.04	0.27	0.04
0.285	0.035	0.31	0.04
0.32	0.035	0.35	0.04
0.355	0.035	0.38	0.03
		0.42	0.04

Fig. A1. Water Tolerance of Laboratory Petrol Blend with Two Blending Agents.

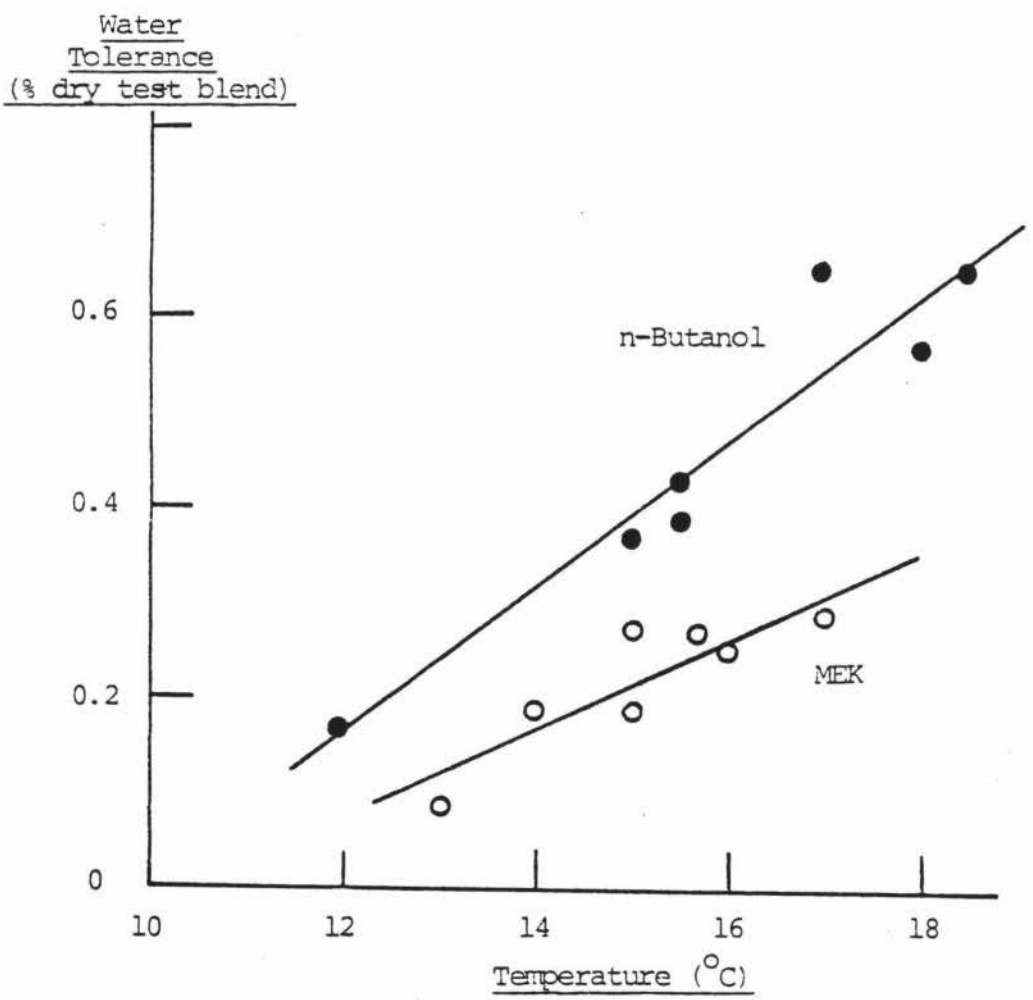


Table A1.3 WATER TOLERANCE (% DRY TEST BLEND)
AT 15°C FOR DIFFERENT BASE BLENDS AND
BLENDING AGENTS

Base Blend	Blending Agent		
	None	MEK	n-Butanol
M15	ca. 0.025(0.02)*	0.21 (0.18)*	0.38
M85	1.0	-	2.2

* Results of Judd (1979) are given in brackets.

iso-butanol is 0.32% (v/v). Piperidine at 0.40% (v/v) has the greatest blending quality of all the agents Judd and Graham (1979) tested, but is only marginally better than n-butanol, and because of butanol's greater ease of production butanol would be preferred for large scale blending. The French government have recently taken this view when planning their nation's fuels future (Prince, 1982).

In conclusion then, it seems that n-butanol is a potentially useful blending agent for methanol/petrol blends.

APPENDIX TWO: CELL COUNT ERROR ANALYSIS

A2.1 The Theoretical Model

The approximate volume of a single cell of *Clostridium acetobutylicum* or *Cl. butylicum* is:

$$\text{Volume of cell} = \pi \times (\text{diameter})^2 \times (\text{length})/4$$
$$= \pi \times (0.1 \times 10^{-6})^2 \times (2 \times 10^{-6})/4 \text{ m}^3$$
$$= 2 \times 10^{-20} \text{ m}^3$$

Hence the number of "samples" (i.e. possible sites which a cell may occupy) taken in counting the cells in a single small haemocytometer square (representing a volume of 2.5×10^{-13} m^3) is

$$\text{Number of sites} = \frac{\text{Total Volume}}{\text{Site Volume}}$$
$$= \frac{2.5 \times 10^{-13} \text{ m}^3}{2 \times 10^{-20} \text{ m}^3 \text{ site}^{-1}}$$
$$= \text{ca. } 10^7 \text{ sites}$$

which is the maximum possible number of cells per small square.

The basic theoretical distribution describing the probable number of cells per square in this case is the Binomial Distribution, where the characteristic recorded is whether a cell is present or not in any particular site. However, in cases where the number of sites is large and the proportion of "successes" is small as is the case here (in this case the actual average number of cells per square divided by 10^7), a Poisson Distribution may be used (Clarke, 1969). Clark (1969) suggested using the Poisson Distribution for cell enumeration but diluted his samples severely. In cases where the actual number of cells per square is greater than 7 the Normal Distribution may also be used (Mendenhall and Reinmuth, 1978).

If the Poisson Distribution is assumed to apply, then the variance of cell counts (s^2) should equal the mean number $\bar{x}$ expected per square (Clarke, 1969). If the variance due to random fluctuation in the actual number of cells per square is assumed to be the only source of error, then a linear regression of s^2 against $\bar{x}$ should give a straight line of intercept zero and a slope of unity. In fact experimental data fitted to the model:

$$s^2 = a + b\,\bar{x}$$

gives an intercept (a) of -13.6 ± 3.4, and slope of 1.93 ± 0.09 (Table A2.1). The data used is shown in Fig. A2.1. The 99.9% confidence t-statistic is 3.373 for 120 df and 3.291 for infinite df, so the 99.9% confidence t-statistics for 394-396 df will be between 3.373 and 3.291. Hence, the pure Poisson ratio does not accurately fit the real situation.

A2.2 A Practical Model

Inclusion of human counting error, absolute error (e_a), and fractional error (f_a), results in a variance relationship

$$s^2 = \bar{x} + e_a{}^2 + f_a{}^2\,\bar{x}^2$$
$$= e_a{}^2 + \bar{x} + f_a{}^2\,\bar{x}^2$$

which is a quadratic in $\bar{x}$. If s^2 is then regressed against $\bar{x}$ and $\bar{x}^2$ for the model:

$$s^2 = c + d\,\bar{x} + e\,\bar{x}^2$$

then c, d, and e evaluated from the data in Fig. A2.1 by least squares regression gives results as shown in Table A2.1. The constant term representing $e_a{}^2$ appears to

Table A2.1 TABLE OF REGRESSION RESULTS FITTING
A MODIFIED POISSON MODEL

Coefficient	Variable	Value	Std error	t-statistic	d.f.
a	constant	-13.4	3.386	-3.96	395
b	$\bar{x}$	1.93	0.09392	20.52	
c	constant	1.431	4.274	0.33	394
d	$\bar{x}$	0.8081	0.2261	3.57	
e	$\bar{x}^2$	0.01170	0.002168	5.39	
f	$\bar{x}$	0.8701	0.1298	6.70	395
g	$\bar{x}^2$	0.01123	0.001658	6.77	
-	$\bar{x}$	1	(defined)	-	396
h	$\bar{x}^2$	0.00976	0.0007706	12.67	

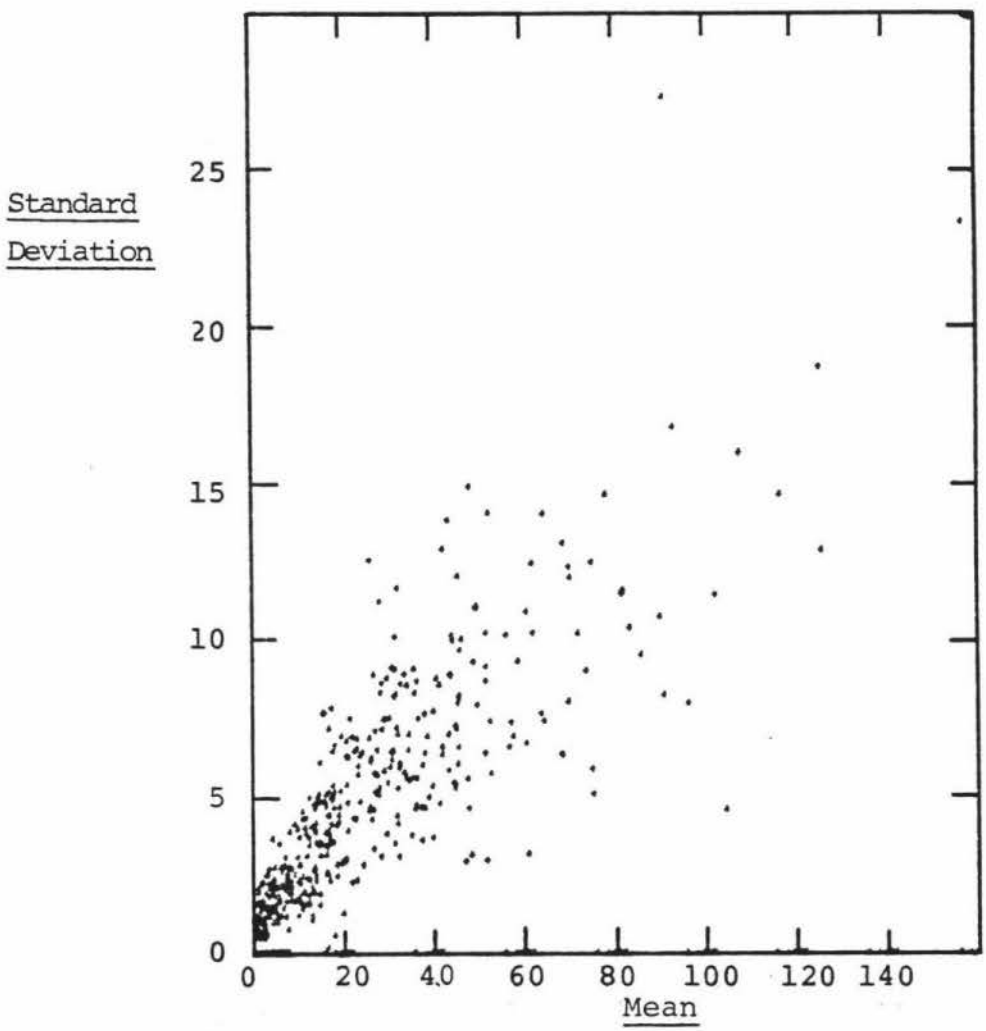


Fig. A2.1. Standard Deviation vs. Mean (Raw Data). (Six Squares Counted.)

be trivial (as its t-statistic is well below even the 80% value of 1.29 with 120 d.f.). The regression was then repeated as:

$$s^2 = \bar{x} + f_a{}^2 \bar{x}^2$$

in the form

$$s^2 = f \bar{x} + g \bar{x}^2$$

calculated with no-intercept linear regression, and values of f and g as shown in Table A2.1 were obtained. Consideration of the standard error for f shows that it is unreasonable to assume that f differs significantly from unity, which is the value suggested by a Poisson type variance structure.

If f is then assumed to be unity, the variance model takes the form

$$s^2 = \bar{x} + f_a{}^2 \bar{x}^2$$

giving

$$s^2 - \bar{x} = h \bar{x}^2$$

where h, evaluated using no-intercept linear regression, is also given in Table A2.1.

Assuming the model to hold, then:

$$\begin{aligned} f_a &= h^{0.5} \\ &= (0.00976)^{0.5} \\ &= 0.099 \end{aligned}$$

i.e. $f_a = 10\%$

which is the error from human sources in addition to the expected variation described by the Poisson distribution.

It is unlikely that the error in the cell counts would come from any source other than human counting. Problems of non-homogeneity within a single drop of culture are unlikely (even if the drop is not representative of the culture as a whole) i.e. there is no evidence to suggest that the cells are not randomly distributed horizontally in the counting volume. Possible error induced by rapid cell movement while counting was confined to a relatively small fraction of the sample population and is unlikely therefore to have a strong effect on f_a. It appears therefore that the cell count error (variance) structure follows a Poisson distribution with an added fractional error term accounting for the lack of human repeatability in counting. The expected variance may then be estimated by:

$$s^2 = \bar{x} + 0.01\ \bar{x}^2 \qquad : f_a = 0.10$$

The error in estimating s^2 from this equation is about 40 (found from the standard deviation of s^2 about the regression line). The residuals about the regression line were checked and were approximately normal and of almost constant magnitude when plotted against $\bar{x}^2$ through the mid range of experimental values. As $\bar{x}^2$ tended to zero the residual distribution shrunk, but above about $\bar{x} = 8$ was reasonably constant.

A2.3 Experimental Design

The design of experiments involving cell counts traditionally requires specification of the number of squares to be counted (nc) for a given acceptable error. Since

$$s^2 = E + 0.01\ E^2 \qquad : E = \text{expected number of objects per square.} = \bar{x}$$

(assuming all experimenters can count to 10% accuracy) then the fractional error (expressed as fraction of the mean) is

$$(s/E)^2 = E^{-1} + 0.01$$

This clearly shows the dependence of the accuracy of enumeration on the average number of objects counted. If the number of squares counted (nc) is included in the expression, then:

$$(s/E)^2 = (E^{-1} + 0.01)/nc$$

As an example of the variation in the enumeration error for varying E but fixed nc, the approximate error involved using a haemocytometer and counting six squares (nc = 6) can be seen in Fig. A2.2.

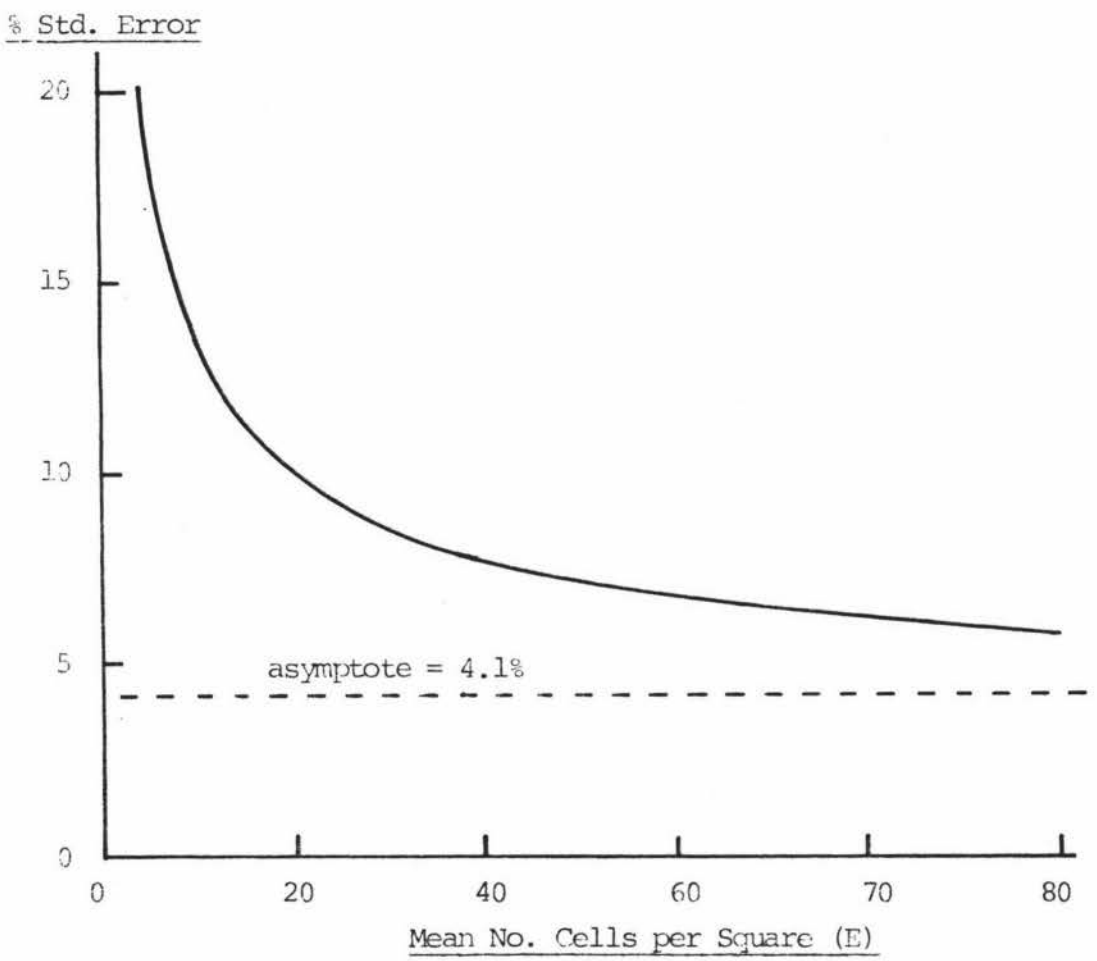
A2.4 Cell Enumeration for Regression Analysis

If least squares regression is used to fit a population model to cell data, then a transformation must be performed on the data to make the error envelope parallel to the regression line throughout the entire range of data (i.e. that the variance be constant about the regression line). Assuming that the variance of the population sampling estimates is given by

$$s^2 = (E + 0.01\ E^2)/nc$$

where s is standard error of the mean of haemocytometer cell counts, E is the expected number of cells per square, and n the number of squares counted then the 68% confidence level envelope $Y_{.68}$ about the regression line may be written as:

Fig. A2.2. Standard Error vs. Object Concentration for Cell Enumeration Using a Haemocytometer (6 squares counted)



$$Y_{.68} = E \pm s$$

$$= E \left[1 \pm nc^{-0.5} \left(E^{-1} + 0.01\right)^{0.5}\right]$$

where $\pm$ is included to represent both the upper (+) and lower (-) error bonds. Traditionally, the logarithm of the cell population is used, giving:

$$\ln Y_{.68} = \ln E + \ln \left[1 \pm nc^{-0.5} \left(E^{-1} + 0.01\right)^{0.5}\right]$$

however, it can be seen that the error term (ln e) (where e is the error which represents s)

$$\ln e = \ln \left[1 \pm nc^{-0.5} \left(E^{-1} + 0.01\right)^{0.5}\right]$$

is not independent of cell population (E) (as shown by Table A2.2 for two values of nc) and as such the assumption behind the least squares technique is violated. In fact the assumption may often be applied in a relaxed fashion, and as such this violation is often overlooked and large values of nc have traditionally been recommended as a partial solution since ln e tends to zero as nc increases, in spite of E. This practice, however, can be improved upon. Two improvements over this technique will now be outlined. The error analysis presented here is applicable to any binomial enumeration technique fulfilling the assumptions for a Poisson distribution approximation, but does not include a factor for systematic errors.

One improvement is to specify the total number of cells counted (given approximately by the product nc E) and not to specify a fixed nc.

The advantage of this can easily be shown.

Table A2.2 VALUES OF FRACTIONAL ERROR (f) AND ln e FOR VARYING CELL CONCENTRATIONS (E) FOR FIXED nc OR nc E

E	nc = 6		nc = 15		nc E = 50		nc E = 100	
	lne	f	lne	f	lne	f	lne	f
1	0.344	0.41	0.231	0.26	0.133	0.14	0.096	0.10
2	0.256	0.29	0.169	0.18	0.134	0.14	0.096	0.10
3	0.214	0.24	0.141	0.15	0.134	0.14	0.097	0.10
4	0.189	0.21	0.124	0.13	0.135	0.14	0.097	0.10
5	0.171	0.19	0.112	0.12	0.135	0.14	0.098	0.10
10	0.127	0.14	0.082	0.09	0.138	0.15	0.100	0.10
15	0.107	0.11	0.069	0.07	0.141	0.15	0.102	0.11
20	0.095	0.10	0.061	0.06	0.144	0.15	0.104	0.11
30	0.082	0.08	0.052	0.05	0.149	0.16	0.108	0.11
40	0.074	0.08	0.047	0.05	0.155	0.17	0.112	0.12
50	0.068	0.07	0.044	0.04	0.160	0.17	0.116	0.12
60	0.065	0.07	0.041	0.04	0.165	0.18	0.119	0.13
100	0.056	0.06	0.036	0.04	0.182	0.20	0.132	0.14

$$s^2 = (E + 0.01\ E^2)/nc$$

$$(s/E)^2 = (E^{-1} + 0.01)/nc$$

or $$f^2 = (E^{-1} + 0.01)/nc$$

and since $nc = nc\,(E/E)$

then

:s = absolute standard error

:E = expected number of cells

:nc = number of squares counted

:f = fractional standard error

$= s/E$

$$f = (1 + 0.01\ E)^{0.5}(nc\ E)^{-0.5}$$

Repeating the earlier calculation to find the error bounds:

$$Y_{.68} = E \pm f\ E$$

$$= E\ (1 \pm f)$$

$$= E\ (1 \pm (1 + 0.01\ E)^{0.5}(nc\ E)^{-0.5})$$

$$\ln Y_{.68} = \ln E + \ln (1 \pm (1 + 0.01\ E)^{0.5}(nc\ E)^{-0.5})$$

Values of $\ln e = \ln (1 \pm (1 + 0.01\ E)^{0.5}(nc\ E)^{-0.5})$ are shown in Table A2.2 for two values of nc E. Both f, and therefore ln e, vary very much less.

Another method which forces ln e to be constant is to allow nc to vary depending on the number of cells per square observed. The required accuracy is preset, and the corresponding nc for this error is determined.

$$f^2 = \frac{1}{nc\ E} + \frac{0.01}{n}$$

so $$nc = (E^{-1} + 0.01)/f^2$$

where it is assumed human enumeration error is ± 10% for a single square count. Fig. A2.3 shows this relationship graphically for different errors (approx. 95% confidence

level). Of course nc should be integer and non zero.

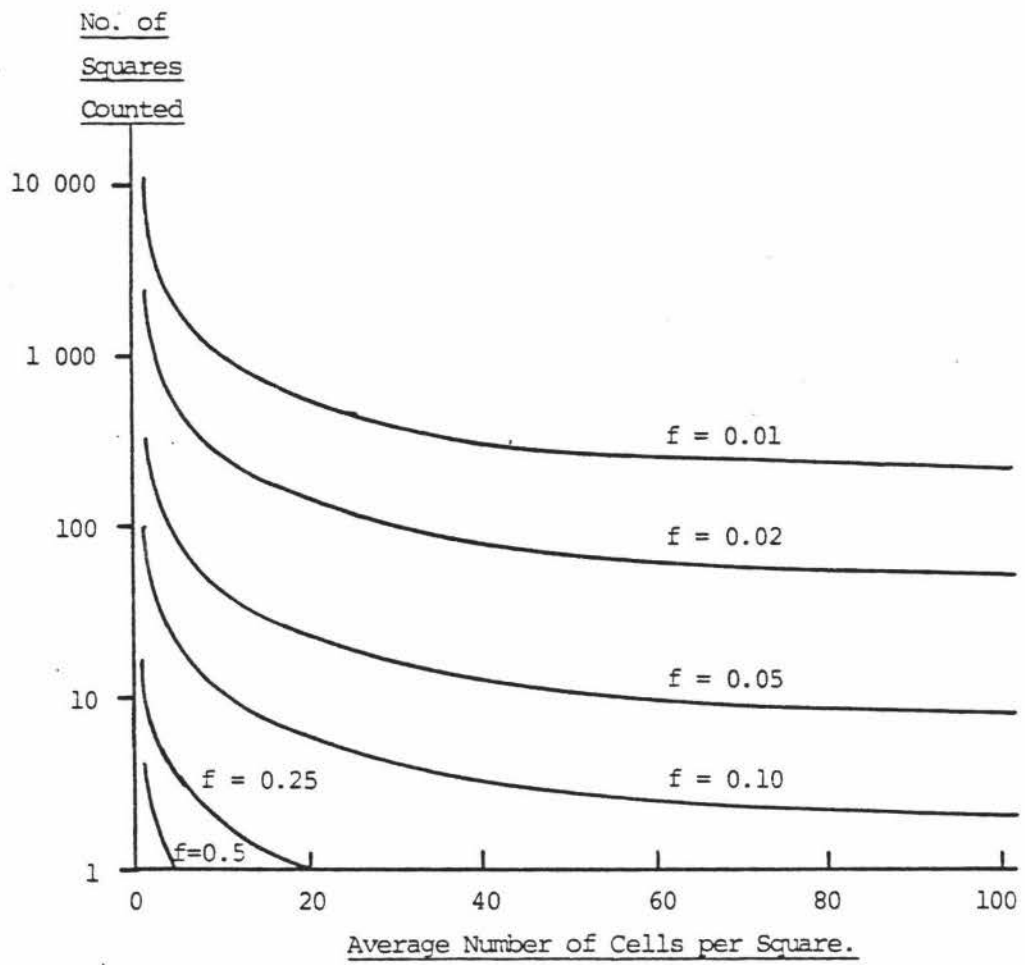
A2.5 Results and Discussion

The conclusion to include only a fractional error for human non-repeatability instead of a fractional and absolute error was surprisingly simple (Table A2.1 and text) since the t-statistic of 0.33 (394 degrees of freedom) for the absolute error was so small. The closeness of the Poisson term ($f = 0.87 \pm 0.13$) to unity was also surprising. These results suggest that the simple model of the error structure presented here is remarkably accurate.

The two improved cell enumeration methods presented are not much more difficult to use than the more traditional method of specifying the number of squares counted. The first involves specifying the total number of cells counted instead of the number of squares, and the number of cells in each square as well as the number of squares counted is recorded. The final square may either be fully counted or neglected. The final concentration is then estimated from the average number of cells per square, and the error in this estimate evaluated from the standard deviation of the counts divided by the square root of the number of squares counted. The second method produces a virtually constant error for any cell concentration, but requires continuous reassessment of the number of squares required to be counted while counting. This extra effort may be considered worthwhile in some circumstances where great accuracy is required.

In conclusion then, the traditional method for cell enumeration of specifying the number of squares counted produces an error in the estimation which varies with the estimate unnecessarily. Specification of the number of cells counted is shown to produce superior estimates for no extra effort. A third, even more accurate method is also presented.

Fig. A2.3. Number of Squares vs. Average Number of Cells Counted at Various Levels of Error (f).



APPENDIX THREE: DETERMINATION OF α AND ß

A3.1 Two Methods of Parameter Evaluation

The constants α and ß which appear in the productivity equation

$$q = \alpha\mu + ß$$

are normally found by numerically evaluating

$$\alpha = \frac{1}{N}\frac{dN}{dt}$$

$$\text{and } q = \frac{1}{N}\frac{dP}{dt}$$

from experimental curves smoothed by eye. Graphs of versus t and q versus t, and of q versus N are then drawn. The first two curves give a qualitative method of checking data quality (as they typically rise and fall from a single peak only), and the second shows the linearity of the relationship $q = \mu\alpha + ß$ and allows α and ß to be evaluated.

Unfortunately however, this method relies heavily on smoothing by eye in order to evaluate the differentials. This is particularly unreliable if noise is present in the data, and undesirable if data is not plentiful since the numerical evaluation of differences also removes one degree of freedom from the data.

Alternatively the equation may be integrated:

$$q = \alpha\mu + ß$$

$$\frac{1}{N}\frac{dP}{dt} = \alpha\frac{1}{N}\frac{dN}{dt} + ß$$

$$\frac{dP}{dt} = \alpha\frac{dN}{dt} + ß N$$

$$P = \alpha N + ß \int N dt$$

:q = rate of production
:μ = growth rate
: α,ß = constants
:N = cell population
:P = product concentration
:t = time

and evaluated between t = 0 and the time of interest. With this expression, raw experimental data (i.e. experimental values of B and N) are used for evaluation of α and β, and only a single numerical integration need be performed with the greater accuracy accompanying numerical integration and without loss of a degree of freedom. If the data are evenly spaced in time, then the integration can easily be done using Simpson's Approximation. This is a second order numerical integration which takes some account of curvature by, effectively, integrating a quadratic equation fitted across successive groups of three data points. If the data are not evenly spread then the integrals may be evaluated for an eye fitted curve (still more reliable than numerical differentiation of an eye fitted curve), or a population model may be fitted to approximate the N versus t data, and then analytically or numerically integrated to the desired accuracy.

For the same set of population and product data, evaluation of α and β using the integrated equation will give more reliable results than evaluation by the differential method provided the population model is the same eye-fitted curve as used for the differential method, or a model of "superior" fit. Evaluation of α and β is easily done by solving the following pair of simultaneous equations:

$$\Sigma x^2 + \beta \Sigma x y = \Sigma x z \qquad :x = N$$

$$\Sigma x y + \beta \Sigma y^2 = \Sigma y z \qquad :y = \int_{t_o}^{t_i} N \, dt$$

$$:z = P_i$$

Unfortunately this method gives no easy method of checking data quality as in the case of the differential method which gives a graphical representation. Reduction of the simultaneous equations to a single dimension by dividing each equation by N (or alternatively by $\int N dt$) may be practical and

would provide a means of checking data quality, however this practice may accentuate noise present in the data.

A3.2 The Logistic Population Model

The Logistic Equation (Bailey and Ollis, 1977) was chosen as a possible model to approximate the growth data observed. This equation may be approximately derived as follows.

A general form of equation describing growth may be approximated by

$$\frac{1}{N}\frac{dN}{dt} = k\ (1 - f\ (I))$$

where the inhibition function f(I) is some function reducing the growth rate and dependent on the inhibition parameter (I), k is maximum growth rate (which occurs when no inhibition is present), and N is the cell population parameter.

The inhibition function is assumed to be first order and linear with respect to inhibitor (I), i.e. the inhibition is directly proportional to inhibitor concentration.

Inhibitor production is approximated by the equation

$$q = \mu\alpha + \beta$$

i.e. $$\frac{1}{N}\frac{dI}{dt} = \alpha\ \frac{1}{N}\frac{dN}{dt} + \beta$$

as suggested by the Luder, King-Piret Model, and may be integrated as earlier (Section A3.1) to

$$I = \alpha N + \beta \int N dt$$

These assumptions may be amalgamated to produce the following growth curve (where b is the inhibition constant):

$$\frac{1}{N}\frac{dN}{dt} = k\ (1 - b\ [\ \alpha N + \beta\ \int N\ dt])$$

This model may be considered as one of three cases.

Case 1 $\quad \beta \int N dt >> \alpha N$

In this case, where inhibitor production is not growth related to any great extent, then

$$\frac{1}{N}\frac{dN}{dt} = k\ (1 - b\beta \int N dt)$$

will be a reasonable approximation.

Case 2 $\quad \beta \int N dt << \alpha N$ (The Logistic Equation)

In this second case, where inhibitor production is strongly growth related, then

$$\frac{1}{N}\frac{dN}{dt} = k\ (1 - b\ \alpha\ N)$$

may be expected to give reasonable approximations, at least until late in the fermentation when $\int N dt$ continues to grow but N is approximately constant or relatively small. (The effect of dynamic equilibrium due to cell death is ignored.) This equation is a Riccarti form of equation (Bailey and Ollis, 1977) and may be integrated to:

$$N = \frac{N_o \exp\ (kt)}{1 - b\ \alpha\ N_o\ (1 - \exp\ (kt))}$$

$$= \frac{N_o \exp\ (kt)}{1 - (N_o/N_s)\ (1 - \exp\ (kt))}$$

where $N_s = 1/b\,\alpha$ in order that N rise assymptopically to N_s, the stationary population. This is known as the Logistic Equation.

Case 3

If neither term dominates then the full form of equation should be used.

A3.3 Example of the Two Methods

To allow comparison of the differential and integral methods, both were applied to the cell population (Table 3.19) and butanol productivity data for Run A of Chapter 3. Table A3.1 shows the data and results of the calculations for both methods of calculation. Table A3.2 gives the units for Table A3.1. Values of t, N, and B were smoothed for the numerical differentiations using three point moving averages instead of using values from a hand fitted curve (producing t_{sm}, N_{sm}, and P_{sm}). Values of N_{sm} late in the fermentation were then adjusted by eye in order that they rose monotonically and assymptopically to N_s. N_s was evaluated as the mean of the population values for t greater than 16 hrs. Numerical differentiation was then performed using the following formula:

$$\mu_{i-1/2} = \frac{N_i - N_{i-1}}{LN}\,\frac{1}{t}$$

$$= \frac{\ln\,(N_i/N_{i-1})}{t}$$

$$q_{i-1/2} = \frac{B_i - B_{i-1}}{LN}\,\frac{1}{t}$$

$$t_{i-1/2} = (t_i + t_{i-1})/2$$

where:

$$LN = \frac{N_i - N_{i-1}}{\ln(N_i / N_{i-1})}$$

$$t = t_i - t_{i-1}$$

TABLE A3.1 PRODUCTIVITY DATA

t	t_{sm}	$t_{i-1/2}$	N	N_{sm}	B	B_{sm}	$\mu_{i-1/2}$	$q_{i-1/2}$	N_{log}	$\int N dt$
0	0	0	7.3	7.3	0	0	-	0	7.3	0
0.4	0.2	0.1	29	18	0	0	4.5	0	-	-
1.3	0.6	0.4	45	27	0	0	1.0	0	-	-
2.5	1.4	1.0	48	41	0	0	0.52	0	-	-
3.8	2.5	2.0	60	51	0	0	0.20	0	-	-
5.2	3.8	3.2	45	51	0	0	0	0	48	113
7.7	5.6	4.7	59	55	0	0	0.04	0	113	303
9.2	7.4	6.5	108	71	0	0	0.14	0	184	523
9.9	8.9	8.2	195	121	0	0	0.36	0	230	677
10.5	9.9	9.4	213	172	0	0	0.35	0	271	822
12.0	10.8	10.4	407	272	0	0	0.51	0	379	1321
13.6	12.0	11.4	480	367	0	0	0.25	0	555	2074
14.6	13.4	12.7	767	551	0	0	0.29	0	655	2679
15.3	14.5	14.0	900	716	0.15	0.15	0.24	0.216	718	3140
15.8	15.2	14.9	973	880	0.19	0.19	0.29	0.072	769	3571
16.5	15.9	15.6	1287	1053	0.12	0.21	0.26	0.030	823	4105
19.5	17.3	16.6	1113	1070	0.24	0.24	0.01	0.020	987	6850
20.4	18.8	18.1	873	1093	0.28	0.28	0.01	0.025	1015	7772
21.3	20.4	19.6	1320	1093	-	-	0	-	1036	8706
22.5	21.4	20.9	697	1093	0.74	0.74	0	0.162	1056	9931
33.5	25.8	23.6	1330	1093	2.0	2.0	0	0.262	1093	21841
57.8	37.9	31.9	(1033)	1093	2.4	2.4	0	0.030	1093	48342
130	73.8	55.9	(560)	1093	3.4	3.4	0	0.025	-	-

TABLE A3.2 UNITS FOR TABLE A3.1

Variable	Symbols	Units
time	t, t_{sm}, $t_{i-1/2}$	hours
cell count (c.c.)	N, N_{sm}, N_{log}	10^6 cells/ml
integral c.c.	$\bar{N}$	10^6 cell hours/ml
butanol concentration	B, B_{sm}	g/l
growth rate	$\mu_{i-1/2}$	$hour^{-1}$
productivity	$q_{i-1/2}$	10^{-6} g/10^6 cell hours

and the subscript i-1/2 represents an averaged value over the interval i-1 to i. The values of t, N, and B used in the equations were in fact the smoothed values (t_{sm}, N_{sm}, and B_{sm}).

The raw experimental data for t and N was used to evaluate N_{log} (the estimate from the logistic population model), and $\int Ndt$ was evaluated using Simpson's Approximation with eight equal time intervals.

Fig A3.1 shows μ and q plotted against time for the values calculated above, and Fig. A3.2 shows q against μ.

The data shown in Fig. A3.2 is badly scattered. If the data were approximately colinear then a least squares regression line could be evaluated to give α (the intercept) and β (the slope). (The method is outlined further in Bailey and Ollis, 1977.) The initial very high, decreasing, growth rate is due to an artifact in sampling, as mentioned earlier in Chapter 3 (refer Sect. 3.3.3.3), but the remainder of the curve could be considered almost typical for the fermentation. However, the smoothing performed by eye to produce a monotonically increasing cell population of the data which had already been smoothed using moving averages in fact hides the negative growth rates which would otherwise result from the large population fluctuations. In fact, therefore, the curve has been drastically smoothed and this fact must be borne in mind when interpreting the data.

The curve of q vs t shows two peaks. This is most unusual. The fact that the dip is coincidental with the fall off of growth may be significant.

The same data was then processed by the method based on integration of the Luder King-Piret Model presented earlier and using the Logistic Model to model the population. The Logistic Model was fitted by minimising the

Fig. A3.1. μ, and q vs. Time.

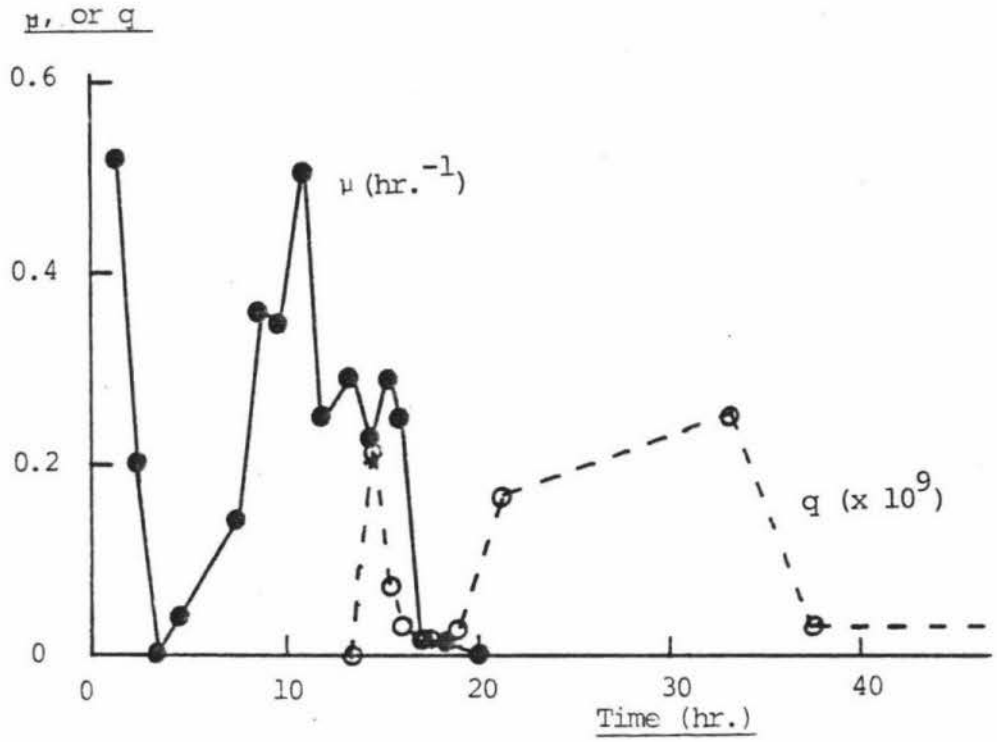
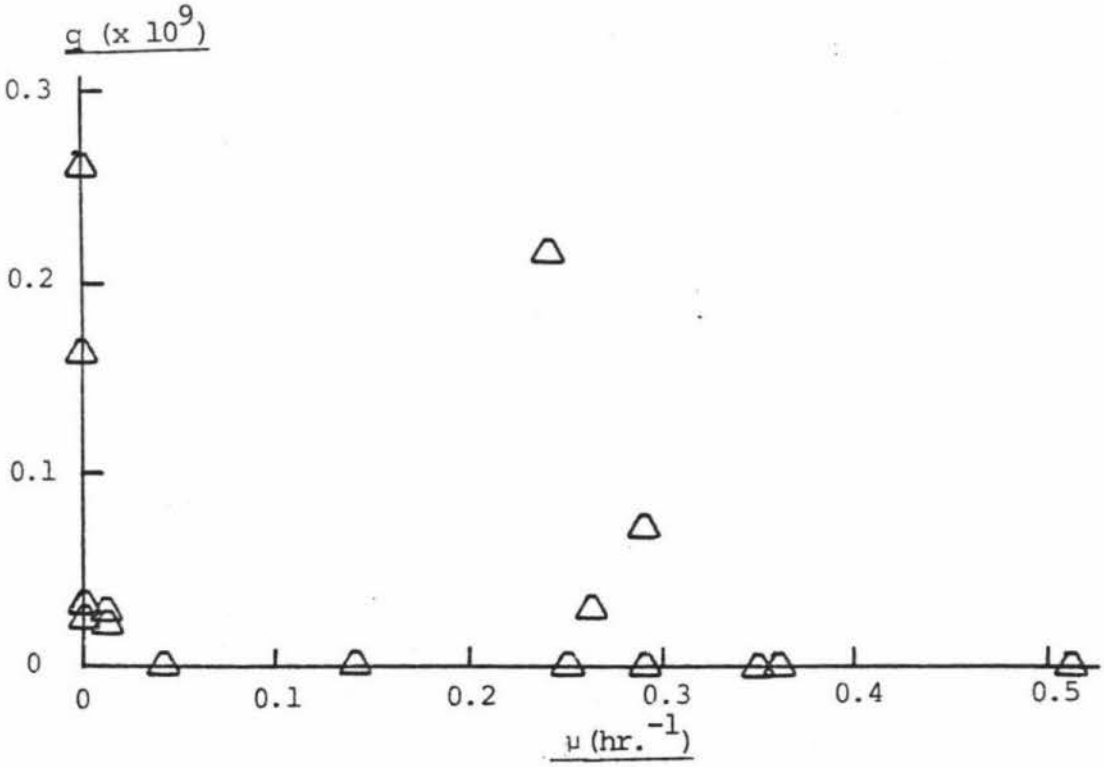


Fig. A3.2. q vs. μ.



summation of different statistics measuring the deviation. These statistics were:

a) $(O - E)^2/E$

b) $(O - E)^2/O$

c) $(O - E)^2$

and were minimised by varying k, or k and N_s. In view of the results of Appendix 2, these statistics (in particular a) and b)) were considered worthwhile trying instead of ordinary least-squares. Due to the final results of this study, however, the method of optimisation is unimportant in the present case. Future work of this type should be conducted using the cell enumeration method improvements suggested in Appendix 2 and ordinary least squares on ln N. Statistic c) suffers the problem of unbalanced dependence on data with large values of N and was not further used. Statistic a) (the Chi-Square Statistic) was used in cases where only k was being varied. In cases where both k and N_s were varied to minimise the test statistic, the Chi-Square statistic gave a strongly biased estimate of N_s, so statistic b) was used. The biasing of N_s by the Chi-Square estimate is because larger estimates of N_s will lower estimates of E and therefore in turn lower the Chi-Square value. The method is not of direct interest as in practice N_s should be clearly defined by the experimental data, and k minimised using least squares regression on the natural log of the cell number. The cell number should be estimated as outlined in Appendix 2. For the following calculations, only k was varied using the Chi-Square statistic and N_s was estimated as for the differential method. Fig A3.1 shows the experimental data and Table A3.1 the calculations involved. The data are the raw experimental data for Run A of Chapter 3 (Table 3.19).

Because of the scatter in the data the logistic model is seen to fit as well as any other model i.e. the scatter is

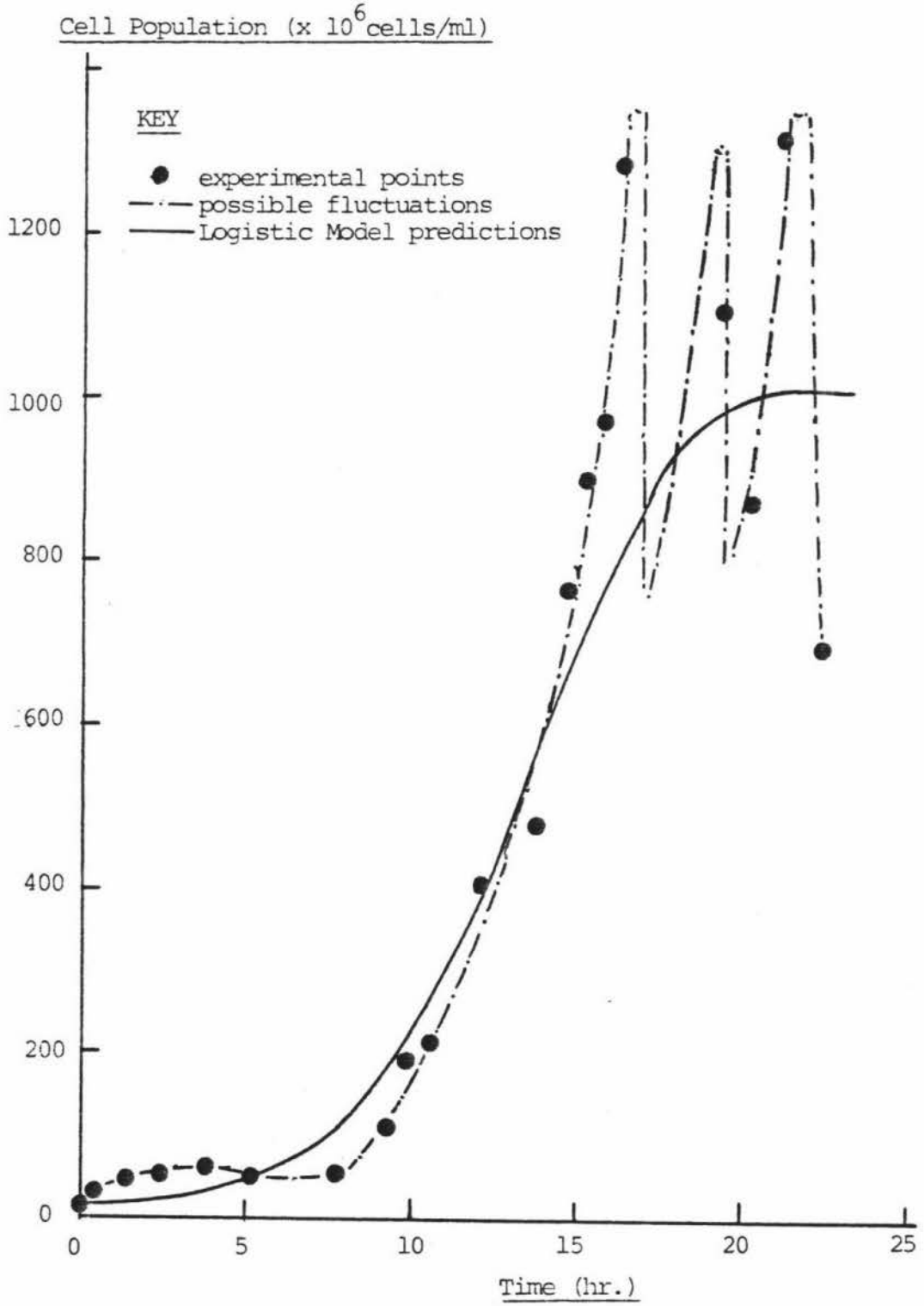
so severe the model is not really tested for goodness-of-fit. This is except to say, that if the fluctuations are real and not due to mere experimental scatter (Section 3.3.3.4) then the model is far too simple, but is as good as any other based on the principal of monotonic increase in population until the end of the stationary phase.

Because of the scatter in the data no meaningful comparison of the two methods of calculation of α and β can be made. The scatter in Fig. A3.2 reveals the uncertainty of estimates of α and β by the traditional method, and Table A3.3 shows the varied results for α and β produced by the integral method. Table A3.3 is comparable with Tables 3.3 and 3.4, although no meaningful relationships are obvious. The values of k in Table A3.3 are the maximum growth rates determined by the Logistic Equation. They should therefore be equivalent to μ of Table 3.4. Comparison shows some discrepancies. These are due principally to uncertainty in evaluation of a suitable N_o and therefore k and μ as discussed in Section 3.3.3.3. Values for N_o were estimated by linear extrapolation back to t = 0 of log N versus t over the exponential growth phase. In some cases the error involved in this procedure was large, leading to considerable error in some estimates. Fortunately the estimates of α and β are not unduly sensitive to k.

A3.4 Inadequacies of the Logistic Equation

Close examination of Fig. A3.3 shows that the Logistic Model overestimates the cell population early in the fermentation, and undershoots later in the exponential phase. Due to scatter it is hard to tell the result during the stationary phase. This observation is illustrated graphically in Fig. A3.4 which is a scatter diagram showing the normalised deviation [(O - E)/E] against time. The overall increasing trend is emphasised by shading and was seen in similar diagrams for the other runs in Chapter 3.

Fig. A3.3. Cell Population Data for Run A (Chapter 3).*



* See also Sect. 3.3.3.4, Appendix 4, and especially Fig. 4.

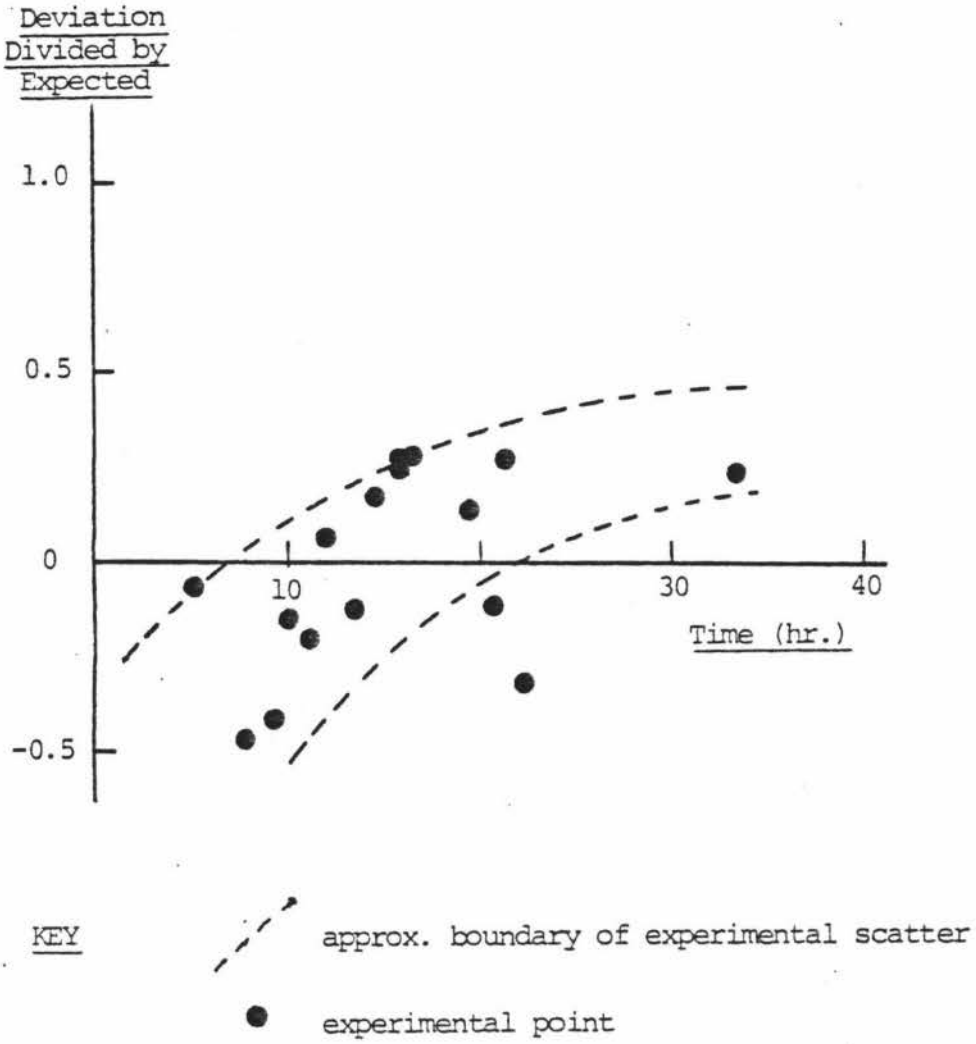
TABLE A3.3 RESULTS OF INTEGRATION METHOD FOR THE RUNS FROM CHAPTER 3

Run	Press	N_s	k*	α	ß	no. pts
A	101	1,093	0.37	0.03	0.054	18
B	101	1,068	-	-	-	-
C	101	1,373	-	-	-	-
D	140	1,977	-	-	-	-
E	157	994	0.39	-1.15	0.181	6
F	160	1,875	0.28	0.59	0.056	7
G	201	1,685	0.30	1.15	0.075	7
H	201	1,110	0.21	-	-	5
I	207	1,183	0.29	1.37	0.049	11
J	250	1,317	- **	1.51	0.09	8

* k expressed here is evaluated for the Logistic Equation, whereas in Table 3.4 μ was evaluated from a graph of ln(N) versus time.

** The results for α and ß were insensitive to inoculation size estimate, but k* was not.

Fig. A3.4. Scatter Diagram for the Logistic Model Approximation



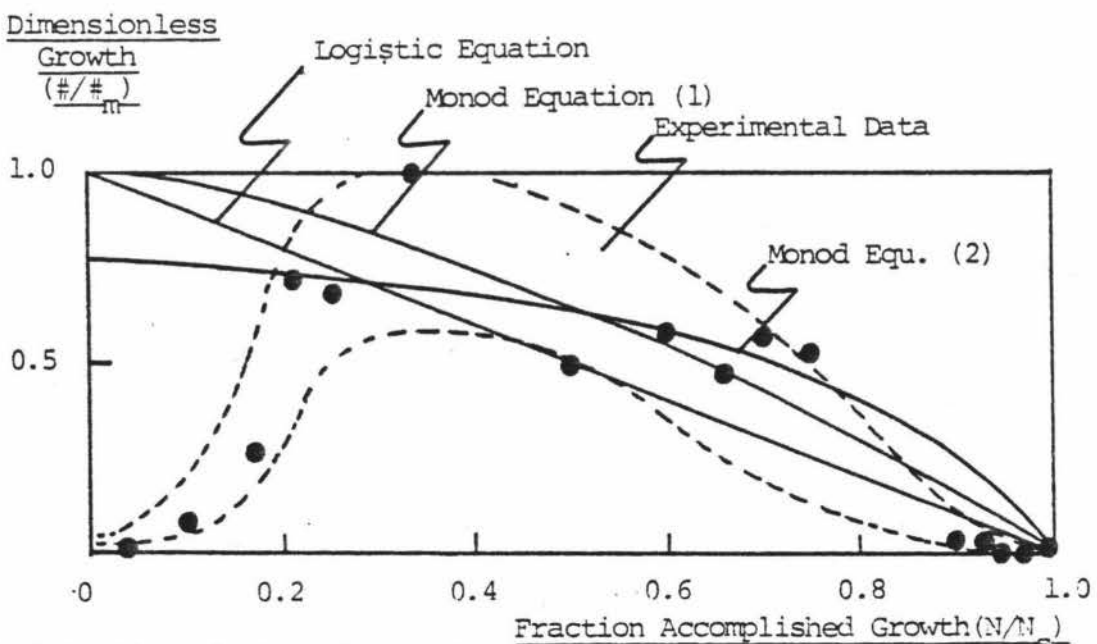


Fig. A3.5. Dimensionless Growth Curve

The cause of this effect is seen in Fig. A3.5. Fig. A3.5 is a graph of dimensionless growth rate (i.e. instantaneous growth divided by maximum observed growth rate) against the fraction of growth accomplished (i.e. the cell population divided by the maximum observed population, the stationary phase population). This particular graph will be useful in evaluating different models. The figure shows a basic inadequacy in matching experimental data of both the Logistic and Monod Equations (whether substrate limited, product inhibited, or both) and which is common to many population models. Experimental data from Chapter 3 showed a tendency to start at a low or zero growth rate then rise to a maximum growth rate at a population of about one third the stationary population. The growth rate then declined to about a half of the maximum growth rate at 60% of the stationary population. Thus the experimental data showed a single maxima and two points of inflection. In contrast, both the Logistic and Monod Equations show their highest predicted growth rates at the beginning of fermentation, and their rates monotonically decrease to zero without showing any stationary values or points of inflection.

The rate equation for the Logistic Model was given at the beginning of Section A3.2, and k is replaced by μ_m.

The equivalent form of the Monod Equation, assuming inhibition is the limiting factor (and assuming the power model for inhibition), can be rearranged in terms of the fractional accomplished growth as follows.

$$dN/dt = \mu_m N (1 - I/I^*)^n$$

:I = inhibitor
:I* = limiting inhibitor concentration

and assuming the inoculum and initial product concentrations are low, and that the Luder King-Piret Model holds

and may be integrated to give inhibitor concentrations (Section A3.1), then:

$$\mu / \mu_m = 1 - ((\alpha N + \beta \int N \, dt)/I^*)^n$$

and if n = 1 and ß = 0 as is assumed by the Logistic Model (i.e. the inhibitor is purely growth related) (Section A3.2), and I* is rewritten as N_s where N_s is the final inhibited cell population, then:

$$\mu / \mu_m = 1 - (N/N_s)$$

which is in fact the same as the rate equation used to derive the Logistic Equation (where $N_s = 1/(b\alpha)$ (Section A3.2, Case 2). Thus it can be seen that the Logistic Equation derives from the more general Monod Equation for product inhibition after suitable simplifying assumptions. In fact, when the integral term in the Monod Equation is dropped in order to avoid the added complication of time, the inhibitor must be assumed growth related and the Monod Equation differs from the Logistic Equation only by including the inhibition power factor n. If n is only a little greater than unity then the curve of μ / μ_m will be slightly bowed, as shown in Fig. A3.5 for n = 1.5 (Monod Equ. (1)). Inclusion of non growth related inhibition makes the picture very much more complex, but the resultant expression would still start at $\mu/\mu_m = 1$ at $N/N_s = 0$.

If the Monod Equation is used assuming substrate limited growth, then:

$$\mu/\mu_m = S/(S + K_m) \qquad : S_o - S = K'(N - N_o)$$

$$= \frac{(S_o - K'(N - N_o))}{(S_o - K'(N - N_o) + K_m)}$$

$$= \frac{K' (N_s - N)}{K'(N_s - N) + K_m}$$

$$= \frac{(1 - N/N_s)}{(1 - N/N_s) + K_m/(K'N_s)}$$

where S is the substrate concentration, K′ the Overall Yield Coefficient, K_m the Monod Constant, the residual concentration after fermentation is assumed to be very much smaller than S_o, and the subscript $_o$ indicates initial conditions. This equation has been drawn in Fig. A3.5 for $K_m/(K'N_s) = 1/3$ (Monod Equ. (2)). However, again the curve is monotonically decreasing, and can be shown to always be so as long as $K_m/(K'N_s)$ is positive i.e. if $f = N/N_s$, then:

$$\frac{d(\mu/\mu_m)}{df} = \frac{-(1 - f + K_m/(K'N_s)) + (1 - f)}{(1 - a + K_m/(K'N_s))^2}$$

$$= \frac{-K_m/(K'N_s)}{(1 - a + K_m/(K'N_s))^2}$$

and since the denominator will always be positive then the expression is shown to be monotonically decreasing. The equation has the advantage of starting at some growth rate lower than the maximum growth rate, however in most practical cases $K_m/(K'N_s)$ will be very much less than 1/3 and the curve will hug the upper limit of $\mu/\mu_m = 1$ until late in the fermentation before dropping to $\mu/\mu_m = 0$ at $N/N_s = 1$. Hence this model is not much better in practical cases.

A combined Monod Equation using both substrate limiting and product inhibiting kinetics would fare little better as it too would monotonically decrease.

The basic fault in the standard models is therefore the inability of any to show an increasing growth rate early in the fermentation. It might be argued that the lag phase exists for only a short period of time, but this is

not always the case.

In the present case, the abrupt change to the stationary mode and the ensuing scatter/fluctuations (refer Section 3.3.3.4) coupled with the above discussion show the Logistic Model to be as good as any other model. The model presented in Appendix 4 will model the population fluctuations, but is not intended to model the lag phase.

A3.5 Conclusions

Unfortunately, due to the nature of the experimental data, it is impossible to make definitive comparison of two methods of parameter evaluation presented in Section A3.1. For the same reason it is impossible to compare the more general Monod Equation with its specific case the Logistic Equation. However, the work is useful in pointing towards a possibly superior method of parameter evaluation, and also presents a basis for comparison of mathematical models.

APPENDIX 4: VIRIAL POPULATION KINETICS IN A BACTERIAL MONOCULTURE

The purpose of this Appendix is to show that a lysogenic bacteriophage could cause the population fluctuations seen in Chapters 3 and 5.

A4.1 Background

Many workers mention the extreme sensitivity of the fermentation to phage attack (Beesch, 1952; Compere and Griffith, 1979; Hastings, 1978; Ogata and Hongo, 1979; Ross, 1961; Spivey, 1978). Ogata and Hongo (1979) describe in some detail the history of the phage problem, the infection properties, and the characteristics of an infected fermentation, and also recommend procedures for protection.

It appears that both temperate as well as virulent phages are a problem. A virulent phage causes cell lysis soon after infection, typically after 0.5 - 1 hr. (Ogata and Hongo, 1979). The phages are strain specific (Ogata and Hongo, 1979), which suggests that a bank of strains with different immunities should be maintained in an industrial plant. Beesch (1952) makes the interesting comment that he had never observed a phage which would effect both starch and sugar fermenting organisms, implying a possible influence of substrate on culture response to phage attack. Beesch also states specifically that the mode of entry of the phage to the fermentation was "a matter of wide speculation". The phage appearance was probably a result of lysogenic phages (Ogata and Hongo, 1979). Ogata and Hongo present a good review of clostridial phages and the reader is referred to their paper as an excellent review for background knowledge as well as much specific detail and some practical suggestions for industrial fermentation processes. Some phage characteristics from the literature will now be discussed.

The characteristic of lysogeny would confer a vastly increased survival potential on the virus, especially if the lysogen form can sporulate and lie dormant in this highly resistant state until conditions are again favourable for host growth. In the case of vegetative cells, lysogeny also has its advantages. Early in a batch culture (which is probably more typical of natural situations in many cases such as a rotting animal carcass or a stagnant pool) the host population is low, so lysogeny would tend to be advantageous to the parasite as it allows its host to multiply. The result is that any newly formed parasitic entity has a greater chance of reinfection with the high host population. If the parasite also had a characteristically low probability of successful reinfection, then this sort of survival pressure would be even more brutally enforced (i.e. the virus population would drop to zero if the delay period was not suitably long).

Defective phages have also created some interest. Ogata and Hongo (1979) mention phage-like particles, some of which have been identified as defective phages. These particles "may be related to high molecular weight bacteriocin", and in at least one case the defective phage mimics the natural autolysin of the host but is not entirely under cell control. It appears in this case that lysis of mature infected cells occurs when cell wall synthesis is reduced due to the cell reaching maturity, but the lytic effect of the bacteriocin continues unchecked. Consequently the cell lyses. Premature lysis of these cells can be induced by antibiotic inhibition of cell wall production mimicking maturation (Ogata and Hongo, 1979). Barber et al. (1979) and Webster et al. (1981) have isolated and characterised a bacteriocin and an autolysin respectively from cultures of Cl. acetobutylicum. Both these lytic agents were also species specific, limiting their lytic attack to just a few species each. It might be that phage inactivation results

in the loss of the phage's reproductive abilities but initially without loss of its invasion and lytic abilities, causing the phage particle to mimic bacteriolysin and autolysins. It would appear that the two phenomena may be linked, although no proof is available. It is distinctly noticeable that the model presented in this Appendix can give cell growth characteristics and bacteriocin production characteristics very similar to those shown by Barber et al. (1979), showing a peak in cell concentration followed by a decline in cell population with simultaneous rapid increase in bacteriocin production.

Also of interest in the papers of Barber et al. (1979) and Webster et al. (1981) is that both groups of workers looked for plasmid DNA in their strains but found none. The importance of the lack of plasmid DNA is that, in order for the rapid deterioration seen by Kutzenok and Ascher (1952) to be due to genetic deterioration, the altering characteristic would almost certainly have to be plasmid coded on a plasmid with a high rate of defective transference. If however there were no plasmids, or the genes supposedly mutating were chromosomally coded rather than plasmid coded (as they must have been in the case of Barber et al. (1979) and Webster et al. (1981)), it is highly unlikely that the degenerative process was purely genetic in nature. It is possible that the plasmid differentiates for a crucial part of the cell's life, but this was thought unlikely. Another point which could suggest involvement by phage or similar agent is the comment by Kutzenok and Aschner (1952) that individual cells are either healthy or degenerate, and the overall culture characteristics depend on the relative proportions. This would certainly be so in the case of phage infection.

This then raises the interesting point that Kutzenok and Ascher (1952) identified a seriological difference in the

normally present in the organism coding for an autolytic agent, and which evolved from, or into, a phage (Stent and Callendar, 1978). This might explain the omnipresence the lysogenic clostridial phages seem to possess.

Whatever the case, due to the capacity of phages to be site specific on the chromosome (as in the lambda coliphage), to be capable of moving between both chromosomal and plasmid DNA types (as in the lambda coliphage), to be capable of lysogeny (Ogata and Hongo, 1979), host-modification (as in Corynebacterium diphtheriae), and removal of bacterial genetic information (as when phages act as vectors for transduction), then it is quite possible that the culture deterioration seen by Kutzenok and Aschner (1952) and in Chapter 5 is indeed phage mediated and the symptoms caused by some combination of phage characteristics.

Serial transfer of a phage infected culture (lysogenic or otherwise) could give results as seen by Kutzenok and Aschner (1952) if the proportion of infected cells in each successive inoculum were increasing. Heatshocking would cause lysogenic bacteria to express themselves. Thus if the model is correct, heatshocking would result in a healthier fermentation by causing expression of lysogenic phages early in the fermentation when the population of prey cells is low and conditions predominantly unfavourable for phage multiplication. This obviously provides a possible mechanism for culture purification by serial transfers of cultures after heatshocking as suggested by Hastings (1978). Transfer is of course best made when the viral population is low.

The experiments of Chapter 5 showed initial improvement due perhaps to progressive culture purification. The cause of the subsequent loss of characteristics and viability might be best explained by phage mutation to a mutant better adapted to withstand heatshocking with low

prey levels. This mutation could be for a lower rate of expression with heatshocking, or a higher rate of successful attack (for any reason). It is possible that the culture is mutating, but unlikely that the mutant aids phage multiplication.

The rise in cell population after 150 hr. (Table 5.2) could be explained by increased culture resistance to the phage by mutation as suggested by Hastings (1978), and the eventual loss of culture activity due to substrate exhaustion.

The above discussion suggests that the symptoms of degeneration seen by Kutzenok and Aschner (1952) could be explained in terms of phage attack rather than genetic mutation. Equally perhaps autolysin/bacteriocin involvement is possible, but this could also be related to phage attack. Unfortunately no direct evidence is available, only indirect evidence in progressive culture failure and large cell population fluctuations. A model will now be outlined which reproduces some of the essential characteristics of these population fluctuations. While further refinement of the model is possible, the essential features have been established in its present form.

The concepts behind the model may be described as follows. If the virus particle has a high probability of success of reinfection once liberated, then a high titre of virus could effectively kill the entire prey population. If, however, the probability of successful reinfection is much lower, then a high titre of virus will merely reduce the prey population to a level where the rate of infection and viral reproduction is <u>less</u> than the rate of bursting and viral inactivation before infection of a new cell - hence the viral population will fall. Once the virus population is again low, the host (prey) population will again increase and the cycle will repeat itself. Theoretically this cycle could go on indefinitely in chemostat. (In

practice viral and/or cell mutation would alter the pattern even if all other conditions were held constant.) In batch culture the cycle would alter due to inhibition of some sort and/or substrate limiting the growth of host cells.

A4.2 The Model

If it is assumed that the virally infected cells act as a predator species (YY) and the healthy bacteria a prey species (Y), then the culture might be expected to behave similarly to the predator/prey model of Lotka and Voltera (Bailey and Ollis, 1977). This forms the basis of the model. A third population (YL) is added to simulate the lysogen concentration.

The equations are:

$$dY/dt = MU \times Y - YYV \times NV \times PS \times Y$$
$$dYY/dt = YYV \times NV \times PS \times Y - YYV + YL \times PLB - YY\ (PBL + DR)$$
$$dYL/dt = YYV \times PBL - YL \times PLB$$
$$YALL = Y + YY + YL$$

where YYV is the population concentration of YY a fixed time (DE) before the moment of interest. This delay factor (DE) is the time taken from infection to cell burst. During a time period from inoculation equal to this delay time (i.e. t = 0 to t = DE), YYV is defined as zero. In order that YY appears as a continuous curve rather than spikes at regular intervals, YYV has arbitrarily been evaluated over two successive time intervals with a weighting of 2:1 for the earlier:later value. The variables appearing in the equations are:

Y = concentration if uninfected cells.
YY = concentration of infected cells undergoing active phage replication.
YL = concentration of lysogenic cells.

YYV = concentration of infected cells (YY) of age DE.
DE = time taken from infection to expression for a non-lysogenically infected cell.
MU = growth rate of non-infected cells.
NV = average burst size of cell.
PS = probability of success of infection of an uninfected cell for a single phage particle.
PLB = probability of conversion from lysogenic to active state of infection.
PBL = probability of conversion from active to lysogenic state of infection.
DR = death rate of active infected cells.
YALL = total number of cells available for counting in a haemocytometer.
t = time.

These equations have made two important basic assumptions. First, the effective growth rates of the different populations are significantly different. The non-infected cells were assumed to grow at rate MU which is approximately the growth rate observed during exponential growth of the organism in monoculture under otherwise identical conditions. The active infected cells are assumed not to grow, but to die at a rate DR which may be significantly greater in magnitude than MU. This death rate must be included in order that YL does not grow too rapidly and to allow YY to drop fast enough to prevent Y from dropping too low. The lysogenic population has been assumed to have a negligible growth rate (or death rate). These assumptions could possibly be mirrored in practice if infection prevented the cell from metabolising at a high enough rate for growth or repair, and phage replication put a maximal strain on the cell which often resulted in cell death. The second assumption made was that infection of a cell prevented reinfection (whether the cell was lysogenic or active in phage replication). This immunity to superinfection is well known (Stent and Calendar, 1978).

Other than these two assumptions, the equations are quite straight forward. The product NV x PS may be thought of as the average number of cells reinfected per cell burst, and neither NV nor PS appear on their own. Ogata and Hongo (1979) give some typical values for NV, 20 was chosen arbitrarily. All other values were chosen arbitrarily in order that a graph of YALL against time showed characteristics similar to those seen in Chapters 3 and 5. Solving the above three simultaneous differential equations for different values of the constants proved difficult and is outlined in the following section.

A4.3 Solution of the Model and Discussion.

The three differential equations are difficult to solve either analytically or by numerical methods. No solution was found analytically for the different population parameters against time alone. Bailey and Ollis (1977) present a transcendental solution for the Lotka-Volterra two equation model, but this solution is not easy to use for the purposes of this Appendix. Initially a 4th Order Runga-Khutta routine was used, however, because of the very high gradients and rates of change of gradients at different points in the different populations, the routine was unsuitable. The problem lay in the use of a fixed step length. If a small enough step length were used the program would have long execution time and create unacceptable roundoff errors. The program was seen to produce oscillations, but these were due to numerical instability. In order to obtain an acceptable solution, a 4th Order Runga-Khutta routine incorporated in a special differential equation solving software system was used. This system is called ISIS (Hay, 1977). The program used is given in Table A4. The following values gave Fig. A4:

inoculum YZ = 6
initial Y = 5.7 (i.e. 95% of inoculum)
initial YY = 0.3 (i.e. 5% of inoculum)

initial YL = 0
initial YYV = 0
DE = 0
MU = 0.4
NV = 20
PS = 0.001
PLB = 1×10^{-6}
PBL = 0.5
DR = 15 (i.e. almost 40 times MU)

where one "cell" in the model represents 10^6 bacteria.

While some attempt was made to make these values typical, the emphasis was on demonstrating that the model could in fact fluctuate in a manner similar to the cell populations seen in Chapter 3. The significant points reproduced are the time of the first peak (ca. 15 hrs), the height of the peak (several thousands), the height of the troughs (about 500), and the period of oscillation (about 7 hours). While these are only similar to those seen experimentally (Fig. A3.2), they are sufficiently close to demonstrate the model. Further effort to perform a multiple variable optimisation to get a closer approximation to the experimental data was considered unwarranted as the original objective was fulfilled, and the data insufficient to give reliable results.

Perhaps the variable most noticeably in error was the assignment of DE = 0. In fact DE is more typically about 0.2 to 1.0 hr. (Ogata and Hongo, 1979). Increasing DE from zero to these more typical values results in very much lower troughs. This is thought to be overcome by better estimates of other variables.

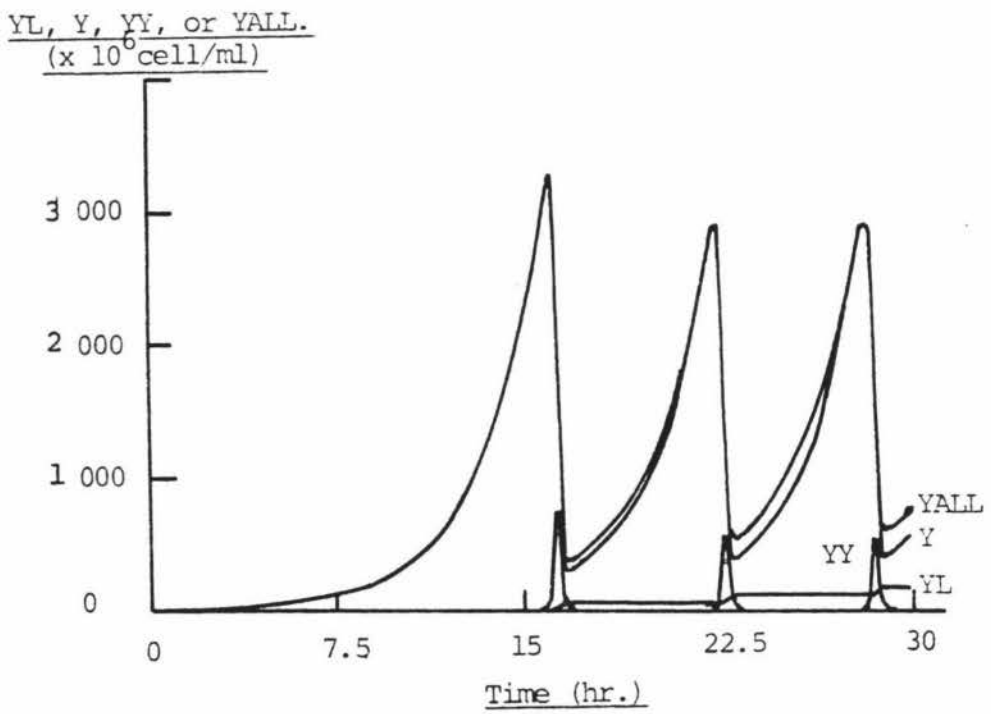
Several modifications of the model have been proposed, but none fit the data significantly better, and better knowledge than was readily available would be required to confirm which model was more fundamentally sound. Some

Table A4 PROGRAM FOR ISIS SOLUTION OF VIRAL POPULATION MODEL

```
    DIMENSION YDEL(100)
        CONSTANT DR=.5
   CONSTANT CINT=0.035,YZ=6,FL=0.05,PBL=0.5,PLB=1E-6
   CONSTANT NV=20
    MU=M
10  YL=FL*YZ
    Y=YZ-YL
    NB=1   ;  NT=1
    N=IVIX(DE/CINT+0.5)+1
    YYV=0
    YY=PLB*YL
    SIM
    STOP


   DYNAMIC
    IF(LCOM)GOTO 20
    GOTO 30
20  YDEL(NT)=YY
    NT=NT+1
    IF(NT.GT.N)NT=NT-N
    YYV=0
    IF(NT.NE.NB) GOTO 30
    YYV=(YDEL(NB)*2+YDEL(NB+1))*0.33333
    NB=NB+1
    IF(NB.GT.N)NB=NB-N
30  CONTINUE
    Z=YYV*NV*PS*Y
     YY'=Z-YYV+YL*PLB-YY*(PBL+DR)
    Y'=Y*MU-Z
       YL'=YYV*PBL-YL*PLB
    YALL=YL+YY+Y
    PREPARE YL,Y,YY,YALL,T
 PLOT T,Y,0,40,0,4000
TFIN     =    30.000
AERR     =    10.000
DE       =  0.00000
RERR     =     5.0000
M        =  0.40000
PS       =  0.10000E-02
DR       =    15.000
```

Fig. A4. Simulation of Population Fluctuations.



distinction might be made on the basis of the probability of success (PS), which is very low, or DE, but it was decided further work was unwarranted.

A4.4 Conclusions

On the basis of the proposed model, it would appear that the cell population fluctuations observed in Chapter 3 could be caused by a lysogenic phage infection.

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