ETHANOL PRODUCTION BY

Bacillus stearothermophilus

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by

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ABSTRACT

Bacillus stearothermophilus strain *lld*-15, a lactate dehydrogenase mutant, was studied with reference to its potential for producing ethanol from renewable resources. Batch fermentation experiments were conducted in order to identify the products of sucrose fermentation and to determine cultural conditions favouring high ethanol yields. Product identification was facilitated by the development of a gas chromatographic technique for the rapid resolution of acidic fermentation products. Acid conditions led to increased ethanol production and it was found that the increased ethanol yields must have been achieved by the operation of one or more of three enzymes, formate dehydrogenase, pyruvate decarboxylase and pyruvate dehydrogenase, although none of these enzymes had previously been found in anaerobically grown *B. stearothermophilus*. Enzyme assays on crude cell extracts of wild-type and *lld*-15 strains of *B. stearothermophilus* showed greater levels of pyruvate dehydrogenase in anaerobically grown *lld*-15 as compared with the wild-type and it is suggested that this led to the increased ethanol production in *lld*-15. Acetate and formate, both anaerobic fermentation products of *lld*-15, were implicated in terminating growth of the strain. Under some conditions *lld*-15 tended to be replaced in continuous culture by mutants having some of the characteristics of the wild-type including low ethanol yields. Conditions under which strain *lld*-15 could be maintained indefinitely in continuous culture were however found. The possibility of using *B. stearothermophilus* for the production of ethanol from cheap raw materials was investigated with cultures of strain *lld*-15 and organisms able to attack the wood components D-xylose and cellulose. The potential of *B. stearothermophilus* as an ethanol producer is compared with both traditional ethanologens and the recently discovered ethanol-producing thermophiles.

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CHAPTER 1

INTRODUCTION

1.1 Ethanol Production by Fermentation

One effect of the 'energy crisis' of the early 1970's was the realization by the most heavily industrialized nations of the world of their reliance on imported petroleum as the primary source of liquid fuels, and that political implications aside, world petroleum reserves would one day become depleted. This period effectively marks the start of the current interest in the production of alternative liquid fuels. One area which has attracted much interest (Bu'Lock, 1979) is the fermentative production of ethanol from renewable resources (biomass).

Then, as now, industrial ethanol was derived primarily from ethylene, a petroleum product, with the fermentation route accounting for only a small part of the total world production. Fermentation ethanol production is however of fundamental importance to the alcoholic beverages industries. The process, whether for industrial ethanol or beverage production, comprises fermentation, usually by yeast of the genus Saccharomyces, of a strictly limited range of mono- and di-saccharides and hydrolyzed starches in a batchwise operated vessel. In the case of industrial ethanol production the ethanol has to be separated from the aqueous fermentation broth; this is conventially achieved by distillation. Whilst considerable advances have been made in the control and operation of the process, in the design and construction of capital equipment and in the selection and maintenance of improved yeast strains, the basic process steps have remained unchanged for centuries.

When this process is evaluated from the standpoint of fuel rather than beverage production, different criteria have to be applied, and as a consequence, limitations in the traditional fermentation process become apparent.

Following a brief consideration of the energetics of ethanol production, the remainder of this section is devoted to a short review of some of the proposals made to overcome these limitations through the use of alternative ethanol-water separation technologies, new fermenter configurations and finally to strategies permitting more efficient exploitation of biomass.

Where the intended use of ethanol is as a fuel, it is of paramount importance to ensure that its manufacture will result in overall net energy production. Whilst certain strategies in areas where environmental conditions are favourable and land is plentiful, such as Brazil, clearly do lead to net energy production (Goldemberg, 1978) it is true to say that certain 'fuels from biomass' programmes would seem to offer at best only marginal benefits. Confusion even exists as to how energy inputs should be accounted for: two studies, Chambers et al. (1979) and Weisz and Marshall (1979), investigating ethanol production from grain in the United States reach different conclusions about the benefits.

It is however generally agreed that the process of separating ethanol from aqueous fermentation broths is an energy intensive one (Scheller, 1978) and much effort has been directed towards reducing this energy input. Proposals aim at improving the efficiency of the distillation process (Black, 1980), using membranes Gregor (1979), dehydrating the ethanol with polymers (Pitt et al., 1980) solvent extraction (Finn and Feldman, 1978) and the use of dry plant material as dessicant (Ladisch and Dyck, 1979). Whilst some of these processes could immediately be applied in industry others remain costly and require fundamental investigation.

Batch operated plants suffer from low ethanol productivities and an obvious way to increase productivity is to employ continuous culture. Certain process refinements such as the recycling of cells back into the fermenter have also been proposed. Fermenter configurations capable of high throughputs can be achieved by immobilizing microbial cells onto inert carrier materials. Of particular interest in the context of thermophiles is 'vacuum fermentation'. The principle here, first proposed by Boeckler (1948) but recently applied by Cysewski and Wilke (1977) and Ramalingham and Finn (1977), is to conduct the fermentation under sufficient vacuum to cause boiling at the fermentation temperature. This results in relatively low liquid phase concentrations of ethanol - and hence reduced ethanol toxicity for the producing micro-organism - and high vapour phase ethanol concentrations. The effect of this is to achieve separation of the ethanol product simultaneously with its manufacture.

Table 1.1 compares the ethanol productivites of the types of fermenter mentioned above: the advantages of changing from batch to continuous operation are clear enough, with the more complex

System	Feed Sugar Conc. (g/l)	Ethanol Conc. (g/l)	Ethanol Productivity (g/l-hr)	Reference
Batch	250	109	2.7	DelRosario et al. (1979)
Continuous	100	41.0	7.0	Cysewski and Wilke (1977)
Continuous with Cell Recycle	150	60.5	32.0	Ghose and Tyagi (1979)
Immobilized Cell	200	100	40.0	Chibata and Tosa (1980)
Vacuum with Cell Recycle	334	160	82.0	Cysewski and Wilke (1977)

Table 1.1 Fermenter Ethanol Productivities of Various Cultures Systems for Yeast

process refinements contributing further to increased productivity. It should also be noted that the removal of ethanol from the fermenter permits concentrated feeds to be processed. The continuous cultivation of micro-organisms raises problems such as the maintenance of sterility over long periods and the possbility of strain degeneration. Notwithstanding, Panuschka (1983) claimed that there were already 5 plants in the world continuously producing ethanol from sugars, and it is certain that this type of operation will see increasing application in industry.

The traditional raw materials for ethanol production are sugarcane juice, molasses and hydrolyzed starches (e.g. from maize or sorghum). These represent a significant fraction of the cost of the end product, ethanol, as Table 1.2 shows, and therefore the application of more efficient process technology would have only a limited effect in reducing the cost of ethanol. This is not so for cheaper and more readily available raw materials such as wood and straw. The latter are composed primarily of hemicelluloses, cellulose and lignin. The hemicelluloses are linear and branched heteropolymers of D-xylose, L-arabinose, D-mannose, D-glucose and D-glucuronic acid. Cellulose is a high molecular weight linear $\beta(1\rightarrow4)$ linked polymer of D-glucose. Whilst lignin, a material highly resistant to chemical and microbiological attack is a complex three-dimensional polymer of sinapyl, coniferyl and p-coumaryl alcohols.

Lignin occurs in close association with cellulose and the tendency in the past was to regard lignin as a nuisance as it prevented access to the less refractive cellulose. This view has changed and lignin is now regarded as having considerable industrial potential (Crowder and Eudy, 1978). In terms of liquid fuel production however, lignin is seen primarily as a source of methanol rather than ethanol and will not be considered further here.

Saccharomyces spp. are unable to ferment either hemicelluloses or cellulose. Proposals to exploit these abundant resources have brought about a departure from the traditional view of fermentative ethanol production described above. Although cellulose may be hydrolyzed chemically to yield glucose which can be fermented by traditional methods, much interest has recently focussed on the biological hydrolysis of cellulose and a number of promising approaches have been identified and are considered further in Chapter 8.

Raw Material	Raw Material Costs (%)	Capital Costs (%)	Operating Costs (%)
Sugarcane	62-78	10-21	11-26
Molasses	50-84	4-22	10-41
Maize	53-69	7-20	23-30
Sorghum	55	12	33
Wood	10-23	27-31	51-59
Wheat Straw	9-23	12-27	51-57

Table 1.2 Distribution of Ethanol Production Costs (Paul, 1979)

The hydrolysis of hemicelluloses yields C5 sugars such as L-arabinose and D-xylose which Saccharomyces is unable to ferment. These C5 sugars constitute a significant fraction of plant biomass as Table 1.3 shows so that the conversion of hemicellulose hydrolyzates to ethanol would be wasteful of resources. However, micro-organisms which possess the ability to ferment C5 sugars are known and the characteristics of some of the more promising ones are described in Chapter 8.

An alternative to the use of micro-organisms possessing the ability to ferment a wide range of substrates but which may be inefficient ethanol producers is to genetically modify yeast to allow them to ferment non-traditional biomass resources. In essence this entails cloning genes into the species of interest from other organisms. For example, xylose isomerase catalyzes the conversion of D-xylose, which Schizosaccharomyces pombe is unable to ferment, to D-xylulose which is fermented. Ueng et al. (1985) succeeded in transferring the gene coding for xylose isomerase from E. coli to S. pombe and in achieving its expression in the latter. Cellulose utilization by Saccharomyces would require the inter generic transfer of a number of genes (Sacco et al., 1984).

1.2 Metabolic Pathways Leading to Ethanol

The ability to produce ethanol from glucose is widely distributed amongst micro-organisms. The yields vary considerably, from almost the 2 moles of ethanol per mole of glucose fermented characteristic of yeast, to the very much smaller amounts formed by many bacteria. These large variations are attributable to the operation of different metabolic pathways. Dawes (1963) identified 4 different routes leading to ethanol and classified each into types: Type 1 is a combination of glycolysis and pyruvate decarboxylase. This pathway is widespread in fungi including yeasts, but a few bacteria also possess this route. Two moles of ethanol can be formed per mole of glucose (see Figure 1.1).

Type 2 is a combination of glycolysis and pyruvate formate lyase and is typical of the enterobacteria and some clostridia. In some cases the formate is broken down into hydrogen and carbon dioxide. The maximum possible ethanol yield is 1 mole per mole of glucose (Fig. 1.2).

Type 3. Zymomonas mobilis gives a similar fermentation balance to

Material	Pentosans %	Hexosans %	Lignin %
Corn stover	15.0	35.0	15.0
Corn cobs	28.1	36.5	10.4
Wheat straw	19.0	39.0	14.0
Rice straw	17.0	39.0	10.0
Oat husks	29.5	33.7	13.5
Bagasse	20.4	41.3	19.9
Pine Wood	7.4	52.3	26.6
Oak Wood	19.6	44.8	24.8

Table 1.3 Chemical Analyses of Agricultural Residues and Wood (% Dry Weight) (Sitton et al., 1979).

Glossary: Pentosans = D-xylose + L-arabinose; Hexosans = D-glucose + D-mannose + D-galactose.

Corn stover - The stalks and leaves of corn after the ears are harvested.

Bagasse - The crushed juiceless remains of sugarcane.

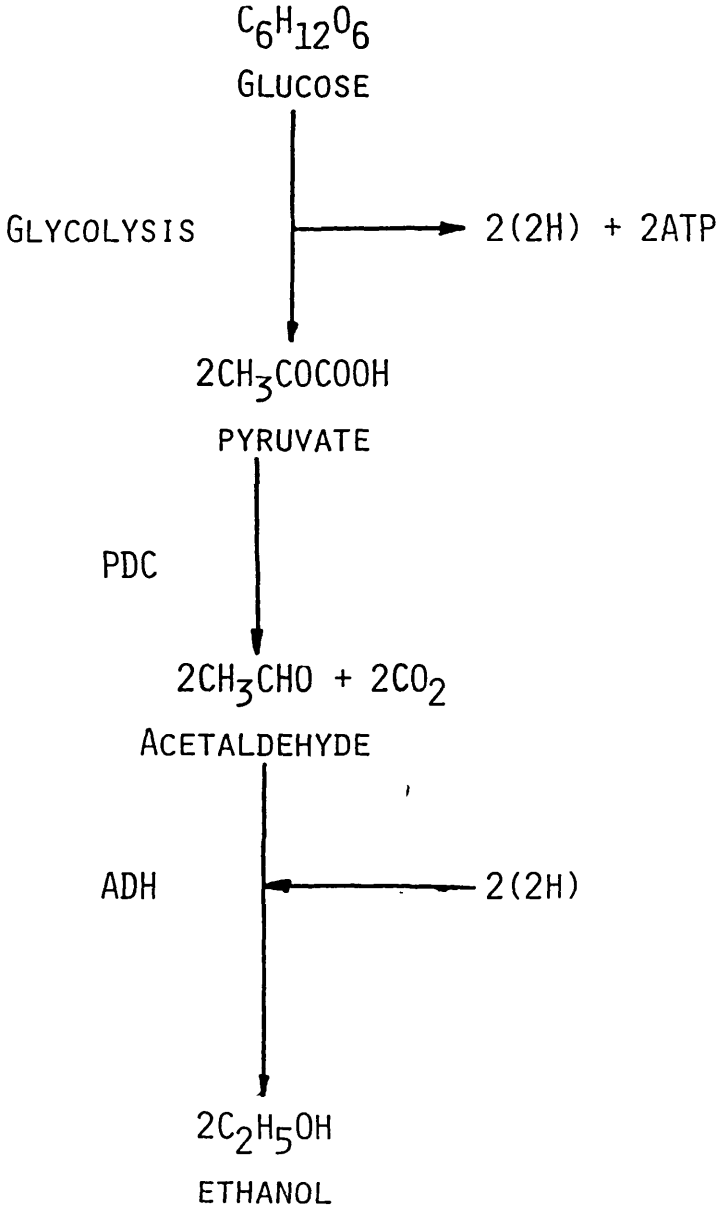


Figure 1.1 Metabolic Pathways Leading to Ethanol - Type 1 Glycolysis and Pyruvate Decarboxylase

PDC: Pyruvate Dehydrogenase, ADH: Alcohol Dehydrogenase.

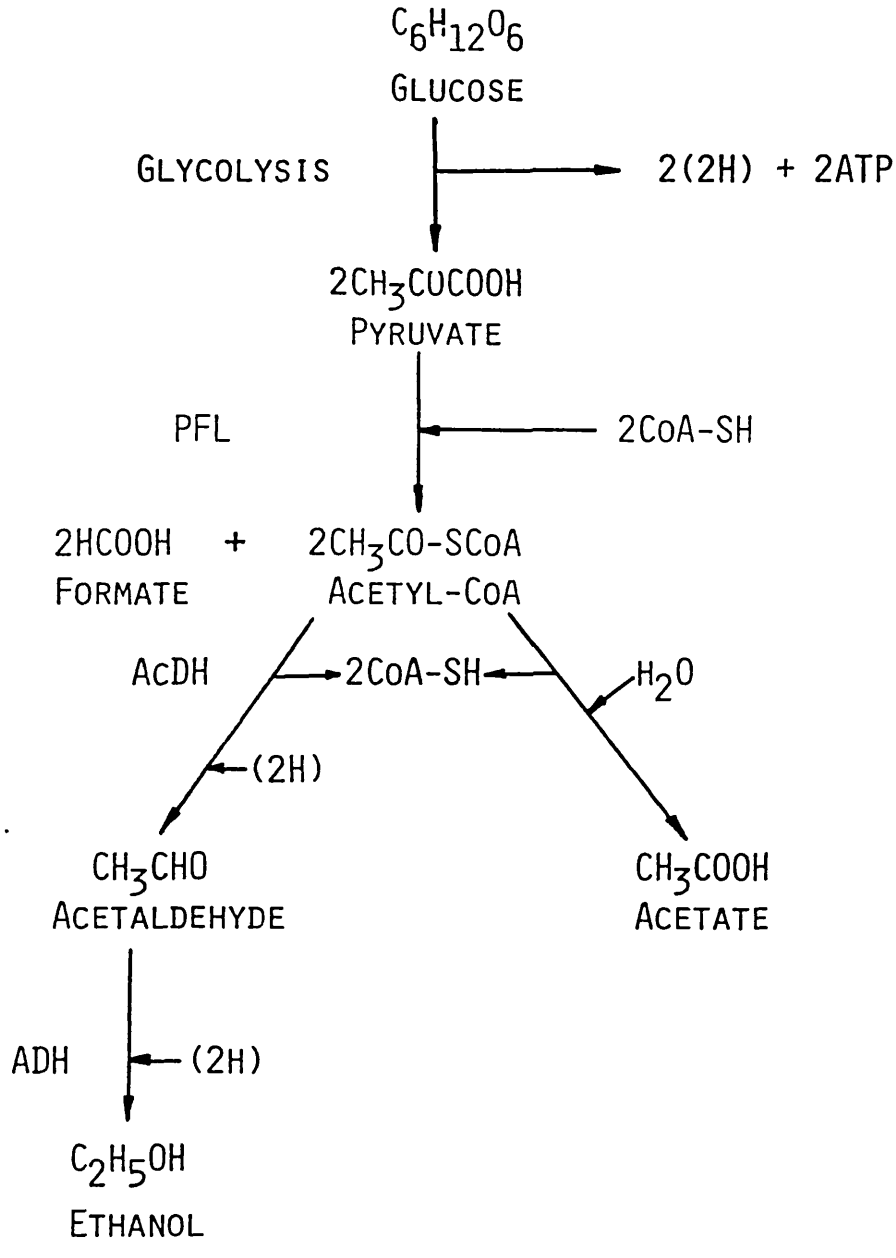


Figure 1.2 Metabolic Pathways Leading to Ethanol - Type 2 Glycolysis and Pyruvate Formate Lyase

PFL: Pyruvate Formate Lyase, AcDH: Acetaldehyde Dehydrogenase

ADH: Alcohol Dehydrogenase

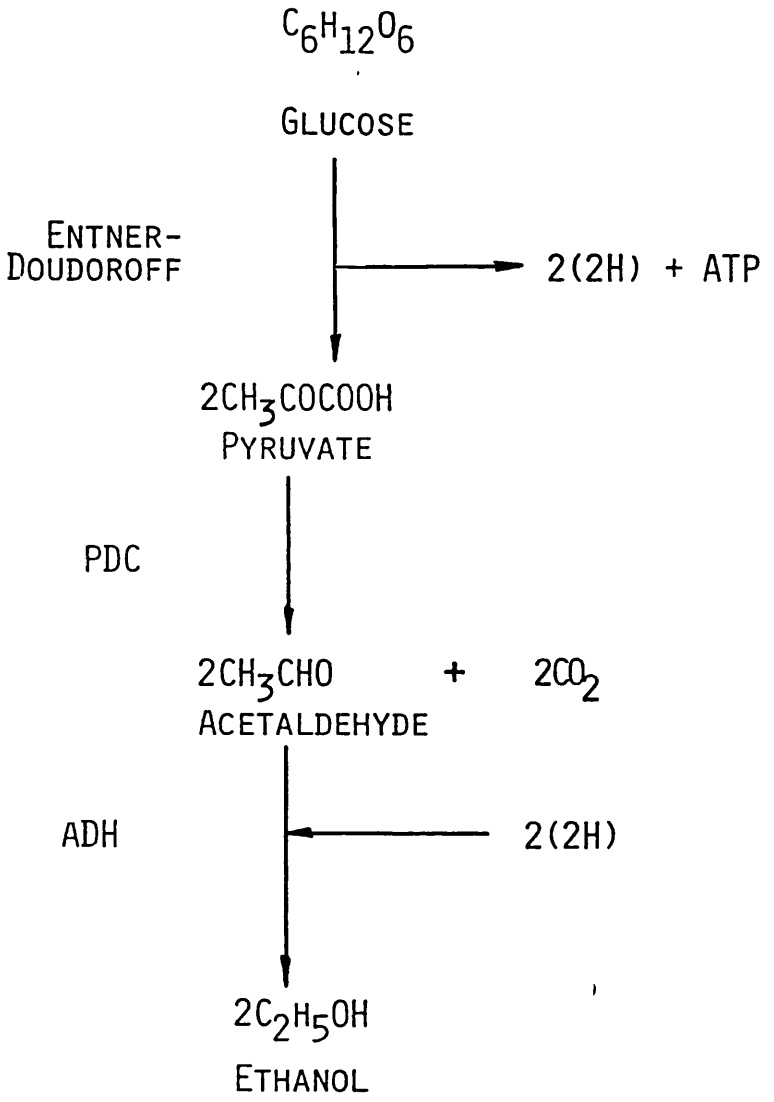


Figure 1.3 Metabolic Pathways Leading to Ethanol - Type 3 Entner-Doudoroff and Pyruvate Decarboxylase

PDC: Pyruvate Decarboxylase, ADH: Alcohol Dehydrogenase

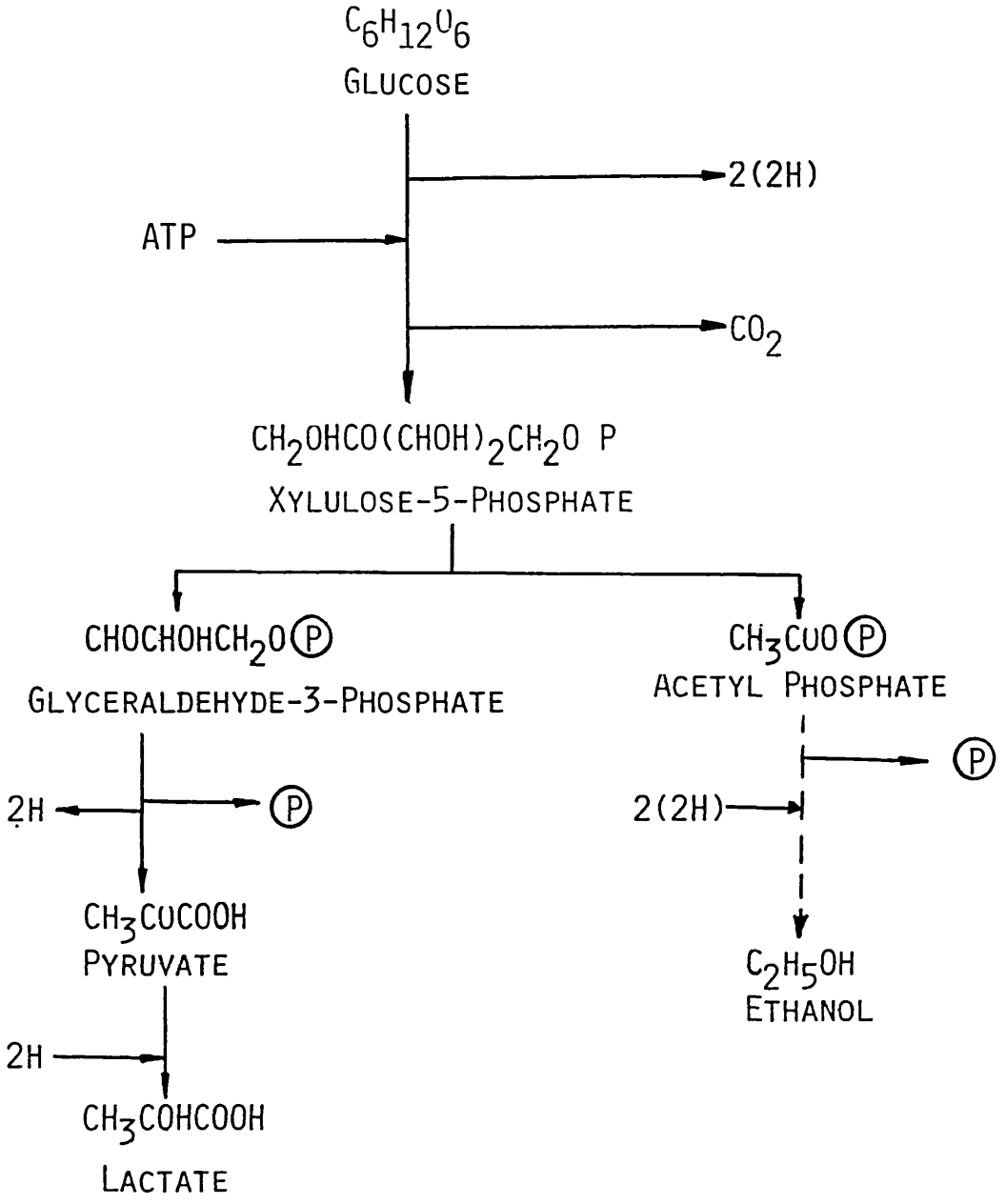


Figure 1.4 Metabolic Pathway Leading to Ethanol - Type 4 Phosphoketolase

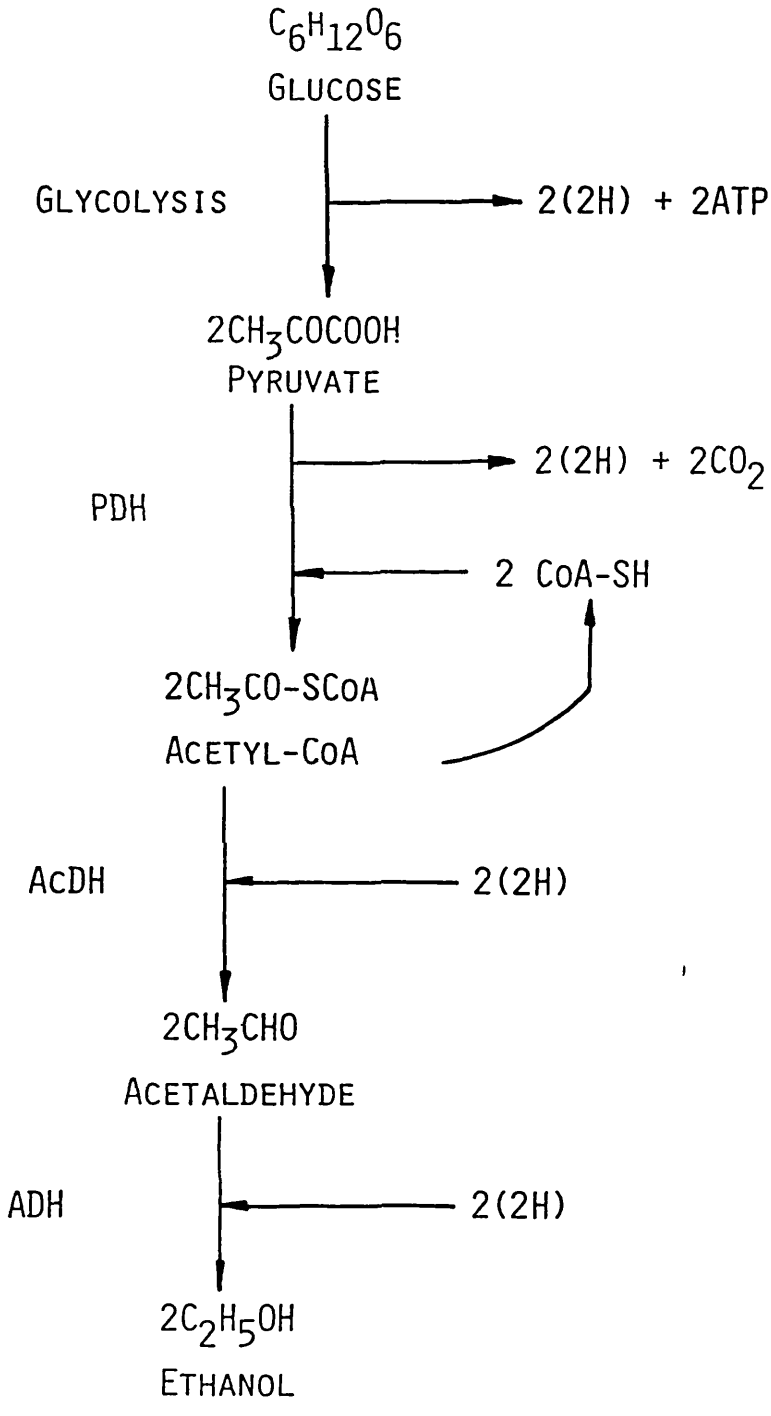


Figure 1.5 A Hypothetical Pyruvate Dehydrogenase Pathway to Ethanol - Type 5

PDH: Pyruvate Dehydrogenase, AcDH: Acetaldehyde Dehydrogenase
ADH: Alcohol Dehydrogenase

yeast except that pyruvate is formed from glucose via the Entner-Doudoroff pathway and only 1 mole of ATP is formed (Figure 1.3).
Type 4. This pathway is characteristic of heterolactic organisms: glucose is converted to xylulose-5-phosphate which is split by the enzyme phosphoketolase to acetyl phosphate and glyceraldehyde-3-phosphate, the former undergoing reduction to ethanol (Figure 1.4).

One further route to ethanol can be envisaged and consists of a combination of glycolysis and pyruvate dehydrogenase. Pyruvate formed by glycolysis is converted to acetyl-CoA and carbon dioxide through the action of pyruvate dehydrogenase, the former is then reduced to ethanol: two moles of ethanol are thus produced per mole of glucose. This pathway will be designated 'Type 5' and is shown in Figure 1.5.

1.3 Thermophiles and Ethanol Production

The concept of vacuum fermentation was described above as being particularly attractive in the context of thermophilic ethanologens. This was appreciated by Ramalingham and Finn (1975) who writing of potential industrial applications, claimed that operation at higher temperatures would result in more favourable process economics through a reduction in energy costs. In fact, any separation process which relies on the high volatility of ethanol relative to water would, in theory at least, benefit from operation at 'thermophilic' temperatures.

Atkinson *et al.* (1975a) used *B. stearothermophilus* to ferment sucrose and achieved separation of ethanol, one of the minor fermentation products, by sparging the fermenter with an inert gas. Admitting that an industrial process based on their strain of *B. stearothermophilus* would not be economically viable, they proposed that a search be made for thermophilic organisms capable of converting sucrose to ethanol in high yields. Ramalingham and Finn wrote similarly of 'thermotolerant yeasts' and though such organisms have since been isolated (Jeffries, 1982), the use of yeast has one disadvantage; yeast are eukaryotic organisms and as such are less well able to tolerate high temperatures than the structurally simpler prokaryotes which comprise all bacteria. Indeed, it appears that for the latter, existence is only limited by a requirement for water in the liquid state. Baross and Deming (1983) claimed to have isolated bacteria capable of growth at 250°C from the surface of the sea bed some 2500m below sea level;

their conclusions have however been questioned (Trent, Chastain and Yayanos, 1984). Bacteria living at temperatures slightly in excess of 100°C are however now well authenticated (Stetter, 1982).

Though interest in thermophiles centred initially on their use in vacuum fermentation processes, many other advantages have been claimed for them (Wiegel, 1980; Sonnleitner and Fiechter, 1983; Payton, 1984) and some of these claims will be considered further in Chapter 10.

Since about 1975, two strategies have emerged for obtaining high ethanol-producing thermophiles: screening for novel thermophilic species and genetic modification of known thermophiles. Screening for new species has been vigorously pursued and a number of interesting thermophiles have been isolated as Table 1.4 shows; some of the properties of these organisms will be considered further in Chapter 10.

The alternative strategy of selecting improved strains of B. stearothermophilus was pursued at Imperial College and has proved to yield promising results.

1.4 Bacillus stearothermophilus

The species named 'stearothermophilus' was first proposed by Donk (1920) for a thermophilic bacillus isolated from a tin of spoiled sweet corn. The strain was further described as having a maximum growth temperature of 76°C and as being facultatively anaerobic. Donk's work was carried out at the National Canners' Association (NCA) in Washington, D.C. and his original strain now bears the classification NCA 26.

In a systematic study of bacilli implicated in the spoilage of tinned foods, Cameron and Esty (1926) examined over 200 cultures. Some 60 proved to be mesophiles (growth at 37°C, no growth at 55°C); almost 100 were facultative thermophiles (growth at 37°C and 55°C); and 55 qualified as obligate thermophiles (no growth at 37°C, growth at 55°C). These latter were further subdivided into 4 groups one of which ('Group 100') consisted of cultures which actively hydrolyzed starch.

The period following these early publiations saw the species name B. stearothermophilus being alternately restricted and extended in its application; the 6th edition of Bergey (Breed, Murray and Hitchens, 1948) recognizing 20 thermophilic species of Bacillus,

Organism	Cardinal Temperatures °C Min.	Opt.	Max.	Specific Growth Rate on Glucose (hr^{-1})	Ethanol Yield (M/M Glucose)	References
Clostridium thermohydrosulfuricum 39E	40	65	75	0.55	1.98	Zeikus *et al.* (1981) Ng *et al.* (1981)
Clostridium thermohydrosulfuricum JW102	42	68	77	0.87	1.39	Wiegel *et al.* (1979)
Clostridium thermocellum LQR1	40	62	70	0.44	0.73	Zeikus *et al.* (1981) Ng *et al.* (1981)
Clostridium thermosaccharolyticum	-	-	-	-	1.09	Lee and Ordal (1967)
Thermoanaerobacter ethanolicus JW200	37	69	78	0.44	1.78	Weigel and Ljungdahl (1981)
Thermoanaerobium brockii HTD4	40	70	80	0.69	0.94	Zeikus *et al.* (1981) Lamed and Zeikus (1980)
Thermobacteroides acetoethylicus HTB2	40	65	80	1.38	1.09	Zeikus *et al.* (1981) Ben-Bassat and Zeikus (1981)

Table 1.4 Characteristics of Various Thermophiles

whilst the 7th edition (Breed, Murray and Smith 1957) listed B. stearothermophilus as the sole thermophilic representative of the genus. By contrast, Gordon and Smith (1949) classified 230 thermophilic bacilli into two species, B. stearothermophilus and B. coagulans, and . resolved a previous source of confusion by noting that certain mesophilic bacilli, B. subtilis, B. brevis and B. circulans occasionally gave rise to thermophilic variants.

Walker and Wolf (1971) performed an extensive investigation of over 200 Bacillus thermophiles and showed that the strains could be divided into 3 distinct major groups: Group 1 strains were characterized by the ability to form gas from nitrate under anaerobic conditions and to weakly hydrolyze starches. These strains did not grow or grew poorly in anaerobic glucose broth. Group 2 organisms were relatively inert, they did not hydrolyze starch, gelatin or casein or reduce nitrate. In general the temperature range for growth was lower than for the other groups, none grew at 70°C. Organisms in Group 3 were characterized by a strong amylase reaction and were unable to reduce nitrate anaerobically, but grew well anaerobically on glucose.

In a more recent study Sharp et al. (1980) attempted to classify a small number of Bacillus thermophiles according to genotypic (DNA hybridization and % G+C content) in addition to phenotypic characters. They showed that 8 of the strains examined displayed characteristics which enabled them to be placed in the 3 main taxonomic groups suggested by Walker and Wolf whilst 2 strains showed characteristics of both Groups 1 and 3. Lastly they demonstrated that examination of genotypic data showed taxonomic groupings which differed from those based on phenotypic characterization.

The compilers of the 8th edition of Bergey, Buchanan and Gibbons (1974) considered B. stearothermophilus to be the only truly thermophilic species in the genus Bacillus, however they expressed the generally felt uncertainty about the demarcation of B. stearothermophilus in their concluding paragraph on the organism.

Future classifications may be made with reference to the groupings of Walker and Wolf (1971), indeed, Wolf and Sharp (1981) have suggested that Group 1 strains should be reclassified B. kaustophilus a species name originally proposed by Prickett (1928).

The organism from which was derived the LDH^- mutant used in

this study, NCA 1503, is one of the Group 100 cultures of Cameron and Esty (1926) and a Group 3 culture of Walker and Wolf (1971) and is generally held to be an authentic strain of B. stearothermophilus.

In contrast to the debate in the literature over the taxonomic definition of B. stearothermophilus, detailed study of this organism's metabolism of carbohydrates has been largely neglected; the only work of significance is that of McKray and Vaughn (1957) who studied the fermentation of glucose by a number of strains of B. stearothermophilus. All the strains behaved similarly in producing lactate as the major fermentation product along with smaller amounts of formate, ethanol and acetate. Moreover, none of the strains produced carbon dioxide. McKray and Vaughn made no investigation into the metabolic pathways of glucose fermentation, however from their yield data it is possible to deduce the scheme shown in Figure 1.6. The intermediate pyruvate is produced by glycolysis and can then either undergo NADH-linked reduction to lactate through the action of lactate dehydrogenase (LDH) or conversion to acetyl-CoA and formate catalyzed by pyruvate formate lyase (PFL). The NADH balance for operation of the latter alone then requires the formation of 1 mole of ethanol and 1 mole of acetate for every 2 moles of formate produced. The pathway leading to ethanol is the 'Type 2' pathway of Dawes (1963) described above.

Under aerobic conditions, pyruvate is converted to acetyl-CoA and carbon dioxide by the pyruvate dehydrogenase (PDH) enzyme complex (Perham and Wilkie, 1980). The acetyl-CoA then enters the tricarboxylic acid (TCA) cycle, Figure 1.7.

1.5 Objectives of this Work

The research programme at Imperial College for the conversion of sucrose to ethanol envisaged the use of a suitably modified strain of B. stearothermophilus in a novel type of fermenter-separator which was based on the principle of a distillation column tray. B. stearothermophilus was chosen because it grows rapidly on sucrose both aerobically and anaerobically, which implies a high rate of sucrose uptake. For such a process to be competitive with those based on efficient ethanol producing organisms such as yeast and Z. mobilis, B. stearothermophilus must be able to produce yields approaching 4 moles of ethanol per mole of sucrose fermented.

Such yields could be achieved through the operation of the

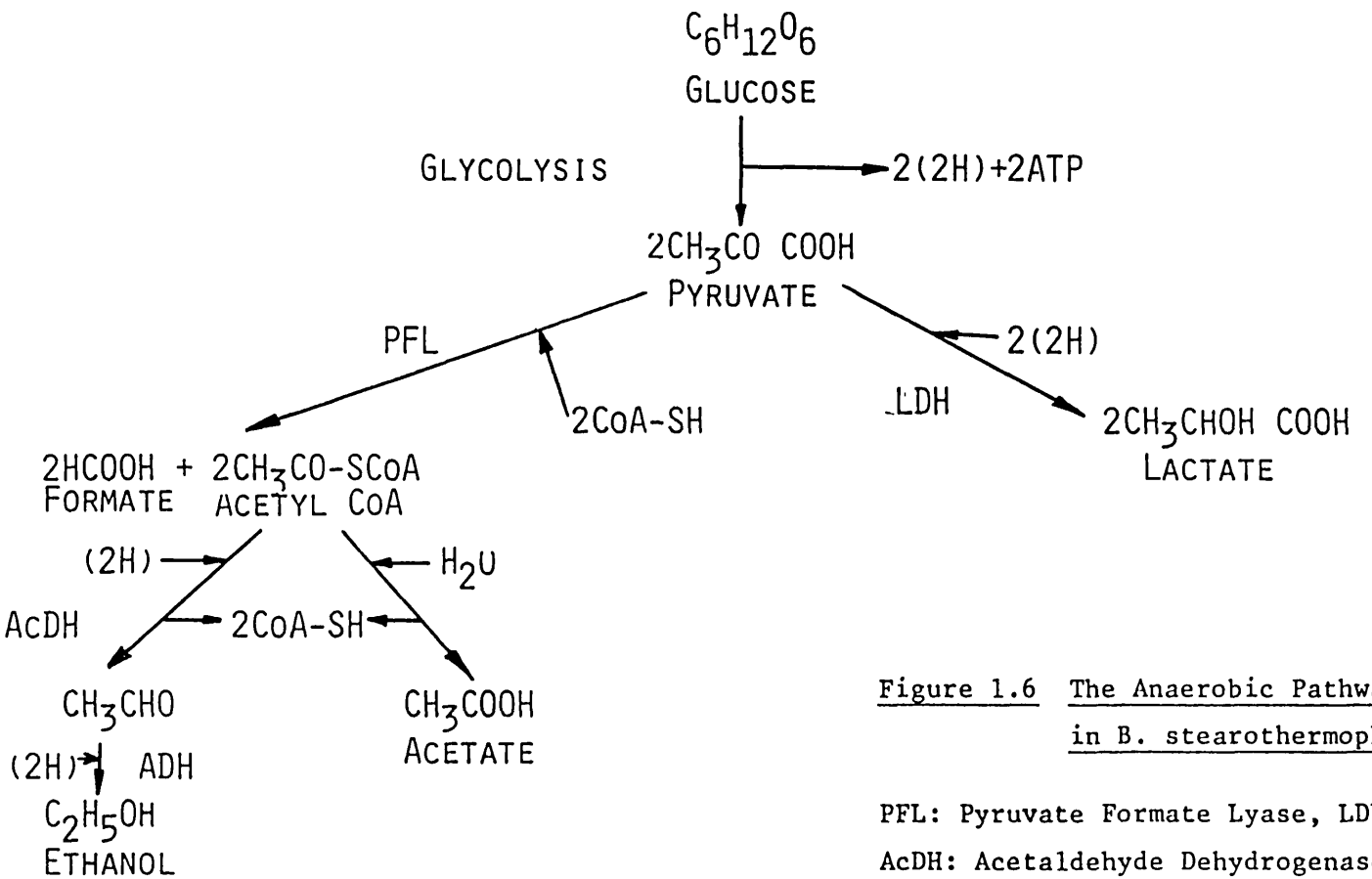


Figure 1.6 The Anaerobic Pathways of Glucose Metabolism in B. stearothermophilus

PFL: Pyruvate Formate Lyase, LDH: Lactate Dehydrogenase, AcDH: Acetaldehyde Dehydrogenase, ADH: Alcohol Dehydrogenase.

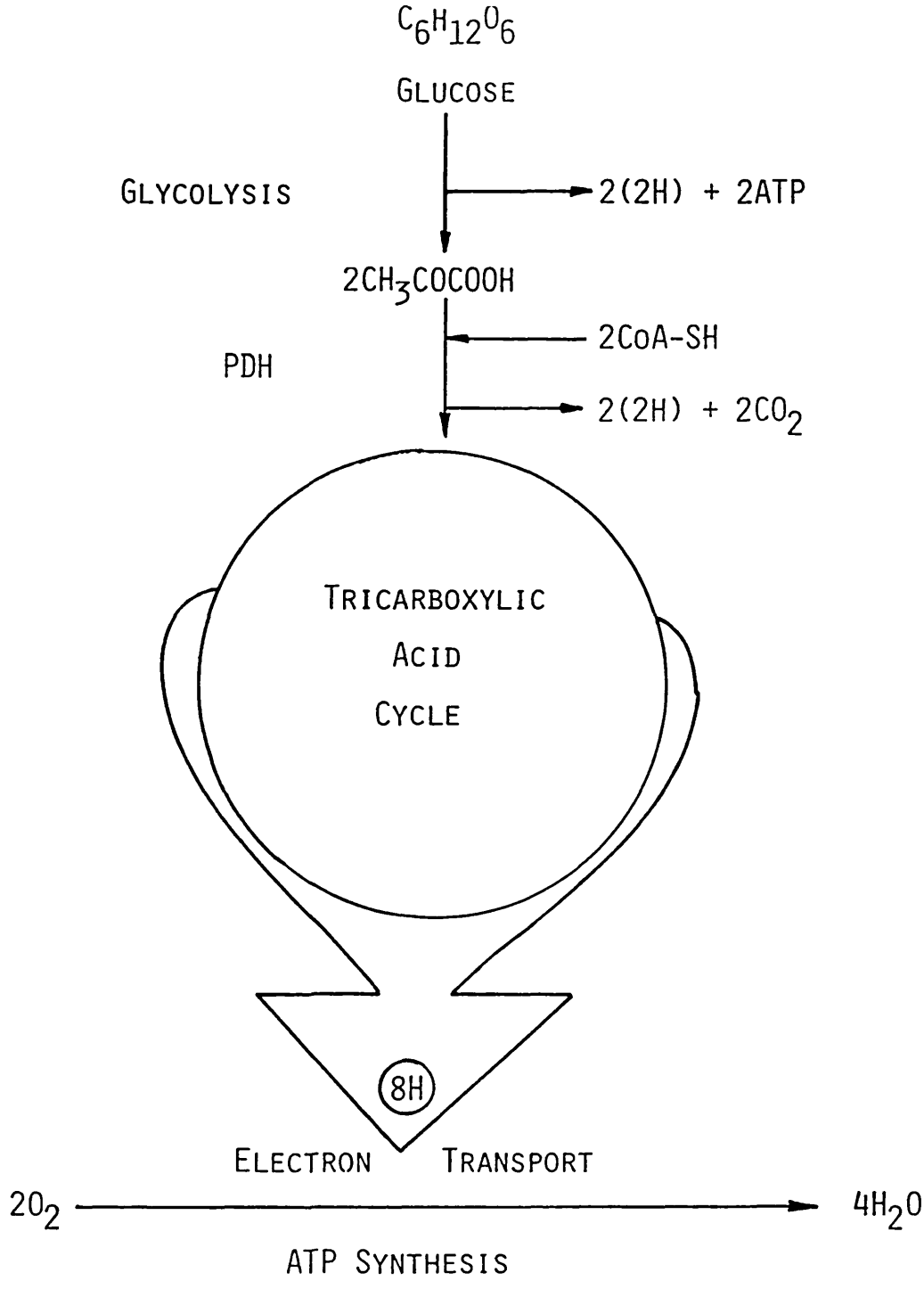


Figure 1.7 Aerobic Glucose Metabolism via PDH in B. stearothermophilus

hypothetical pyruvate dehydrogenase pathway (Type 5, Figure 1.5). Some evidence exists to suggest that pyruvate dehydrogenase is inactive under anaerobic conditions in E. coli due to unfavourable NAD/NADH ratios (Hansen and Henning, 1966). However, B. S. Hartley (personal communication) reasoned that mutants altered in this control property might be selected if competing anaerobic pathways were eliminated.

As stated earlier, wild-type B. stearothermophilus produces lactate from sugars in high yields and the first objective of the programme was to isolate mutants defective in lactate dehydrogenase (LDH). This work was successfully completed and has recently been described (Payton and Hartley, 1985). The next phase, that of isolating mutants lacking pyruvate formate lyase (PFL) was however unsuccessful. This work examines some of the characteristics of B. stearothermophilus 11d^{-15} one of the LDH^{-} mutants obtained by Payton and Hartley, especially in relation to its potential for producing ethanol.

CHAPTER 2

GENERAL METHODS

2.1 Introduction

This chapter contains descriptions of methods common to many of the experiments contained in this work. In some cases it has been felt more appropriate to include a short methods section in later chapters, particularly where a technique was used for only one or a small number of experiments. All gas chromatographic techniques are dealt with in Chapter 3.

This chapter is divided into three sections: microbiological techniques and culture media are described in Section 2.2: Section 2.3 contains specifications of fermenters and associated equipment and accounts of operating procedures: analytical techniques are described in Section 2.4.

2.2 Microbiological Techniques and Culture Media

2.2.1 Storage of Cultures

Preparation of stock ampoules from source cultures (see Appendix 1) involved inoculating a 100 ml quantity of the appropriate growth medium with the source culture and incubating at the optimum growth temperature for 24 hours. 10ml of sterile glycerol were added to the flask which was then gently agitated to ensure even distribution. The glycerol was required as a 'cryoprotectant' to prevent the formation of intracellular ice crystals which can cause cell disruption. Approximately 0.3 to 0.5ml of bacterial suspension was aseptically transferred to sterile plastic ampoules using Pasteur pipettes. 50 ampoules were normally prepared at a time. The ampoules were sealed, placed on metal holders which were then tied, wrapped with a 1½" layer of cotton wool and aluminium foil and placed in a freezer at -20°C. The cotton wool served to protect the culture against too sudden cooling. After 24 hours at -20°C the ampoules were transferred to large Dewar flasks containing liquid nitrogen. Viability and sterility tests (see Sub-section 2.2.4) were performed after 24 hours storage under liquid nitrogen.

2.2.2 Preparation of Agar Slopes

Agar slopes were prepared only from ampoules stored under liquid nitrogen and never by sub-culturing from established slopes.

After allowing the ampoule to thaw at room temperature, a sterile Pasteur pipette was used to deposit approximately 0.1 ml of microbial culture on the surface of the agar slope. The slopes were then rotated gently to ensure maximum coverage of the agar surface. A small sample of bacterial suspension from the opened ampoule was used to perform sterility tests. The slopes were then incubated at the optimum growth temperature of the organism for 24 hours. Slopes prepared in this way were stored in a refrigerator at 4°C for up to a maximum of two months.

2.2.3 Preparation of Liquid Inoculum for Fermenters

Table 2.1 indicates the media used for the storage and culture of the thermophiles employed in this work. Media compositions are given in Sub-section 2.2.5.

Organism	Storage Medium	Growth Medium	Growth Temp.°C
B. stearothermophilus 11d-15	TYE Agar	BSM	60
B. stearothermophilus NCA 1503	BST Agar	BSM	60
C. thermocellum NCIB 10682	CM3 liquid medium	CM3	60

Table 2.1 Storage and Culture Media

Except in the case of *C. thermocellum* (see Section 8.2), liquid inoculum was prepared from agar slopes. The procedure involved first suspending the bacteria from the surface of the agar into 9 ml of sterile physiological saline. 1 ml of the cell suspension was then pipetted into the growth medium. Cultivation was carried out in 500 ml Erlenmeyer flasks containing 100 ml of growth medium. Flasks were incubated on a shaking incubator operating at 200 rpm and radius of gyration 3.2 cm at the organisms' optimal growth temperature. Sterility tests were performed on a small sample from the saline suspension.

2.2.4 Sterility Tests

Sterility tests were performed at all stages of inoculum preparation and during continuous culture experiments where sterile

medium had to be stored for relatively long periods of time (see 2.3.3). Two procedures were used. First, approximately 0.5 ml of the sample was pipetted into each of two test-tubes containing 10 ml of Sterility Testing Broth the composition of which is given below. One tube was incubated at 24°C whilst the second tube was incubated at 37°C. The broths were examined after 24 hours incubation: microbial growth was indicated if a colour change of the bromo cresol indicator from purple to yellow had resulted from metabolic production of acids. Secondly, approximately 0.1 ml of sample was deposited on the surface of each of three agar plates, of composition given below, using a sterile Pasteur pipette. The inoculum was streaked over the surface of each plate with a flame-sterilized platinum wire loop and the plates were incubated at each of the following temperatures: 24, 37 and 60°C. The plates were examined after an incubation period of 24 hours for growth of colonies.

2.2.5 Culture Media

Unless otherwise stated, all ingredients are of 'GPR' grade and were purchased from BDH Chemicals Ltd., Poole, Dorset.

(a) Sterility Testing Broth

	% (w/v)
Glucose	1.00
Nutrient Broth (Oxoid)	0.80
Yeast Extract (Oxoid)	0.50
Bromo-cresol purple (2% solution)	1 ml per litre

Distilled water to volume.

pH adjusted to 7.0 with H_2SO_4/NaOH

Autoclaved for 20 minutes at 121°C, 15 psi.

(b) Sterility Testing Agar

	% (w/v)
Glucose	1.00
Yeast Extract (Oxoid)	0.50
Nutrient Agar (Oxoid)	2.30

Distilled water to volume

pH adjusted to 7.0 with H_2SO_4/NaOH.

Autoclaved for 20 minutes at 121°C, 15 psi.

(c) B.S.T. Medium (Sargeant et al., 1975)

	% (w/v)
Sucrose	Variable
Tryptone (Oxoid)	2.00
Yeast Extract (Oxoid)	1.00
K_2SO_4 anhyd.	0.13
Na_2HPO_4 anhyd.	0.25
$MgSO_4.7H_2O$	0.027
$MnCl_2.4H_2O$	0.0015
$FeCl_3 \cdot 6H_2O$	0.0007
Citric Acid	0.032

Distilled Water to Volume

pH adjusted to the required value by the addition of KOH or H_2SO_4.

100 ml quantities of medium were sterilized by autoclaving at 15 psi. for 20 minutes.

(d) BSM Medium.

BSM medium was used for growing B. stearothermophilus without pH control e.g. for inoculum. The composition was similar to that of BST medium, except that sucrose was omitted and the buffering capacity was increased by replacing Na_2HPO_4 with KH_2PO_4, which was added at a concentration of 1.0% (w/v).

(e) MST Medium.

This minimal medium was based on Medium D of Atkinson et al. (1975b).

	% (w/v)
Sucrose	1.0
$NaH_2PO_4.2H_2O$	0.312
NH_4Cl	0.535
KCl	0.0373
Na_2SO_4	0.146
Biotin	0.00003
Trace Element Solution (see below)	1.0% (v/v)

Distilled Water to Volume

pH adjusted to 7.0 by the addition of KOH or H_2SO_4.

100 ml quantities of medium were sterilized by autoclaving at 15 psi. for 20 minutes. The trace element solution had the following composition:

	% (w/v)
Citric Acid	4.2
$ZnSO_4 . 7H_2O$	0.071
$FeCl_3 . 6H_2O$	0.270
$MnCl_2 . 4H_2O$	0.100
$CuSO_4 . 5H_2O$	0.012
$CoCl_2 . 6H_2O$	0.024
H_3BO_3	0.003
$MgSO_4 . 7H_2O$	3.050
$CaCO_3$	0.100
$Na_2MoO_4 . 2H_2O$	0.004

(f) CM3 Medium.

CM3 Medium of Weimer and Zeikus (1977) was employed with the modification to the reductants solution proposed by Ait et al. (1979). The Basal Medium had the composition

$(NH_4)_2SO_4$	1.3 g
$K_2HPO_4 . 3H_2O$	2.9 g
KH_2PO_4	1.5 g
$MgCl_2 . 6H_2O$	1.0 g
$CaCl_2$	0.15 g
Cellulose powder (Whatman)	10.00 g
Cellobiose	2.00 g
$FeSO_4$ (5% soln.)	0.025 ml
Yeast Extract (Difco)	2.0 g
Deionized Water	1 litre
pH adjusted with NaOH	7.8
Resazurin (0.2% soln.)	1 ml

The basal medium was dispensed as 100 ml amounts in screw-capped bottles, the mixture was agitated to ensure that the cellulose powder was distributed evenly. The medium was sterilized by autoclaving at 15 psi. for 15 minutes. The reductants solution had the composition:-

L-Cysteine hydrochloride	0.5 g
Sodium bicarbonate	5.0 g
Deionized water	50.0 ml

The reductants solution was sterilized by membrane filtration. Complete medium was prepared by aseptically adding 5 ml of fresh reductant solution to each 100 ml bottle of Basal Medium.

(g) CM3(M) Medium

The composition of CM3(M) Medium differed from CM3 only in the omission of cellobiose from the basal medium.

(h) BST Agar.

Composition was as for BST liquid medium (see above) but with the following additions:

	% (w/v)
Sucrose	1.0
Agar (Oxoid No.3)	2.0

pH adjusted to 7.0 by the addition of KOH or H_2SO_4. 100 ml quantities of medium were sterilized by autoclaving at 15 psi. for 20 minutes.

(i) Tryptone - Yeast Extract Agar (TYE).

	% (w/v)
Tryptone (Oxoid)	0.80
Yeast Extract (Oxoid)	0.50
NaCl	0.50
Agar (Oxoid No.3)	1.50

Distilled Water to Volume. Sterilization as for BST agar.

(j) RCM 3

This medium was used in the resting cell suspension experiments and contained no nitrogen source.

	% (w/v)
Sucrose	5.0
3-(N-Morpholino)propanesulphonic acid	2.05
Glycerophosphate	0.79
NaCl	0.90
K_2SO_4 anhyd.	0.13
$MgSO_4.7H_2O$	0.027
$CaCl_2$ anhyd.	0.025
$MnCl_2.4H_2O$	0.0015
$FeCl_3.6H_2O$	0.0007

Washed cell experiments were not performed under aseptic conditions and therefore RCM3 was not sterilized prior to use.

2.3 Fermenter Culture

2.3.1 Introduction

This section is divided into three parts: the first part deals with batch fermentation procedures and the second part with chemostat operation. In both cases a description of the fermenters and associated equipment is given first and is followed by details of autoclaving, operating and monitoring procedures. The inoculation of the fermenters is considered separately in the third part as the procedures adopted depended on the type of fermenter and not on the mode of operation.

All batch fermentations were conducted in a single type of fermenter whereas two different types of fermenter, the choice of which was dictated by availability and experimental requirements, were used in continuous cultivation experiments.

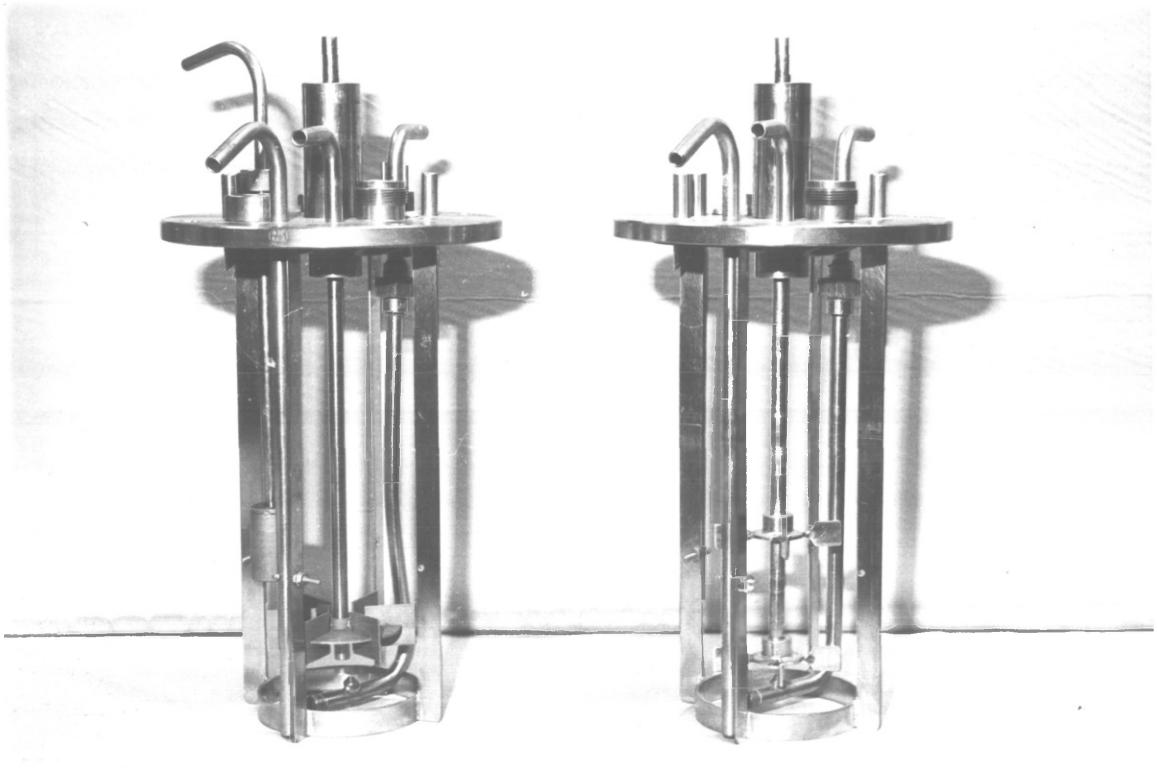
2.3.2 Batch Experiments

(a) Fermenters

5 litre 'Labroferm' fermenters manufactured by the New Brunswick Scientific Co. Inc., N.J., U.S.A. were used. The fermenters were of standard design: viz. top-driven impeller shaft with two sets of impellers, single-orifice sparger located centrally below the bottom impeller and four vertical baffles. Further features included probe, inoculation and addition ports, sampling tube and thermometer well. The fermenter vessel itself was manufactured from Pyrex glass; all the other fermenter parts coming into contact with the culture fluid such as the impellers, baffles, etc., were fabricated from stainless steel. A view of the fermenter can be seen on the right hand side of Fig. 2.1 which shows the configuration of the baffles and the arrangement of impellers.

(b) Assembly Unit

During operation the fermenters were placed in an 'assembly unit' also manufactured by New Brunswick. The unit consists of a combined water bath and impeller drive mechanism. When positioned in the bath each fermenter was connected to the agitator drive by a flexible rubber coupling. The unit could contain up to three fermenters and is shown in Figure 2.2: the fermenter on the right hand side of the figure is being used in a batch fermentation experiment and that on the left hand side for continuous cultivation (see 2.3.3).



A B

Figure 2.1. Impeller and Baffle Configurations for Continuous (A) and Batch (B) New Brunswick Fermenters

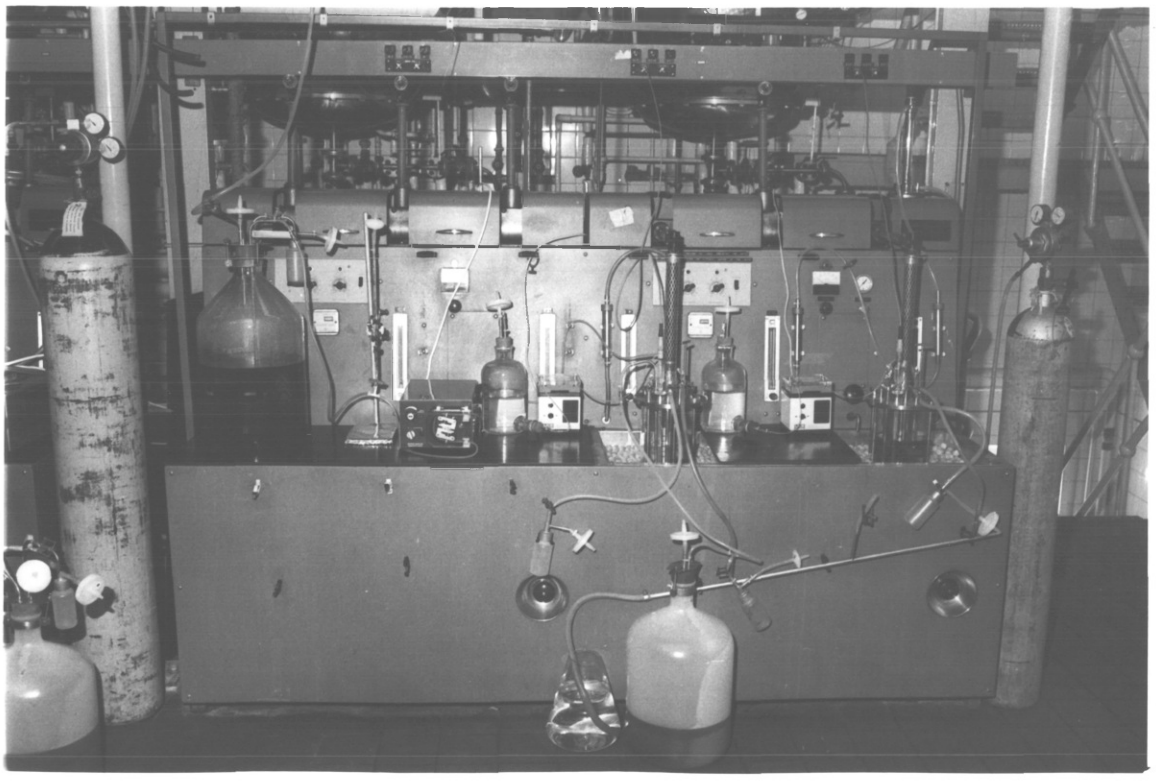


Figure 2.2 New Brunswick Fermenters in Position in the Water Bath Unit

The temperature of the water in the bath was maintained at the desired value by a conventional control thermostat: a circulation pump situated below the water bath drew water over a heating element before returning it to the bath. Under normal conditions of operation, the temperature of the water in the bath was some 3°C above the temperature of the medium in the fermenter. Light plastic balls were floated onto the surface of the water to reduce evaporative losses and temperature control was achieved to an accuracy of ± 1°C. Air was introduced to the fermenters through a pressure regulator, needle valve, flowmeter and a removable stainless steel filter (see below) mounted on the control panel of the unit. Air flow to each individual fermenter was adjustable up to 8.8 litres per minute.

(c) Control of pH

A schematic diagram of the pH control system used in conjuction with the New Brunswick fermenters is shown in Figure 2.3. A steam sterilizable combined glass and reference electrode ('Ingold' Model No. 465, Pye Unicam Ltd., Cambridge, Cambs.) with a pH measuring range of 0 to 12 mounted through the top plate of the fermenter was connected via an amplifier module (Model 9820 with automatic and manual temperature compensation, EIL Ltd., Chertsey, Surrey) to a combined controller and recorder unit (Clearspan P120L, Foster Cambridge Ltd., Huntingdon, Cambs.). 'One-way' pH control was used in all experiments as the tendency in both aerobic and anaerobic fermentations was for the pH to decrease as a result of acid production. In these circumstances, the action of the controller was to activate a peristaltic pump (Perpex Model 10200, LKB-Produkter AB, Bromma, Sweden) which caused the addition of sterile potassium hydroxide solution (30% w/v) to be made to the fermenter until the measured pH corresponded to the set pH.

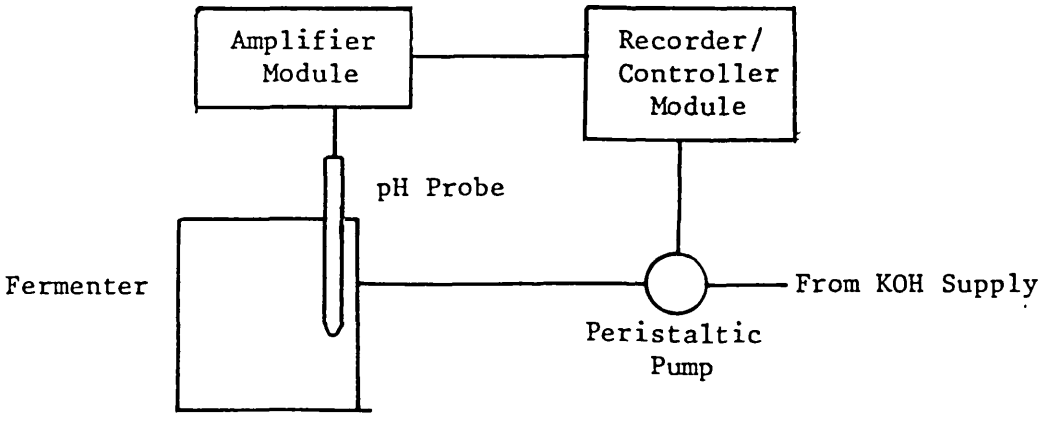


Fig. 2.3 Schematic Diagram of pH Control System.

(d) Autoclaving, Medium Preparation and Operation

Media containing sucrose are prone to caramelization and other undesirable interactions during heat sterilization, and in order to avoid such risks the sucrose component of the medium was autoclaved separately. The sucrose was dissolved in a volume of water at pH 4 equal to 20% of the fermenter working volume (normally 3.4 litres) and autoclaved for 1 hour at 15 psi. in glass screw-topped bottles which were later aseptically connected to the fermenter. The fermenter was charged with the nitrogenous and mineral constituents of the medium dissolved in a volume of water equal to 80% of the fermenter working volume and autoclaved for 1 hour at 15 psi. The time required for the steam pressure in the autoclave chamber to reach 15 psi was approximately 3 minutes. The autoclave chamber exhaust valve was regulated so as to allow a chamber evacuation period, following sterilization, of approximately 1½ hours. This relatively slow exhaust rate was imposed to reduce the risk of medium losses by flashing due to sudden decreases in chamber pressure. On removal from the autoclave, the fermenter was transferred rapidly to the drive assembly unit. Air, sterilized by passage through a stainless steel filter packed with glass wool, was supplied at 1 litre/min. to each fermenter and connection was made to the agitator drive unit. The impellers were run at a speed of 350 rpm. The air outlet tube of the fermenter was connected to a manifold tube, the single open end of which emerged into a glass container filled with disinfectant solution to a level of about 5 inches. This figure represents the over pressure relative to atmosphere which was maintained in the fermenter in order to reduce the risk of contamination. The disinfectant served two functions. First, to prevent contamination of the fermenter via the air outlet in the event of a gas supply failure, and secondly to reduce the risk of releasing aerosols containing viable organisms into the environment. Assembled in this way, the fermenter was left overnight to cool sufficiently to permit filling of the water bath without risk of cracking the pyrex glass vessels.

The passage of air through the hot culture medium resulted in high evaporative losses which were made up by the aseptic addition of sterile distilled water after the sucrose addition. For fermentation experiments carried out under anaerobic conditions the air supply to the fermenter was interrupted and an oxygen-free nitrogen supply was connected to the sparger. The flow of nitrogen gas to the sparger was maintained for one hour before inoculating the fermenter to ensure

that all traces of oxygen had been stripped from the culture medium.

In some experiments the supply of nitrogen gas to the fermenter was via the sparger and in order to reduce evaporative losses from the fermenter a stainless steel total reflux condenser (Length 12", I.D. 0.25", LH Engineering Co. Ltd., Stoke Poges, Bucks.) was incorporated into the gas outlet line. Chilled water at 11°C was circulated through the outer jacket of the condenser. A glass U-tube trap, completely submerged in an acetone-carbon dioxide ice mixture was installed further downstream to collect any uncondensed substances.

In the early experiments this procedure was not adopted - instead nitrogen was admitted to the fermenter headspace at a low flow rate (200 ml/min.). In order to further reduce evaporative losses, the impeller was operated at 200 rpm which resulted in only minimal disturbance of the surface of the medium. Some degree of agitation was necessary for adequate temperature and pH control and to prevent settling of the bacterial cells and the speed chosen was found to result in the maintenance of constant pH and temperature.

In both cases nitrogen was supplied from a single cylinder via a manifold. Rotameters were incorporated into the gas line to permit the flow to be accurately monitored.

The constituents of complex culture media undergo interactions during autoclaving which result in a change in the pH of the medium, so that although the pH of the medium has been brought to the desired level prior to autoclaving, its value after autoclaving had to be determined by removing a sample of medium from the fermenter and measuring its value on a laboratory pH meter. This value was subsequently used to calibrate the fermenter pH probe. After aseptically connecting the sterile KOH supply to the fermenter, the pH controller was activated and the fermenter inoculated (see 2.3.4.)

The removal of samples from the fermenter during the course of a fermentation was achieved by temporarily clamping shut the air exhaust tube from the fermenter. The build-up of pressure in the fermenter headspace caused the culture liquid to rise into the sample line and thence into a sterile sample bottle. Normally, between 15 to 20 ml volume of sample were taken. To ensure that representative samples were taken, the fermenter sampling line was cleared by blowing back via the filter on the sample bottle attachment with either air or nitrogen gas depending as to whether the fermentation was being

conducted aerobically or anaerobically. Filters fitted to the sample bottle attachment (and also to the sterile alkali supply) were of the hydrophobic PTFE membrane type with a pore size of 0.45 μm (Gelman Sciences Inc., Michigan, U.S.A.). All connecting lines were of silicone rubber. Temperature, impeller speed and gas flow rate were routinely monitored every 2 hours on a 24 hour basis during fermentation experiments.

2.3.3 Continuous Culture

(a) Fermenters

Two 5-litre capacity New Brunswick Fermenters were modified for continuous culture experiments. The modifications provided an additional port for the continuous supply of medium and a medium take-off line. The hood located at the extreme end of this line can be seen on the left hand side of Figure 2.1. As a relatively low working volume of 2 litres was used, agitation was provided by a single turbine impeller. The associated hardware and instrumentation used with this fermenter was identical to those used in batch fermentation experiments (see 2.3.2.)

Series 2000 fermenter manufactured by L. H. Engineering Co. Ltd., Stoke Poges, Bucks. were also used. The design incorporated all the conventional features. In addition to single orifice sparger, turbine impellers and vertical baffles, this model featured a medium outlet weir and heat exchange finger. The fermenter vessel was of pyrex glass all other fittings were of stainless steel. A working volume of 750 ml was used in all experiments. The instrumentation used with this fermenter was manufactured by L. H. Engineering Co. Ltd., Stoke Poges, Bucks. and supplied as standard units with the series 2000 fermenter. This comprised a Model 2100 measurement amplifier and Model 2215 dual point on/off pH controller, and a Model 2200 measurement amplifier and Model 2115 dual point on/off temperature controller. The temperature control for this fermenter was more accurate than the New Brunswick system. The LH design featured a heat exchange 'finger' through which was circulated chilled water and which was used to cool the fermenter contents down rapidly after auto-claving. Medium temperature was monitored with a platinum resistance thermometer with digital display. During operation, heat transfer to the fermenter was by direct contact of the medium with an immersion

heater located inside the fermenter. Temperature was controlled to within ± 0.1°C of the set point value.

(b) Autoclaving, Medium Preparation and Operation

The procedures followed for autoclaving and preparing the fermenters for inoculation were similar to those described earlier for batch experiments: after autoclaving, the LH fermenter was transferred to its upright stand shown in Figure 2.4.

The procedures differed in that for continuous culture experiments, culture medium was prepared and stored in plant scale fermenters. The laboratory scale fermenters were filled with water prior to autoclaving in order to protect the pH electrode. This water was subsequently removed via the sampling line before filling the fermenters with sterile medium from the pilot scale fermenter.

Both 50 litre, Figure 2.5, and 300 litre, Figure 2.6, vessels were used according to experimental requirements. In both cases medium sterilization was by live steam injection. (Allowances for the condensation of steam during autoclaving were made at the medium batching stage). For the reasons stated earlier in 2.3.2(b), sucrose was autoclaved separately from the other medium components and then aseptically combined with the latter on cooling. After sterilization for 1 hour at 15 psi the medium was cooled to approximately 11°C by the passage of chilled water from a refrigerator unit through heat exchanger coils located around the outer walls of the fermenter vessel. Medium was stored at this temperature during the entire course of the experiment. As further precautions against contamination, the pH of the medium was brought down to 6 by the aseptic addition of sterile sulphuric acid, and a fermenter headspace pressure of 12 psi was maintained by sparging sterile air through the medium. The supply of sterile medium to the fermenter was via a 20 litre glass aspirator bottle. The aspirator was autoclaved separately and was connected in line between the medium storage fermenter and the laboratory scale fermenter. The filling of the aspirator with medium was carried out periodically as required. The connecting line linking the storage fermenter and aspirator was of silicone rubber, (sterilized by autoclaving for 1 hour) all connections were made with stainless steel 'Herbert' couplings.

The aspirator had provision for the removal of samples for sterility testing. Also included was a gas sparger line so that in

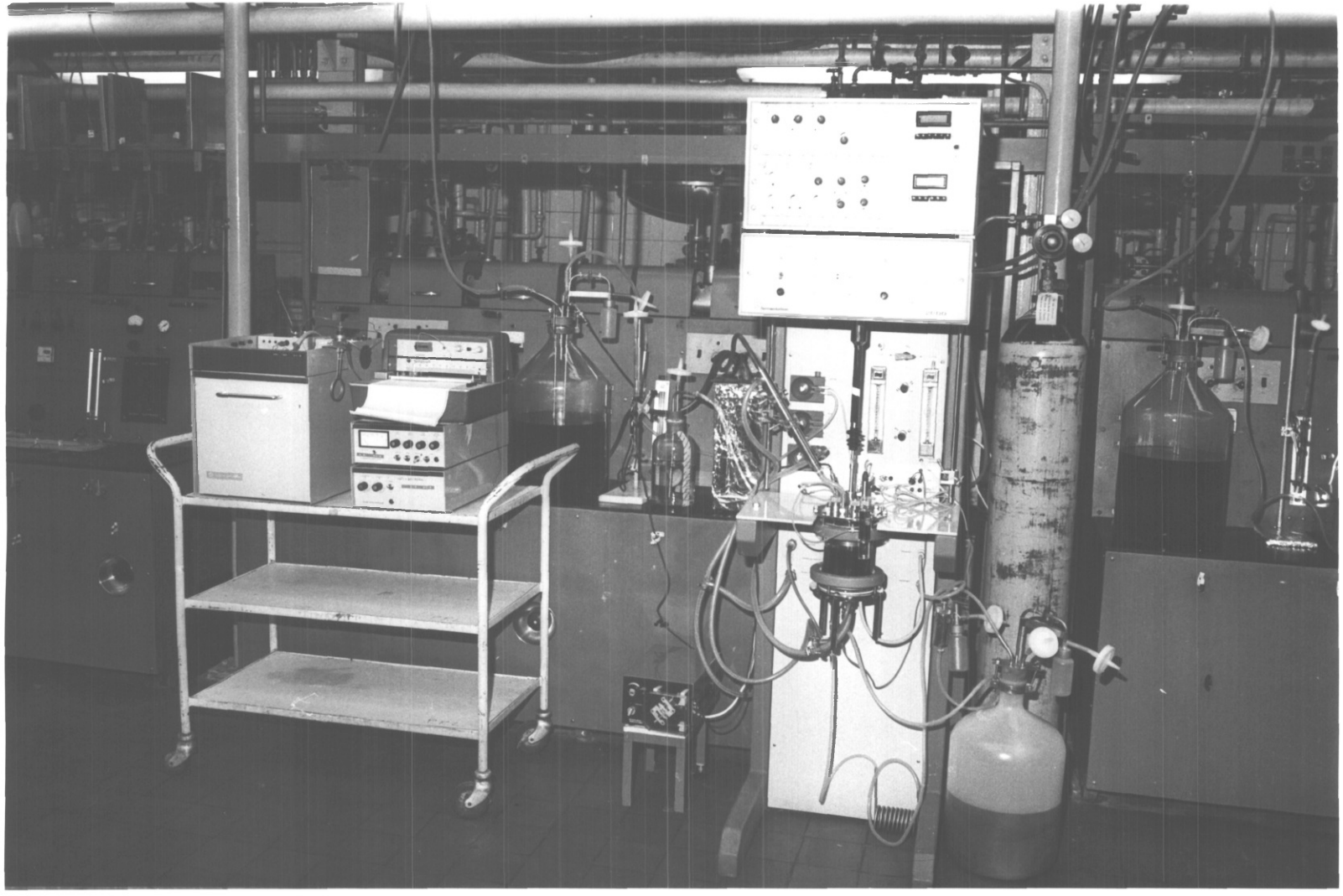


Figure 2.4. 1 litre LH Fermenter and Associated Instrumentation: a Gas Chromatograph used for Fermenter Exit Gas Analysis can be seen to the left of the figure

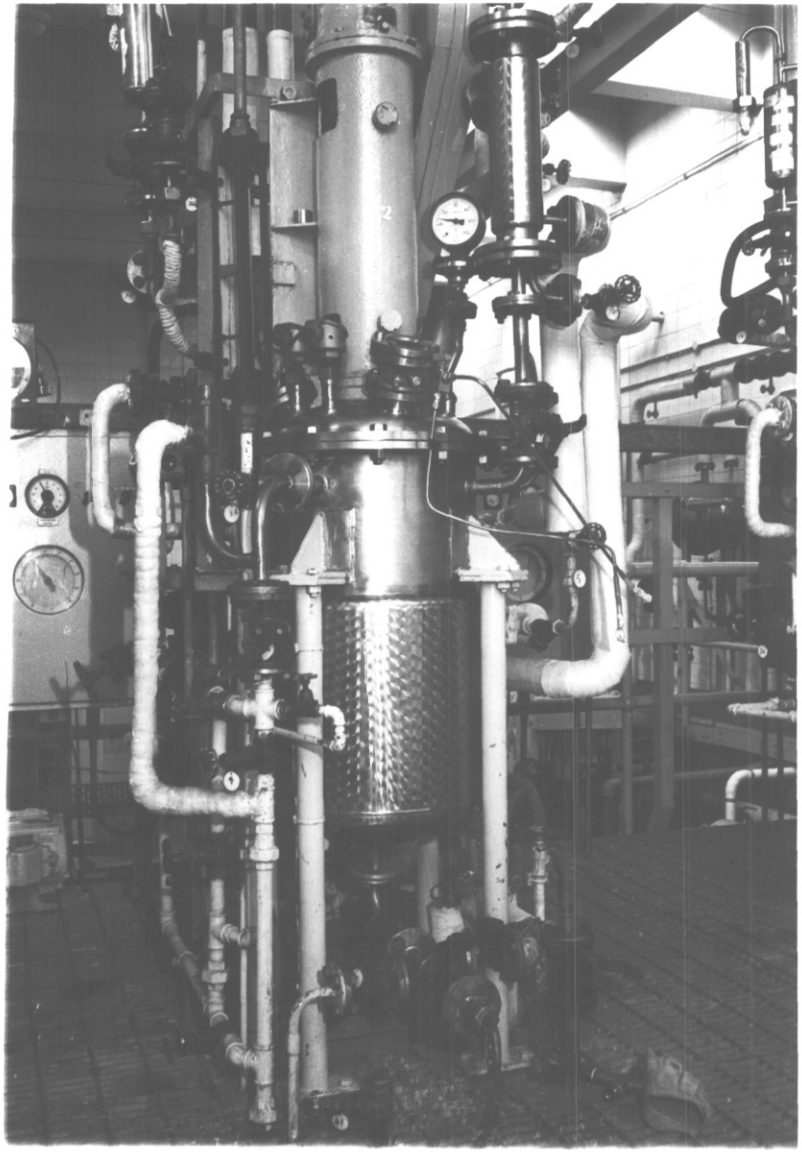


Figure 2.5. Plant Scale 50 litre Fermenter

Figure 2.6 Plant Scale 500 litre Fermenter

experiments performed under anaerobic conditions the medium supply could be continuously sparged with oxygen-free nitrogen.

The medium addition pump (Model 501s, Watson Marlow Ltd., Falmouth, Cornwall) was installed in line between the medium aspirator and the fermenter, Figure 2.7. Also shown in Figure 2.7. is the graduated tube arrangement used in the calibration of the pump and to ensure constant medium delivery.

With tubing clips A and B open, the tube was filled with medium by decreasing the level of the graduated tube relative to the height of liquid in the medium aspirator. Clip A was then closed, the peristaltic pump was switched on and the rate of decrease of liquid level in the tube was determined. If the measured delivery rate was not that required, the pump speed was altered accordingly and the calibration process repeated.

During operation the liquid level in the medium aspirator was recorded 2-hourly and adjustments to the pump speed were only made if the pump delivery rate differed from the required valve by more than 5%.

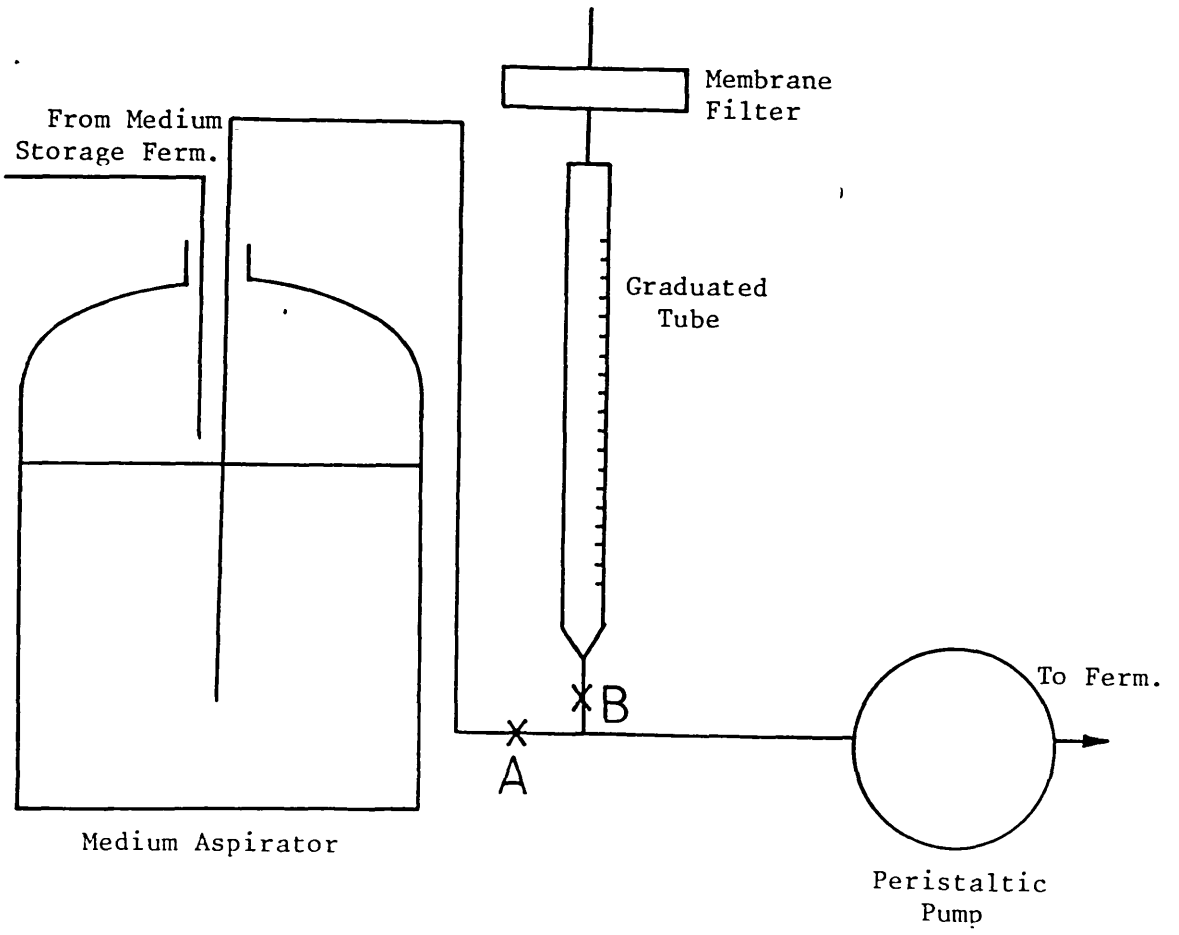


Fig. 2.7 Calibration of Peristaltic Pump

The LH fermenter featured a standard gravity type overflow weir and in order to maintain constant fermenter volume, the overflow line was unclipped once the medium feed pump had been turned on. In contrast, spent medium flowed out under pressure via the top plate in the New Brunswick Fermenter. The fermenter contents were pressurized by partially clamping shut the gas exit line. Culture samples were taken directly from the fermenter for sterility testing. The procedure used for both types of fermenter was identical to that described in 2.3.3. However, in order to minimize the risk of contaminating the fermenter vessel all samples taken for cell density or product analyses were overflow samples. In order to prevent evaporative losses of volatile products from the liquid sample during the sampling procedure, the overflow stream leaving the fermenter was cooled prior to entry into the sample bottle. This was achieved by passage of the overflow through a stainless steel coil (total length 4 feet) which was inserted in-line between the fermenter and sample point and which was kept submerged in ice during sampling.

Spent culture medium was collected in a harvest reservoir of 20 litres capacity. Gas metered to the fermenter was vented to atmosphere via a membrane filter installed in the gas exit line of the harvest reservoir. When full, the reservoir was aseptically emptied and the contents autoclaved and disposed of.

After inoculation of the fermenter, (2.3.4), samples were taken approximately every 2 hours for cell density determinations. When cell density readings indicated that the late logarithmic phase of growth had been reached, the medium addition pump was activated. A period of 24 hours was allowed to elapse after the continuous addition of medium had commenced before taking samples for full analyses. During the course of the experiment a minimum period equivalent to 5 fermenter volume changes was allowed before sampling following a change in operating conditions. Thereafter, the establishment of steady state was confirmed when the difference in absorbance between samples taken at intervals of 2 hours was less than 5%. A sample was taken immediately prior to imposing a change in operating conditions. Sterility tests (see Sub-Section 2.2.4) were performed daily during continuous culture experiments. Samples were taken from the medium storage fermenters, medium aspirator, continuous culture fermenter and harvest reservoir.

2.3.4 Fermenter Inoculation

(a) New Brunswick Fermenter

Inoculum was dispensed into the fermenters from a glass inoculation bulb. The model used comprised an elongated glass graduated vessel of approximately 3 inch diameter and 12 inch length which was connected to a stainless steel hood by a 6 inch length of rubber hose. Prior to autoclaving, the bulb opening was sealed with a cotton wool plug and the hood wrapped in cotton wool and aluminium foil. Inoculum was poured aseptically into a sterile inoculation bulb with the rubber hosing clamped tightly shut and with the hood still wrapped up. The bulb was then transferred to the fermenter, the inoculation port cover was removed and flamed whilst the hood was unwrapped, and carefully placed over the open port. The hose clip was then loosened to permit inoculum to enter the fermenter via the rubber hose and hood (the port cover being continuously flamed during this operation). After transferring the required amount of inoculum, (normally 100 ml per 3.4 litres of medium), the fermenter port cover was replaced. Where more than one fermenter was to be inoculated, the inoculum flasks, prepared as detailed in Subsection 2.2.3, were combined together in a single inoculation bulb. The above described procedure was followed for each fermenter.

(b) L. H. Fermenter

25 ml of inoculum was dispensed into the fermenter from a 250 ml Erlenmeyer flask with side arm. Attached to the side arm was a length of silicone tubing whose free end terminated in a stainless steel male coupling. Inoculation of the fermenter was brought about by aseptically connecting the Erlenmeyer flask to a female coupling on the fermenter and tipping the flask slightly to cause the bacterial suspension to flow into the fermenter. After inoculation the silicone tube was clamped shut and the flask was separated from the fermenter.

2.4 Analytical Techniques

2.4.1 Cell Density Measurement

The absorbance of bacterial samples were measured against medium blanks using an SP6 - 400 spectrophotometer (Pye Unicam Ltd., Cambridge, Cambs.) at 600 nm.

Plastic cuvettes of 4 ml volume and 1 cm light path were used.

2.4.2 Sample Preparation

Bacterial cells were separated from culture medium by centrifugation prior to routine analysis.

25 ml aliquots of bacterial cell suspension were centrifuged at 15,000 rpm at 10°C for 20 minutes using an RC5 Superspeed Refrigerated Centrifuge (Du Pont (UK) Ltd., Hitchen, Herts.) fitted with a GSA model rotor.

2.4.3 Determination of Sugars

Nelson's colourimetric modification of Somogyi's titrimetric method was used throughout, following the procedure of Hodge and Hofreiter (1962). Although the range of the colourimetric method is more limited, it extends from 600 to 5 µg per sample which nevertheless represents a wider range than usual for colourimetric methods. All reagents used were analytical grade.

The low alkalinity copper reagent was prepared as follows:- 12 g of potassium sodium tartarate and 24 g of sodium carbonate were dissolved in about 250 ml of distilled water. A solution of 4.0 g of cupric sulphate pentahydrate in water was added with stirring followed by 16g of sodium hydrogen carbonate. A solution of 180 g of anhydrous sodium sulphate in 500 ml of water was boiled to expel air; the two solutions were then combined and diluted to 1 litre. After standing for one week the clear supernatant solution was used.

The arsenomolybdate reagent was prepared as follows:- To 25g of ammonium molybdate in 450 ml of water was added 21 ml of 96% sulphuric acid, followed by 3.0 g of disodium hydrogen arsenate heptahydrate dissolved in 25 ml of water. The mixed solution was incubated for 24 hours at 35°C and stored in a brown glass-stoppered bottle.

(a) Method for Reducing Sugars

Between 1 and 5 ml of sample prepared as described above (see Sub-section 2.3.2) and containing not more than 0.6 mg of D-glucose or its equivalent was pipetted into a graduated test tube; an equal volume of the low-alkalinity copper reagent was then added. The sample tubes and a distilled water blank tube were stoppered and then heated in a vigorously boiling water bath for 10 minutes. After cooling, 1 ml of arsenomolybdate reagent was added to determine 0.1 mg or less of D-glucose and 2 ml was added for 0.1 to 0.6 mg of D-glucose.

The tubes were shaken in order to bring about the complete dissolution of the cuprous oxide formed and then the volume of each tube was made up to 25 ml with distilled water. After standing for at least 15 but not more than 40 minutes, absorbances were read at 500 nm against the blank. The sugar content of the sample was computed from a curve of sugar concentrations versus absorbances previously established with standard glucose solutions.

A calibration curve was prepared each time a new stock of reagents were made up. A typical calibration curve is shown in Figure 2.8.

(b) Method for Sucrose

Sucrose is not a reducing sugar and consequently the determination of sucrose by the Nelson-Somogyi procedure requires a preliminary hydrolysis stage. Between 1 and 2.5 ml of sample containing not more than 0.6 mg of sucrose were pipetted into a test tube to which was added half the sample volume of 1 M hydrochloric acid. The sample tubes and a similarly acidified water blank tube were stoppered and then heated in a vigorously boiling water bath for 5 minutes. After cooling, a volume of 1 M sodium hydroxide solution equal to the volume of 1 M hydrochloric acid added was pipetted into each tube. The procedure followed thereafter was identical to that employed for the determination of reducing sugars described above. The sucrose content of the sample was computed from a curve of sucrose concentration versus absorbance previously established with standard sucrose solutions. A calibration curve was prepared each time a new stock of reagents was made up.

2.4.4 Lactic Acid Determination

The enzyme lactate dehydrogenase (LDH) catalyzes the oxidation of lactate to pyruvate in the presence of excess nicotinamide adenine dinucleotide (NAD) with the concomitant reduction of NAD to NADH. An estimate of lactate concentration can be obtained by measuring the increase in absorbance in the UV region due to production of the strongly absorbing NADH.

All reagents used in this method were purchased from the Sigma Chemical Co. Ltd., Poole, Dorset.

The commercial preparation of LDH used in this assay was isolated from beef heart and was suspended in ammonium sulphate solu-

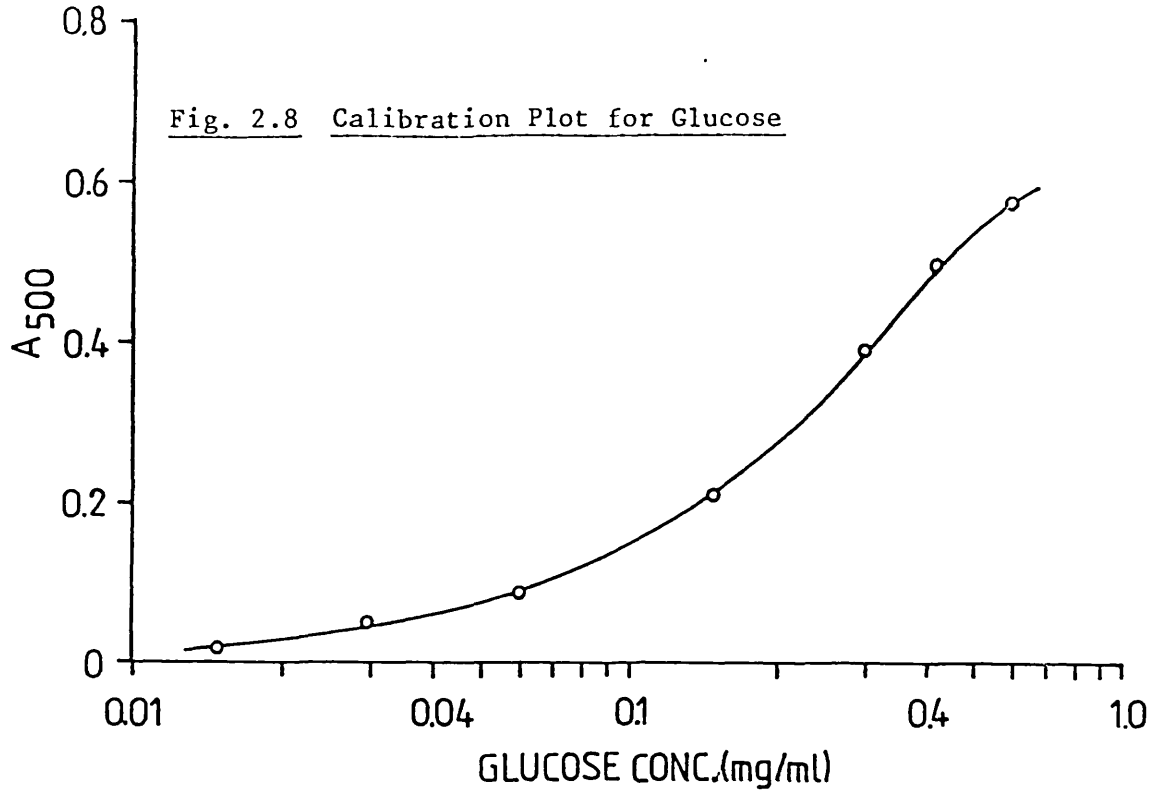


Fig. 2.8 Calibration Plot for Glucose

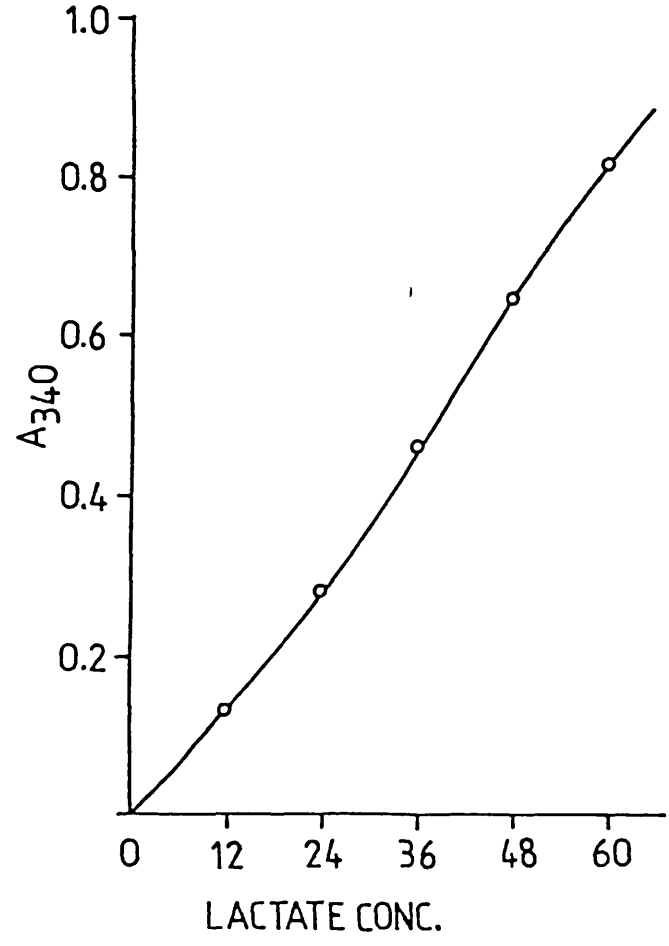


Fig. 2.9 Calibration Plot for Lactate

tion. The activity of the enzyme was approximately 1000 units/ml (1 unit will reduce 1.0 µmole of pyruvate to lactate per minute at pH7.5 and 37°C.

The buffer used in this assay consisted of a commercial prepared glycine buffer (pH9.2) - and for convenience glass vials containing 10 mg of NAD were used.

A preliminary stage in the assay procedure was the precipitation of proteinaceous matter from the culture supernatants.

2.0 ml of culture supernatant containing less than 1.2 mg of lactate were pipetted into a centrifuge tube containing 4.0 ml of 8% (w/v) perchloric acid at 4°C. After shaking vigorously for 30 seconds, the mixture was maintained at 4°C for five minutes to ensure complete protein precipitation. The mixture was then centrifuged at 3000 g for 10 minutes.

To each vial containing NAD was pipetted:-

2.0 ml Glycine Buffer
4.0 ml Distilled Water
0.1 ml LDH

The vial was then sealed and inverted several times to ensure the complete dissolution of the NAD. 2.8 ml of this buffered LDH/NAD mixture was combined with 0.2 ml of deproteinized culture supernatant in a 4 ml plastic cuvette with 1 cm light path. The cuvette was sealed and then inverted several times to ensure good mixing of the reagents. Cuvettes were incubated for 30 minutes in a water bath at 37°C. Absorbance was read at 340 nm on a spectrophotometer against a reference cuvette containing 0.2 ml distilled water and 2.8 ml buffered NAD/LDH mixture.

The lactate concentration of the culture supernatants was computed from a curve of absorbance at 340 nm versus lactate concentration established with standard lactate solutions. A typical calibration curve is shown in Figure 2.9.

2.4.5 Carbon Dioxide Measurement

The percentage of carbon dioxide in the exit gas stream from fermenters was measured using an automatic infra-red gas analyzer manufactured by ADC Co. Ltd., Hoddesdon, Herts. The instrument had four measurement ranges covering 0.1, 1.0, 5.0 and 10.0% v/v carbon dioxide. The instrument was calibrated with a mixture comprising

0.5% carbon dioxide in nitrogen (BOC Special Gases, Morden, Surrey) and was certified as being of the stated composition to within an accuracy of ± 5%. The infra-red analyzer was connected to a chart recorder ('Euroscribe', A. Gallenkamp and Co. Ltd., London, E.C.2).

2.4.6 Elemental Analysis

Elemental analyses were performed on a Model 1102 Elemental Analyzer manufactured by Carlo Erba S.p.A., Milan, Italy.

Approximately 0.4 mg of sample to be analyzed was accurately weighed into a small tin bucket using an electronic microbalance (Model 4125 Sartorius, Belmont, Surrey.) The accuracy achievable with this balance was of the order of ± 0.001 mg. Approximately equal amounts of cyclohexanone -2,4-dinitro phenyl hydrazone standard were similarly weighed into at least two further buckets. The tin buckets were then crimped sealed and loaded into the sample holder which is in the form of a wheel. The wheel was then placed in the analyzer. Finally, a mechanism was activated which caused the wheel to revolve slowly and to automatically discharge single samples into the oxidation tube at preset intervals of time. Rapid pyrolysis of sample occurred in the oxygen-enriched atmosphere existing inside the oxidation tube. The decomposition products formed were carried away from the oxidation tube to a tube packed with chromic and cobalt oxides which serve as catalysts to convert the decomposition products to carbon dioxide; water and nitrogen oxide. The mixture was then passed to a tube packed with reduced copper, maintained at an elevated temperature, and in which the reduction of nitrogen oxide to nitrogen occurred. From there, the mixture was carried into a chromatographic column where the separation of the carbon dioxide, water and nitrogen was achieved. Finally, the separated products passed over a thermal conductivity detector.

The elemental composition of the sample was determined by comparing the heights of the peaks displayed on the recorder chart with the corresponding peaks of the standard.

CHAPTER 3

GAS CHROMATOGRAPHY OF FERMENTATION PRODUCTS

3.1 Introduction

Gas chromatographic (GC) methods for the identification and quantitative determination of a range of fermentation products are described in this chapter. The methods dealing with neutral fermentation products such as ethanol are direct applications of existing methods and are included without further discussion. Some adaptations of existing techniques were made in order to develop a method of analysis for fermentation gases of interest and are briefly described. Finally, the work leading to the development of a convenient method for the simultaneous determination of a number of acidic fermentation products is considered in some detail.

3.1.1 Gaseous Fermentation Products

The gases of interest in the context of this work were hydrogen and carbon dioxide and a method was sought which would permit the detection of these gases in nitrogen-rich mixtures. No such methods were found in the literature, but Cross (1966) achieved good separation of a number of flue gas components, including nitrogen and carbon dioxide on a Poropak Q column under isothermal conditions, and it was decided to adapt his method.

3.1.2 Acidic Fermentation Products

Mention was made in Section 1.4 that the expected acidic fermentation products of *B. stearothermophilus* 11d-15 would include formate and acetate. In addition to producing these acids, wild-type *B. stearothermophilus* produces lactate as its main fermentation product. The requirement was therefore for a method that would permit the determination of formate, acetate and lactate. Inclusion of the latter was felt to be important not only for experiments with wild-type *B. stearothermophilus* but also in those with strain 11d-15 where the appearance of lactate would indicate that either strain reversion to wild-type or contamination had occurred. A survey was made of analytical methods in the literature. Colourimetric methods are available for these acids, but are time consuming and often lack specificity. Good enzymatic techniques are also known, but there is the disadvantage of the high costs of reagents, the cost for a single

acetate determination being about £1 in 1985. GC, on the other hand, offers the prospect of making simultaneous determinations of the individual components in a mixture of related species at relatively low running costs.

Interest in GC detection of fermentation products stems mainly from studies concerned with the identification of micro-organisms. Many methods have relied on the detection of acidic products as the review of Wade and Mandle (1974) shows. The preparatory procedures involved vary. Carlson (1973) proposed the straightforward injection of aqueous culture broth into the chromatograph, whereas Lindner and Truger (1979) advocated the preparation of succinimido-methyl esters. Although Carlson's method appears at first sight to be attractive, only volatile fatty acids of chain length 2 to 6 and the so-called 'non volatile' acids, lactate and succinate, are detectable. Formate is undetectable by his, or any other method in which flame ionization detectors (FID) are used. The method most commonly used to overcome this limitation is to prepare derivatives of the acids. Methyl esters in particular have attracted much attention because they are relatively easy to prepare. Drucker (1970), proposed extraction of the acids in ether followed by drying and reaction with a methanol-borontrifluoride mixture, however a much more convenient method is that of Moore and Holdemann (1972) in which the aqueous fermentation samples were reacted with sulphuric acid and methanol at a slightly elevated temperature before extracting the methyl esters into chloroform. This procedure was optimized for both volatile and non-volatile acids by Bohannon *et al*. (1978) and was used throughout this present work. The procedure is described below. Although a number of column packings have been claimed as being suitable for the separation of methyl esters (Drucker, 1981a) little retention time data has been published on the methyl esters of the acids of specific interest here.

In the present work, as described below, a number of column packings at different operating conditions were used in order to obtain good resolution of mixtures of methyl formate, methyl acetate and methyl lactate.

3.2 Methods

3.2.1 Apparatus and General Techniques

(a) Chromatographs

A GCD chromatograph (Pye Unicam Ltd., Cambridge, Cambs.) equipped with dual flame ionization detectors was used for the determination of all non-gaseous fermentation products. For gases, a series 104 gas chromatograph (Pye Unicam Ltd.) equipped with a thermal conductivity detector and a gas sample valve was used.

(b) Column Packings

All of the chromatograph packings used were obtained from Phase Separations Ltd., Queensferry, Clywd, and were packed into either 1 metre or 1.5 metre long glass columns of 2 mm bore.

(c) Product Estimations

The volume of each liquid sample injected into the chromatograph was 1 μL. For gases the volume of sample injected was 1 ml. Determinations of product concentration were made on the basis of a comparison of the peak height of the sample with the peak height of a standard. Aqueous solutions of standards were prepared at concentrations between 0.05 and 0.1% v/v or w/v. For gases a calibration gas mixture (BOC Special Gases, Morden, Surrey), comprising 2% carbon dioxide and 2% hydrogen was used.

(d) Sample Preparation

Aqueous fermentation samples were prepared as described in Sub-section 2.4.2.

(e) Methylation Procedure

The technique employed for methylating culture broth samples for the determination of acidic fermentation products was identical to that of Bohannon *et al*. (1978): 2 ml of sample were combined with 2 ml of methanol and 0.75 ml of 50% (v/v) aqueous sulphuric acid in 1 oz. screw-capped bottles that were subsequently incubated in a water bath at 50°C for 30 minutes. After cooling in a refrigerator at 4°C for approximately 30 minutes, 1 ml of deionized water and 0.5 ml of chloroform were added to each tube for extraction by vigorously shaking for 5 minutes. Methylated samples were stored at 4°C until required.

3.2.2 Chromatographic Procedures

The chromatographic procedures used in this work for the detection and quantification of gaseous, neutral and acidic fermentation products are summarized in Tables 3.1 to 3.4.

Analysis	Column Packing	Carrier Gas	Carrier Gas Flowrate (mls/min)	Column Temp. °C	Detector Current mA
Carbon Dioxide	POROPAK Q	Helium	40	30	100
Hydrogen	POROPAK Q	Nitrogen	40	30	100
Ethanol & Water in Nitrogen	POROPAK Q	Helium	50	2 mins at 30°C increased at 32°C/ min to 180°C.	100

Table 3.1 Operating Conditions for Various Gas Chromatographic Analyses.

Method	Column Packing	Column Length m	Nitrogen Carrier Flowrate ml/min.	Injector Temp. °C	Detector Temp. °C	Column Temp. °C	Internal Standard
A	10% DEGA 2% Phosphoric Acid on Diatomite C	1.0	35	225	250	100	-
B	Poropak PS	1.5	35	225	250	135	-
C	Poropak QS	1.5	50	225	250	175	Isopropanol

Table 3.2 GC Methods for Ethanol Determination

Analysis	Column Packing	Nitrogen Carrier Flowrate ml/min.	Injector Temp. °C.	Detector Temp. °C.	Column Temp. °C.	Retention Time Min.
Acetone	POROPAK Q	25	120	150	100	17
Butanol	POROPAK Q	25	120	150	100	39
Ethyl Acetate	POROPAK R	25	190	225	175	1.83
Acetal-dehyde	POROPAK R	25	190	225	175	0.39

Table 3.3 Retention Times and Operating Conditions for the Gas Chromatographic Detection of Non-Acidic Fermentation Products.

Column Packing		Column Temperatures (°C)	Injector Temperature (°C)	Detector Temperature (°C)	Nitrogen Flowrate (ml/min)
Poropak R		160	300	350	25
Poropak Q		160	300	350	25
Chromosorb W		160	300	350	25
10% DEGA and 2% Phosphoric Acid on Diatomite C	Method I	1 min. at 20°C increasing to 100°C at 32°C/min.	150	200	30
	Method II	1 min. at 20°C increasing to 130°C at 16°C/min.	225	250	20

Table 3.4 Column Packing and Operating Conditions for the Gas Chromatographic Separation of Acidic Fermentation Products

3.3 Results

3.3.1 Ethanol

Ethanol determinations were initially carried out using a Diatomite C column. The discovery that the Diatomite column could be used for ethanol determinations was only made after it had been extensively employed for the determination of acidic fermentation products.

Sharp and reproducible ethanol peaks were routinely obtained under isothermal operating conditions. With time, however, a heavy deposit of carbon and tar-like material built up on the inside of the column and caused a partial discoloration of the column packing. As this residue threatened to become deposited on the detector (which would then require dismantling and cleaning) the glass column was emptied of packing, thoroughly cleaned and then re-packed with a new batch of packing. The new column packing behaved differently under conditions which had been used routinely with the first batch of packing. Poor peak reproducibility was manifested and it became apparent that the water component of the injected samples was eluting from the column irregularly. Two explanations could account for this. First, the repeated prior use of the column for the determination of acidic fermentation products may have modified the characteristics of the packing in some way, making it fortuitously suitable for ethanol determination, or secondly, the physico-chemical characteristics of the two batches of packing were different. Diatomite C is a naturally occurring substance and the material marketed by suppliers of chromatographic packings comes from a variety of sources (D.B. Drucker, personal communication). Ethanol determinations for experiments 151 to 176 were made using the Diatomite C packing (Method A in Table 3.2). Ethanol determinations for continuous culture experiments 183 and 185 were made using a Poropak PS colunn (Method B). Ethanol determinations for the remainder of the experiments were made using a Poropak QS column (Method C) which gave excellent reproducibility.

3.3.2 Gaseous Fermentation Products

Fig. 3.1a shows the chromatogram obtained on injecting a carbon dioxide-nitrogen-hydrogen mixture into a Poropak Q column operating under conditions identical to those used by Cross; whilst good separation of carbon dioxide and nitrogen was achieved, hydrogen was not resolved. The absence of a detector response to hydrogen was attributed to the similarities between the thermal conductivities of

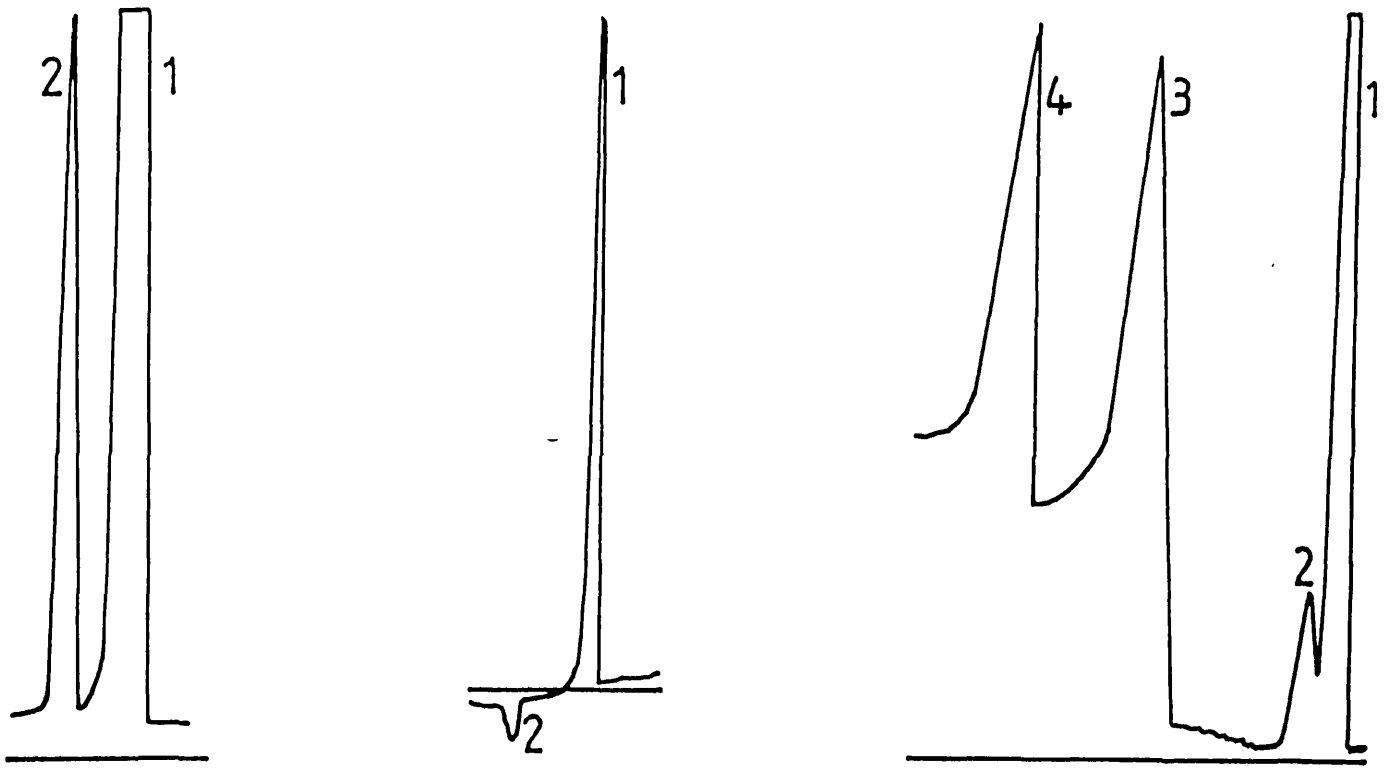


Fig. 3.1 Gas Chromatographic Resolution of Gases and Volatile Products on Poropak Q. (a) 1. Nitrogen 2. Carbon Dioxide T=30°C, Helium Carrier. (b) 1. Hydrogen 2. Carbon Dioxide T=30°C, Nitrogen Carrier (c) 1. Nitrogen 2. Carbon Dioxide 3. Water 4. Ethanol Temperature programmed 2 min at 30°C increasing to 180°C at 32°C/min, Helium Carrier.

hydrogen and the helium carrier gas. With nitrogen as carrier gas (Fig. 3.1b) hydrogen was clearly resolved whereas carbon dioxide was only poorly resolved. Moreover, the nitrogen present in the gas mixture was not 'seen' by the detector.

It was therefore decided to use helium as the carrier gas for the determination of carbon dioxide, and nitrogen as the carrier gas for the determination of hydrogen (see Table 3.1).

Gas chromatographic analyses of fermenter exit gases were performed only during continuous fermentation experiments in which steady state was maintained for a period of several hours. It took between 15 and 20 minutes to change the carrier gas supply to the chromatograph from nitrogen to helium or vice-versa and to allow equilibration of the detector. As this was negligible in comparison to the period over which steady state was maintained, analyses of fermenter exit gas with both nitrogen and helium as carrier performed within approximately 20 minutes of one another were taken as having been obtained simultaneously.

A modification of the techniques described above was used to detect traces of ethanol and water which may have become volatilized into the exit gas (see Table 3.1 and Fig. 3.1c).

3.3.3 Acidic Fermentation Products

Fig. 3.2a shows the chromatogram obtained on injecting a water 'blank' (distilled water subjected to the methylation procedure) onto the Poropak R column at 160°C. The peak labelled '2' is probably ethanol which is present in all grades of chloroform (including the analytical standard) as stabilizer.

Fig. 3.2b shows the chromatogram obtained on injecting a methylated mixture of formate, acetate and lactate under the same conditions. Although good separation of methyl acetate and methyl formate was achieved, methyl lactate was not resolved. There was no detector response to methyl lactate even 30 minutes after the elution of the chloroform peak, (the recorder output during these 30 minutes has been omitted from Fig. 3.2b). The peak labelled '1' is an unidentified impurity present in formate. Similar results were obtained with Poropak Q and Chromosorb W and consequently, a fourth packing, 10% DEGA and 2% Phosphoric acid on Diatomite C, as used by Drucker (1970), was investigated.

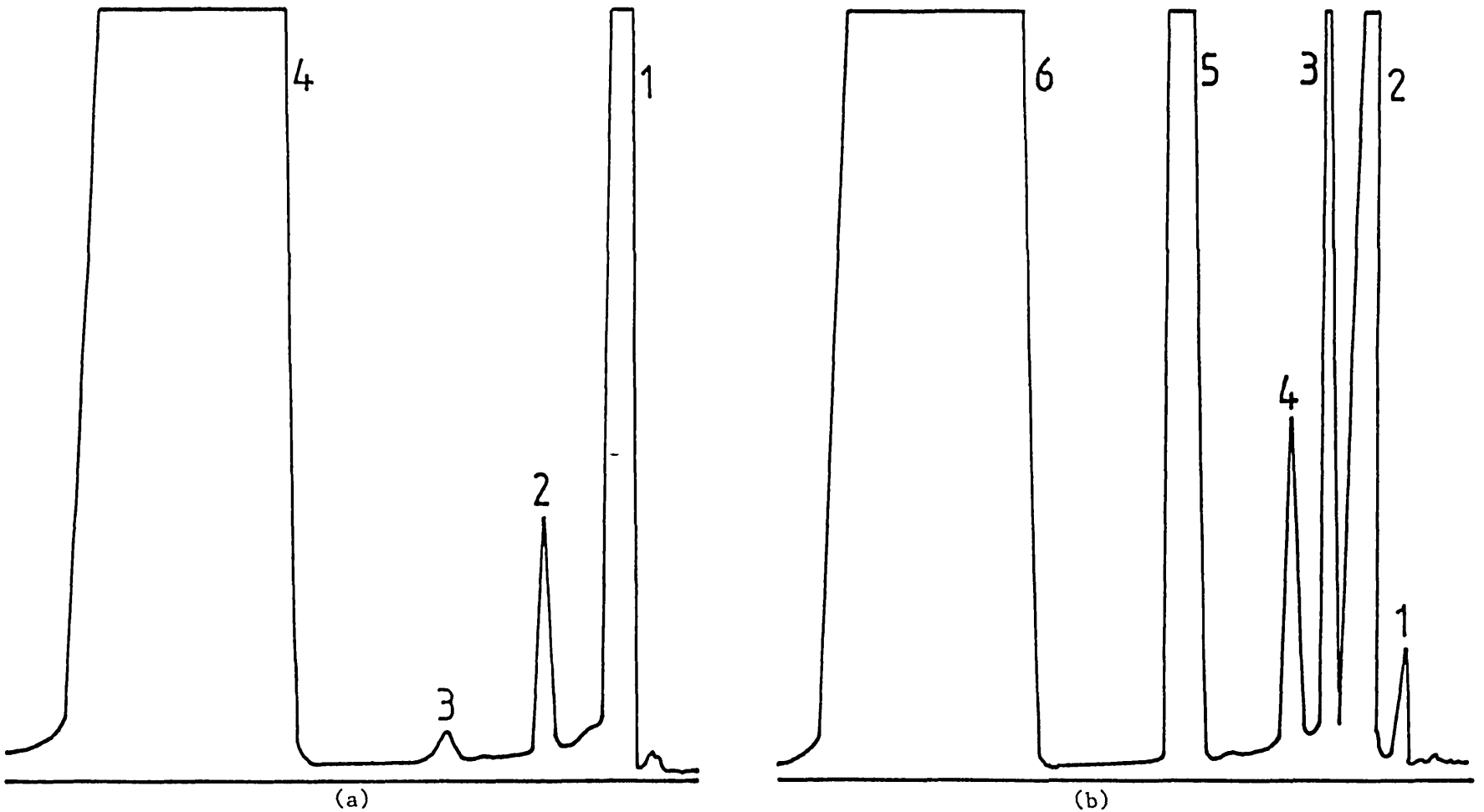


Figure 3.2 Gas Chromatographic Resolution of Methyl Esters on Poropak R at 160°C (a) (Blank) 1. Methanol 2. Unknown 3. Unknown 4. Chloroform. (b) 1. Unknown 2. Methanol. 3 Methyl Formate 4. Unknown. 5 Methyl Acetate 6. Chloroform.

Fig. 3.3a (reproduced from Drucker, 1970) shows the resolution of a mixture of a number of methyl esters of fatty acids. Although good separation was achieved, the broad asymmetric lactate peak would make quantitative estimation of the acid difficult. In contrast to Ducker's methylation procedure, the technique adopted here involved extraction of the methyl esters into chloroform as a final stage (see Sub-section 3.2.1). In order to establish whether chloroform would co-elute with any of the compounds of interest, a mixture of methyl formate, methyl acetate and methyl lactate was injected onto the Diatomite column at the operating temperature (56°C) advocated by Drucker, but with an increased carrier gas flow rate (50 ml/min) in order to reduce the retention time of methyl lactate.

Fig. 3.3b is the chromatogram obtained. Satisfactory separation of methyl formate, methyl acetate and chloroform was achieved. However, the increased carrier gas flow rate was of little effect in reducing the retention time of methyl lactate which eluted from the column 15.9 minutes after injection of the sample. An increase in the column temperature to 100°C (Fig. 3.4a) resulted in a lower retention time for methyl lactate of 3.45 minutes but in a loss of resolution between methyl acetate and chloroform.

The final strategy adopted was to employ a 'programmed' increase in temperature; a low initial temperature would promote good separation of the more volatile esters from each other and from chloroform whereas the higher temperatures at the end of the temperature programme would result in lower overall sample retention times and a 'sharpening' of the peaks of the less volatile esters.

A delay of 1 minute at 20°C followed by an increase at the rate of 32°C/min. to a final temperature of 100°C resulted in the chromatogram shown in Fig. 3.4b. Methyl formate and methyl acetate are well resolved and the retention time of methyl lactate is increased slightly to 4.8 mins.; however, the peak still retains a relatively large height:base ratio. Further investigations showed that still better separation could be achieved by decreasing the carrier gas rate to 30 ml/min. Using this method (Method I, Table 3.4) formate, acetate and pyruvate could be reliably determined with an accuracy of ± 5%. However the precision was not as good for lactate and lactate determinations were performed enzymatically (see 2.4.4). With time it became possible to identify high molecular weight acids not considered by Drucker (1970) and Figure 3.5a shows the separation of a mixture of 7 methyl esters.

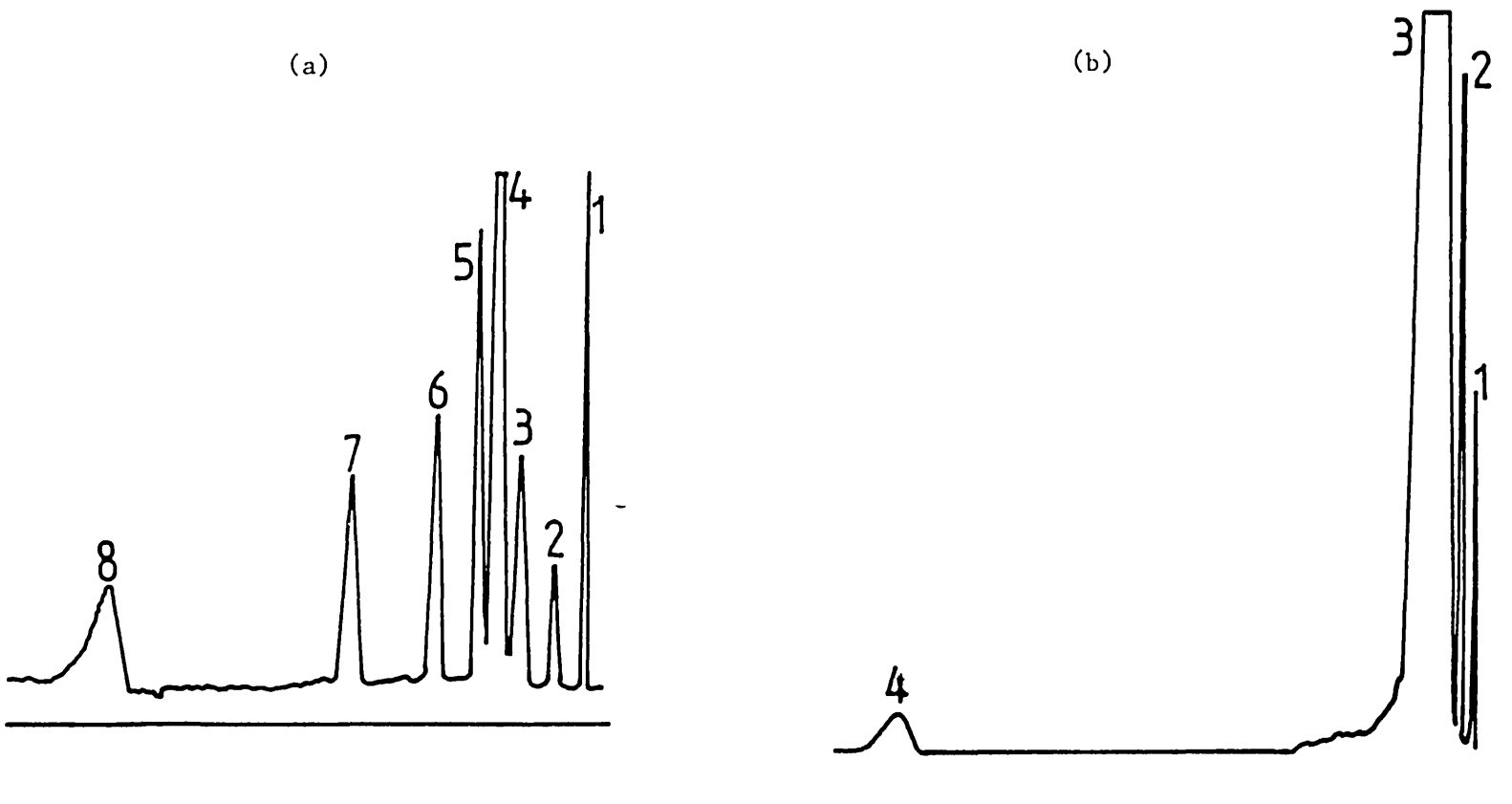


Figure 3.3 Gas Chromatographic Resolution of Methyl Esters on 10% DEGA and 2% Phosphoric Acid on Diatomite C at 56°C (a) (Reproduced from Drucker, 1970) Methylation with methanol-boron trifluoride. 1 Reagent 2. Methyl Formate 3. Methyl Acetate 4. Methanol 5. Methyl Butyrate 6. Methyl Valerate 7. Methyl Hexoate 8. Methyl Lactate (b) Methylation with methanol-sulphuric acid. 1 Methyl Formate 2. Methyl Acetate 3. Chloroform + Methanol 4. Methyl Lactate.

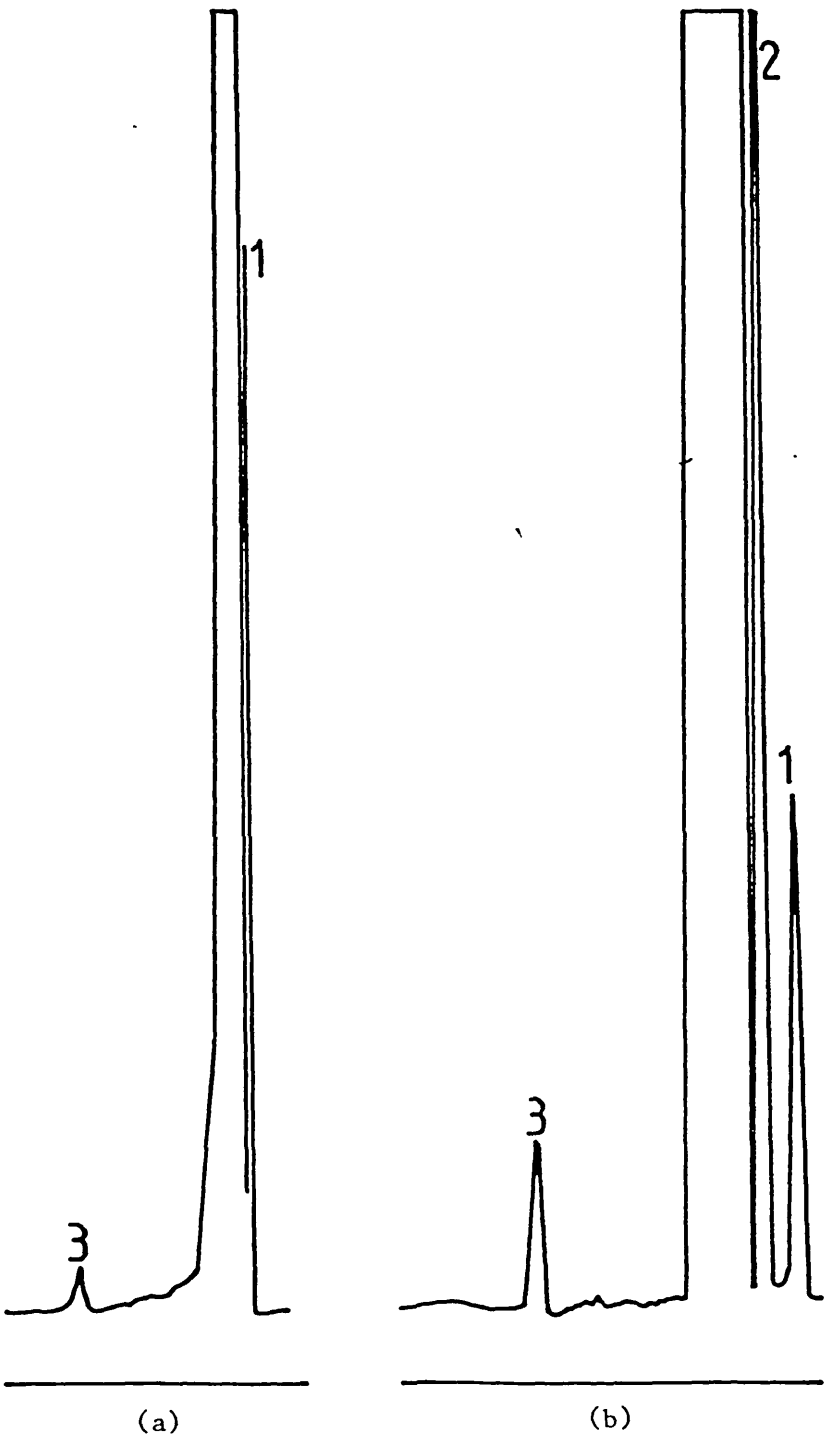


Figure 3.4 Gas Chromatographic Resolution of Methyl Formate (1) Methyl Acetate (2) and Methyl Lactate (3) on 10% DEGA and 2% Phosphoric Acid on Diatomite C (a) at 100°C (b) Temperature programmed, 1 min at 20°C increasing to 100°C at 32°C/min.

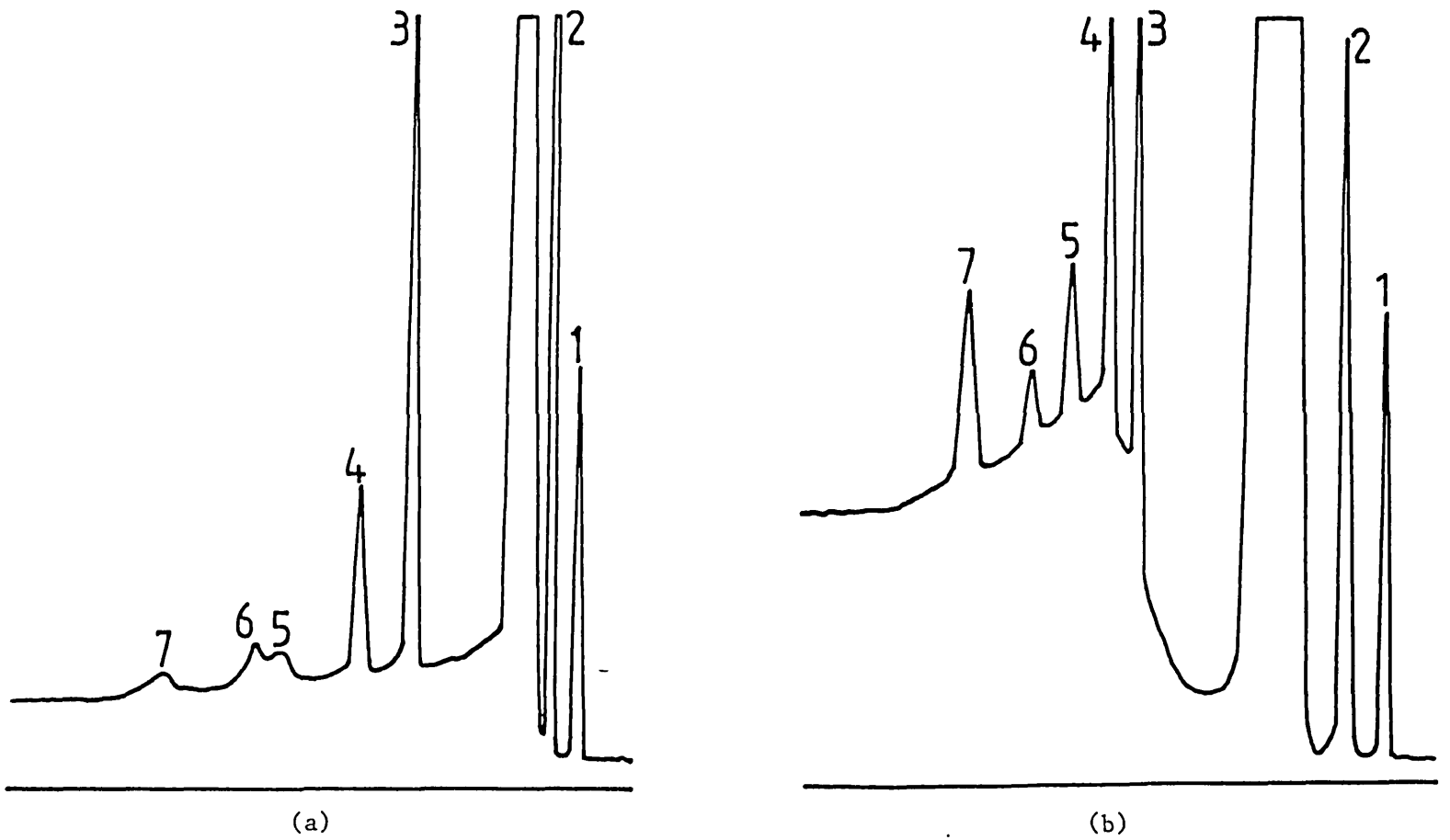


Figure 3.5 Gas Chromatographic Resolution of Methyl Formate (1) Methyl Acetate (2) Methyl Pyruvate (3) Methyl lactate (4) Methylated Dimer of Pyruvate (5) Methyl Propionate (6) Methyl Butyrate (7) on 10% DEGA and 2% Phosphoric Acid on Diatomite C. (a) Temperature Programmed, 1 min at 20°C increasing to 100°C at 32°C/min carrier flowrate 30 ml/min. (b) Temperature Programmed, 1 min at 20°C increasing to 130°C at 16°C/min carrier flowrate 20 ml/min.

It was later discovered that still better resolution could be achieved by decreasing the carrier gas rate to 20 ml/min. and changing the temperature programme to 1 min. at 20°C followed by an increase at the rate of 16°C/min. to 130°C (Method II). The improved results obtained using this method are shown in Figure 3.5b. Under these conditions all the acidic products, including lactate and succinate, could be determined with an accuracy of ± 8%. This slight decrease in accuracy for the determinations of formate, acetate and pyruvate was more than compensated for by the ability to perform acceptable lactate and succinate determinations.

The retention times of a number of methyl esters relative to methyl lactate (retention time 9.19 mins.) obtained under the modified operating conditions are listed in Table 3.5. Figs. 3.6 to 3.8 have been included to illustrate the versatility of the method developed in this work.

Figs. 3.6a and b show the product 'spectra' of *B. stearothermophilus* 11d-15. The chromatogram of Fig. 3.6a was obtained by following procedure II in Table 3.4. In Fig. 3.6b, however, the chloroform layer containing the dissolved methyl esters was concentrated by partial evaporation prior to analysis. This was done in order to reveal whether products not previously detected were present in the chloroform extract. Although none were revealed, the procedure could be of use in detecting fermentation products present in trace amounts.

Figs. 3.7a, 3.7b and 3.8a show the methyl esters of acids produced by *B. stearothermophilus* NCA 1503, *C. thermocellum* and *E. coli* respectively. Fig. 3.8b was obtained by injecting a methylated sample of natural yoghurt formed by the action of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* on skimmed milk.

3.4 Conclusion

The GC technique developed in this work has been published, Drummond and Shama (1982), and permits the rapid resolution of the methyl esters of a large number of commonly encountered acidic fermentation products on a single column. Alternative methods of resolving a comparable range of methyl esters require either the use of more than one column (Bohannon *et al.*, 1978) or the use of capillary columns. Whilst the latter offer the prospect of achieving high resolution of both acidic and neutral fermentation products,

Acid	Retention Times Relative To Methyl Lactate
Formic	0.156
Acetic	0.283
Pyruvic	0.903
Lactic	1.000
Oxalic	1.156
Propionic	1.251
Methyl Malonic	1.271
Iso-Butyric	1.297
Malonic	1.396
N-Butyric	1.464
Fumaric	1.491
Iso-Valeric	1.556
Succinic	1.687
N-Valeric	1.831

Table 3.5 Retention Time Data* of Methyl Esters of Volatile and Non-Volatile Acids (R.T. of Methyl Lactate = 9.19 mins.).

*Operating Conditions:-
Temperature Programme: 1 min at 20°C increasing to 130°C at 16°C/min. Carrier flowrate 20 ml/min.

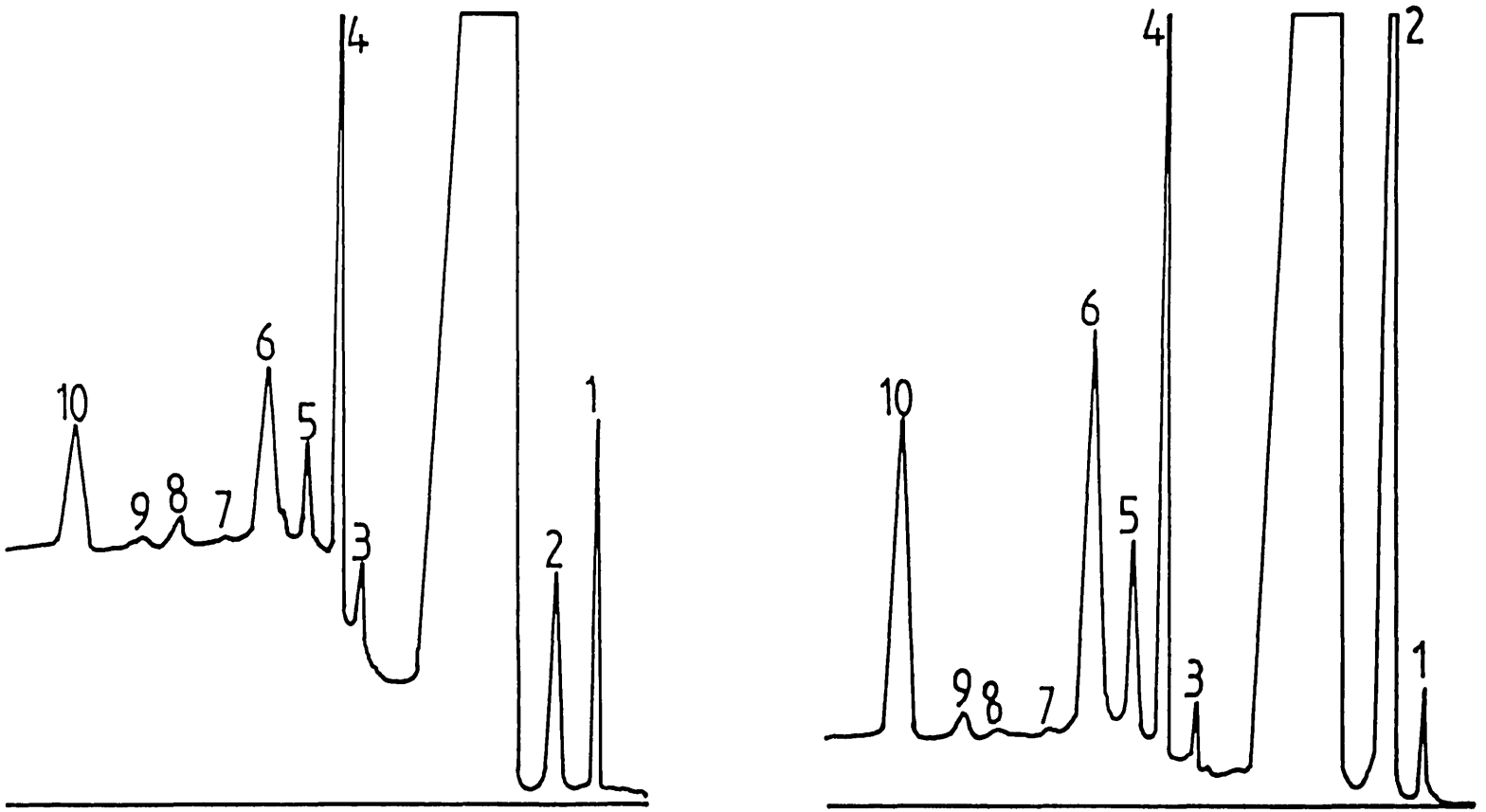


Figure 3.6 Methyl Esters of Acids Produced by B. stearothermophilus 11d15 (conditions as in Fig. 3.5b)
1. Methyl Formate 2. Methyl Acetate 3. Unknown 4. Methyl Pyruvate 5. Methyl Lactate
6. Dimethyl Oxalate 7. Methyl Propionate 8. Dimethyl Malonate 9. Dimethyl Fumarate
10. Dimethyl Succinate. (a) Direct injection of chloroform layer as described in Section 3.2.
(b) Chloroform layer concentrated prior to injection.

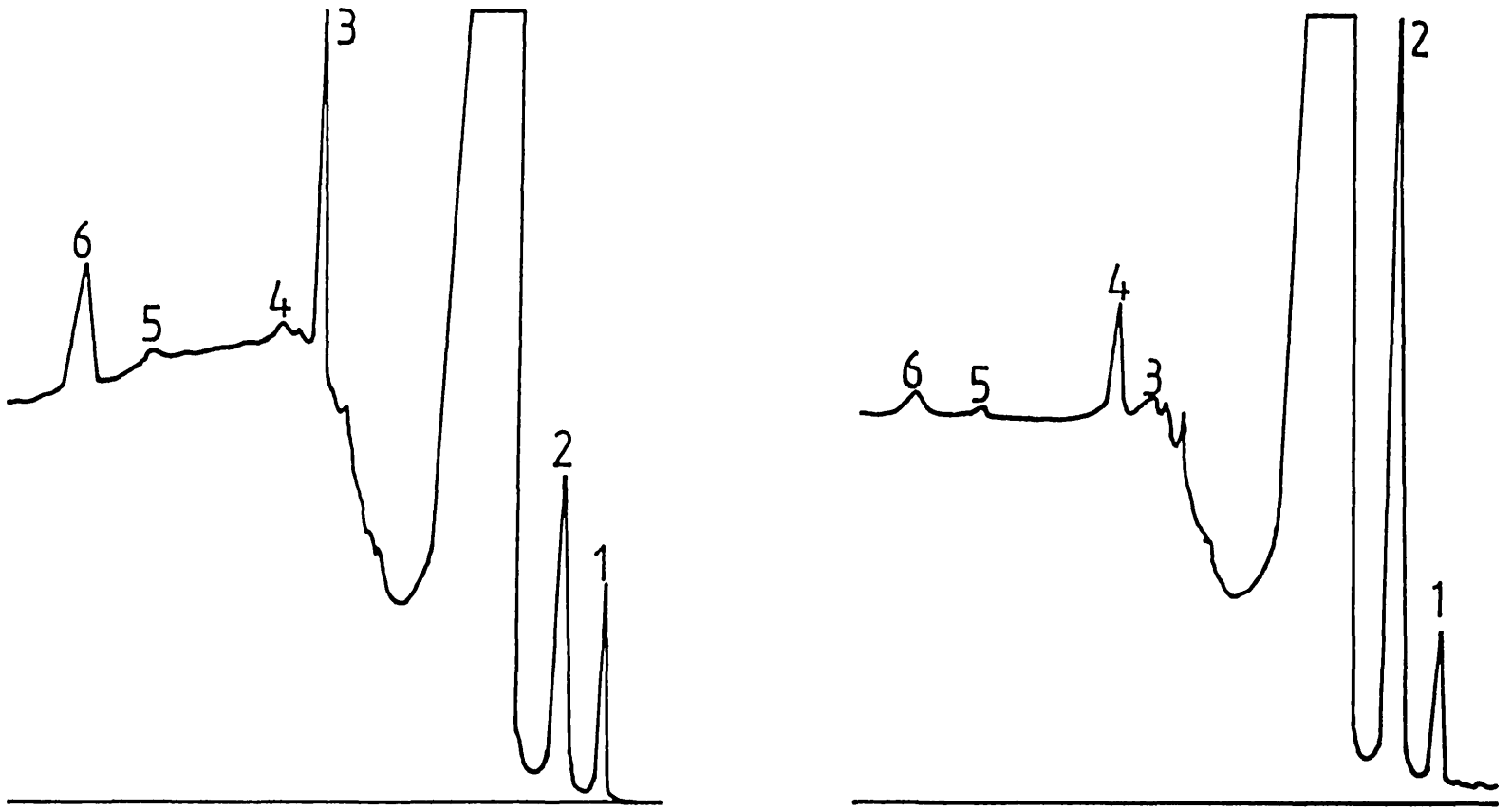


Figure 3.7 (a) Methyl Esters of Acids Produced by B. stearothermophilus NCA 1503 (Conditions as in Fig. 3.5b) 1. Methyl Formate 2. Methyl Acetate 3. Methyl Lactate 4. Dimethyl Oxalate 5. Dimethyl Fumarate 6. Dimethyl Succinate (b) Methyl Esters of Acids Produced by C. thermocellum 1. Methyl Formate 2. Methyl Acetate 3. Methyl Lactate 4. Dimethyl Oxalate 5. Dimethyl Fumarate 6. Dimethyl Succinate.

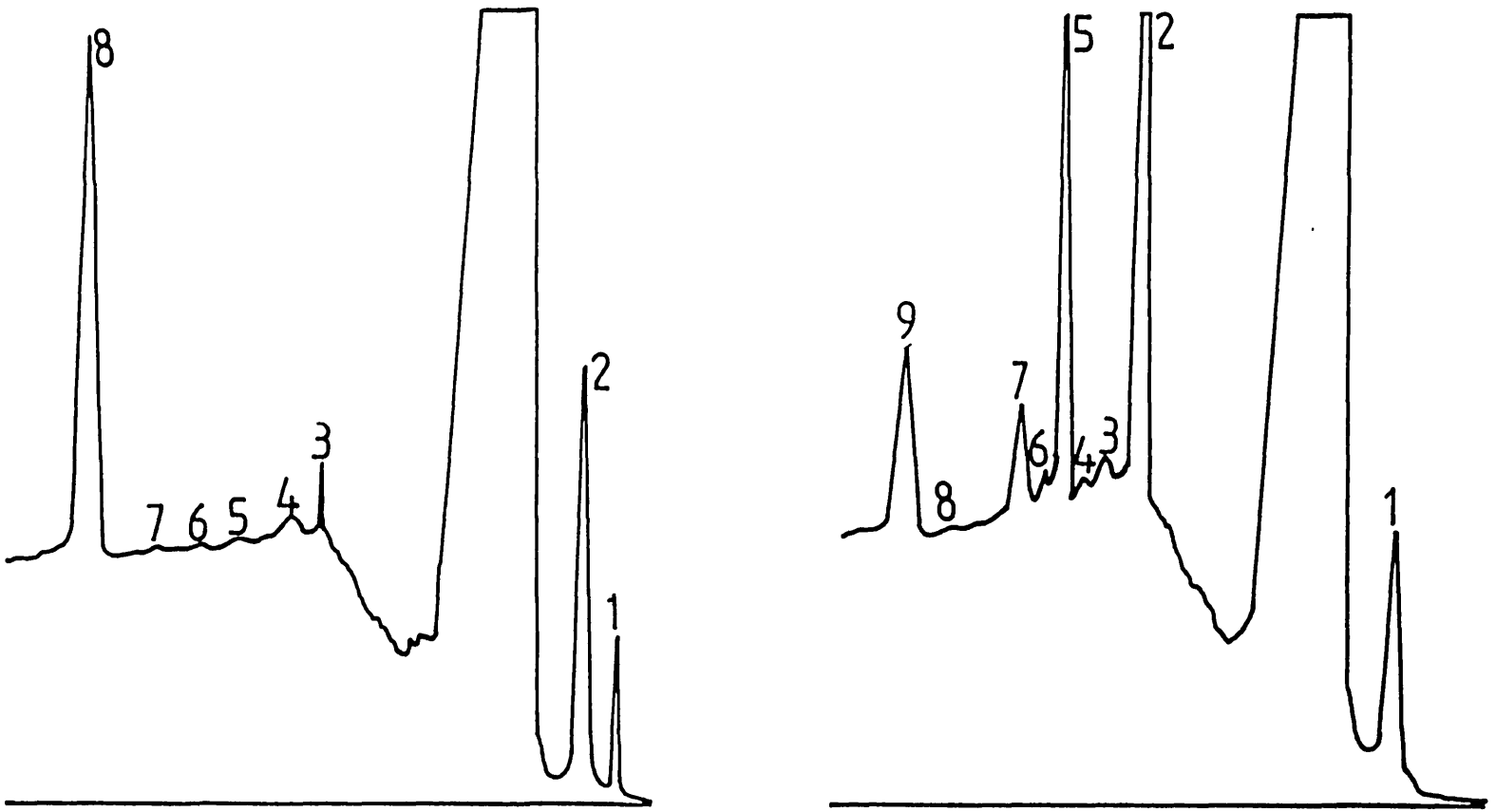


Figure 3.8 (a) Methyl Esters of Acids Produced by E. coli. (Conditions as in Fig. 3.5b). 1. Methyl Formate 2. Methyl Acetate 3. Methyl Lactate 4. Unknown 5. Methyl Propionate 6. Dimethyl Malonate 7. Dimethyl Fumarate 8. Dimethyl Succinate (b) Methyl Esters of Acids Occurring in a Natural Yoghurt. 1. Methyl Acetate 2. Methyl Lactate 3. Dimethyl Oxalate 4. Unknown 5. Methyl Propionate 6. Methyl Isobutyrate 7. Dimethyl Malonate 8. Dimethyl Fumarate 9. Dimethyl Succinate.

(Drucker, 1981b) the relatively lower cost and greater durability of packed columns make the technique described in this work competitive with this development.

CHAPTER 4

NUTRIENT REQUIREMENTS OF B. stearothermophilus

4.1 Nutritional Experiments on B. stearothermophilus

The growth requirements of thermophilic bacilli, and in particular B. stearothermophilus, have been the subject of much study. Cleverdon et al. (1949) investigated the vitamin requirements of 12 strains of B. stearothermophilus. Using a vitamin-free casein hydrolyzate medium they found that growth was stimulated in all 12 strains by nicotinic acid, biotin and thiamine both at 55 and 65°C. However, 5 of the strains required only biotin and nicotinic acid, 2 required only biotin, and the remaining 5 - which included B. stearothermophilus NCA 1503 - had an absolute requirement for all 3 vitamins. With the exception of one strain no effect of temperature on vitamin requirements was found.

As part of a study aimed at elucidating the molecular nature of thermophily, Campbell and Williams (1953) examined the growth requirements of obligately and facultatively thermophilic strains of B. coagulans and of B. stearothermophilus. Their study included strains examined by Cleverdon and his co-workers. A comparison of nutritional requirements specified by the two groups of workers shows discrepancies - Campbell and Williams claimed that strain NCA 1503 had an absolute requirement for nicotinic acid and biotin (and also the amino acid valine) irrespective of growth temperature. The situation was further complicated by Baker et al. (1960) who claimed that strain NCA 1503 displayed a requirement for either vitamin B_{12} or methionine.

One methodological weakness common to all the studies referred to above was that cultures were incubated statically. This results in poor aeration and conditions may even arise where the oxygen availability becomes so low that a change occurs from aerobic to anaerobic metabolism. For facultative anaerobes such as B. stearothermophilus anaerobic growth requirements are generally more extensive than aerobic ones. Failure therefore to maintain a constant supply of oxygen to the cultures may, in part at least, explain the disparities in the observed growth requirements.

Rowe et al. (1975) published details of defined and minimal media capable of supporting the growth of strains NCA 1503 and NCA 1508. They criticised earlier studies for failing to account

for nutrient carry-over with inocula and claimed that the increases in absorbance reported in these studies were partly the result of sporulation rather than cell growth. In their experiments Rowe and his co-workers eliminated the effects of nutrient carry-over with inocula and ensured that cultures were vigorously aerated. Both strains of B. stearothermophilus grew well in a medium containing 20 amino acids, biotin, thiamine and nicotinic acid. They found that manganese, not deliberately added to previously formulated media, stimulated growth. They also claimed that of the 20 amino acids present in their defined medium only glutamate, histidine, iso-leucine, methionine and valine were absolute requirements of strain NCA 1503. The claim made for manganese was supported by Sargeant et al. (1975) who published details of a complex medium for NCA 1503 which included manganese.

At about the same time Atkinson et al. (1975b) claimed to have grown NCA 1503 in continuous culture in a sucrose ammonium chloride medium supplemented only with biotin and minerals. Atkinson's work contained few experimental details but he later revealed (personal communication) that his results had been achieved using a 'medium shift' technique. This is a technique in which an organism growing in a chemostat is first supplied with a complex growth medium which is then gradually diluted with minimal growth medium until all traces of the former have been washed but and the organism is growing solely on minimal medium. An attempt to culture strain 11d-15 by this method is described below. Identical growth conditions to the ones used by Atkinson and his co-workers were adopted, and as they had cultured their strain aerobically, it was decided to do similarly with 11d-15 and to determine subsequently whether anaerobic growth requirements were different.

4.2 Medium Shift Experiment

The task of constructing fermentation balances for a micro-organism is greatly facilitated by using a defined medium. The use of an undefined medium containing many complex constituents, some of which may serve both as sources of carbon and nitrogen, may lead to uncertainties in interpreting product yields or in elucidating the pathways of energy metabolism.

The ability to culture B. stearothermophilus 11d-15 in a medium such as Atkinson's in which sucrose serves both as principal carbon source and sole energy source, in which ammonium chloride

serves as principal nitrogen source and in which all remaining nutritional requirements are met by biotin and minerals, would be particularly useful.

Figure 4.1 shows the results of culturing strain 11d-15 in a chemostat in which complex BST medium was gradually replaced by minimal MST medium according to the strategy of Table 4.1. A gradual decrease in cell density is apparent over days 1 to 13 followed by a dramatic washout on day 14.

4.3 Conclusion

The cause for the dramatic drop in cell density cannot be stated with certainty. The most likely explanation is that during the process of diluting the complex medium, one or more nutrients became growth limiting and no longer capable of sustaining growth. According to Monod's model of microbial growth kinetics, a region exists in which growth rate is more or less a linear function of the concentration of the growth limiting nutrient. Growth rate in a chemostat is determined by the dilution rate, however if the concentration of a growth-limiting nutrient decreases to such a level where the organisms' growth rate falls below that of the dilution rate washout of the culture occurs. A second possibility is that phage infection occurred. Although phages infecting thermophiles are known (Ljungdahl, 1979) this seems an unlikely cause of the washout as such phenomena have not previously been reported, and were not encountered elsewhere in this work.

Although the situation regarding the true growth requirements of B. stearothermophilus NCA 1503 is confused, the claim made by Atkinson et al. to have grown this strain in minimal medium supplemented only with biotin is difficult to explain. If sustained growth was maintained it might be argued that a selection pressure had been applied for a variant requiring sucrose and biotin as sole organic nutrient requirements and that such a variant had arisen through spontaneous mutations but even that seems improbable.

There is no reason to suppose that the growth requirements of strain 11d-15 are different from those of strain NCA 1503 from which it was derived. S. Amartey (personal communication) attempted to grow the former aerobically in a vitamin-free casein hydrolyzate supplemented with glucose, thiamin, biotin and nicotinic acid, the level of casein corresponding to the concentrations of individual

Time hr.	Dilution Factor
0 - 14	1
15 - 58	3.87×10^{-1}
59 - 102	5.81×10^{-2}
103 - 146	8.71×10^{-3}
147 - 190	1.31×10^{-3}
191 - 234	$1.96 - 10^{-4}$
235 - 278	2.94×10^{-5}
279 - 322	4.41×10^{-6}
323 - 366	6.61×10^{-7}

Table 4.1 Medium Dilution Strategy

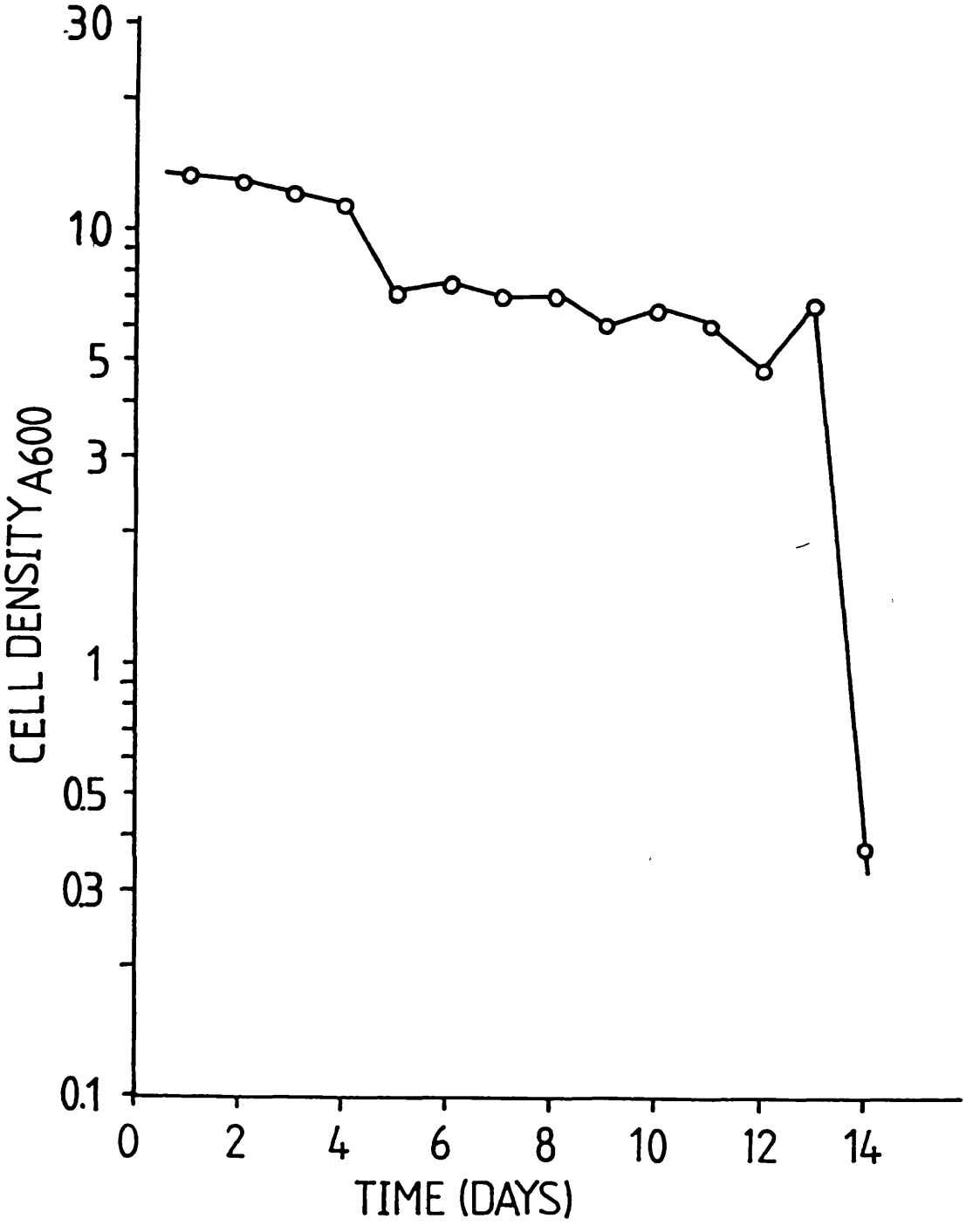


Figure 4.1 Cell Density against Time During Medium Shift Experiment

Fermenter: - Modified 5 litre New Brunswick, Working Volume: 2.0 litres. Operating Conditions:- Continuous, Aerobic. Dilution Rate: 0.2 hr^{-1}. Air flowrate to sparger: 2.0 litre/min. Impeller speed 350 rpm. pH controlled by the addition of 30% KOH soln. Antifoam: Polyglycol P2000. Medium: Gradual Shift from BST to MST (See Table 4.1) Inoculum: 3% (v/v) of fermenter working volume. Feed sucrose concentration: 10 g/l T: 60°C. pH: 7.0

amino acid published by Rowe et al. (1975). The results were unsatisfactory. If judged solely on the basis of the drop in medium pH - a criterion used by Cleverdon et al. (1949) then good growth had been achieved; however the cells had conglomerated to form a single mass, the cause of which was not determined.

The trend in industrial fermentations towards continuous culture was mentioned in Chapter 1 and in view of the industrial potential which B. stearothermophilus has for ethanol production, it might be more pertinent to conduct future nutritional studies on strain 11d-15 under continuous culture conditions and in this connection a recently described technique shows promise: Kuhn et al. (1979) used a continuous culture technique to design a defined medium for the thermophile B. caldotenax. The chemostat was supplied with a medium containing nutrients at concentrations such as to sustain a low level of growth. After steady state had been established individual amino acids, vitamins and minerals were injected into the chemostat: if the injection of a compound caused an increase in cell density, then its concentration in the incoming medium was permanently increased. If, on the other hand, injection of a particular compound did not cause an increase in cell density then the concentration of that compound was decreased in the incoming medium or eliminated from it altogether.

CHAPTER 5

GROWTH AND PRODUCT FORMATION IN BATCH CULTURE

5.1 Introduction

The first objective of the strategy given earlier in Chapter 1 for selecting high ethanol yielding strains of B. stearothermophilus was accomplished by Payton and Hartley (1985) who isolated mutants defective in LDH. These workers were however unsuccessful in obtaining PFL^- derivatives of these strains. In this chapter attempts are described to increase the yields of ethanol produced by strain 11d-15 (a mutant in LDH) by manipulating environmental conditions. Experiments were performed in batch fermenters under pH controlled conditions and the variables examined were pH, temperature and initial sucrose concentration. Data from these experiments is displayed in graphical form with the concentrations of fermentation products and biomass plotted against time. From this data are derived product yields, carbon recoveries, O/R indices (the ratio of the oxidation states of oxidized to reduced fermentation products), specific growth rates and specific ethanol productivities.

Although no measurements of carbon dioxide evolution were made in the initial experiments, a method of estimating carbon dioxide yields is presented. Two further batch experiments were conducted in which carbon dioxide measurements were made and in which the spent culture broths were subjected to a more complete GC analysis.

Complex BST medium was used in the experiments described in this chapter as it was known to support the growth of B. stearothermophilus 11d-15. At the lowest concentration of sucrose used here -23.5g/litre - sucrose contributes approximately 44% of the total carbon content of the medium and tryptone and yeast extract the remaining 56%. The assimilation of individual medium components by cultures is however not determined solely on the basis of their elemental composition: Stanier et al. (1971) stated that for complex sugar-containing media for chemoheterotrophs, the production of biomass is primarily at the expense of amino acids, peptides and other complex constituents of the medium whilst energy for biosynthesis is derived from the sugar substrate.

Two sets of experiments were performed to test this possibility. The first involved the use of radioactively labelled isotopes and

sought to compare the uptake of tritiated amino acids and ^{14}C-labelled sucrose by B. stearothermophilus 11d-15. This experiment also permited an estimate to be made of the proportion of cell carbon derived from sucrose. In the second, attempts were made to separate the process of fermentation from that of growth and examine the former in isolation by using washed cell suspensions.

5.2 Methods

5.2.1 The Uptake of Radioisotopes

One batch fermentation was conducted in which the relative uptake of radioisotopes by B. stearothermophilus 11d-15 was investigated. The radioisotopes were obtained from Amersham International plc, Amersham, Bucks. In this experiment 250 μCi of uniformly labelled ^{14}C sucrose (specific activity 552 μCi/μmole) were used in conjunction with 250 μCi of a tritiated amino acid mixture of the following composition and activity.

Composition of mixture by activity 1 mCi of the mixture contains		Specific activity of component amino acids	
L-[2,3-^{3}H]Alanine	95 μCi	57	Ci/mmol
L-[5-^{3}H]Arginine	70 μCi	10.8	Ci/mmol
L-[2,3-^{3}H]Aspartic acid	100 μCi	34	Ci/mmol
L-[G-^{3}H]Glutamic acid	90 μCi	22	Ci/mmol
[2-^{3}H]Glycine	60 μCi	15	Ci/mmol
L-[2,5-^{3}H]Histidine	15 μCi	50	Ci/mmol
L-[4,5-^{3}H]Isoleucine	60 μCi	111	Ci/mmol
L-[4,5-^{3}H]Leucine	125 μCi	56	Ci/mmol
L-[4,5-^{3}H]Lysine	50 μCi	40	Ci/mmol
L-[4,^{3}H]Phenylalanine	75 μCi	22	Ci/mmol
L-[3,4(n)-^{3}H]Proline	55 μCi	54	Ci/mmol
L-[3-^{3}H]Serine	30 μCi	30	Ci/mmol
L-[3-^{3}H]Threonine	55 μCi	17.5	Ci/mmol
L-[3,5-^{3}H]Tyrosine	60 μCi	51	Ci/mmol
L-[3,4(n)-^{3}H]Valine	60 μCi	38	Ci/mmol

Radioactive concentration : 1.0 μCi/ml.

In order to minimize exposure of the culture to the radioisotopes, B. stearothermophilus 11d-15 was first cultured in a batch fermenter under pH controlled conditions until the cells were in the mid-logarithmic phase of growth; at this point approximately 100 ml of culture were aseptically transferred to a second fermenter which contained medium with radioisotopes. When the cell density in the second fermenter had reached a maximum, a 300 ml sample of culture broth was taken and divided into 50 ml portions; these were then

centrifuged at 25,000g for 15 minutes at 4°C. The supernatant fractions were combined and filtered by passage through a membrane filter of 2 μm pore-size and stored at 4°C. Following this the cells were resuspended in an equal volume of fresh BST medium without radioisotopes and re-centrifuged at 25,000g for 15 minutes at 4°C and stored at -20°C until needed. In order to provide a control, approximately 4 grams of cell paste from a previous experiment (234) were suspended in the filtered supernatant and allowed to stand at 4°C for 30 minutes. This suspension was then centrifuged and washed as described above. In order to estimate the quantity of radioisotopes taken up by the bacterial samples, the cell pastes had first to be solubilized; 0.75 ml of tissue solubilizer (Soluene 350, U.T. Packard, Ltd., Caversham, Berks.) was added to each sample of cell paste in a glass scintillation vial (the actual quantities of cell pastes weighed out are shown in Appendix 2, Section 1), the mixtures were then incubated at 60°C for 2 hours. The cell homogenates were then bleached in order to reduce the effects of colour quenching on counting efficiency. This was achieved by adding 0.15 ml of iso-propanol followed by 0.2 ml of hydrogen peroxide and incubating at 37°C for 15 minutes. Finally, 10 ml of a commercially prepared scintillation 'cocktail' (Insta-Gel, U.T. Packard Ltd.) was added to each vial, the vials were then stoppered and stored overnight in the dark at 4°C. Each sample was then counted twice for 4 minutes using a Kontron Model SL30 counter (Intertechnique Ltd., Portslade, Sussex) with preset channels for ^{3}H and ^{14}C double-counting. Quenching effects were estimated by adding to the samples first ^{14}C, and then ^{3}H standards of known specific activities and recounting after the addition of each standard.

5.2.2 Batch Fermentation Procedures

For the majority of experiments only initial and final sucrose determinations were made, but for experiments 151 and 176 sucrose determinations were performed throughout the course of the experiment. In all experiments where the supply of nitrogen gas was to the fermenter headspace (i.e. experiments in which neither condensers nor U-tube traps were employed) the ethanol concentration data were corrected for evaporative losses as described in Appendix 2, Section 2. In addition, all product data were corrected for the dilution effects of potassium hydroxide solution added to maintain constant pH (see Appendix 2, Section 3). An elemental analysis of batch-cultured cells

of B. stearothermophilus 11d-15 was performed on a cell sample taken at peak cell density during experiment 151:: 25 ml of culture broth was centrifuged at 25,000g for 15 minutes at 4°C, the culture medium supernatant was discarded and the bacterial pellet was washed twice with distilled water. After washing, the cells were resuspended in approximately 10-15 mls of distilled water, transferred to a circular crystallizing dish and dried overnight at 105°C. The cell carbon, hydrogen and nitrogen content was determined using approximately 1 mg of dried cells as described in Sub-section 2.4.6.

Carbon dioxide production was measured continuously during experiments 239, 240, 263 and 264 with infra-red analyzers of the type described in Sub-section 2.4.5. Carbon dioxide produced during fermentation may either pass out of the culture broth with the nitrogen gas supplied to the sparger or may remain chemically bound in the culture broth. This chemical binding could take many forms; carbon dioxide produced as a result of fermentation would leave the cell in hydrated form the nature of which would depend on pH (Holliday et al., 1973). The hydrated species might then react with alkaline fermentation products such as ammonia or with the hydroxyl ions from the potassium hydroxide added during fermentation. The total quantity of carbon dioxide produced is then the sum of bound and evolved carbon dioxide. The method of estimating the former was as follows:- the area below the curve of % carbon dioxide in exit gas against time (see for example Figure A2.29 in Appendix 2) was cut out and weighed. The ratio of the weight of this area to the weight of a rectangle bounding the curve (i.e. of height equal to the maxima of the curve and of base equal to the time over which carbon dioxide measurements were made) was then determined. The quantity of carbon dioxide which this represented was then computed using the Law of Perfect Gases:-

$$pV = nRT$$

(where p, V, T are respectively the pressure, volume and temperature of the gas, n, the number of moles and R, the gas constant).

Multiplying this quantity by the ratio of area weights gave the quantity of carbon dioxide evolved.

In order to determine bound carbon dioxide, the culture broth was acidified to pH4 by the addition of conc. hydrochloric acid and

the carbon dioxide released was recorded against time and determined as described above.

5.2.3 Resting Cell Suspensions

B. stearothermophilus 11d-15 was cultured anaerobically at 60°C at a controlled pH of 6.5 in a plant fermenter of 300 litres capacity in BST medium containing 5% w/v sucrose. Fifty litres of culture medium at peak cell density were then harvested, chilled to 11°C and centrifuged at an approximate rate of 15 litres/hr at 13,000 g on a Model 6 'Supercentrifuge' (Sharples Co. Ltd., Camberley, Surrey). Centrifugation on this scale could not be performed aseptically and consequently the following operations were all conducted under non-aseptic conditions. The cell paste obtained was then resuspended in an equal volume of wash buffer of composition identical to RCM3 but containing no sucrose and centrifuged as described above. The washed cell paste (c. 132 g) was divided into three equal portions. Two of these portions were each resuspended in 2 litres of RCM3 medium at pH 6.5 and 8.0 at 12°C. The third portion was resuspended in 2.0 litres of wash buffer at 12°C at pH 7.3 and constituted a control. The suspensions were then transferred to 5 litre New Brunswick fermenters and the temperature of the water bath was increased from 15°C to 60°C over a 35 minute period. Carbon dioxide production was monitored throughout the course of the experiment and the yields of carbon dioxide were calculated as described in Sub-section 5.2.2.

5.3 Results

5.3.1 Introduction

This section contains data from experiments which were performed over a long period of time and under slightly differing conditions and in order to simplify the treatment of results, data will be considered in the following order: the experiment in which radioisotopes were used (experiment 238) is presented first as the results obtained were subsequently used in carbon recovery calculations. Experiments 151-176 are considered next followed by experiments 239 and 240 and finally, experiments 263 and 264 in which resting cell suspensions were used.

5.3.2 Radioisotope Experiment

Two samples of cell paste from the radioisotope experiment (238)

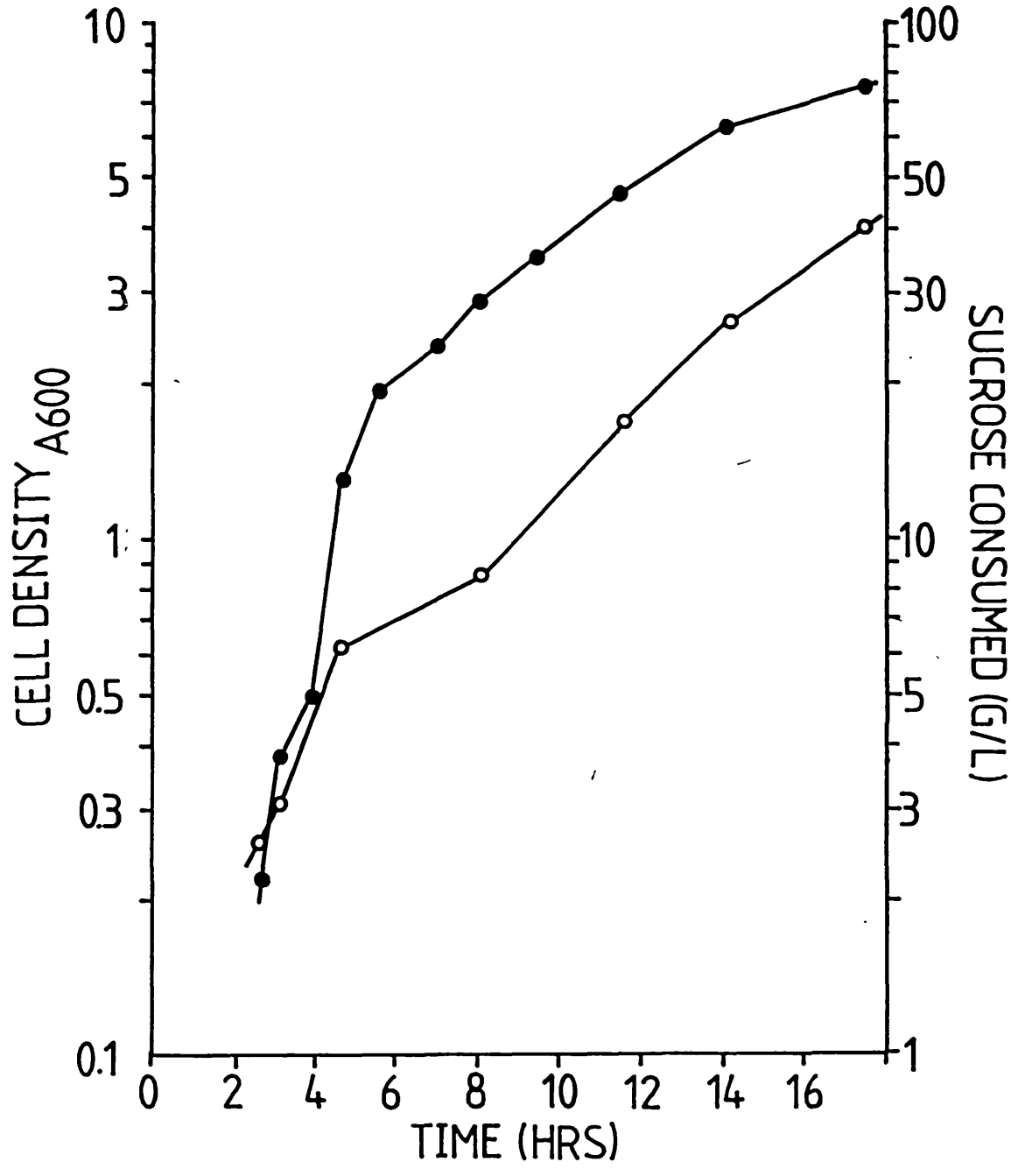


Figure 5.1 Cell Density and Sucrose Consumed Against Time

Experiment 238 (So = 50 g/l, T = 60°C, pH 7.0).
(●) Cell Density, (O) Sucrose Consumed.
Fermenter:- 5 litre New Brunswick, Working Volume: 3.4 litres.
Operating Conditions:- Batch, Anaerobic. Nitrogen flowrate to sparger : 1.0 litre/min. Impeller speed: 200 rpm. pH controlled by the addition of 30% KOH soln. Medium: BST. Inoculum: 3% (v/v) of Fermenter Working Volume

and a single sample of cell paste from experiment 234 (a control) were solubilized and counted. The mathematical treatment of the results are shown in Appendix 2, Section 1. The corrected ratio of ^{3}H to ^{14}C counts for the two samples are 3.84 and 3.31 (mean 3.58). The estimates for the percentage of cell carbon derived from sucrose are 10.4 and 12.3% (mean 11.4%).

Figure 5.1 shows logarithmic plots of cell density and sucrose consumed against time. Over the same time period in which the cell density increased from 0.22 to 6.30 absorbance units, the sucrose consumption was 24.2g. Using the figure of 11.4% as the proportion of cell carbon derived from sucrose, only 1.1% of the sucrose consumed became assimilated as cellular material whilst the remaining 98.9% was utilized as energy source.

5.3.3 Experiments on Product Formation (Runs 151-176)

Figure 5.2 shows the concentrations of fermentation products and biomass against time for experiment 151. Graphs for experiments 152 to 176 are contained in Section 6 of Appendix 2. Cell density absorbance readings were converted to a dry weight basis using a calibration plot established by Philpott (1980) for *B. stearothermophilus* 11d-15 of absorbance versus dry weight: 1 absorbance unit was shown to be equivalent to a cell dry weight of 0.342 g/litre. Figure 5.2 indicates a typical time course of this series of experments and the frequency of sampling. 'One-way' pH control was employed in all these experiments and sampling of the fermenters was continued until the culture pH rose above the controlled pH and it was no longer possible to control the pH. The total quantities of potassium hydroxide added to the fermenters in experiments 151 to 176 are shown in Appendix 2, Section 5. The concentrations of products and biomass at the times corresponding to maximum cell density are shown for the entire run of experiments in Table 5.1; when maximum cell density was maintained over a considerable period, the values shown in the table correspond to data from the middle of the stationary phase of growth. Product yields (Table 5.2) were derived from the data of Table 5.1 and Appendix 2, Section 4. It is immediately apparent from Table 5.2 that experiments carried out under apparently identical environmental conditions e.g. 151, 171 and 176 yielded differing results. The variability in these and other experiments is discussed more fully in Section 5.4. Elemental analysis of a sample of dried cells from experiment 151 gave the following results

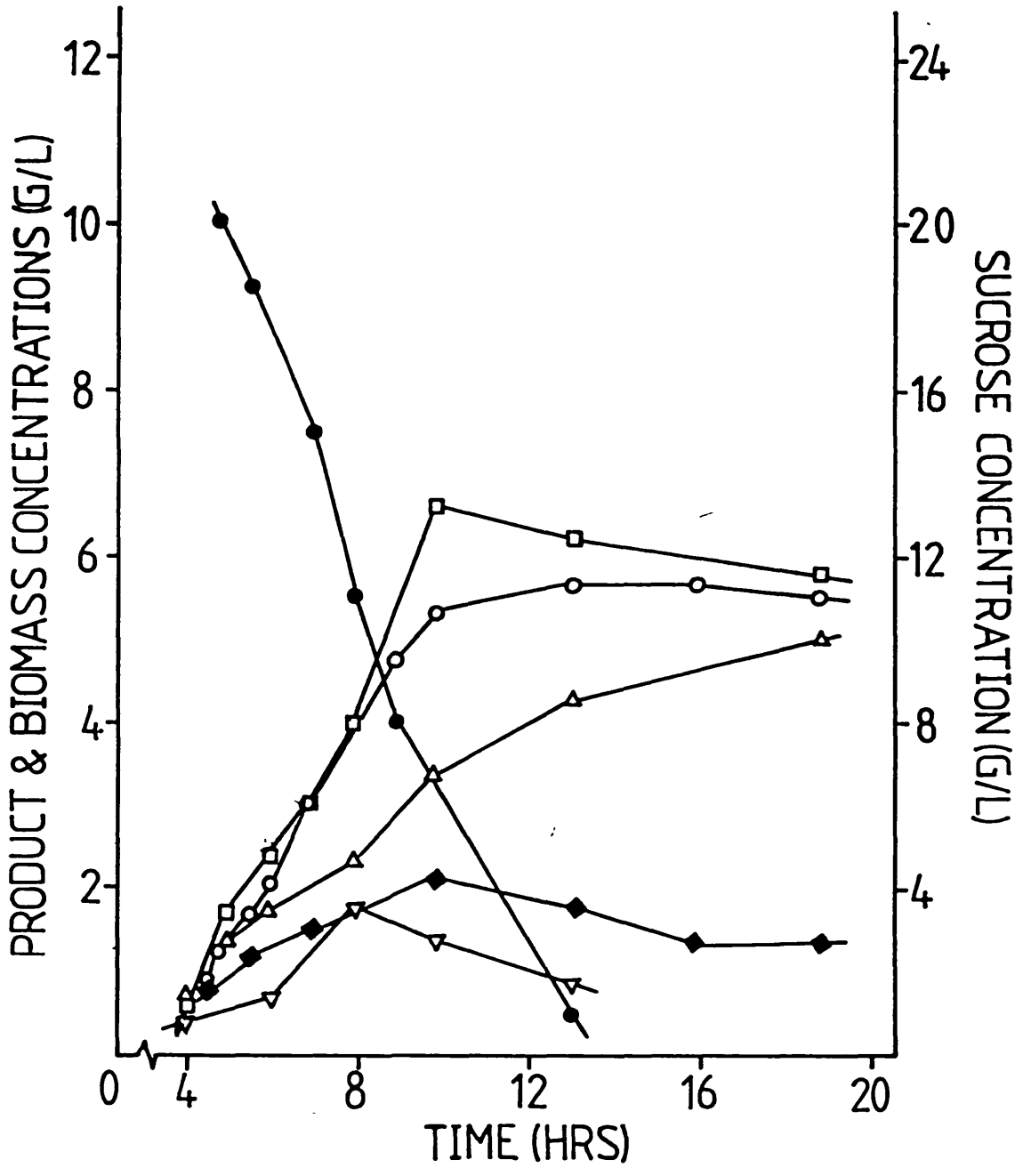


Figure 5.2 Product and Biomass Concentrations against Time

Experiment 151 (So = 23.5g/l, T = 60°C, pH 7.0)
(○) Ethanol, (□) Formate, (△) Acetate, (▽) Pyruvate
(●) Sucrose (◆) Biomass.
Fermenter:- 5 litre New Brunswick, Working Volume: 3.4 litres
Operating Conditions:- Batch, Anaerobic. Nitrogen flowrate to head : 0.2 litre/min. Impeller speed: 200 rpm. pH controlled by addition of 30% KOH soln. Medium: BST. Inoculum: 3% (v/v) of Fermenter Working Volume.

Expt.	Temp °C	pH	So* g/l	Acetate	Formate	Pyruvate	Biomass	Ethanol
151	60	7.0	23.5	3.45	6.65	1.41	2.16	5.40
152	60	6.2	23.5	2.07	3.39	1.93	1.51	7.32
153	60	8.0	23.5	5.78	9.20	0.45	1.55	4.84
154	50	8.0	23.5	3.26	6.51	0.10	2.23	4.05
156	50	7.0	23.5	3.77	4.75	0.10	2.44	4.16
159	70	7.7	23.5	4.20	6.34	0.72	1.08	6.30
160	70	7.0	23.5	1.24	2.66	1.67	1.34	3.98
161	60	7.0	100	5.53	4.58	2.31	1.90	11.13
162	60	7.0	50.0	4.16	8.91	1.84	2.49	15.35
171	60	7.0	23.5	3.35	6.93	2.68	2.11	6.61
172	60	7.0	50.0	6.23	12.27	6.70	2.59	12.58
174	60	6.6	23.5	3.06	5.12	2.13'	1.81	7.79
176	60	7.0	23.5	3.15	6.20	1.36	2.00	7.39

Table 5.1 Product Concentrations (g/l).

*The values shown for S_0 (the initial sucrose concentration) are nominal ones only, the actual values are contained in Appendix 2, Section 4.

Expt.	Temp °C	pH	Acetate	Formate	Pyruvate	Ethanol
151	60	7.0	0.97	2.44	0.27	1.98
152	60	6.2	0.56	1.19	0.36	2.58
153	60	8.0	1.33	2.75	0.07	1.45
154	50	8.0	1.09	2.85	0.02	1.77
156	50	7.0	1.06	1.74	0.02	1.52
159	70	7.7	1.07	2.31	0.26	2.08
160	70	7.0	0.47	1.32	0.43	1.97
161	60	7.0	0.80	0.87	0.23	2.11
162	60	7.0	0.54	1.50	0.16	2.59
171	60	7.0	0.76	2.04	0.41	1.95
172	60	7.0	0.72	1.86	0.53	1.90
174	60	6.6	0.73	1.59	0.35	2.42
176	60	7.0	0.84	2.14	0.25	2.56

Table 5.2 Molar Product Yields (Moles/Mole of Sucrose Utilized).

(all figures in %); carbon 48.8, nitrogen 12.3, hydrogen 7.2. In order to perform carbon recovery calculations it is also necessary to know the percentage of cell carbon derived from sucrose. Because of the high cost of radioisotopes, experiments such as experiment 238 (Sub-section 5.3.2) could not be repeated for all the combinations of environmental conditions used here. However the conditions used in experiment 238 are intermediate in terms of pH, temperature and initial sucrose concentration to those used in experiments 151 to 176, so that the value obtained for the incorporation of sucrose into biomass in experiment 238, was applied in all experiments.

Carbon recovery data is shown in Table 5.3; in all cases the carbon recovery was less than 100% indicating that one or more fermentation products were undetected. The calculated error in the carbon recovery figures is ± 7% and was based on analytical measurement errors, errors in the dilution and ethanol correction factors and errors in the estimation of the initial medium volume.

Another useful indicator of the recovery of fermentation products is the 'O/R index', this is the ratio of the oxidation states of the sum of the oxidized products to the sum of the reduced products. In calculating the oxidation state of a compound, hydrogen is arbitrarily assigned a value of -0.5 and oxygen a value of + 1.0. If neither oxygen nor any other oxidant enter into the sequence of fermentation reactions, the oxidation state of the products should equal that of the energy source; sucrose has an oxidation state of 0 and consequently the expected O/R index for its fermentation products is 1.0. O/R indices for experiments 151-176 are also shown in Table 5.3. In only one experiment, 153, does the index approach the value 1.0.

Growth curves for experiments 152 and 161 are shown in Figures 5.3 and 5.4. Growth curves for the remaining experiments are contained in Appendix 2, Section 4. The curves display a wide variety of shape and the examples shown in Figures 5.3 and 5.4 represent two extremes. In figure 5.3 cell density increases in two stages to the maximum cell density, growth in the first stage being more rapid than in the second stage. However in figure 5.4 cell density increases more uniformly to the maximum cell density. The implications of this are discussed further in Chapter 6.

Initial specific growth rates are tabulated in Table 5.4 for a

Experiment	Carbon Recovery (%)	O/R Index
151	77.8	0.68
152	71.9	0.30
153	71.8	0.97
154	73.6	0.81
156	59.6	0.58
159	82.9	0.62
160	76.9	0.44
161	62.1	0.26
162	90.3	0.32
171	73.5	0.63
172	73.1	0.63
174	75.4	0.40
176	81.7	0.47

Table 5.3 Carbon Recoveries (%) with Respect to Sucrose and O/R Indices of Fermentation Products.

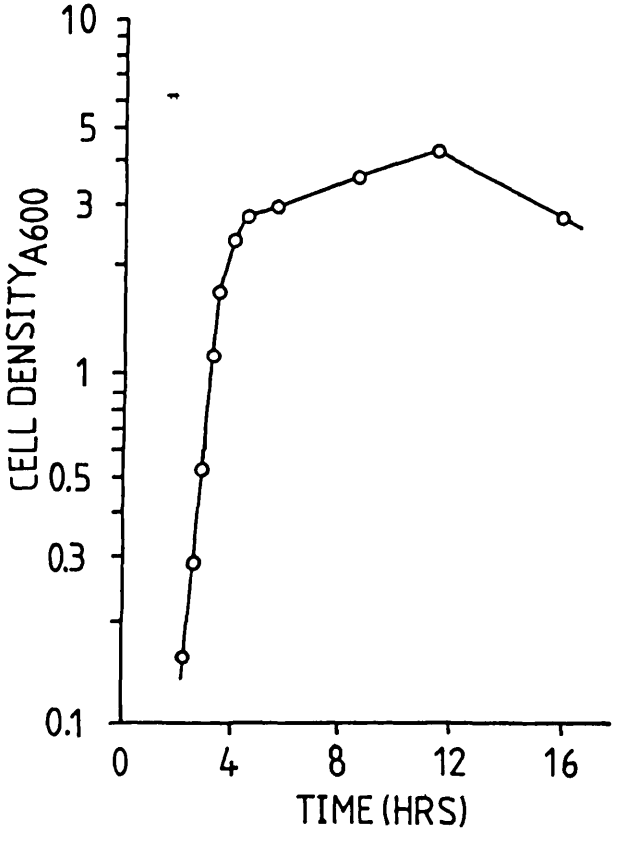


Figure 5.3 Growth Curve for Experiment 152
(So = 23.5 g/1, T = 60°C, pH 6.2)

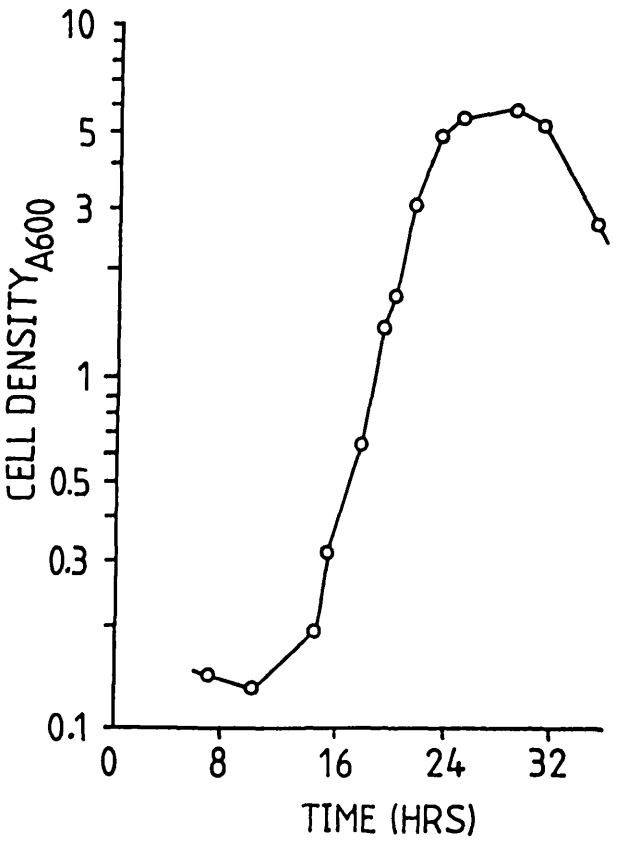


Figure 5.4 Growth Curve for Experiment 161
(So = 100g/1, T = 60°C, pH 7.0)

Fermenter:- 5 litre New Brunswick, Working Volume: 3.4 litres.
Operating Conditions:- Batch Anaerobic, Nitrogen flowrate to head: 0.2 litre/min.
Impeller speed: 200 rpm. pH controlled by the addition of 30% KOH Soln. Medium: BST
Inoculum: 3% (v/v) of Fermenter Working Volume.

variety of temperatures and pHs at a constant initial sucrose concentration of 2.35% (w/v), and in Table 5.5 at a variety of initial sucrose concentrations at a constant pH of 7 and temperature of 60°C. Included also in Table 5.5 are data from control experiments carried out with an initial sucrose concentration of 75g/litre which are described fully in Chapter 6. In order to avoid long lag phases in investigating growth at low pHs (i.e. pH 5.0 and 5.5, experiments 169 and 170), the initial pH of the medium on inoculation was 7.0, and cell density was allowed to reach a value of between 1.0 and 1.5 absorbance units before resetting the set point on the pH control system to the required value. The medium pH then was allowed to decrease naturally through the production of acids by the culture. However, in all cases, the drop in pH was preceeded by a rapid decline in cell density (see Figure A2.28).

Growth rates are seen to be approximately constant over a wide range of environmental conditions ranging from pH 6.2 and 60°C to pH 7.0 and 70°C (Table5.4) and are comparable to those of mesophilic organisms such as E. coli. Lower growth rates were recorded outside this range. The relatively high value for experiment 151 in comparison to the values for experiments 171 and 176, which were carried out under identical conditions is of questionable reliability since for experiment 151 the determination was based on only two readings in the exponential phase. B. stearothermophilus 11d-15 is unable to tolerate a sucrose concentration of 15% (w/v). The large disparity in growth rates between experiments 162 and 172 is discussed in greater detail in Section 5.4.

Specific ethanol productivities were derived from the batch data by dividing ethanol concentrations by the corresponding cell dry weights and plotting the data obtained against time; the slope of the resulting line - computed by the method of least squares - is the specific ethanol productivity. Ethanol productivity data for experiment 151 is shown in Figure 5.5. The early data points (from 3.7 to 5.5 hours) show some scatter, however after approximately 6 hours, the data displays a linearly increasing pattern with time until approximately 16 hours when the specific ethanol concentration levels off. In certain experiments cell density decreased rapidly due to lysis after a maxima had been reached. Under these conditions the specific ethanol concentration would show, when plotted, an

Experiment	Temp °C	pH	Specific Growth Rate hr^{-1}
155	50	6.2	NG
156	50	7.0	0.80
154	50	8.0	0.79
170	60	5.0	NG
169	60	5.5	NG
152	60	6.2	2.1
174	60	6.6	2.1
151	60	7.0	3.15
171	60	7.0	2.17
176	60	7.0	2.24
153	60	8.0	1.65
158	70	6.2	NG
160	70	7.0	1.69
159	70	7.7	0.98

Table 5.4 Specific Growth Rates for Experiments Conducted at Various Temperatures and pHs and at an Initial Sucrose Concentration of 2.35% (w/v)

NG = No Growth

Experiment	S_0 (g/l)	Specific Growth Rate hr^{-1}
151	23.5	3.15
171	23.5	2.17
176	23.5	2.24
162	50.0	0.91
172	50.0	1.87
203*	75.0	0.91
206*	75.0	0.94
216*	75.0	0.92
161	100.0	0.56
173	100.0	NG
163	150.0	NG

Table 5.5 Specific Growth Rates for Experiments Conducted at Various Initial Sucrose Concentrations (S_0) and at a Temperature of 60°C and at pH 7.0.

*From the data of experiments described in Chapter 6.

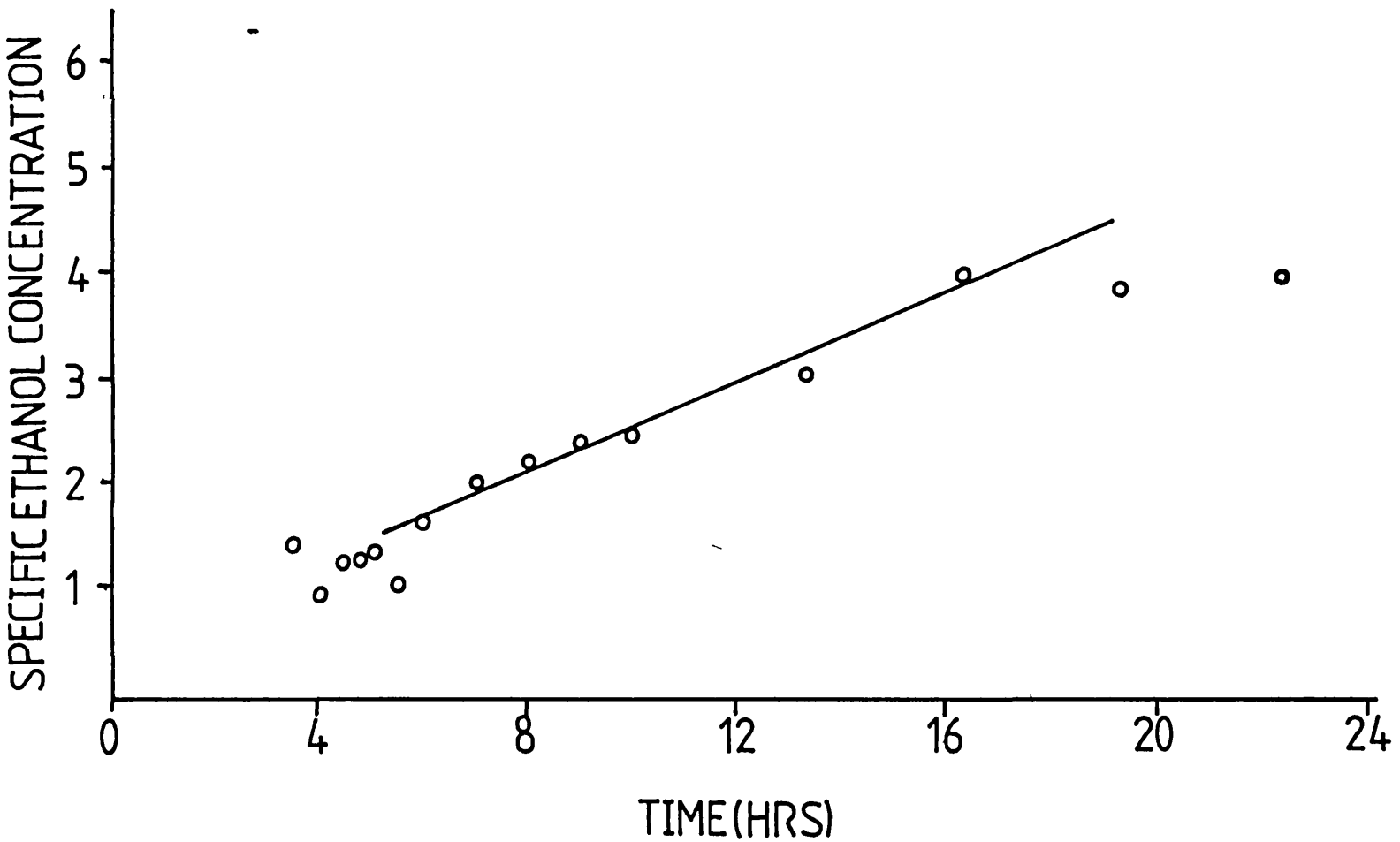


Figure 5.5 Specific Ethanol Concentration (g ethanol/g dry wt. cells) against Time (hrs).

Experiment	Temperature °C	pH	Specific Ethanol Productivity
156	50	7.0	0.14
154	50	8.0	0.07
152	60	6.2	0.46
174	60	6.6	0.32
151	60	7.0	0.22
171	60	7.0	0.31
176	60	7.0	0.24
153	60	8.0	0.42
160	70	7.0	1.01
159	70	7.7	0.97

Table 5.6 Specific Ethanol Productivies (g ethanol/ g dry weight cells-hr) at Various Temperatures and pHs.

apparent increase. Data of this type were not included in the calculations of productivity; all data where the ethanol concentration remained constant to within ± 5% whilst cell density remained constant or decreased were ignored. Table 5.6 shows specific ethanol productivities at a variety of environmental conditions for experiments performed with an initial sucrose concentration of 23.5g/litre. The highest productivities obtained were at 70°C.

Specific ethanol productivities are presented in Table 5.7 for a number of initial sucrose concentrations. The productivity is approximately constant over the range of concentrations studied. Moreover, the productivity is apparently not affected by the final ethanol concentration.

Experiment	S_0 (g/l)	Specific Ethanol Productivity	Final Ethanol Concentration (g/l)
151	23.5	0.22	5.9
171	23.5	0.31	6.6
176	23.5	0.24	7.8
162	50.0	0.39	21.7
172	50.0	0.25	13.9
161	100.0	0.32	11.8

Table 5.7 Specific Ethanol Productivities (g ethanol/g dry weight cells-hr) and Final Ethanol Concentrations (g/l) at Various Initial Sucrose Concentrations for Experiments Conducted at 60°C and pH 7.0.

The natural logarithm of specific ethanol productivity is plotted against the reciprocal of absolute temperature for the data at 50, 60 and 70°C at pH 7.0 in Figure 5.6. The specific productivity for 60°C was taken as 0.23 - the mean of the values for experiments 151 and 176. Specific ethanol productivity is plotted against pH in Figure 5.7.

Productivity reaches a minimum at a pH of approximately pH 7.2. The specific ethanol productivity data for *B. stearothermophilus* 11d-15

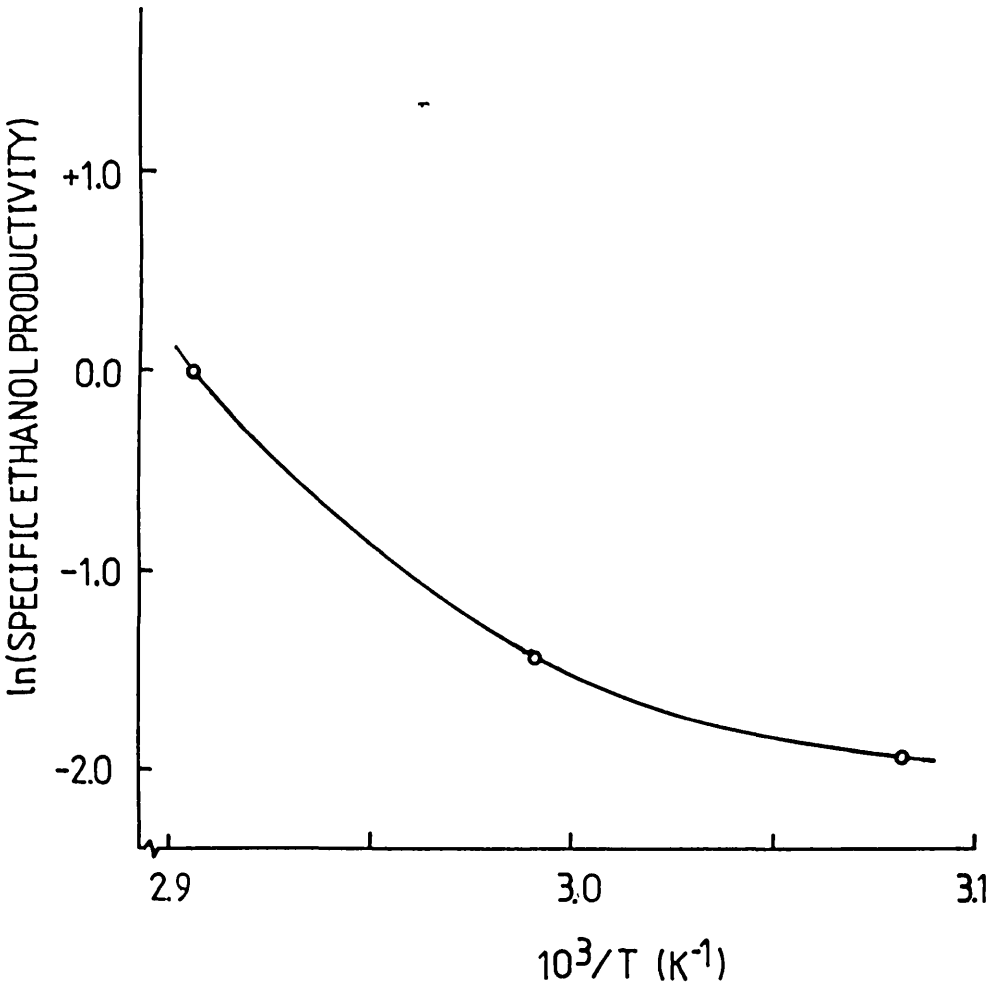


Figure 5.6 Arrhenius Plot of Specific Ethanol Productivity

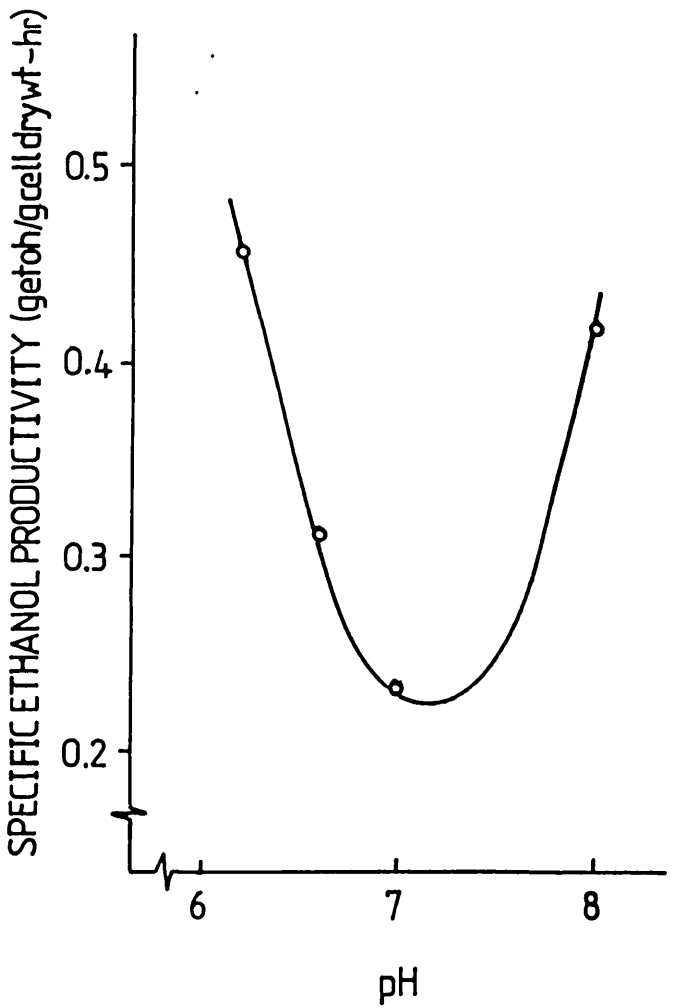


Figure 5.7 Specific Ethanol Productivity against pH

is compared with both mesophilic and thermophilic ethanologens in Chapter 10.

5.3.4 Experiments on Product Formation (Runs 239 and 240)

Product concentrations at peak cell densities, graphs of carbon dioxide evolution against time and growth curves for these experiments are contained in Appendix 2, Section 7. Data from this Appendix was used to compile Table 5.8. It can be seen that both ethanol and carbon dioxide yields are increased by conducting the fermentation at acid pH. The detection of succinate was unexpected as it has not previously been reported as a fermentation product of B. stearothermophilus. Trace quantities of acetaldehyde were also detected. Spent culture broths from both experiments showed a positive reaction in the Voges-Proskauer test which was conducted as outlined by Cowan and Steel (1966). The Voges-Proskauer test detects the presence of acetoin and/or diacetyl, however no attempts were made to conduct assays for these compounds. Carbon recovery at pH8 seems acceptable, although the O/R index (the ratio of the oxidation states of oxidized to reduced fermentation products) at this pH is well in excess of 1.0. Carbon recovery at pH 6.5 suggests that one or more fermentation products detected were derived from sources other than sucrose and this is borne out by the high O/R index.

5.3.5 Washed Cell Experiments

Cells cultured in BST medium were washed and resuspended in a medium of composition similar to BST but from which the tryptone and yeast extract had been omitted. This medium was found to be totally unsuitable as virtually all the cells lysed within approximately 6 hours without fermenting the sucrose. Further experiments were conducted to devise a more suitable medium and in experiments 263 and 264 RCM3 medium was used. In this medium (see 2.2.5) 3-(N-Morpholino) propanesulphonic acid (MOPS) was used as buffer and phosphate was supplied in the form of glycerophosphate so as to prevent precipitation of calcium which earlier tests had revealed was effective in helping to maintain cell integrity. Although an improvement on earlier media, substantial cell lysis still occurred, cell dry weights decreasing by approximately 40% within 2.8 hours. As lysis occurred complex carbon and nitrogen bearing compounds were released into the medium and the surviving cells were observed by microscopic examination to have started dividing. Fermentation data at 2.8 hours are shown in Table 5.9.

Experiment	239	240
pH	6.5	8.0
Product Yields [1]		
Ethanol	3.07	2.29
Carbon Dioxide	5.05[2]	1.83[3]
Formate	1.22	2.87
Acetate	0.25	1.26
Pyruvate	0.38	0.10
Lactate	0.02	0.01
Succinate	0.06	0.03
Acetaldehyde	0.03	0.04
Carbon Recovery %[4]	121.4	103.3
O/R Index	1.91	1.44

Table 5.8 Fermentation Data for Experiments 239 and 240

1 Units: Moles of Product/Mole of Sucrose Utilized

2 Total CO_2 Yield = Evolved CO_2 (5.02) + Bound CO_2 (0.03)

3 Total CO_2 Yield = Evolved CO_2 (1.68) + Bound CO_2 (0.15)

4 Includes 11.4% of Biomass Carbon

Fermenter: 5 litre New Brunswick, Working Volume 3.4 litres. Operating Conditions: Batch, Anaerobic, Nitrogen flowrate to sparger: 1.0 litre/min. Impeller speed: 200 rpm. pH controlled by the addition of 30% KOH soln Medium: BST. Inoculum: 3% (v/v) of Fermenter Working Volume. T: 60°C. S_0: 50g/litre.

Experiment	263	264
pH	6.5	8.0
Product Yields[1]		
Ethanol	2.61	1.82
Carbon Dioxide	2.44[2]	1.50[3]
Formate	1.87	2.33
Acetate	1.18	1.80
Pyruvate	0.03	0.01
Lactate	0.08	0.09
Succinate	0.15	0.05
Acetaldehyde	0.15	0.05
Carbon Recovery %	107.9	96.7
O/R Index	1.30	1.45

Table 5.9 Fermentation Data for Resting Cell Suspension Experiments

1 Units: Moles of Product/Moles of Sucrose Utilized

2 Total CO_2 Yield = Evolved CO_2 (1.59) + Bound CO_2 (0.85)

3 Total CO_2 Yield = Evolved CO_2 (0.17) + Bound CO_2 (1.33)

Fermenter: 5 litre New Brunswick, Working Volume: 3.4 litres. Operating Conditions: Batch, Anaerobic. Nitrogen flowrate to sparger: 1.0 litre/min. Impeller speed: 200 rpm. pH controlled by the addition of 30% KOH soln. Medium: RCM3. Initial biomass concentration: 7.0g/litre (dry weight). T: 60°C. So: 50g/litre

No data is shown for the control experiment (265) in which cells were suspended in a medium similar to RCM3 but which did not contain sucrose. Here no metabolism occurred and the concentrations of fermentation products did not exceed 2 mM.

Although carbon recoveries appear to be acceptable, O/R indices are significantly greater than 1.0.

5.4 Discussion

In their study of glucose fermentation by B. stearothermophilus, McKray and Vaughn (1957) reported that they were unable to detect carbon dioxide as a fermentation product, and moreover their carbon recoveries ranged from 89 to 96%. The data of Tables 5.8 and 5.9 reveal that significant quantities of carbon dioxide were produced by strain lld-15, particularly at acid pH. The high carbon dioxide yields at acid conditions coincide with increased ethanol yields. This suggests three possible pathways by which these products may have been formed; pyruvate might have been channeled through the hypothetical PDH pathway (Figure 1.5) despite the presence of PFL; alternatively formate dehydrogenase (FDH) may have catalyzed the conversion of formate to carbon dioxide and NADH, the latter would be consumed in reducing acetyl-CoA to ethanol; the third possibility is that B. stearothermophilus possesses a PDC enzyme similar to that of yeast and Zymomonas mobilis, (Figure 1.1). All of these possibilities were examined by performing enzyme assays and the results of these investigations are presented in Chapter 9.

The detection of succinate was also unexpected; McKray and Vaughn did not report its presence. There are 2 major metabolic routes to succinate. The first, shown in Figure 5.8 is common to enterobacteria and involves the operation of part of the TCA cycle in reverse; the second pathway (Figure 5.9) has been reported to be present in certain clostridia. No investigations were conducted to determine which of the two pathways exists in B. stearothermophilus.

Tables 5.8 and 5.9 show that small quantities of lactate were detected. S. Amartey (personal communication) has shown that it is the L-isomer and not the D-isomer that is produced. This may indicate that the NAD-linked L-lactate dehydrogenase although defective in strain lld-15 is still able to weakly catalyze the conversion of pyruvate to lactate. Alternatively B. stearothermophilus may possess a flavo-protein linked LDH in common with enterobacteria (Garvie, 1980).

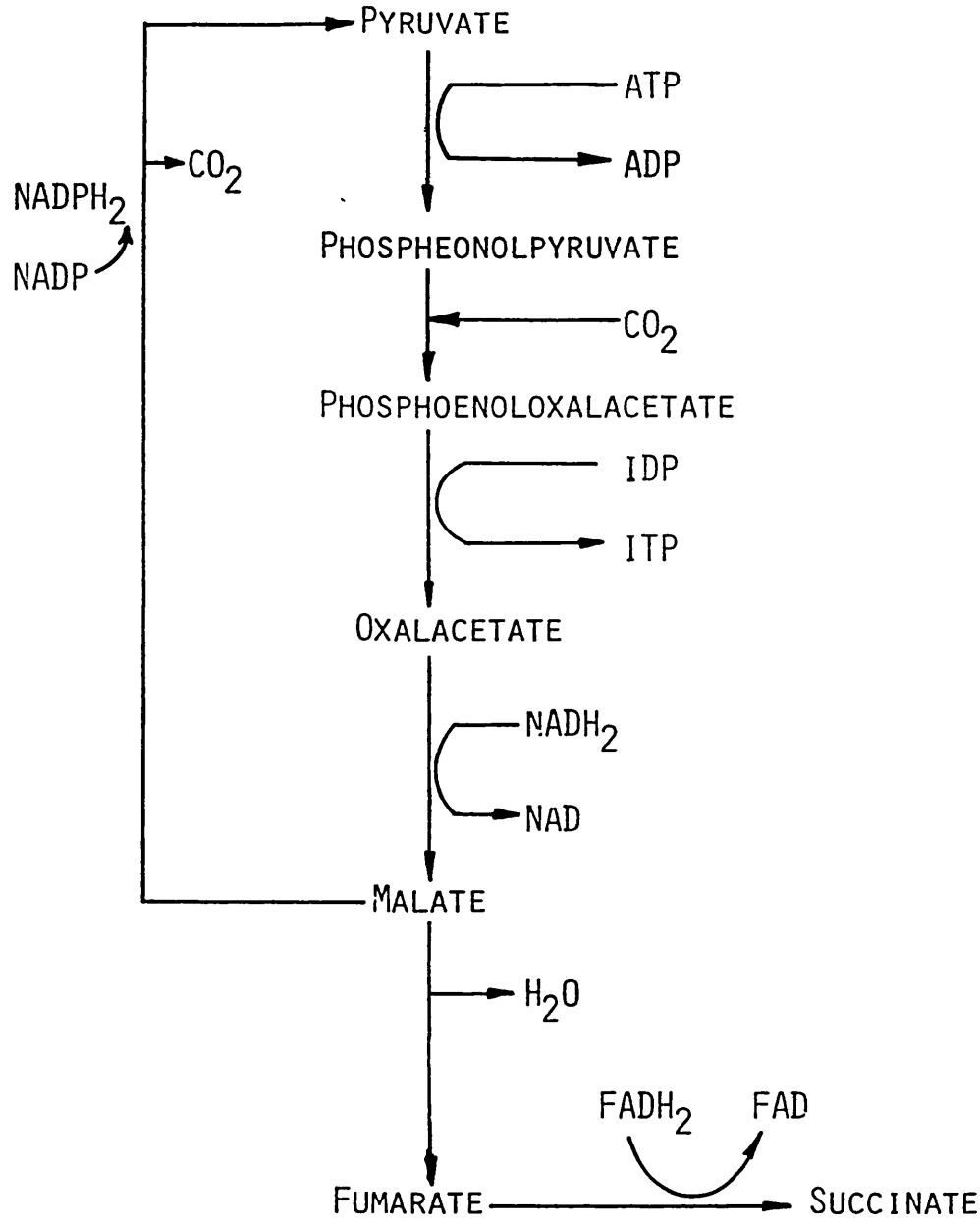


Figure 5.8 The Formation of Succinate from Pyruvate by Enterobacteria using the Reversed TCA Cycle

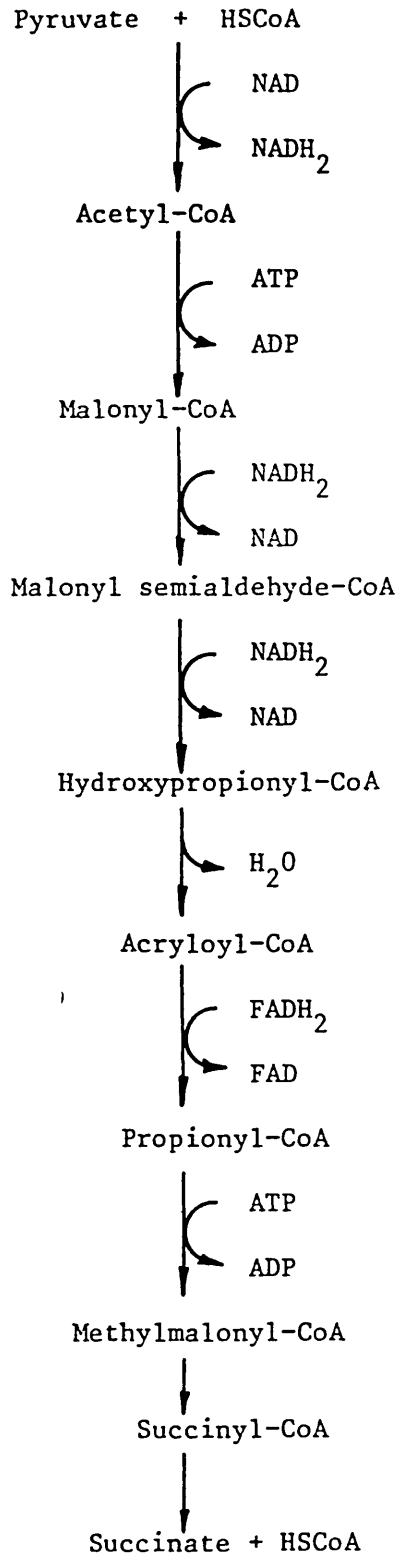


Figure 5.9 The Formation of Succinate from Pyruvate by Clostridium kluyveri

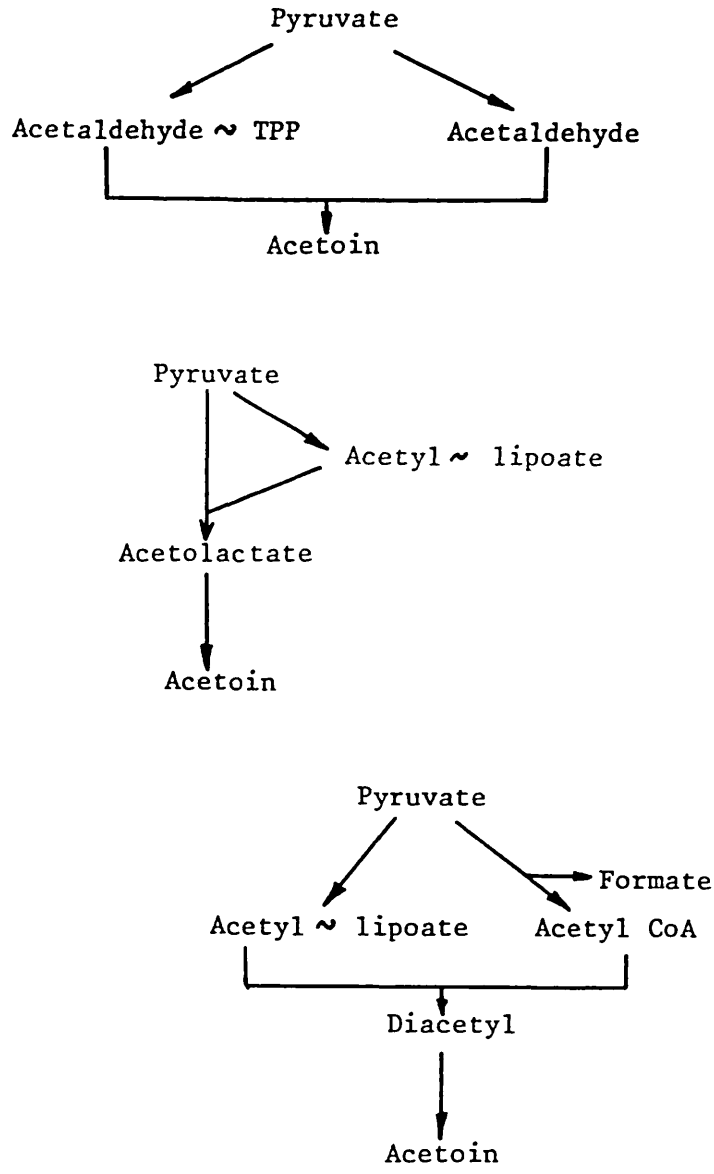


Figure 5.10 Postulated Sequences for the Formation of Acetoin from Pyruvate

Samples of culture broths from experiments 239 and 240 showed a positive reaction to the Voges Proskauer test which indicates the presence of acetoin and/or diacetyl. B. subtilis has also been shown to produce acetoin from pyruvate (Lemoigne et al. 1949). There are three possible routes to acetoin (Figure 5.10) one involving diacetyl as intermediate. The first pathway shown has not previously been found in bacteria. No attempts were made either to conduct quantitative analyses for these products or to determine which pathway B. stearothermophilus possessed.

At the commencement of the batch culture experiments, culture broth samples from experiment 151 were subjected to a number of GC analyses (see Table 3.3) to determine whether acetone, butanol, ethyl acetate or acetaldehyde were present. Only trace quantities of acetaldehyde were detected (see also Table 5.8 and 5.9). Its presence is accounted for by being an intermediate in all pathways leading to ethanol.

In section 5.1 it was stated that fermentation experiments were carried out using BST medium because it was assumed that biomass production would be at the expense of amino acids, peptides and other complex constituents of the medium, whereas sucrose would serve primarily as energy substrate. The results of experiment 238 show that the latter assumption was essentially correct: 1.1% of the sucrose consumed was incorporated into biomass whilst the remaining 98.9% was fermented.

Payne (1980), reviewing the uptake of peptides by bacteria, stated that in certain cases the uptake of peptides surpassed that of amino acids. The ratio of ^{3}H to ^{14}C counts obtained in experiment 238 (3.58) is therefore likely to be an underestimate of the extent to which nitrogen containing carbon sources are assimilated into biomass. There is however no firm evidence that sucrose was the only medium constituent utilized as energy source. In some experiments sucrose was almost completely utilized and in such cases it is possible that the cells which did not lyse survived by exploiting energy sources such as amino acids. However, yields, carbon recoveries, O/R indices, etc., were all calculated using data obtained either at peak cell densities or at the mid-stationary phase of growth (i.e. before lysis occurred) and under conditions where the exploitation of energy sources other than sucrose would have been unlikely.

The carbon recovery figures in excess of 100% with respect to sucrose input obtained in experiments 239 and 240 may not seem to support the hypothesis that sucrose was the only medium constituent fermented. However this hypothesis may be tested by attempting to predict the yields of one of the fermentation products on the assumption that the remaining products are all derived from sucrose. The method described below was used to predict carbon dioxide yields.

PFL catalyzes the conversion of pyruvate to acetyl-CoA and formate. Using brackets to indicate moles, this may be expressed:-

$$(\text{Pyruvate}) = (\text{Acetyl-CoA}) + (\text{Formate})$$

Hence

$$(\text{Pyruvate}) = (\text{Acetyl-CoA}) = (\text{Formate}) \qquad 5.1$$

Acetyl-CoA is then converted entirely into acetate, ethanol or both

$$(\text{Pyruvate}) = (\text{Ethanol}) + (\text{Acetate}) \qquad 5.2$$

or using 5.1,

$$(\text{Ethanol}) + (\text{Acetate}) = (\text{Formate}) \qquad 5.3$$

Formate may be decomposed into H_2 (or $NADH_2$) and CO_2 in equimolar quantities. Hence

1 mole of H_2 or 1 mole of CO_2 represents
1 mole of Formate decomposed 5.4

However, not all the formate may be decomposed,

$$(\text{Formate})_{\text{TOTAL}} = (\text{Formate})_{\text{RESIDUAL}} + (\text{Formate})_{\text{DECOMPOSED}}$$

or using 5.4,

$$(\text{Formate})_{\text{TOTAL}} = (\text{Formate})_{\text{RESIDUAL}} + (H_2) \qquad 5.5$$

Equating 5.3 and 5.4,

$$(\text{Ethanol}) + (\text{Acetate}) = (\text{Formate}) + (H_2) \qquad 5.6$$

If CO_2 is taken into consideration it is possible that some CO_2 (which was derived solely from formate) is consumed in the production of succinate at the rate of 1 mole of CO_2 per mole of succinate.

Therefore

$$(CO_2)_{\text{TOTAL}} = (CO_2)_{\text{RESIDUAL}} + (CO_2)_{\text{CONSUMED IN SUCCINATE PRODUCTION}}$$

Because the total moles of CO_2 formed from formate equal the total moles of H_2 formed from formate

$$(H_2) = (CO_2) + (\text{Succinate})$$

5.6 becomes

$$(\text{Ethanol}) + (\text{Acetate}) = (\text{Formate}) + (CO_2) + (\text{Succinate}) \qquad 5.7$$

It was stated earlier that evidence existed for the operation alongside PFL of either PDC or PDH (the possible existence of FDH has been accounted for above) it can be shown that equation 5.7 would still remain applicable under these conditions.

Rearranging 5.7,

$$(CO_2) = (\text{Ethanol}) + (\text{Acetate}) - (\text{Formate}) - (\text{Succinate})$$

This form of equation 5.7 was used to calculate carbon dioxide yields. Predicted carbon dioxide yields for experiments 151 to 176 are shown in Table 5.10 and for experiments 239 to 264 in Table 5.11. It is apparent that predicted carbon dioxide yields are lower than the measured yields: a theory to account for this disparity is proposed below. The predicted carbon dioxide yields were used in carbon recovery calculations (Table 5.12) and in the calculation of O/R indices (Table 5.13). A substantial improvement in both carbon recoveries and O/R indices is apparent for virtually each experiment when compared to the data of Tables 5.3, 5.8 and 5.9. More complete carbon recoveries would have been obtained had acetoin and diacetyl determinations been performed for all experiments and had succinate determinations been performed for experiments 151 to 176.

It remains necessary to account for the discrepancies between measured and predicted carbon dioxide yields. A possible explanation is that in the process of assimilating amino acids and peptides *B. stearothermophilus* produced decarboxylases which result in the production of CO_2, and that the greater discrepancies between measured and predicted carbon dioxide yields at lower pHs was due to the increased activity of the decarboxylases under those conditions. A method of accounting for such reactions is suggested in Chapter 10.

Experiment	Predicted Carbon Dioxide Yield*
151	0.51
152	1.94
153	0.02
154	0.02
156	0.84
159	0.84
160	1.13
161	2.05
162	1.62
171	0.66
172	0.77
174	1.56
176	1.25

Table 5.10 Predicted Carbon Dioxide Yields (Moles/Mole of Sucrose Utilized).

* Calculated on the assumption that

$$(CO_2) = (\text{Ethanol}) + (\text{Acetate}) - (\text{Formate})$$

Succinate determinations were not performed in these experiments.

Experiment	Measured[1] Carbon Dioxide Yield	Predicted[2] Carbon Dioxide Yield
239	5.05	2.04
240	1.83	0.65
263	2.44	1.77
264	1.50	1.24

Table 5.11 Measured and Predicted Carbon Dioxide Yields (Moles/Mole of Sucrose Utilized)

[1]From Tables 5.8 and 5.9

[2]Calculated on the assumption that

(CO_2) = (Ethanol) + (Acetate) - (Formate) - (Succinate)

Further evidence to support the claim made earlier that B. stearothermophilus 11d-15 makes use anaerobically of one or more pathways in addition to PFL comes from the product ratios of Table 5.14.

If the PFL pathway operated alone the expected ethanol/acetate ratio would be 1.0 and the ethanol/formate ratio 0.5. The data shows however that in virtually all experiments - but most notably 152 and 160 - departures from these expected values occur. A further unexpected result is that these ratios change with time during the course of fermentation. In experiment 171 (Table 5.16) for example, the ratios decrease initially until minimum values approximately corresponding to the expected values are reached, the ratios then increase to 2.57 for the ratio of ethanol/acetate and 0.95 for the ratio of ethanol to formate. For experiment 153 (Table 5.15), however, the ratios initially decrease slightly but finally approach the expected values. Possible causes of this apparent switch in metabolism and attempts to control this switch are considered in greater detail in Chapter 6.

The objective of the fermentation experiments described in this chapter was to identify environmental conditions favouring ethanol production. The variables studied were initial sucrose concentration, temperature and pH. Variations in initial sucrose concentration

Experiment	Carbon Recovery[1] (%)	Corrected C-Recovery[2] (%)
151	77.8	93.0
152	71.9	95.5
153	71.8	78.4
154	73.6	87.3
156	59.6	78.9
159	82.9	95.0
160	76.9	82.1
161	62.1	84.0
162	90.3	88.6
171	73.5	87.6
172	73.1	84.9
174	75.4	96.2
176	81.7	101.8
239	121.4	96.4
240	103.3	93.5
263	107.9	102.3
264	96.7	95.7

Table 5.12 Carbon Recoveries With Respect to Sucrose

[1] From Tables 5.3, 5.8 and 5.9

[2] Calculated using predicted CO_2 yields.

Experiment	O/R Index[1]	Corrected O/R Index[2]
151	0.68	0.94
152	0.30	1.05
153	0.97	0.99
154	0.81	0.82
156	0.58	1.13
159	0.62	1.02
160	0.44	1.02
161	0.26	1.24
162	0.32	0.95
171	0.63	0.97
172	0.63	1.03
174	0.40	1.04
176	0.47	0.96
239	1.91	0.93
240	1.44	0.93
263	1.30	1.05
264	1.45	1.31

Table 5.13 O/R Indices of Fermentation Products

[1]From Tables 5.3, 5.8 and 5.9

[2]Calculated using predicted CO_2 yields

Experiment	Ethanol / Acetate	Ethanol / Formate
151	2.04	0.81
152	4.61	2.17
153	1.09	0.53
154	1.62	0.62
156	1.43	0.87
159	1.94	0.90
160	4.19	1.49
161	2.64	2.43
162	4.80	1.73
171	2.57	0.96
172	2.64	1.02
174	3.32	1.52
176	3.05	1.20

Table 5.14 Ethanol/Acetate Ratios and Ethanol/Formate Ratios

Time (Hours)	Ethanol / Acetate	Ethanol / Formate
3.52	1.07	1.09
4.05	0.96	0.74
5.05	0.96	0.67
6.75	1.08	0.68
7.75	1.17	0.61
9.75	1.04	0.59
10.17	1.09	0.53

Table 5.15 Ethanol/Acetate Ratios and Ethanol/Formate Ratios for Experiment 153

Time (Hours)	Ethanol / Acetate	Ethanol / Formate
5.35	2.17	2.02
7.52	1.65	0.76
8.30	1.17	0.49
9.00	1.39	0.64
10.07	2.10	0.88
10.98	2.03	0.77
12.48	2.57	0.95

Table 5.16 Ethanol/Acetate Ratios and Ethanol/Formate Ratios for Experiment 171

do not appear to affect ethanol yields. An increase in temperature from 50°C to 60°C results in increased ethanol yields at pH 7, Table 5.2 but a further increase to 70°C does not have any marked effect. The strongest factor influencing yield is pH: this is illustrated in Figure 5.11 where the ratio of ethanol to acetate yields are plotted against formate yields for experiments conducted at 60°C and pHs ranging from 6.2 to 8.0. The curve drawn in Figure 5.11 represents pyruvate metabolism by a combination of the PFL pathway and alternative pathways leading to the production of ethanol and carbon dioxide only. The shape of the curve is independent of the actual pathway to ethanol. Use of PFL combined with FDH, PDC or PDH pathways would give identical curves.

Point A in the figure represents pyruvate metabolism solely via the PFL pathway where the ratio of ethanol to acetate is 1.0 and the yield of formate is 4 moles per mole of sucrose. Another important point has the co-ordinates $(0,\infty)$, at this point pyruvate is completely metabolized to ethanol and carbon dioxide as final products. It can be seen that the experimental data points are distributed fairly closely to the curve. Data obtained at acid pHs are grouped in a region of high ethanol to acetate ratios and low formate yields whilst data obtained at high pHs are clustered around point A.

Some experiments carried out under apparently identical conditions yielded results which displayed large disparities. For example experiments 162 and 172 were both carried out at 60°C, pH 7.0 and at an initial sucrose concentration of 50g/litre, but the ethanol yields obtained were 1.9 and 3.6 moles. In addition, the specific growth rates were widely different - 0.91 hr^{-1} for experiment 162 and 1.87 hr^{-1} for experiment 172.

The molar product yields from three experiments carried out at a temperature of 60°C and at pH 7.0 (Table 5.17) serve as a further example of variability. Growth controlling parameters such as pH and temperature were normally maintained to within ± 0.1 pH units and at ± 1°C respectively during the course of the experiments and it is most unlikely that variations of these magnitudes could account for the observed discrepancies. The cultivation of fermenter inoculum was however subject to less stringent control. The inoculum was generally incubated at the growth temperature for approximately 18 hours, but delays in inoculating the fermenters due to operational

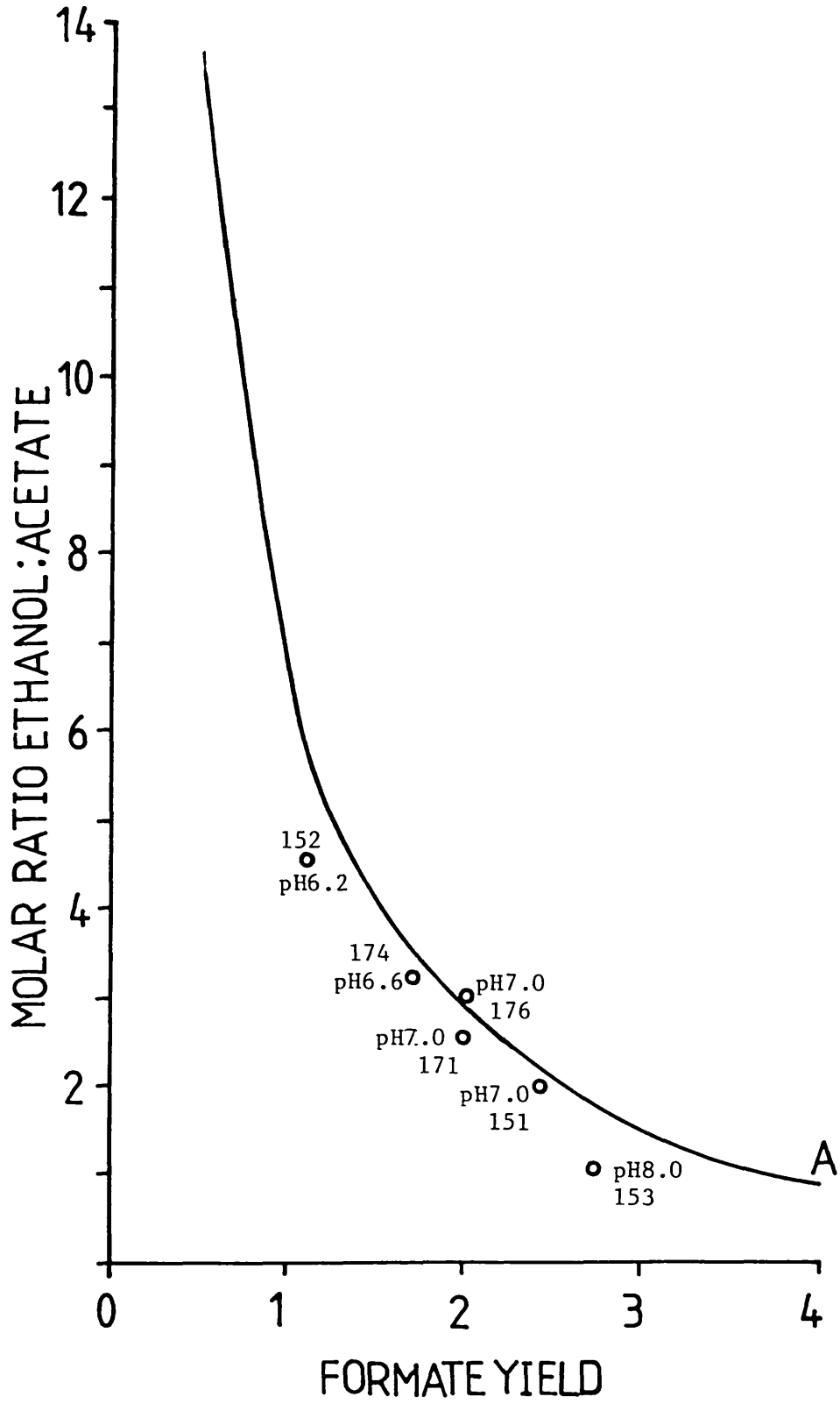


Figure 5.11 Molar Ratio of Ethanol to Acetate against Formate Yield[1] (Moles/Mole of Sucrose Utilized)

[1]For experiments conducted at 60°C and with an initial sucrose concentration of 23.5 g/l.

difficulties or equipment malfunctions occasionally occurred and resulted in a longer (by circa 2 hours) incubation period. It is

Experiment	Acetate	Formate	Pyruvate	Ethanol
151	0.97	2.44	0.27	1.98
171	0.76	2.04	0.41	1.95
176	0.84	2.14	0.25	2.56

Table 5.17 Product Yields (Moles/Mole of Sucrose Utilized) for Experiments Carried Out at a Temperature of 60°C and a pH of 7.0.

possible that variations in inoculum treatment might have resulted in certain cases in conditions favouring the growth of mutants. For example, the acidic fermentation products acetate and formate inhibit the growth of strain 11d-15 (see Chapter 6). Both of these acids have been detected in spent inoculum culture broths and mutants possessing an increased resistance towards them could have arisen during inoculum cultivation and have outgrown 11d-15. Such mutants may have differed from 11d-15 in the fermentation of sucrose but may have been morphologically indistinct from the latter and so have escaped detection. Similar phenomena have been reported, for example Hill et al. (1967) showed that the pathways of glucose metabolism by smooth and rough variants of B. stearothermophilus NCA 1518 differed: the Embden-Meyerhof pathway was more active in the smooth variant whereas the Hexose Monophosphate Shunt was predominant in the rough variant.

Although the operation of unknown factors prevented complete control being exercised over product distributions by manipulating the growth environment most of the data agrees closely with postulated product distributions. This is illustrated in Figure 5.12 which contains not only the data in Figure 5.11 but that from all additional fermenter runs.

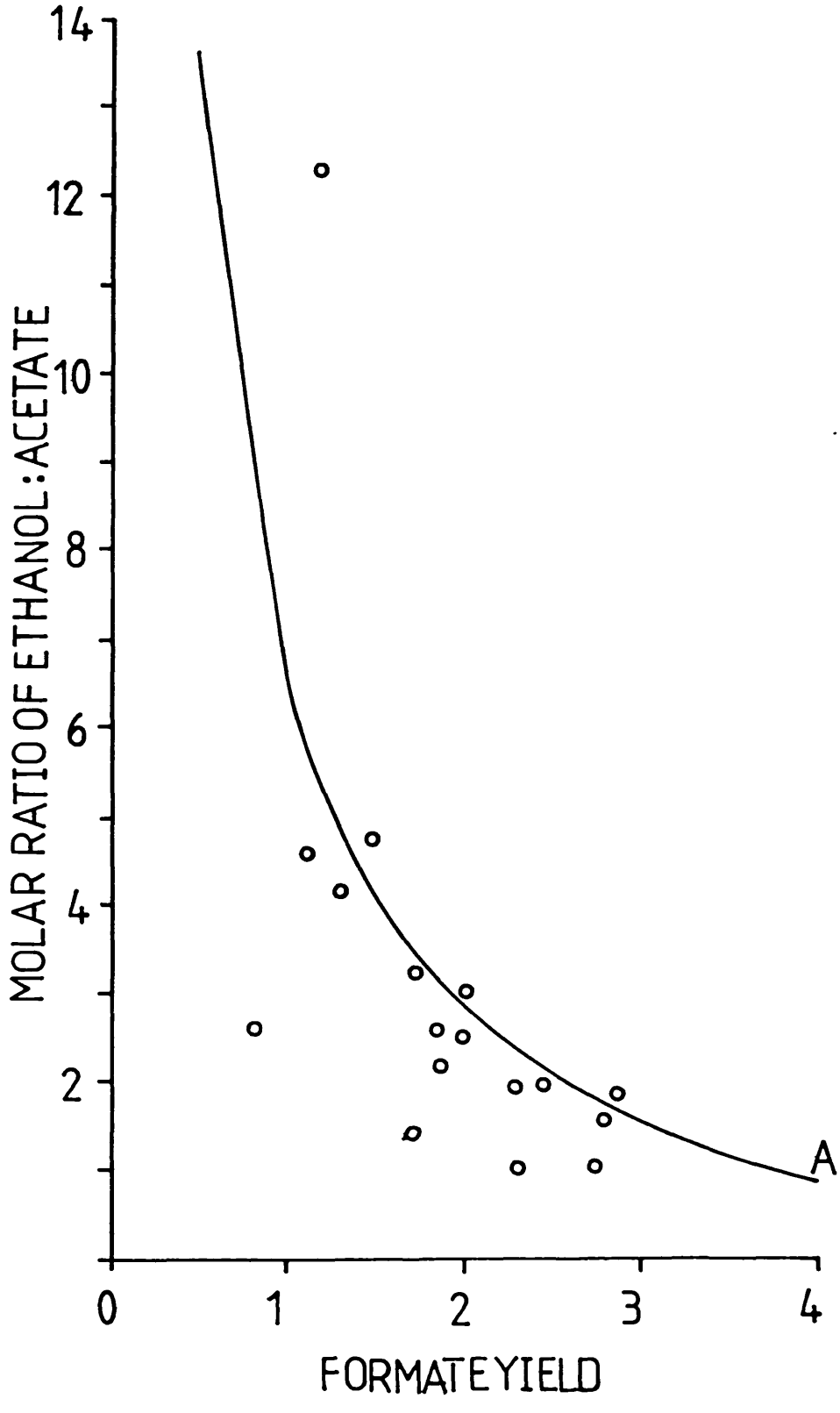


Figure 5.12 Molar Ratio of Ethanol to Acetate against Formate Yield[1] (Moles/Mole of Sucrose Utilized)

[1]For all experiments

5.5 Conclusions

Acceptable carbon recoveries were obtained by excluding from the experimental data the carbon dioxide likely to be produced by decarboxylation reactions unconnected with energy metabolism. However attempts to study energy metabolism uncoupled from growth by using washed cell suspensions were unsuccessful. High ethanol yields and abnormal product ratios indicate that B. stearothermophilus 11d-15 possesses anaerobic pathways of pyruvate metabolism in addition to the PFL pathway. An important variable influencing ethanol yield is pH:acid conditions resulted in high ethanol yields.

CHAPTER 6

GROWTH INHIBITION STUDIES

6.1 Introduction

Analysis of the data from batch fermentation experiments revealed that the ratios of fermentation products changed with time during the course of the experiment. In particular, for experiments performed at acid pHs the ratios indicated that shifts in metabolism towards increased ethanol production were occurring. Figure 6.1 shows the ethanol to formate ratio against time for experiment 152 which was conducted at pH 6.2. Initially the ratio drops slightly to a minimum value of 0.9 at approximately 4.5 hours. After this, it increases steadily to 2.1 at 11.5 hours. Also shown in Figure 6.1 is a logarithmic plot of cell density against time; a period of exponential growth (marked OA in the figure) is followed by growth at a reduced rate. If the shift in metabolism and reduction in specific growth rate are connected, then an understanding of the inter-relationship between the two may suggest a strategy for increasing ethanol yields with B. stearothermophilus 11d-15.

Two hypotheses can be proposed for the observed decrease in growth rate which preceeds the stationary phase. One possibility is that those nutrients which require the least energy for uptake and incorporation into biomass are utilized first, and those assimilated less efficiently later. The other possibility is that growth rate was reduced by toxic materials arising as a result of metabolism.

In order to test the nutrient limitation hypothesis, fermentations were performed using media with different levels of complex constituents. In addition to the use of medium containing 'normal' levels of tryptone and yeast extract, 2% and 1% (w/v), respectively, media containing these ingredients at a half and twice the normal level were used. The inhibition hypothesis was investigated by examining the inhibitory effects of the four main fermentation products, formate, acetate, ethanol and carbon dioxide singly and in various combinations. In order to avoid long lag phases, the fermentation experiments were initiated in the absence of these products which were only added to the fermenters when the cell density reached an absorbance reading of between 1.0 and 1.3 units, corresponding to mid-

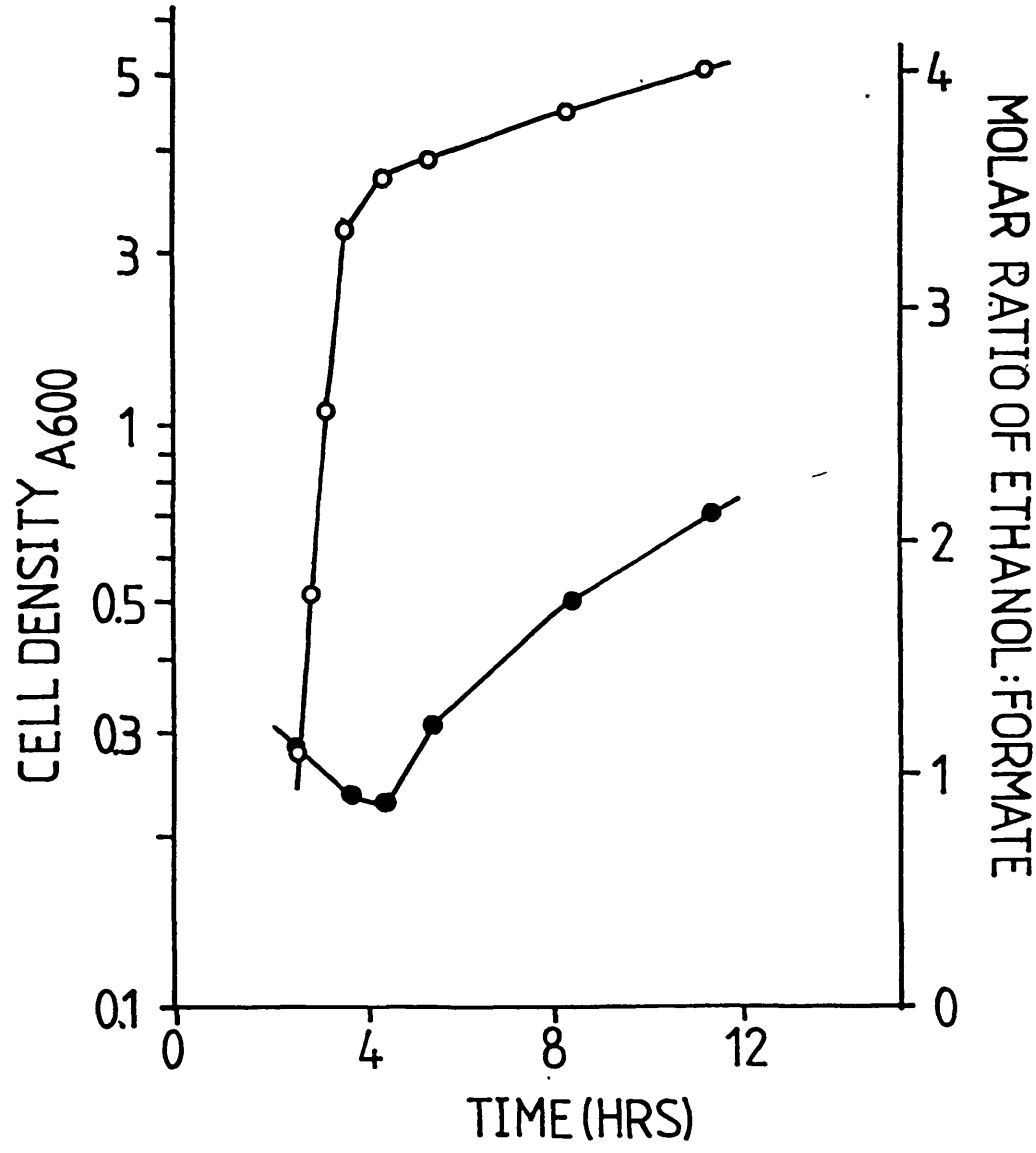


Figure 6.1 Cell Density and Molar Ratio of Ethanol: Formate for Experiment 152

(O) Cell Density, (●) Ratio of Ethanol:Formate.

Fermenter:- 5 litre New Brunswick, Working Volume: 3.4 litres. Operating Conditions:- Batch Anaerobic. Nitrogen flowrate to head: 0.2 litre/min. Impeller speed: 200 rpm. pH controlled by the addition of 30% KOH soln. Medium: BST. Inoculum: 3% (v/v) of Fermenter Working Volume. So: 23.5 g/litre. T: 60°C. pH: 6.2.

exponential phase. The effects of controlling medium pH by adding ammonia rather than potassium hydroxide were also investigated. Data from these experiments is presented in this chapter in the form of plots of cell density and sucrose concentration against time.

6.2 Methods

Experiments were normally performed in groups of three.

The quantities of ethanol, acetate and formate added to the fermenters and the time of addition (mid-long phase at a cell density of between 1.0 and 1.3 absorbance units) was intended to result in an approximate doubling of the concentration of these compounds in the culture medium to give the values at the time at which a decrease in the growth rate had previously been observed. The concentrations of ethanol and formate at that time were approximately equal to each other and double that of acetate. Acetate and formate were added as aqueous solutions of potassium salts, the pH of the solution was adjusted to 7.0 and the total volume made up to 100 ml. Sterilization of the solutions was by membrane filtration. 100 ml of sterile distilled water was added to the fermenters in control experiments. The quantities of acetate, formate and ethanol added to each fermenter were 6.6, 13.2 and 13.2 g respectively.

For experiment 235 6.6g of acetate and 13.2g of formate were dissolved in 150 mls of distilled water and sterilized as described above. The solution of acids was aseptically added to the fermenter at the rate of 1.0 ml/min. by means of a peristaltic pump.

6.3 Nutrient Limitation Studies

Figures 6.2a and 6.2b show cell density and sucrose uptake respectively against time for anaerobic growth of B. stearothermophilus 11d-15 in the 3 types of medium. The maximum cell densities attained in media B and C are similar as are the quantities of sucrose taken up. Specific growth rates in the later stages of growth are respectively 0.14 and 0.18 hr^{-1}. In medium A cell density increases initially at rates comparable to growth in the other two media but the maximum cell density attained is only approximately half that attained in the former and is followed by a rapid decline. The similar maximum cell densities, and growth rates in the latter stages in media B and C strongly imply that cessation of growth was not due to nutrient deficiencies. Growth was clearly inhibited in the most concentrated

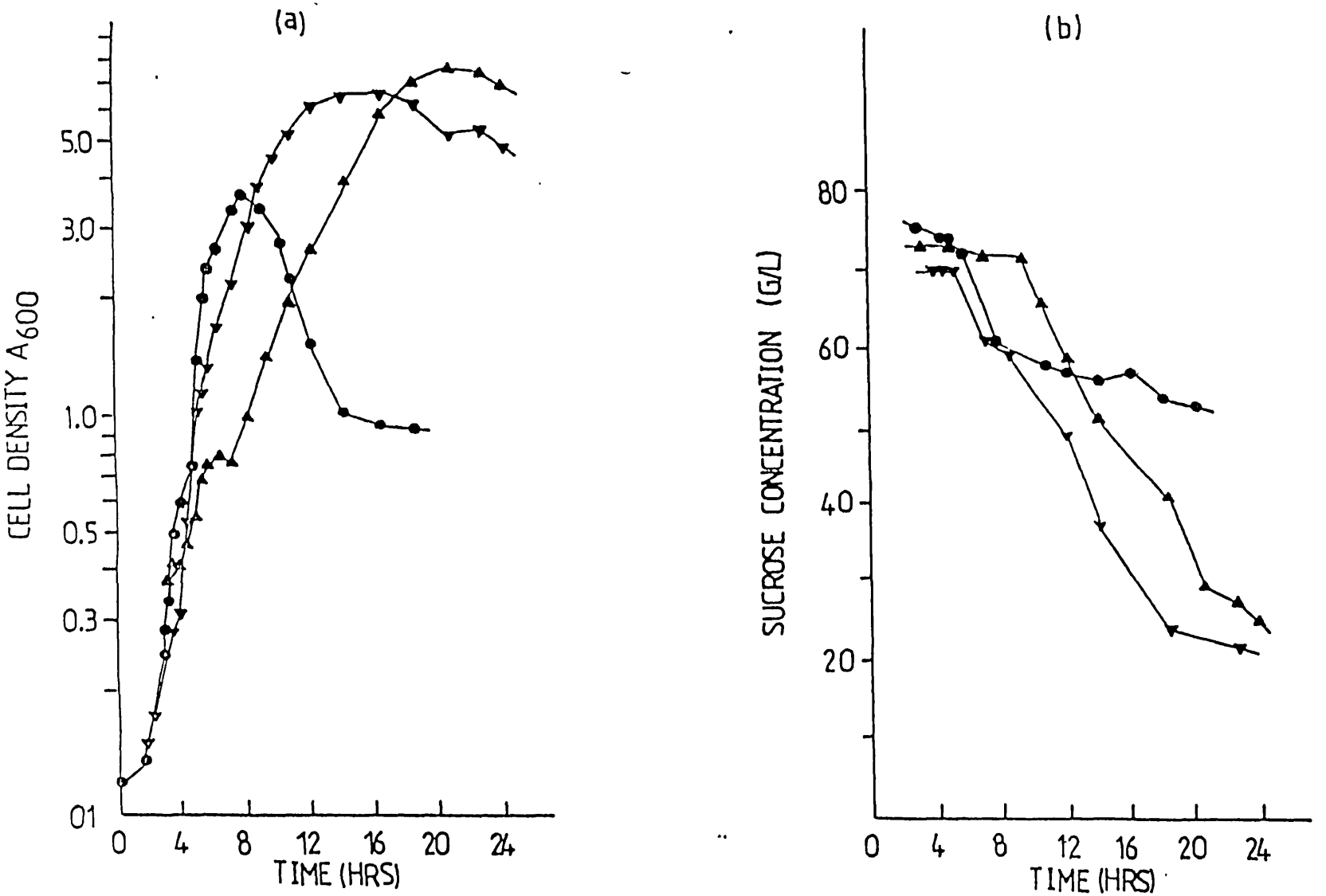


Figure 6.2a and b Growth and Sucrose Uptake in Various Media

(●) Medium A: Tryptone 4%, Yeast Extract 2%. (▲) Medium B: Tryptone 2%, Yeast Extract 1%. (▼) Medium C: Tryptone 1%, Yeast Extract 0.5%. Fermenters: 5 litre New Brunswick, Working Volume 3.4 litres. Operating Conditions: Batch Anaerobic Nitrogen flowrate to head: 0.2 litre/min. Impeller speed 200 rpm, pH controlled by the addition of 30% KOH soln. Inoculum: 3% (v/v) of Fermenter Working Volume. So = 75 g/litre. T = 60°C. pH 7.0.

medium (Medium A).

6.4 Growth Inhibition Studies

6.4.1 Results

The arrows in Figures 6.3 to 6.8 indicate the times of addition of potential inhibitors. Although formate, acetate and ethanol all exerted a growth inhibitory effect (Figures 6.3a and 6.4a) as indicated by reduced maximum cell densities relative to the control experiments, the effect in each case was relatively small. The greatest uptake of sucrose (Figures 6.3b and 6.4b) occurred in the control experiments. The greatest inhibitory effect (per mole of product added) was exerted by acetate and the least by ethanol. The total amounts of acetate, formate and ethanol present in the culture medium at the end of the fermentation were not however determined, so caution is needed in interpreting the experiment. The maximum cell density attained was marginally higher when ammonia rather than potassium hydroxide was used to control pH (Figure 6.5a). Sucrose uptake was also greater (Figure 6.5b).

The small increase in growth rate in the ammonia experiment in the latter stages of growth might indicate weak inhibition by K^+ ions.

Addition of acetate, formate and ethanol in combination to an actively growing culture had a pronounced effect both on cell density (Figure 6.6a) and sucrose uptake (Figure 6.6b): growth ceased approximately 2 hours after addition and a rapid decline in cell density ensued. The final cell density was less than one-half that of the control experiment. Moreoever, sucrose uptake was less than 10% that of the control.

The effect of sparging with carbon dioxide is shown in Figures 6.7a and 6.7b. Sparging was initiated immediately after inoculation. The maximum cell density attained (Figure 6.7a) is marginally below that attained in the (nitrogen sparged) control experiment. Sucrose uptake (Figure 6.7b) was similar for both experiments.

The combined effects of acetate, formate, ethanol and carbon dioxide on cell density and sucrose uptake are shown in Figures 6.8a and 6.8b, respectively, together with data showing the combined effects of carbon dioxide, acetate and formate. It is apparent that the inclusion of ethanol in the mixture of potential inhibitors has no additional inhibitory effect. This holds promise for the development

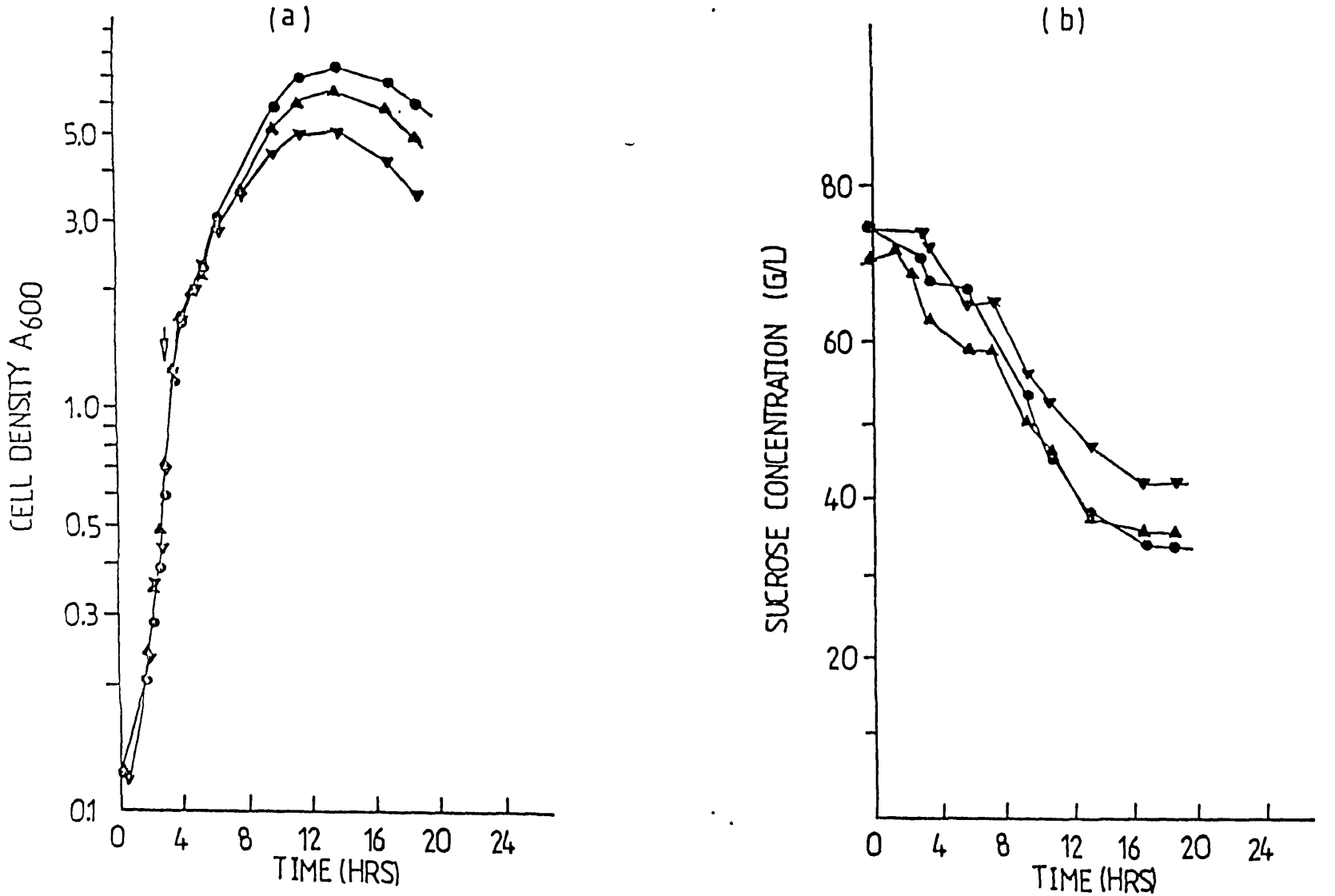
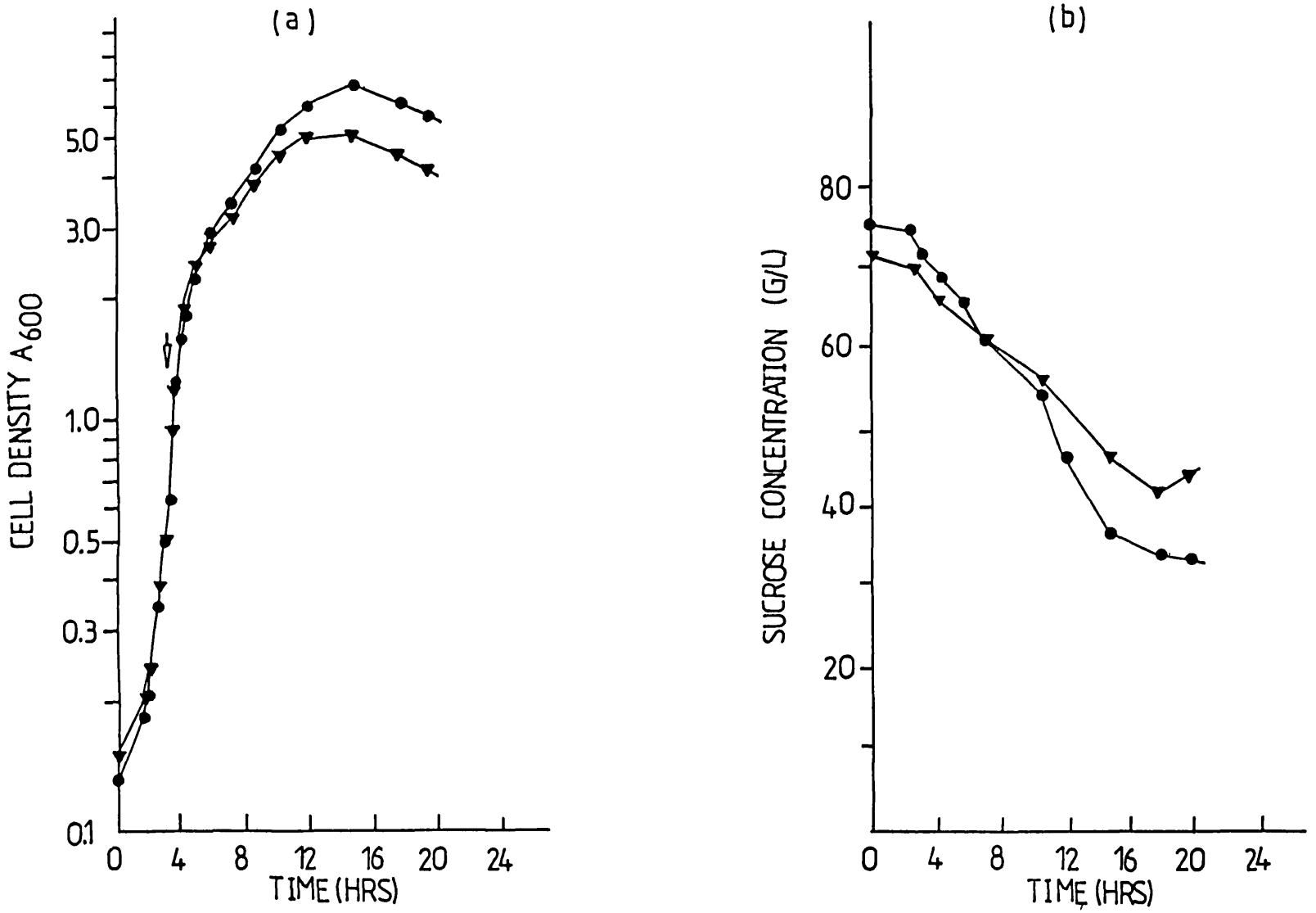
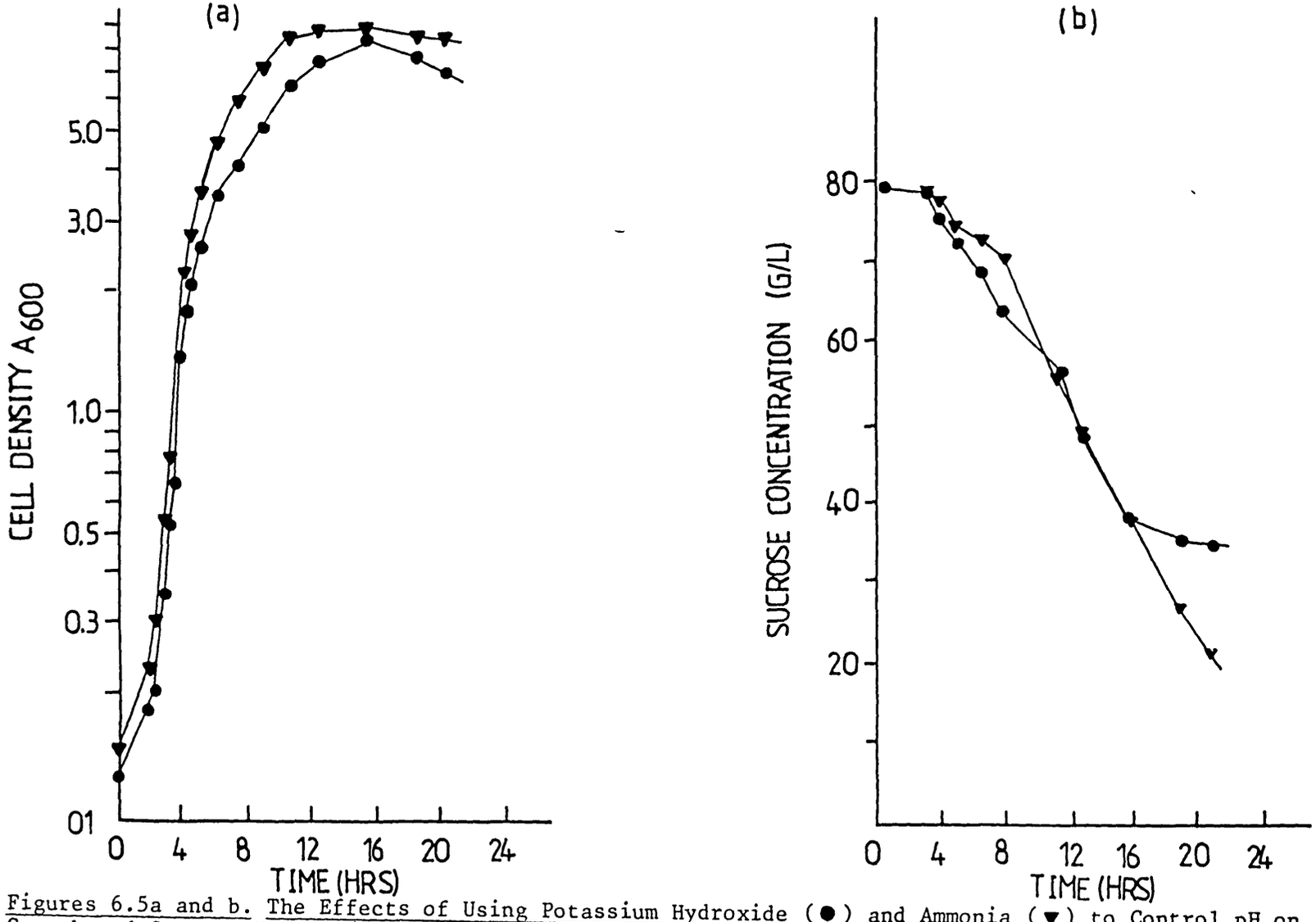


Figure 6.3a and b. The Effects of Potential Innibitors on Growth and Sucrose Uptake

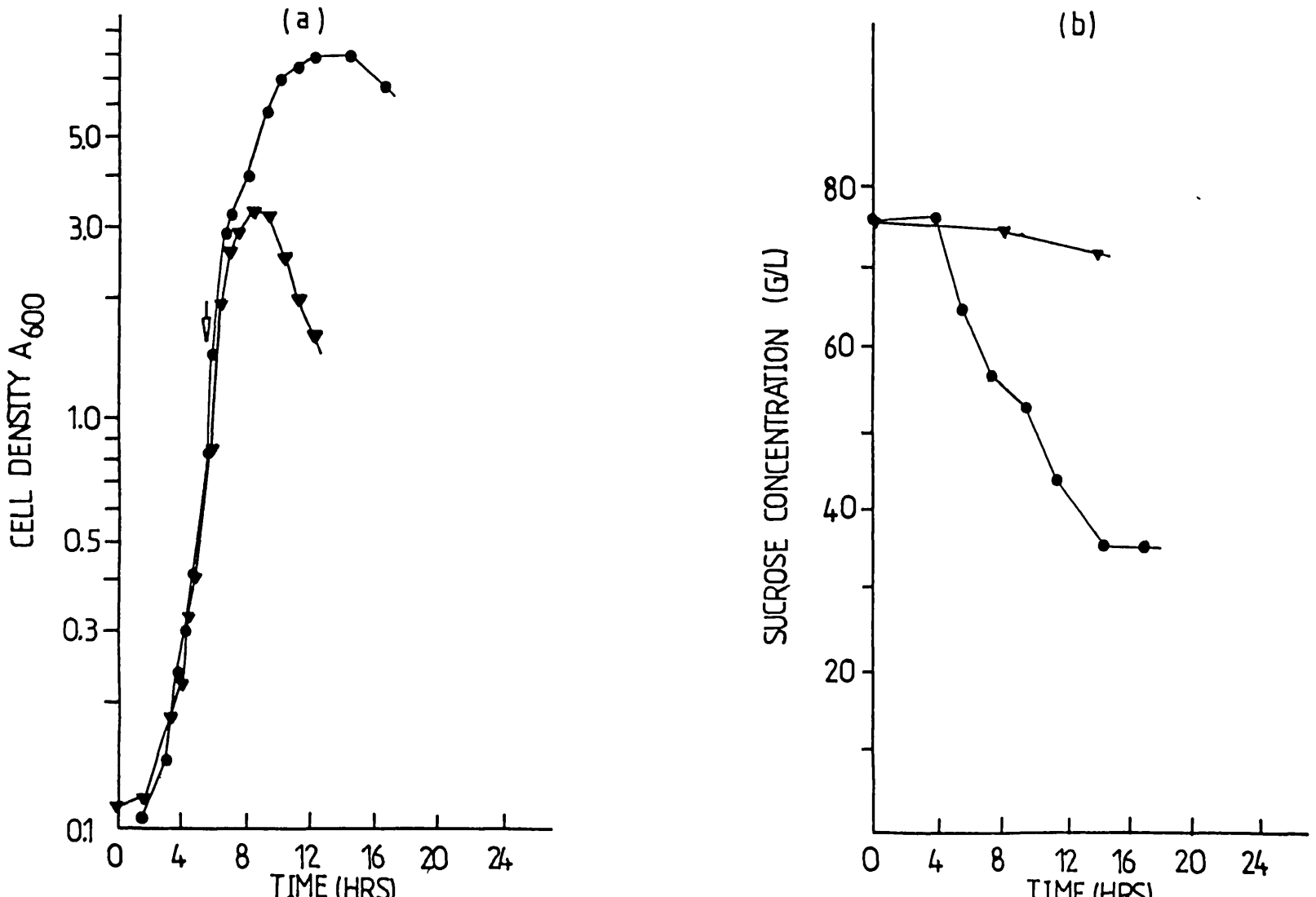
(▲) Acetate, (▼) Formate, (●) Control ↓ Indicates time of addition of fermentation product. Fermenters: 5 litre New Brunswick, Working Volume 3.4 litres. Operating conditions: Batch, Anaerobic Nitrogen Flowrate to sparger: 1.0 litre/min. Impeller speed 250 rpm. pH controlled by the addition of 30% KOH soln. Inoculum 3% (v/v) of Fermenter Working Volume. So: 75 g/litre, T: 60°C. pH 7.0.



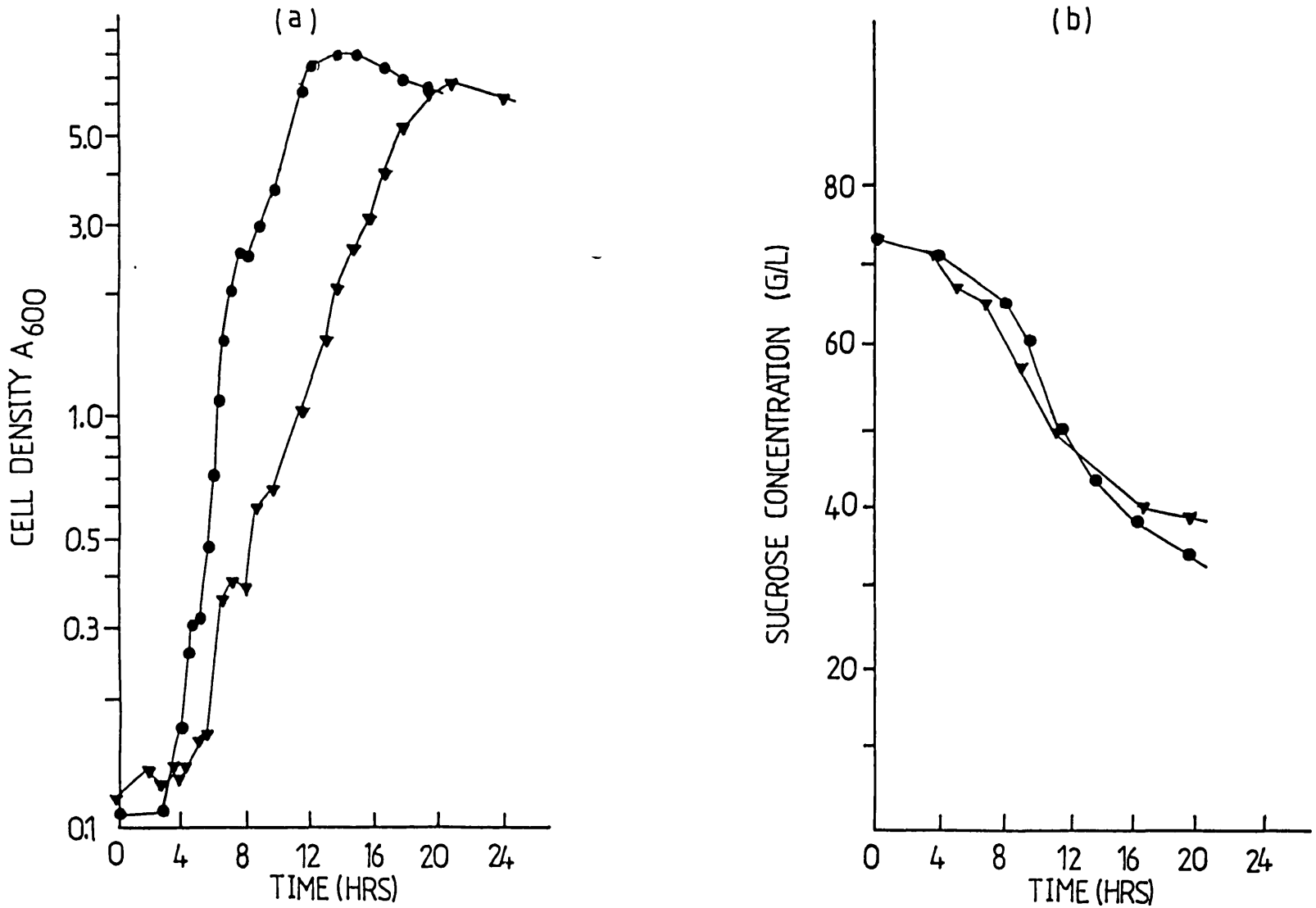
Figures 6.4a and b. The Effects of Potential Inhibitors on Growth and Sucrose Uptake. (▼) Ethanol, (●) Control. (see footnote to Figures 6.2 for Operating Conditions).



Figures 6.5a and b. The Effects of Using Potassium Hydroxide (●) and Ammonia (▼) to Control pH on Growth and Sucrose Uptake (see Footnote to Figure 6.2 for Operating Conditions).



Figures 6.6a and b. The Effects of Potential Inhibitors on Growth and Sucrose Uptake. ▼ Acetate+Formate+Ethanol, ● Control. (See Footnote to Figure 6.2 for Operating Conditions).



Figures 6.7a and b. The Effects of Sparging with Nitrogen (●) and Carbon Dioxide (▼) on Growth and Sucrose Uptake

(See Footnote to Figure 6.3 for Operating Conditions)

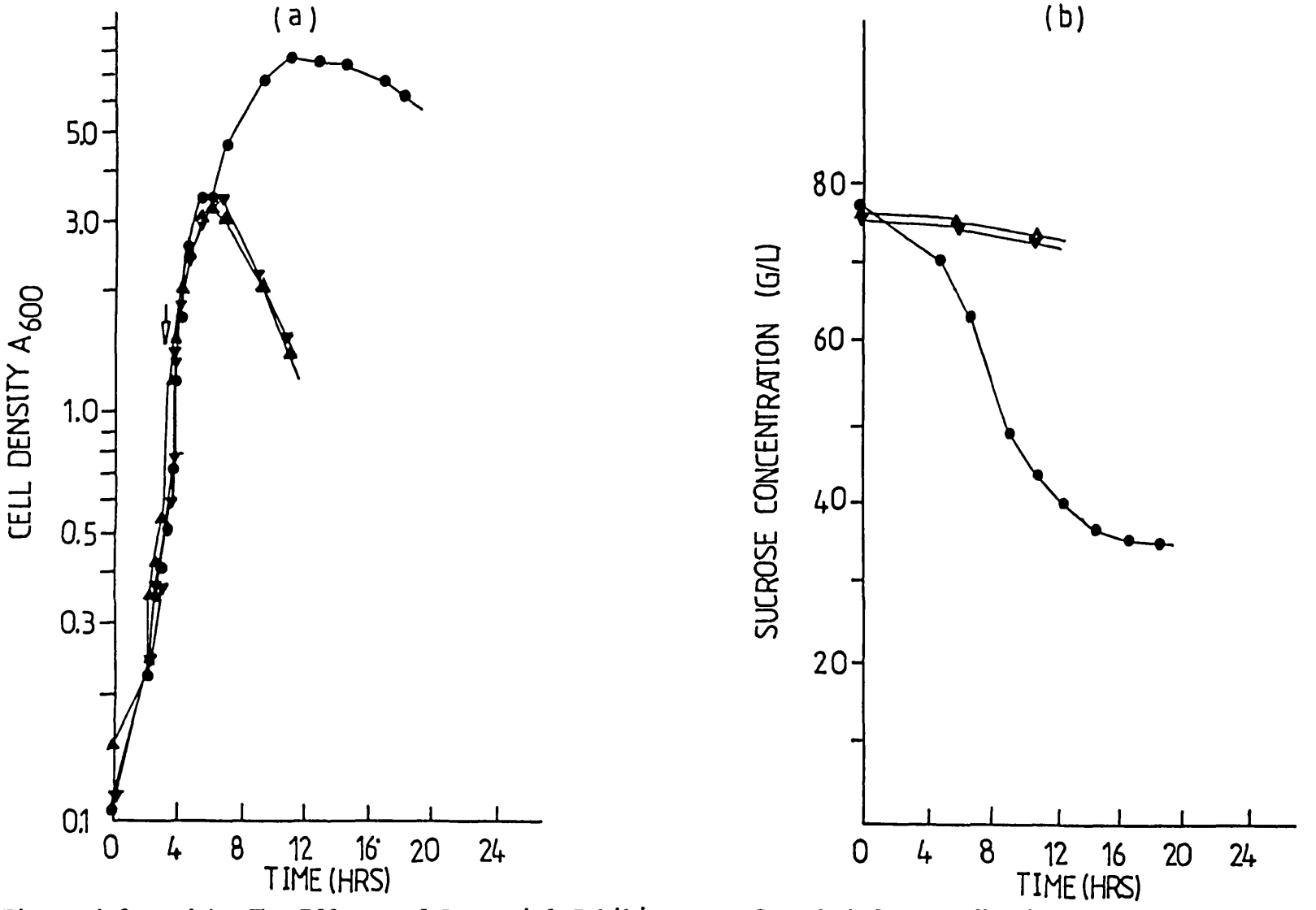


Figure 6.8a and b. The Effects of Potential Inhibitors on Growth & Sucrose Uptake
(▲) Acetate + Formate + Ethanol + Carbon Dioxide, (▼) Acetate + Formate + Carbon Dioxide, (●) Control.

(See Footnote to Figure 6.2 for Operating Conditions)

of high ethanol yielding mutants of B. stearothermophilus.

Data from experiment 235 is shown in Figures 6.9 to 6.12. The most notable feature is that pyruvate is the major fermentation product. The characteristics of the acetate curve (i.e. its slope and maxima) suggest that only added acetate was present in the culture broth and that if any acetate was produced from sucrose its rate of consumption must have equalled or exceeded its rate of production. The addition of the acids was completed 3.6 hours after inoculation and at approximately this time the concentration of acetate begins to decrease. Some formate production did occur after 3.6 hours but only at a very low rate. Ethanol was produced both during the period of acid addition and after addition was completed, however its rate of production after approximately 5 hours is very low. The maximum cell density (Figure 6.9) was reached shortly after 3.6 hours. Sucrose uptake was approximately constant during the first 5 hours but then declined quite sharply. The highest ethanol/formate ratio attained -5.61 occurred between 4.9 and 10.1 hrs. During this time 14.7 mM sucrose and 9.8 mM acetate were consumed against which were produced 2 mM carbon dioxide (evolved carbon dioxide only), 6.5 mM formate, 36.3 mM ethanol (14.6 mM in the culture broth and 22 mM in the cardice trap) and 25.4 mM pyruvate: a carbon recovery (moles produced/moles consumed) of approximately 80%.

6.4.2 Discussion

The exprimental findings with respect to growth inhibiton are summarized in Table 6.1.

The inhibitory effect caused by the addition of ethanol, acetate, formate or carbon dioxide in isolation was relatively low. Identical levels of inhibition were achieved in experiments 215, 219 and 221. Comparison of the results of experiments 215 and 219 shows that the addition of carbon dioxide to acetate, formate and ethanol does not increase inhibition; comparison of experiments 215 and 221 suggests that the observed inhibition was due solely to acetate and formate. However, it was not established whether the increased inhibition caused by both acids together over inhibition by each acid singly was due to a synergistic chemical interaction or was a non-specific effect dependent on total acid concentration.

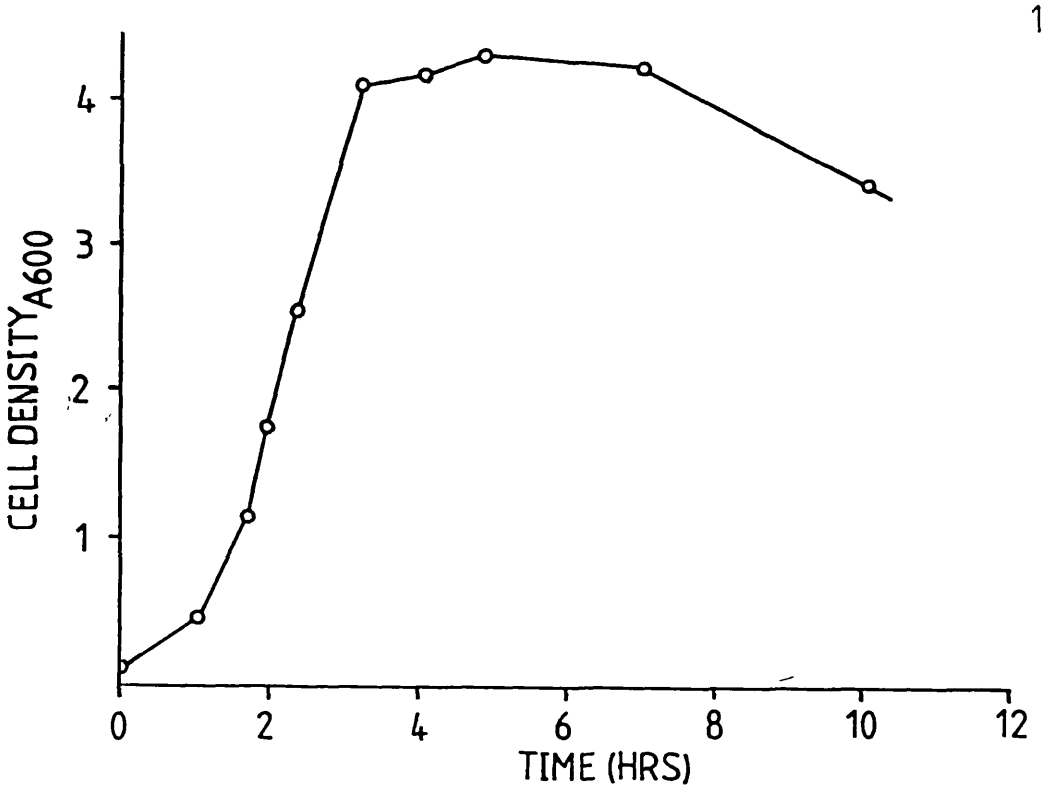


Figure 6.9 Effect of Acetate and Formate on Cell Density

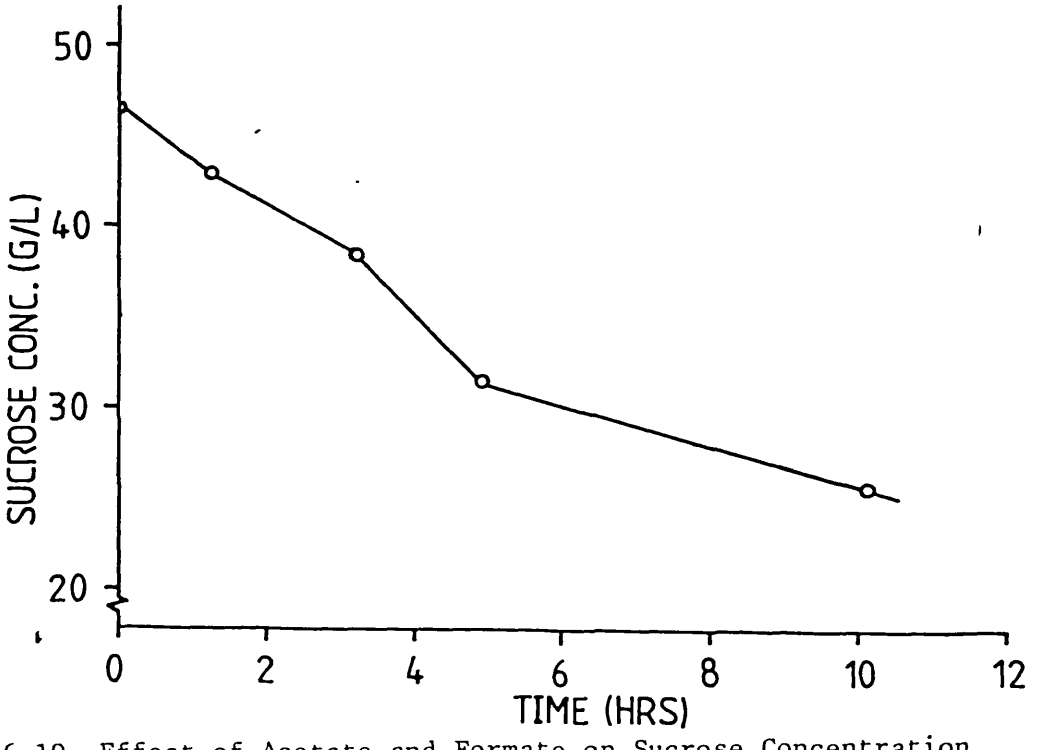


Figure 6.10 Effect of Acetate and Formate on Sucrose Concentration

Fermenter:- 5 litre New Brunswick, Working Volume: 3.4 litres
Operating conditions:- Batch Anaerobic, Nitrogen flowrate to sparger:0.5litre/min
Impeller speed: 200 rpm. pH controlled by the addition of 30% KOH soln.
Medium: BST. Inoculum: 3% (v/v) of Fermenter Working Volume. So: 50 g/litre.
T: 60°C, pH: 6.6. The addition of acetate and formate was made between 1.3 and 3.6 hours.

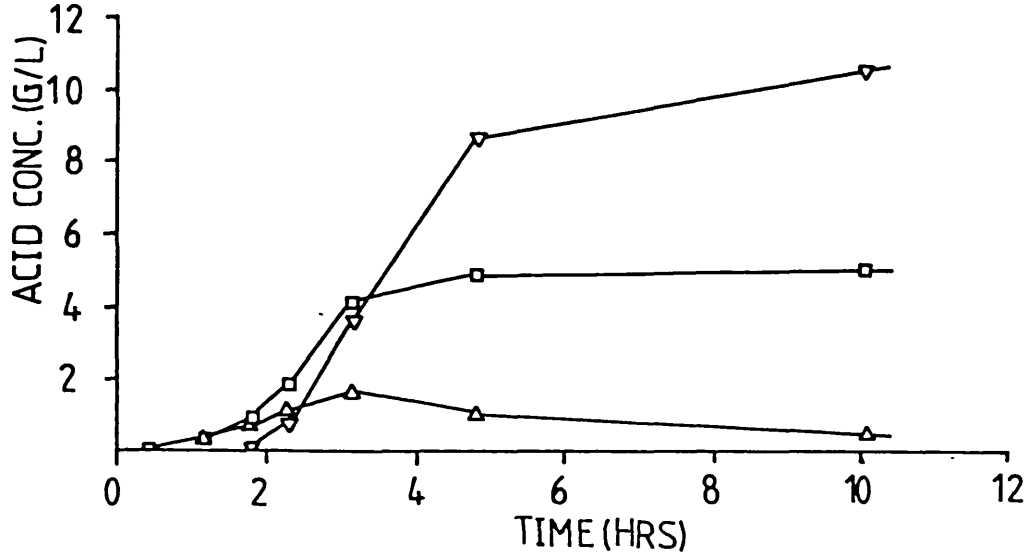


Figure 6.11 Concentration of Acidic Products against Time
(□) Formate, (△) Acetate, (▽) Pyruvate

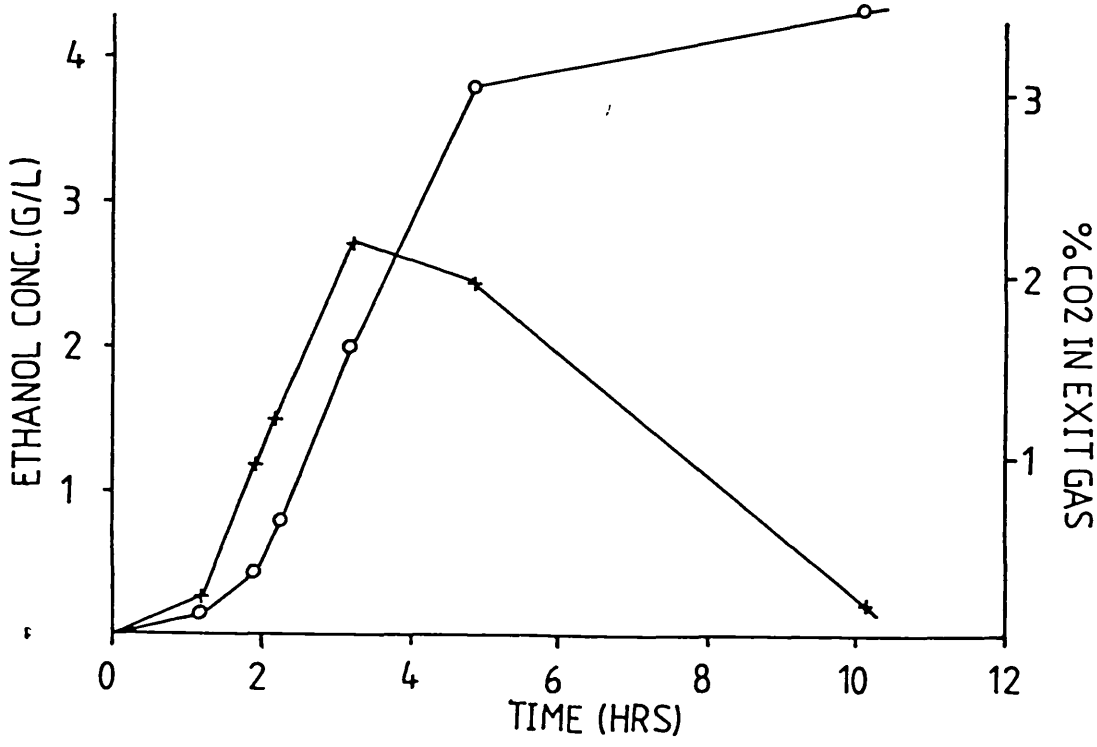


Figure 6.12 Concentration of Ethanol (O) and Carbon Dioxide (+) against Time

Experiment	Additions	Cell Density Ratio*
202	Acetate	0.87
204	Formate	0.70
205	Ammonia	1.17
207	Ethanol	0.75
217	Carbon Dioxide	0.85
215	Acetate, Formate, Ethanol	0.42
219	Acetate, Formate, Ethanol, Carbon Dioxide	0.42
221	Acetate, Formate, Carbon Dioxide	0.44

Table 6.1 Summary of Results

*Max. Cell Density/Max.Cell Density of Control

In the light of the findings that acetate and formate cause cessation of growth of B. stearothermophilus 11d-15 it is appropriate to examine the experimental data of Chapter 5 for evidence of inhibition by these acids. The composition of the culture medium at the point at which a decrease in growth rate is first noted is tabulated for a number of experiments in Table 6.2 to 6.4. The data is tabulated as a function of temperature and pH in the form of 'mole fraction of acetate + formate' which is defined as moles of (acetate + formate)/moles of (ethanol + acetate + formate). Tables 6.2 to 6.4 show that a decrease in growth rate occurs at progressively lower mole fractions of acetate and formate as pH is decreased and temperature is increased. Also shown in these tables are the concentrations of pyruvate (in milli-molar) at the end of the logarithmic phase. The pyruvate concentrations are seen to be inversely proportional to the mole fraction of acetate and formate except for transitions in pH at 50°C where pyruvate concentrations are relatively low.

It was noted in Chapter 5 that product ratios displayed departures from those expected for metabolism via the PFL pathway in transitions from alkali to acid pHs at 60°C and for increases in temperature at pH 7.0. It is therefore possible that the low tolerance of B. stearothermophilus 11d-15 to acetate and formate at acid pHs and high temperatures may have triggered shifts in metabolism which resulted in the production of less acidic end products.

When acetate and formate were exogenously supplied to growing cells of B. stearothermophilus 11d-15 in experiment 235, sucrose uptake continued for a period of time, however most of the sucrose was only partially metabolized, with large quantities of the metabolic intermediate pyruvate being excreted by the cells into the medium. Only a small fraction of the pyruvate was further metabolized to yield ethanol and lesser quantities of formate. Another unexpected phenomenon was that a portion of the exogenously added acetate was taken up by the cells and metabolized. A hypothesis based on these observations and which attempts to account for metabolism by B. stearothermophilus 11d-15 under these 'stressful' conditions is presented below.

Mitchell (1966) proposed the 'chemi-osmotic coupling' hypothesis to explain how the energy yielded by electron transport in

Experiment	pH	Mole Fraction of Acetate & Formate	Conc. Pyruvate mM
152	6.2	0.602	21.7
174	6.6	0.614	12.0
151	7.0	0.699	7.5
153	8.0	0.822	4.0

Table 6.2 Medium Composition at the End of the Logarithmic Phase of Growth at Various pHs (Temperature = 60°C).

Experiment	pH	Mole Fraction of Acetate & Formate	Conc. Pyruvate mM
156	7.0	0.769	1.0
154	8.0	0.835	1.1

Table 6.3 Medium Composition at the End of the Logarithmic Phase of Growth at Various pHs (Temperature = 50°C).

Experiment	Temp. °C	Mole Fraction of Acetate & Formate	Conc. Pyruvate mM
156	50	0.769	1.0
151	60	0.699	7.5
160	70	0.631	13.9

Table 6.4 Medium Composition at the End of the Logarithmic Phase of Growth at Various Temperatures (pH = 7.0)

Mole Fraction of Acetate + Formate =

$$\frac{\text{Moles Acetate + Moles Formate}}{\text{Moles of Acetate + Moles of Formate + Moles of Ethanol}}$$

oxidative phosphorylation is converted into the phosphate bond energy of ATP. According to this model electron transport reactions cause protons to be pumped out of the cell thus generating a proton gradient across the membrane (a 'proton motive force'). This proton motive force is then used to drive the synthesis of ATP by a membrane bound ATPase complex when protons are pulled back into the cell. The anaerobic metabolism of sugars to acidic end products and the transport or translocation of these acids from the cytoplasm to the cell exterior also involves proton transport. Under these conditions bacteria generate a proton motive force via a proton translocating ATPase (Thauer et al., 1977). However not all protons are transferred via this ATPase: Harold and Levin (1974) found that in Streptococcus faecalis, the major product of glucose fermentation, lactate was translocated from the cells in protonated form by a carrier mediated process independent of the membrane ATPase.

It is postulated here that the preponderance of pyruvate in the culture broth of B. stearothermophilus 11d-15 occurred because pyruvate is similarly translocated by a carrier mediated process whereas acetate and formate are not and that their translocation would require the expenditure of ATP. Moreover, it is also postulated that acetate acted as an 'uncoupler' of the phosphorylation of ADP to ATP. Uncouplers are compounds which act by transporting protons across membranes (which are normally proton-impermeable) thereby causing a collapse of the proton gradient. (Lardy and Wellman, 1953). Organic acids such as acetate are soluble in the lipids of the cell membrane (Conway and Downey, 1950) and are known to behave as uncouplers (Herrero, 1983). The cessation of growth and the depression of sucrose uptake in experiment 235 was probably due to the entry of acetate into the cells in a protonated form. The fate of the acetate once inside the cells is uncertain but it may have been metabolized to acetyl CoA.

If this hypothesis is correct then some interpretation of Figure 6.1 becomes possible; during the logarithmic period of growth energy production occurs primarily via the PFL pathway, however at critical acetate and formate concentrations (which depend on environmental conditions) the translocation of these acids out of the cells becomes the rate limiting step in the metabolism of sucrose. These acids begin to accumulate intracellularly and in so doing exert an inhibitory effect on metabolic steps beyond pyruvate more readily

than on those between pyruvate and sucrose. This in turn results in the intracellular accumulation of intermediates one or more of which may cause the activation of an alternative pathway of energy metabolism. Such a pathway is that resulting in ethanol production which as a neutral compound is able to be translocated with relative ease. The corresponding decrease in the channeling of pyruvate down the PFL pathway results in a decrease in ATP production as less acetyl phosphate is converted to acetate, and this is manifested by a decrease in growth rate.

6.5 Conclusion

The cessation of growth in batch culture could be brought about by the addition of acetate and formate and was not caused by nutrient depletion. It was suggested that these acids acted as uncouplers of ADP phosphorylation and that B. stearothermophilus 11d-15 responded to the inhibitory effects of these acids by diverting pyruvate away from further acid production towards increased ethanol production. An attempt to externally manipulate the metabolism of strain 11d-15 was partially successful in that favourable ethanol/formate ratios were obtained but industrially impractical in that large amounts of pyruvate were released into the medium.

CHAPTER 7

CONTINUOUS CULTURE STUDIES

7.1 Introduction

An important characteristic of continuous culture is that it offers, within certain limits, the possibility of adapting organisms to conditions which they would normally not tolerate. This may be achieved by imposing the desired changes on the culture in a step-wise fashion. For example, it was shown in Chapter 5 that increases in ethanol yield could be achieved by culturing B. stearothermophilus 11d-15 under acid conditions, moreover, it was found that 11d-15 grew at pH 6.2 but not at pHs 5.5 or 5.0. It would be of interest to know whether the lower limit of pH tolerance of 11d-15 lay between pH 6.2 and 5.5 or whether the strain could be adapted to grow at pHs below 5.5 and also whether ethanol yields continue to increase at pHs below 6.2. One method of determining this would be to culture 11d-15 in continuous culture at a pH known to support growth and then to impose a small decrease in culture pH. Then after a new steady state had been reached a further decrease in culture pH could be imposed. This process could be repeated until washout of the culture occurred. This method of adapting the culture is one of selection amongst the microbial population for individuals more able to tolerate the new conditions - in the example above, a lower pH - than the population as a whole. In addition, batch experiments are not ideal for characterizing the effects of different environments on parameters of interest, such as specific ethanol productivity, since the medium composition changes continuously with time. Continuous culture techniques permit such investigations to be made under steady state conditions. Changes in the concentration of fermentation products or substrate can be imposed by altering the dilution rate.

The initial objectives of the present continuous culture experiments were to examine the effects of variations in environmental conditions on the metabolism of B. stearothermophilus.

It was also hoped that by adapting the cultures as described above a wider range of environmental conditions could be examined than had been possible with batch culture. The effect of pH on the metabolism of strain NCA 1503 was successfully investigated, but strain 11d-15 in continuous culture consistently showed a tendency towards reversion to a metabolism resembling that of the wild-type. This was manifested by a dramatic increase in the yield of lactate and corresponding decreases in the yields of all other fermentation products. The experiments with 11d-15 described in this chapter consist primarily of investigations into this reversion. The fermentation of sucrose by a revertant strain isolated from a chemostat was compared in batch culture with strains 11d-15 and NCA 1503. The findings (Chapter 6) that acetate and formate inhibit the growth of 11d-15 suggested a successful technique for maintaining strain 11d-15 in continuous culture. Studies were also made on the transient effects arising from the introduction of pulses of sucrose and pyruvate into established chemostat cultures.

7.2 Methods

Isolation of a revertant strain

A culture sample was taken aseptically from the chemostat after reversion had occurred in experiment 241 and streaked out onto BST agar plates. The plates were incubated overnight at 60°C and single colonies were carefully picked off and replated onto BST agar. When the latter had been incubated overnight the plate having the most prolific growth was used to prepare inoculum as described in Sub-section 2.2.3.

7.3 B. stearothermophilus NCA 1503 (Wild Type)

7.3.1 Results

The effects of pH on product formation by B. stearothermophilus NCA 1503 were examined in Experiment 183. Component fluxes (g/hr) of overflow and gas exit streams from the fermenter and of the sucrose feed stream to the fermenter are shown in Table 7.1. The component fluxes shown in the table were averaged over the entire period of steady state operation and were obtained by multiplying averaged component concentrations by the appropriate averaged

Time* hr	pH	Outlet							Inlet
		Formate	Acetate	Lactate	Ethanol	Sucrose	Biomass	Carbon Dioxide	Sucrose
36.5-49.5	7.0	0.21	0.14	4.25	0.03	0.12	0.30	0.05	5.00
111.0-133.0	6.6	0.08	0.06	3.28	0.00	1.21	0.26	0.04	4.76
160.5-182.5	6.35	0.06	0.06	2.70	0.00	2.35	0.22	0.04	5.27
207.5-230.8	8.0	0.26	0.13	4.08	0.00	0.13	0.33	0.04	5.08

Table 7.1 Component Fluxes (g/hr) in Experiment 183

*These figures represent the time after inoculation over which steady state conditions were maintained.

Fermenter:- 1 litre L.H., Working Volume: 0.75 litre
Operating Conditions: Continuous, Anaerobic. Nitrogen flowrate to sparger: 0.25 litre/min. Impeller speed: 250 rpm. pH controlled by the addition of 30% KOH solution. Medium: BST. Medium feed sucrose concentration: 2.35% (w/v). Inoculum: 3% (v/v) of fermenter working volume. Dilution rate: 0.25 hr^{-1}. T: 60°C.

stream flow rates. The former are simply the arithmetic means of 'instantaneous' component concentrations in the samples taken at the beginning and end of each steady state period of operation. Medium and potassium hydroxide solution volumes were recorded whilst taking samples from the fermenter and the average medium and potassium hydroxide solution input rates were obtained by dividing the volumes added by the time interval between samples: the overflow rate is the sum of medium and potassium hydroxide addition rates. The average gas exit flowrate was obtained by calculating the arithmetic mean of the instantaneous flowrate readings made at 2-hourly intervals.

As no calibration curve for absorbance at 600 nm versus cell dry weight was available for strain NCA 1503, the conversion factor for strain 11d-15 was used. The quantities of potassium hydroxide added to maintain a constant pH are shown in Table A3.1 (Appendix 3). Molar product yields (Table 7.2) were derived from Table 7.1. Lactate is clearly the major product at all pHs with ethanol being detectable in only very low yields (0.04 moles per mole of sucrose at pH 7.0). The molar ratios of formate: acetate and lactate: formate as a function of pH are also shown. A decrease in pH from 8.1 to 6.35 results in a decrease in the ratio of formate : acetate and an increase in the ratio of lactate : formate.

In the calculation of carbon recoveries, also shown in Table 7.2, it was assumed that the carbon composition of cells of strain NCA 1503 was identical to that of 11d-15 viz. 48.8% (see 5.3.3) and further that 11.35% of cell carbon was derived from sucrose (see 5.3.2). The calculated error in the carbon recovery figures is ± 5% and was based on analytical measurement errors and errors in the overflow rate. O/R indices (not shown) calculated from the yield data of Table 7.2 were between 3 and 6.

7.3.2 Discussion

The ethanol yields produced by strain NCA 1503 were unexpectedly low by comparison with results from previous studies: Atkinson et al. (1975a) with strain NCA 1503 and McKray and Vaughn (1957) with several strains of B. stearothermophilus reported yields in the region of 0.12 to 0.18 moles of ethanol per mole of glucose which corresponds to between 0.24 to 0.36 moles of ethanol per mole of sucrose. Periodic GC analysis of the fermenter exit gas by the method described

pH	FORMATE	ACETATE	LACTATE	ETHANOL	CARBON DIOXIDE	MOLES FORMATE / MOLES ACETATE	MOLES LACTATE / MOLES FORMATE	CARBON RECOVERY %
6.35	0.15	0.12	3.50	0.00	0.11	1.21	24.15	93.0
6.6	0.15	0.11	3.52	0.00	0.07	1.43	23.44	91.8
7.0	0.31	0.16	3.31	0.04	0.07	1.94	10.68	90.3
8.0	0.39	0.15	3.13	0.00	0.06	2.68	8.13	85.3

Table 7.2 Product Yields*, Product Ratios and Carbon Recoveries for Experiment 183

* Moles per mole of sucrose utilized

in Sub-section 3.3.2 did not reveal the presence of ethanol so that it is unlikely that the disparities were due to the loss of ethanol by evaporation.

At the time experiment 183 was conducted the GC technique used for the assaying of acidic fermentation products had not been refined to permit succinate determinations to be made, however it was later discovered that strain NCA 1503 does produce succinate (see Figure 3.7a and Table 7.4). Undetected succinate might fully explain the high O/R ratios obtained and partly account for the incomplete carbon recoveries. Trace quantities of succinate would have resulted in O/R ratios nearer 1.0.

Further disparities exist between the results of this work and those of earlier studies. Atkinson et al. reported acetate yields of 0.0185 moles per mole of glucose, a figure which is much lower than those shown in Table 7.2. These inconsistencies may have been due to small differences in the methods employed in culturing strain NCA 1503. For example, Atkinson and his co-workers used a temperature of 65°C whereas 60°C was used here, moreover, they sparged their cultures with relatively large volumes of nitrogen in order to remove ethanol (and possibly other volatiles) from the culture environment. In contrast, McKray and Vaughn obtained higher acetate and formate yields than those of Table 7.2. They used uncontrolled pH batch culture and determined the combined quantities of acetate and formate produced by titration and then assumed that these acids were produced in the ratio of 2 moles of formate per mole of acetate. The data of Table 7.2 shows that this ratio approaches 2:1 at pH 7.0, and as pH was continuously changing with time in their experiments their assumption was unjustified.

The presence in the gas phase of carbon dioxide was probably due to the decarboxylation of amino acids - in batch culture experiments with lld-15 the carbon dioxide yields in excess of those predicted from fermentation balances were explained in Chapter 5 as by products of biosynthetic reactions. The comparatively low carbon dioxide yields produced by NCA 1503 are to be expected since its specific growth rate was 0.25 hr^{-1} which is very much

lower than the specific growth rates displayed by 11d-15 under similar environmental conditions (Table 5.4).

The lactate to formate ratios shown in Table 7.2 indicate that at low pHs much of the pyruvate flux was towards lactate production, however as the pH increased greater diversion of pyruvate via the PFL pathway took place. The formate to acetate ratio increased with an increase in pH. At low pHs it appears therefore that a significant portion of the Acetyl-CoA produced was not converted to acetate but channeled towards other products.

7.4 B. stearothermophilus 11d-15

7.4.1 Results

It may be useful to define here terms which are made use of frequently in this chapter. Reversion in a microbial population implies a genetic change resulting in the expression of phenotypic characteristics resembling that of the wild-type. A 'revertant' is a mutant possessing these characteristics. In the context of this work a revertant is a mutant which ferments sucrose to yield principally lactate and lesser quantities of acetate, formate and ethanol. The term is not intended as implying genetic identity with the wild-type.

Several attempts were made to grow B. stearothermophilus 11d-15 in continuous culture under conditions identical to that used for culturing strain NCA 1503, however all of these attempts ended in colonization of the chemostat by lactate producers. Complete takeover by the latter generally occurred between approximately 70 and 100 hours after inoculation of the fermenters. Table 7.3 shows product concentrations for one such experiment. The ethanol concentration is approximately constant over the first 54 hours but declines sharply thereafter. At the same time the acetate and formate concentrations decreased and the lactate concentration increased.

The effects of product concentration on the stability of strain 11d-15 in continuous culture were examined by culturing the organism in chemostats each supplied with BST medium of a different sucrose concentration. The sucrose concentrations examined were 0.5 and 2.5% (w/v). Both chemostats were inoculated simultaneously with the same source of inoculum.

Time hr	pH	Formate	Acetate	Pyruvate	Lactate	Ethanol	Sucrose	Biomass
25	7.0	4.90	2.40	0.39	0.54	6.28	1.18	2.21
45	7.0	5.05	2.40	0.39	0.59	6.96	0.88	2.35
54	7.0	5.66	3.11	0.52	0.71	6.51	0.80	2.17
78	6.6 *	3.07	2.19	ND	9.13	2.70	0.70	1.54
105	6.6	0.79	1.12	ND	17.51	0.61	3.72	1.58

Table 7.3 Concentrations (g/l) of Components in the Overflow Streams in Experiment 185

Fermenter:- 1 Litre L.H., Working Volume: 0.75 litre.
Operating Conditions:- Continuous, Anaerobic, Nitrogen flowrate to sparger: 0.25 ml/min.
Impeller speed: 250 rpm. pH controlled by the addition of 30% KOH soln. Medium: BST.
Medium feed sucrose concentration: 2.35% (w/v).
Inoculum: 3% (v/v) of fermenter working volume. Dilution Rate: 0.25 hr^{-1}. T: 60°C.

ND Not Detected

* pH set point altered from 7.0 to 6.6 at 78 hrs.

Overflow product concentrations for experiment 241, in which the medium feed contained 2.5% sucrose are shown in Figure 7.1. Graphs of sucrose concentration and cell density against time are contained in Section A3.2. The fluctuations in product and sucrose concentrations between 10 and 25 hours are the results of perturbations following the change from batch to continuous operation which was made at approximately 9 hours. At approximately 30 hours after inoculation lactate appeared and increased rapidly in concentration thereafter. Corresponding decreases in the concentrations of all the other products also occurred after 30 hours. Pyruvate was completely depleted from the culture broth by 40 hours. The gas phase carbon dioxide concentration decreased initially but peaked during the period in which the lactate concentration increased. The concentration of succinate (not shown in Figure 7.1) did not exceed 0.75 g/litre.

In experiment 242 medium containing 0.5% sucrose was supplied to the chemostat for approximately 120 hours. During this period product concentrations (Figure 7.2) gas phase carbon dioxide levels, Figure 7.3 and cell density and sucrose concentrations (Section A3.2) changed little. The molar ratios of ethanol to acetate and ethanol to formate were approximately 0.35 and 0.22 respectively. No lactate, pyruvate or succinate production occurred during this period. At 128 hours the concentration of sucrose in the incoming medium was increased to 2.1%. Product concentrations rose accordingly but declined as the concentration of lactate rose and takeover by lactate producers occurred. A sharp rise then decline in the gas phase carbon dioxide concentration occurred during the period of takeover (Figure 7.3).

Figure 7.4 shows product concentrations against time for experiment 244. The chemostat was continuously supplied with medium containing 0.5% sucrose but at 27 hours a sterile solution of sucrose (50 g in 100 mls) was aseptically introduced into the chemostat. Graphs of sucrose concentration, cell density and gas phase carbon dioxide levels are contained in Section A3.2. After 27 hours the concentration of all products increased to a maximum then decreased as steady state was once more attained. No lactate was produced following the introduction of extra sucrose and the

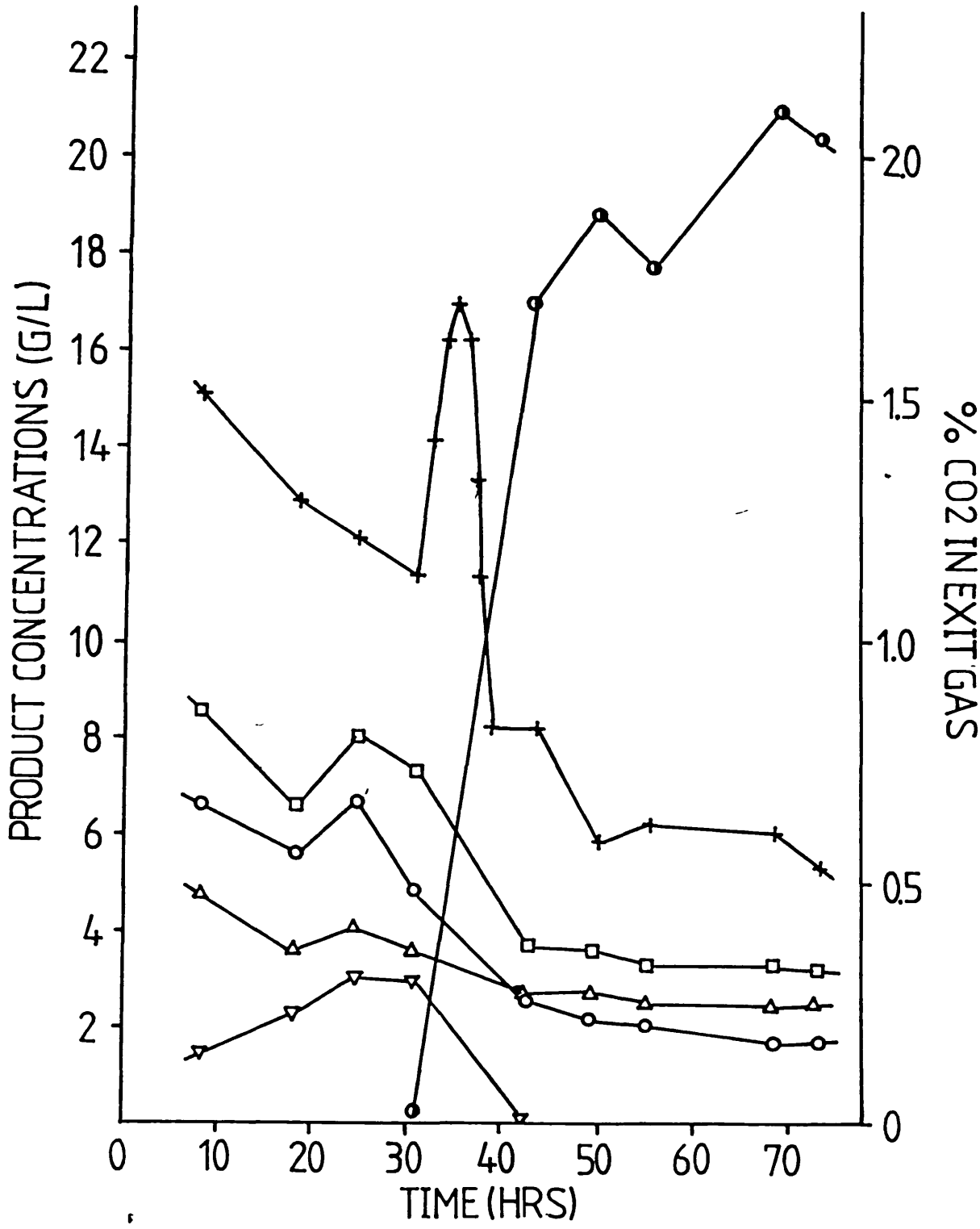


Figure 7.1 Product Concentrations against Time for Experiment 241
(o) ethanol, (□) Formate, (△) acetate, (▽) pyruvate, (●) lactate, (+) carbon dioxide
Fermenter: 5 litre New Brunswick, Working Volume: 2.0 litres. Operating conditions: Continuous. Anaerobic. Nitrogen flowrate to sparger: 1.5 litre /min. Impeller speed 200 rpm. pH controlled by the addition of 30% KOH soln. Medium: BST. Inoculum: 3% (v/v) of Fermenter Working Volume. Dilution Rate: 0.25 hr^{-1} T: 60°C. pH: 7.0. Medium feed sucrose concentration: 25 g/l.

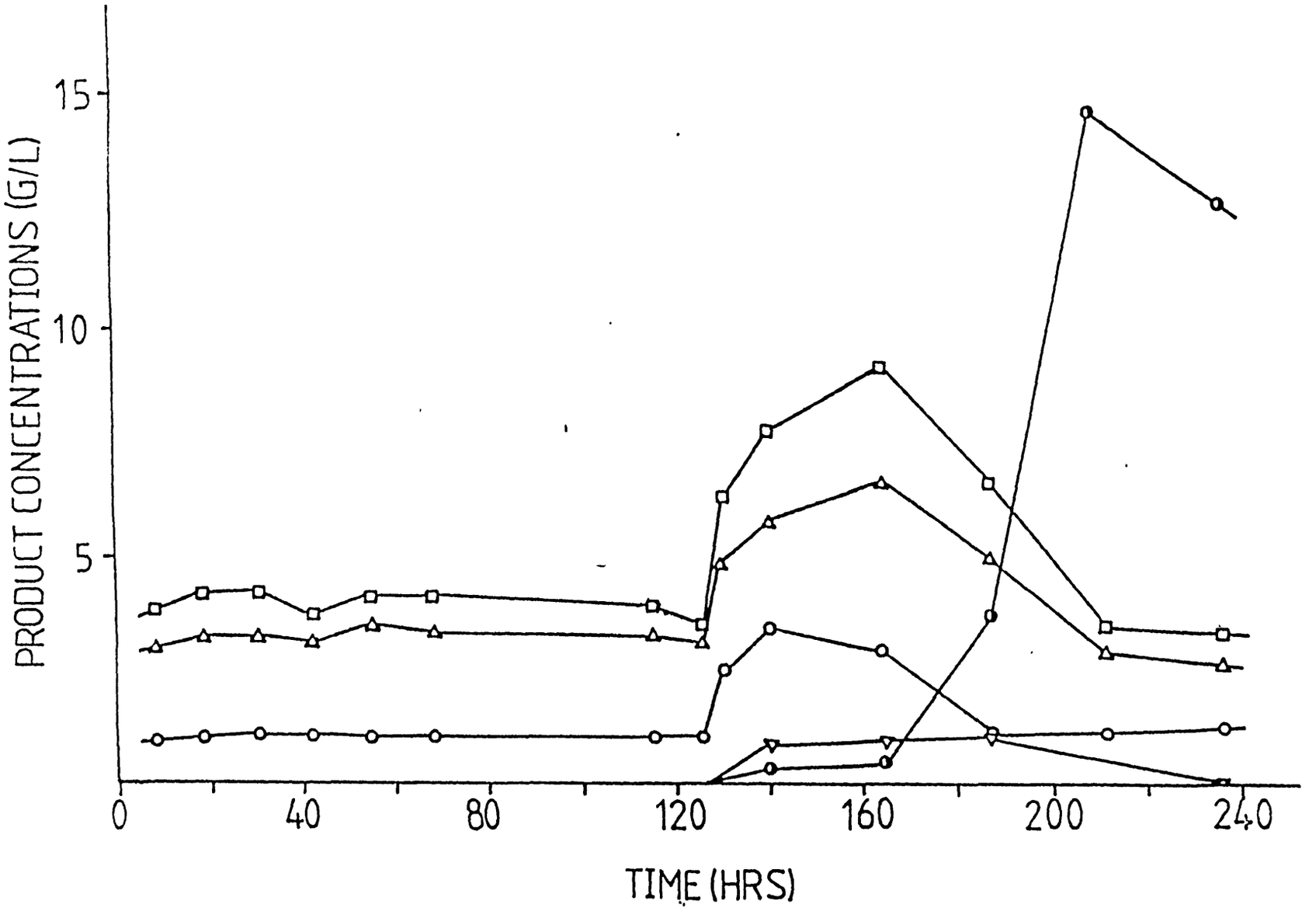


Figure 7.2 Product Concentrations against Time for Experiment 242

(○) Ethanol; (△) Acetate, (▽) Pyruvate; (□) Formate, (●) Lactate.
Fermenter: 5 litre New Brunswick, Working Volume: 2.0 litres. Operating Conditions: Identical to those in footnote to Figure 7.1 except that from 0-128 hours the medium feed sucrose concentration was 5.0 g/l and from 128 hrs. onwards it was 21.0 g/l.

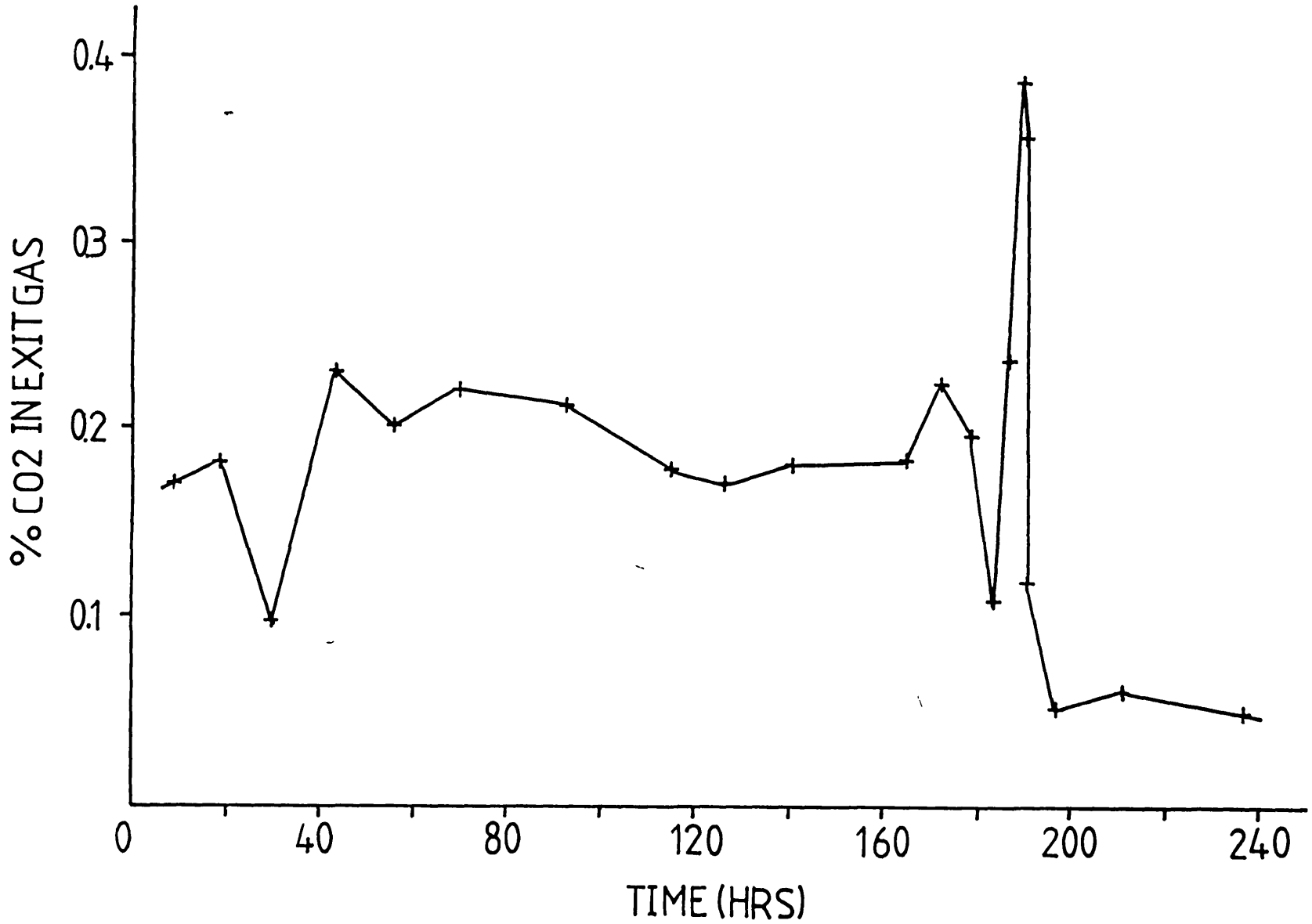


Figure 7.3 Gas Phase Carbon Dioxide Concentration against Time for Experiment 242

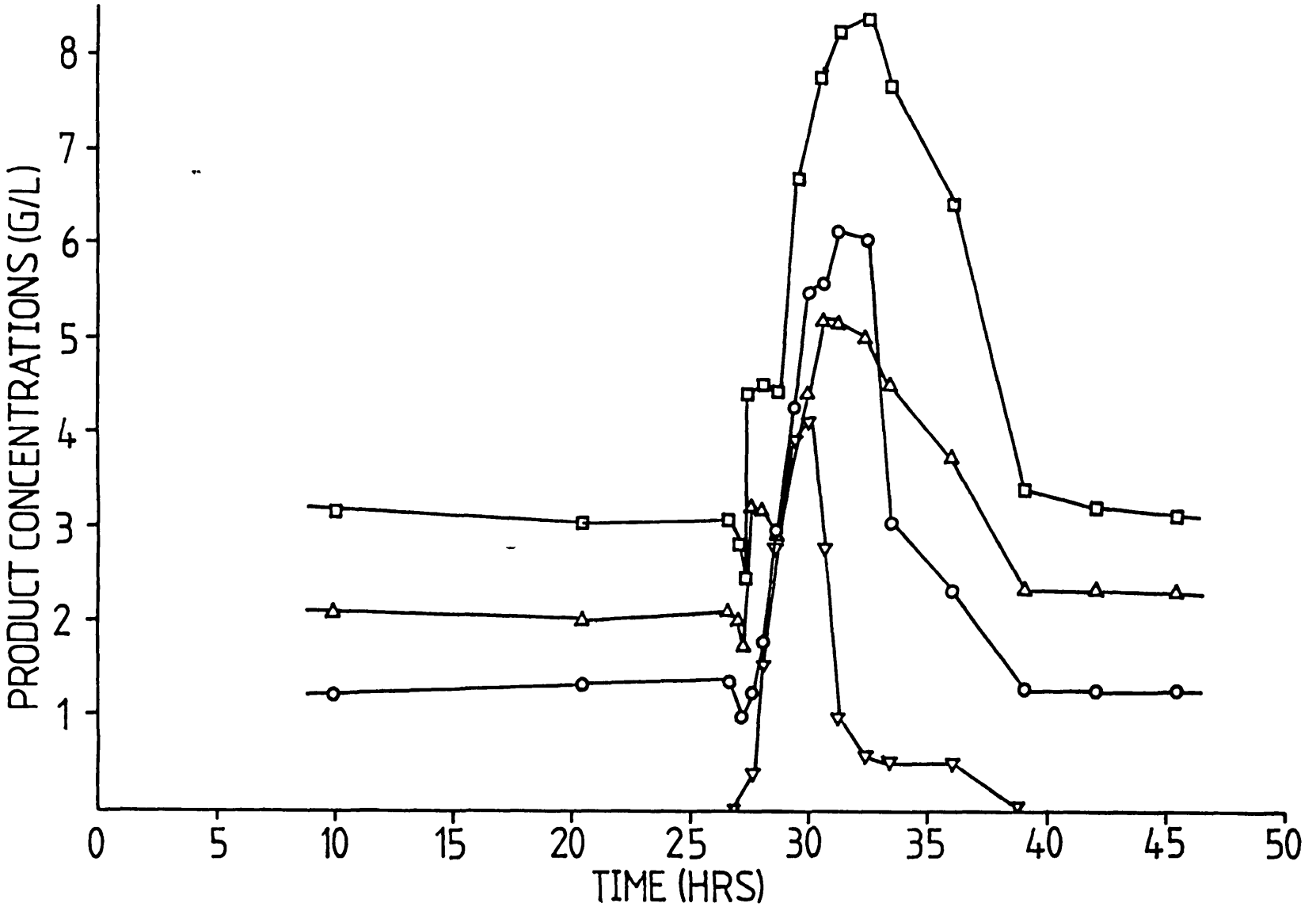


Figure 7.4 Product Concentrations against Time for Experiment 244
(○) Ethanol, (□) Formate, (△) Acetate, (▽) Pyruvate
Fermenter: 5 litre New Brunswick, Working Volume: 2.0 litres Operating Conditions:- Identical to those for Experiment 241 (see footnote to Figure 7.1) except that the medium feed sucrose concentration was 5.0g/1 and that at 27 hours 50g of sucrose were introduced into the fermenter.

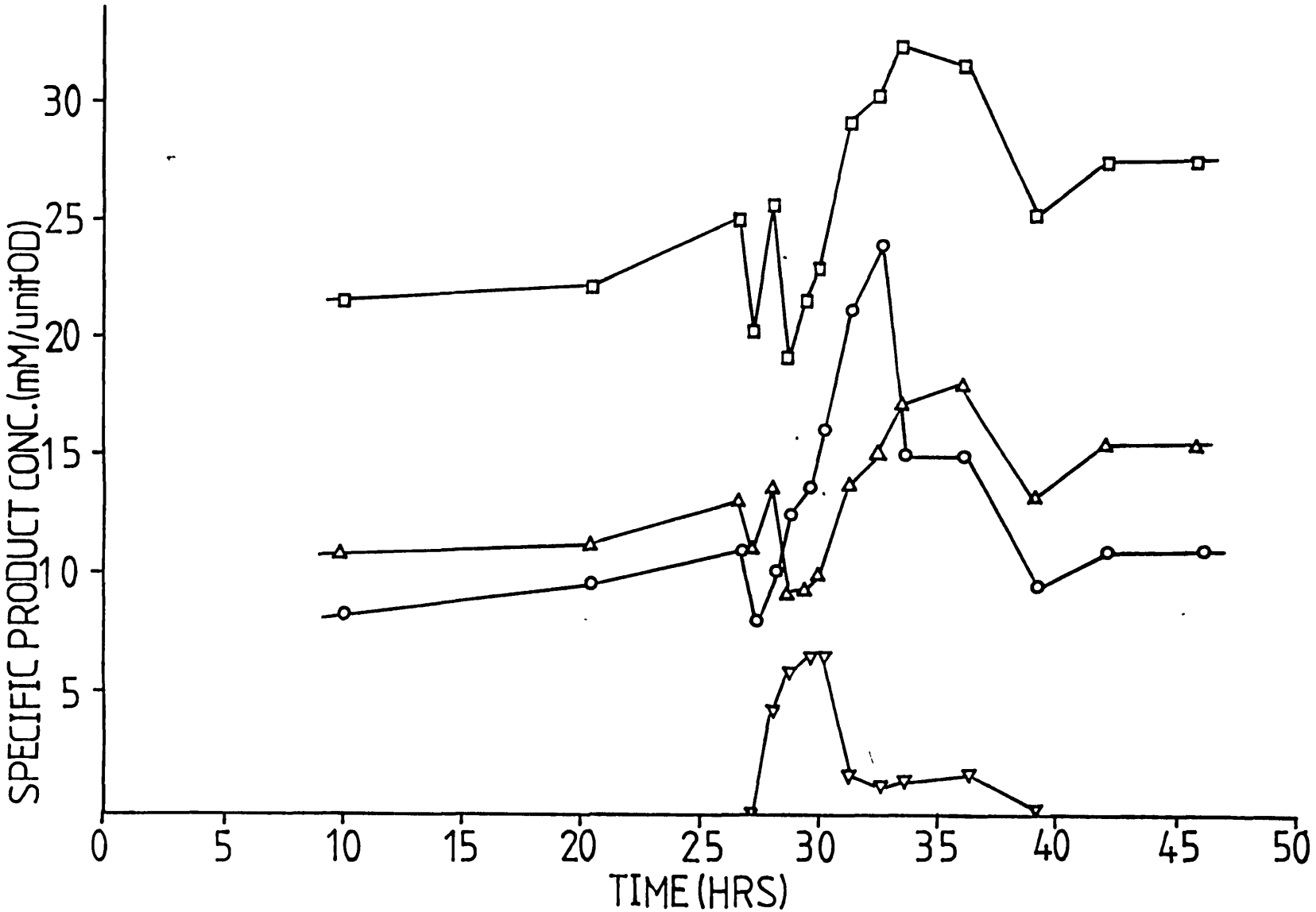


Figure 7.5 Specific Product Concentrations against Time for Experiment 244
(O) Ethanol, (□) Formate, (△) Acetate, (▽) Pyruvate.

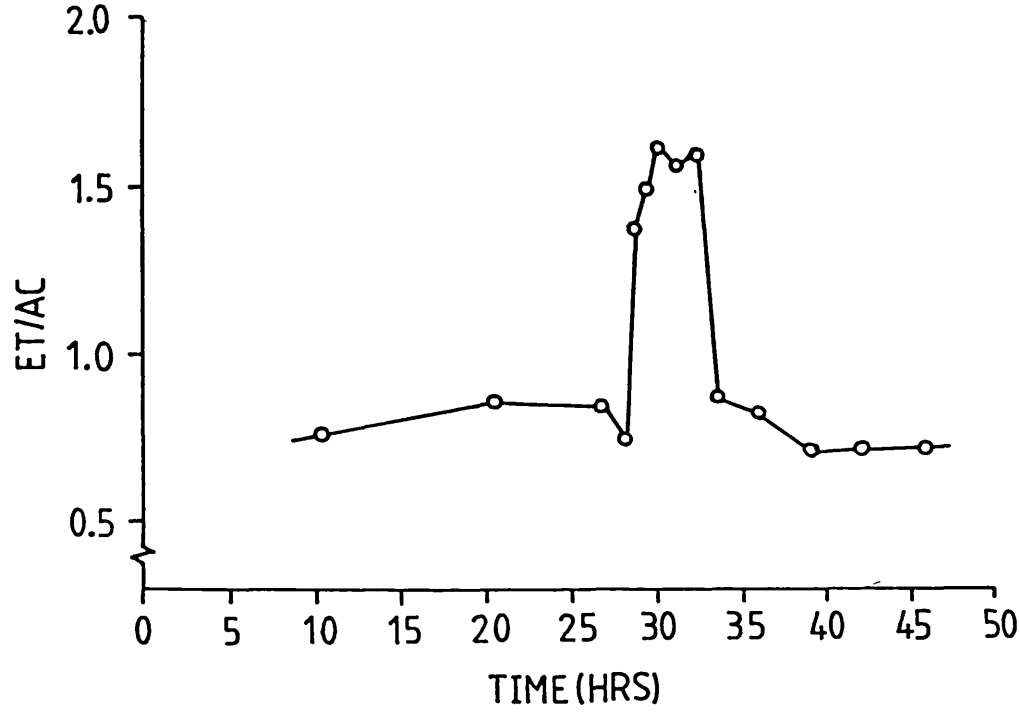


Figure 7.6 Ethanol to Acetate Ratio (Et/Ac) against Time for Experiment 244

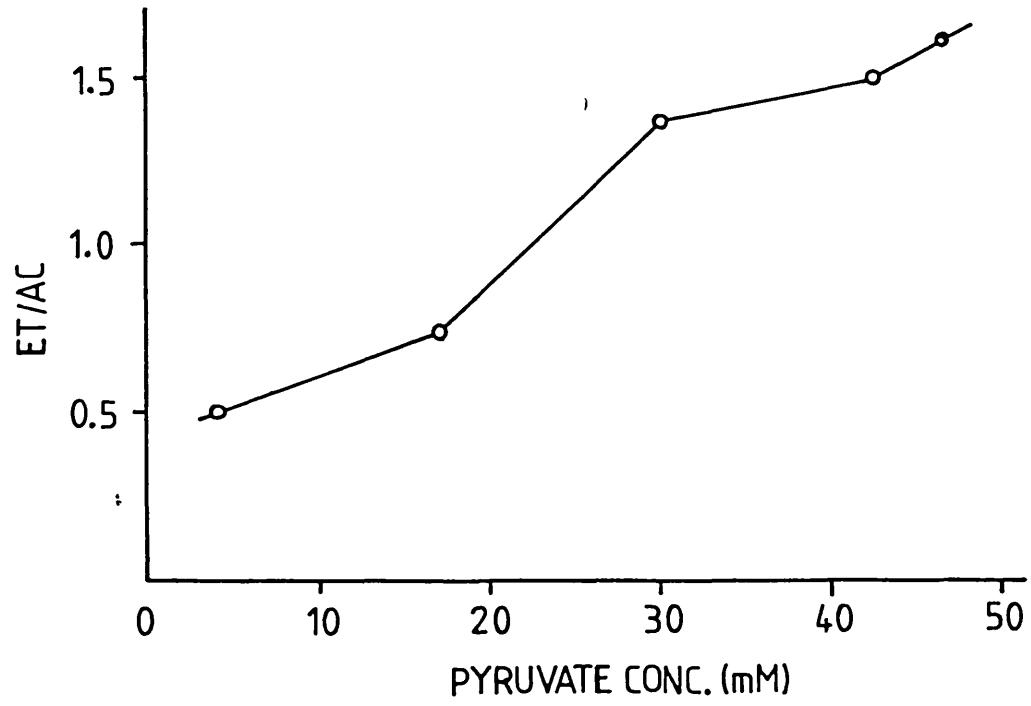


Figure 7.7 Ethanol to Acetate Ratio (Et/Ac) against Pyruvate Concentration for Experiment 244

culture did not revert. Specific product concentrations against time are shown in Figure 7.5 and indicate that the introduction of additional sucrose increased specific productivities. Figure 7.6 shows the ratio of ethanol to acetate plotted against time; the maximum value attained was 1.61. The correlation between pyruvate concentration during the period of unsteady state cell density increase and the ratio of ethanol to acetate is shown in Figure 7.7.

Product concentrations at peak cell densities for batch fermentation of sucrose by strains 11d-15 (Experiment 250), NCA 1503 (Experiment 251) and the revertant (Experiment 256) isolated during experiment 241 are shown in Table 7.4. Growth curves for the 3 strains of B. stearothermophilus are contained in Section A3.3. The reverant and strain NCA 1503 show similar product profiles; strain NCA 1503 produced slightly less lactate than the revertant but apparently channeled relatively more pyruvate via the PFL pathway as indicated by the higher yields of formate, acetate and ethanol. The product ratios for both strains are approximately 2:1:1 respectively. The ethanol yield of strain 11d-15, although greater than those of the other 2 strains, is relatively low compared to yields from previous experiments conducted under similar conditions (see Section 5.4 for a discussion of possible causes of this phenomenon.)

7.4.2 Discussion

B. stearothermophilus 11d-15 reverted to wild-type in all experiments where the concentration of sucrose in the medium feed was equal to or greater than 2.1%. Strain 11d-15 can be maintained stably in continuous culture without reversion taking place in media containing sucrose at a concentration of 0.5%.

A hypothesis to explain reversion of 11d-15 in continuous culture can be put forward on the basis of the result of Subsections 6.4.1 and 7.4.1 where the growth inhibitory effects of acetate and formate were demonstrated. It is suggested that in media containing sucrose at a concentration equal to or greater than 2.1% acetate and formate accumulated to such levels as to prevent 11d-15 from multiplying at a specific growth rate equal to the fermenter dilution rate (0.25 hr^{-1}) and that as a consequence the 11d-15 culture washed out. This permitted spontaneous revertants to lactate production to rapidly colonize the

PRODUCT mM	STRAIN		
	REVERTANT	NCA 1503	11d-15
FORMATE	34.3	83.8	147.7
ACETATE	20.7	35.7	65.6
PYRUVATE	-	1.6	23.5
LACTATE	230.5	231.0	1.9
SUCCINATE	5.0	1.2	8.9
ETHANOL	21.6	38.7	106.4
SUCROSE UTILIZED mM	78.3	82.7	77.2

Table 7.4 Sucrose Fermentation by B. stearothermophilus strains

Fermenters:- 5 litre New Brunswick, Working Volume: 3.0 litres
Operating Conditions: Batch, Anaerobic. Nitrogen flowrate to sparger: 0.5 litre/min.
Impeller speed: 200 rpm. pH controlled by the addition of 30% KOH soln. Medium: BST
Inoculum: 3% (v/v) of Fermenter Working Volume. Initial Sucrose Concentration: 25g/l T: 60°C. pH 7.0

chemostat. Two pieces of evidence support the theory that reversion involved takeover by a mutant population rather than the phenotypic adaptation of strain 11d-15. First, cells isolated in the period following the appearance of lactate also produced lactate when cultured under batch conditions and in general metabolized sucrose similarly to wild-type B. stearothermophilus (Table 7.4). Secondly, the rapid increase then decline in gas phase carbon dioxide levels observed during reversion (see Figures 7.1 and 7.3) may be indicative of rapid biosynthesis, since it was suggested in Chapter 5 that 'excess' carbon dioxide production occurred as a result of the decarboxylation of amino acids during biosynthesis. The disparities in the gas phase concentrations of carbon dioxide in experiments 241, 242 and 244 during growth on 0.5% sucrose were probably caused by minor differences in the pHs of the culture broths. Better agreement might have been obtained if the concentration of carbon dioxide in the aqueous phase had also been accounted for.

In batch experiments with strain 11d-15 the ratios of ethanol to acetate and ethanol to formate were found to be dependent on environmental conditions (see Chapter 5) but were in all cases equal to or greater than the ratios expected from metabolism via the PFL pathway (i.e. 1.0 and 0.5). By contrast in continuous culture on media containing 0.5% sucrose the ethanol to acetate and ethanol to formate ratios were approximately 0.35 and 0.22 respectively. This strongly suggests that under these conditions alternative energy sources to sucrose were being exploited.

When additional sucrose was introduced into the chemostat in experiment 244 it rapidly stimulated cell growth and productivity. However, the appearance of pyruvate in the culture broth suggests that its dissimulation may have become rate limiting and that it accumulated intra-cellularly and passed (or was transported) through the cell membrane. The graph of ethanol/acetate ratio against pyruvate concentration, Figure 7.7 provides additional evidence to that presented in Chapter 6 that pyruvate plays an important role in the metabolic switch towards increased ethanol production. Addition of exogenous pyruvate to a culture of 11d-15 did not cause signficant changes in product ratios suggesting that these changes are induced only by intracellular pyruvate levels.

7.5 Conclusion

The technique of continuous culture was successfully employed to investigate the effects of pH on the metabolism of *B. stearothermophilus* strain NCA 1503. It was found that increases in pH resulted in increased metabolism via the PFL pathway.

Reversion of strain *lld*-15 in continuous culture impeded attempts to investigate the effects of environmental conditions on its metabolism of sucrose. Evidence was presented to show that the level of sucrose in the medium feed indirectly determined the stability of *lld*-15 in continuous culture. The concentration of sucrose determines the final concentrations of all fermentation products and on the basis of the findings reported in Chapter 6 that acetate and formate inhibited the growth of *lld*-15 it was suggested that the concentrations of these acids were the ultimate determinants of culture stability. Stable operation of a chemostat supplied with medium containing 0.5% sucrose was demonstrated.

CHAPTER 8

XYLOSE AND CELLULOSE FERMENTATION WITH B. stearothermophilus

8.1 Introduction

8.1.1 Non-Traditional Substrates for Ethanol Production

The prospects of decreasing the cost of fermentation ethanol by substituting traditional feedstocks with cheaper and more abundant ones, such as agricultural residues, were discussed briefly in Chapter 1. However estimates of the quantities of agricultural residues generated (see for example Sitton et al., 1979) may give a distorted view of process potentials since in many cases they are too widely dispersed for practical use; moreover, some agricultural practices require that a portion of the residues be returned to the land. Nonetheless circumstances can be envisaged in which surplus agricultural material can be gathered at sufficiently low cost to present real opportunities for exploitation as a source of fuels. Agricultural residues are composed primarily of cellulose and hemicelluloses. Unfortunately, however, the yeast species traditionally used to produce ethanol are unable to ferment these materials and brief mention was made in Chapter 1 of attempts to genetically modify yeast to permit them to ferment a range of substrates. The alternative strategy of exploiting micro-organisms naturally possessing the ability to ferment non-traditional raw materials is considered below with reference to D-xylose and cellulose.

8.1.2 D-xylose

Although the five carbon sugar fraction of hemicellulose is composed of a number of components, D-xylose is the single most abundant one (Lee et al.1979a) and the majority of studies aimed at utilizing hemicellulose hydrolyzates more efficiently have focussed on D-xylose.

A key to the identification of yeast compiled by Barnett and Pankhurst (1974) lists 434 yeast species all of which are classified as being unable to ferment D-xylose. It has since emerged that yeast possessing D-xylose fermenting ability do exist, some examples are Pachysolen tannophilus (Schneider et al., 1981), Candida sp. (Gong et al., 1981a) and Kluyveromyces marxianus (Margaritas and Bajpai, 1982). Kurtzman (1983) conducted a survey of yeast for D-xylose fermenters in which species were selected for testing on the basis of two criteria;

first, that they were able to assimilate oxidatively D-xylose and secondly, that they possessed D-glucose fermenting ability. He reasoned that species unable to ferment hexoses would not be able to ferment pentoses. To further reduce the amount of testing a sub set of species was chosen that was comprised of species isolated from woody habitats. The majority of the species tested were members of the Pichia, Hansenula and Candida genera. Kurtzman found that of the 54 species examined only Pachysolen tannophilus could ferment D-xylose.

Toivola et al. (1984) examined 193 yeast species, 173 of which were contained in the key of Barnett and Pankhurst, for D-xylose fermenting ability. In contrast to the procedures used in earlier studies fermentation ability was assessed on the basis of gas chromatographic detection of ethanol in addition to the visual detection of gas production. They found that ethanol production was negligible in most strains, 19 were found to produce between 0.1 and 1.0 g of ethanol per litre and that 6 strains - Brettanomyces naardensis, Pachysolen tannophilus, Candida shehatae, C. tenuis, Pichia segobiensis and Pichia stipitis - produced more than 1 g of ethanol in fermentation tests with 2% D-xylose. They concluded that the disparities between their findings and those of earlier studies were caused by the use in the latter of the insensitive gas production test.

Wang et al. (1980a) found that D-xylulose fermenting ability was more widespread among yeasts than the ability to ferment D-xylose. The enzyme xylose isomerase catalyzes the conversion of D-xylose to D-xylulose and Wang et al. (1980b) showed that the former could be fermented by Schizosaccharomyces pombe in the presence of a commercial preparation of xylose isomerase. This finding was made the basis for a proposal for the industrial conversion of D-xylose to ethanol using yeast (Gong et al. 1981b) however this approach has been largely superseded following the recent announcement of the successful cloning of the gene coding for xylose isomerase into S. pombe (see Section 1.1).

Fusarium spp. have long been known to ferment D-xylose (White and Willamen, 1928) and have recently received attention in the context of ethanol production (Batter and Wilke, 1977, Viikari et al. 1981). A major disadvantage in the use of fusaria is that they are capable of only low ethanol productivities. Some of the thermophilic ethanologens listed in Table 1.5, viz Clostridium thermohydrosulfuricum, Clostridium thermosaccharolyticum and Thermoanaerobacter ethanolicus, ferment D-xylose in addition to other pentoses and hexoses. The use of these

organisms in an industrial operation would confer on it an adaptability which would allow the processing of a range of feedstocks.

8.1.3 Cellulose

Humphrey (1975) estimated that of the total annual global carbon dioxide fixation of 3.3×10^{11} tonnes, approximately 2.0×10^{10} tonnes is converted into cellulose of which some 4.0×10^{9} tonnes are 'readily' available for conversion to ethanol and industrial chemicals. This and similarly attractive estimates of cellulose availability have resulted in numerous proposals for processes for converting cellulose to ethanol. It is possible to divide these processes into 3 categories. The first are 2-stage processes. In the first stage, suitably pre-treated biomass is hydrolyzed by cellulose-degrading enzymes (cellulases) either in the presence or absence of cellulolytic organisms. Ethanol production occurs in the second stage when the hydrolyzate is fermented by a suitable ethanologen. One example of this type of operation is that described by Mandels (1974) in which the fungus Trichoderma reesei is the source of the cellulases and S. cerevisiae is used to ferment the hydrolyzate.

The second type of process envisages the use of an organism which possesses both the ability to hydrolyze cellulose and to produce ethanol. To date however no organisms having both these characteristics at high activity have been isolated. Organisms such as Clostridium thermocellum (Weimer and Zeikus, 1977) and the Monilia isolate described by Gong et al. (1981c) possess relatively good cellulose degrading abilities but only produce low ethanol yields. The genetic modification of a good ethanologen, such as S. cerevisiae, to enable it to hydrolyze cellulose was briefly mentioned in Section 1.1 and holds future promise, however there remain many technical difficulties to be overcome in reaching this objective.

The third process category is one in which the process of cellulose hydrolysis and ethanol production, although performed by different organisms, are carried out simultaneously in a single stage. Numerous combinations of organisms have been advocated.

Wang et al. (1979) achieved a 30% increase in ethanol yield obtained from cellulose with C. thermocellum by co-culturing with C. thermosaccharolyticum and Ng et al. (1981) a 100% increase by co-culturing C. thermocellum with C. thermohydrosulfuricum. More recently, Saddler and Chan (1984) advocated the use of a consortium of all three organisms in fermenting pre-treated wood chips to ethanol.

Reference to Table 1.2 shows that for cellulosic substrates process costs represent a greater fraction of the end cost of ethanol than with sugar or starch containing feedstocks. Therefore in the conversion of cellulosic substrates to ethanol the adoption of more sophisticated processing technology such as continuous operation or fermentation under vacuum will have a correspondingly greater impact on reducing the cost of ethanol.

It is difficult to predict which systems and which organisms will find the greatest industrial application. Probably the best characterized cellulolytic organism in the context of ethanol production is T. reesei and pilot studies based on this fungus have reached advanced stages. However, there has recently been much interest shown in thermophilic cellulolytic clostridia and processes based on these organisms will almost certainly feature strongly in the future.

8.1.4 Objectives

The possibility of using B. stearothermophilus for the conversion of D-xylose and/or cellulose containing raw materials has not previously received attention; limited studies are reported here using B. stearothermophilus 11d-15. The first experiment described in this chapter is the batch cultivation (under pH controlled conditions) of B. stearothermophilus 11d-15 with D-xylose as substrate. Similar methods of analyzing the experimental data are employed as were used for sucrose fermentations (Chapter 5). The specific ethanol productivity of strain 11d-15 is calculated for later comparison with data for other organisms in Chapter 10. The second experiment reported in this chapter was designed to establish whether B. stearothermophilus 11d-15 could be co-cultured with C. thermocellum and in particular whether the ethanol yields achieved by the mixed culture with cellulose as substrate are greater than those for C. thermocellum mono-cultures. This experiment was conducted in small scale batch culture without pH control. The data obtained is then compared to that of previously investigated mixed culture fermentations featuring C. thermocellum.

8.2 Methods

8.2.1 Inoculum

The medium conventionally used for culturing C. thermocellum - CM3 medium - contains cellobiose. The presence of this disaccharide would have interfered with determinations of the quantities of reducing

sugars liberated from the cellulose by the cellulases. Consequently, a medium deficient in cellobiose - CM3(M) - was used in all mixed culture experiments. Transfer of C. thermocellum cultures from storage under liquid nitrogen to CM3 (M) medium resulted in either no growth or in very poor growth, therefore on transfer from storage C. thermocellum was first cultured in CM3 medium for 48 hours at 60°C. 2 mls of this culture were then transferred to CM3(M) medium which after 48 hours incubation at 60°C was used as inoculum (2% v/v) for mixed culture experiments. The cultivation of B. stearothermophilus 11d-15 inoculum was as described in Sub-section 2.2.3: a 2% (v/v) inoculum level was used.

8.2.2 Culturing Procedures

Procedures for the cultivation of C. thermocellum mono-cultures or C. thermocellum - B. stearothermophilus co-cultures were identical: bottles of CM3 or CM3(M) medium (with loose screw-caps) were placed in a glass anaerobic jar from which the air was evacuated and replaced with carbon dioxide three times. The anaerobic jar was then incubated at 60°C.

8.2.3 Thin Layer Chromatography (TLC)

Hough and Jones (1962) tabulated R_f values for a number of sugars using different solvent systems for the technique of paper chromatography. The solvent mixture giving the best separation between cellobiose and glucose (ethyl acetate: pyridine: water, in the ratio of 10 : 4 : 3) was adopted in this study for use with plates.

1 µl quantities of sample and marker sugars were carefully spotted onto Kieselguhr Plates ("Kiesel Gel" 60F 20 x 20 cm: layer thickness 0.25 mm E. Merck and Co. Darmstadt, Germany). The plate was then placed in a glass chromatography tank into which the solvent mixture had previously been poured, and the plate was left to develop for approximately 2½ hours. After development, the plate was dried in a current of air and then sprayed with an ammoniacal solution of silver nitrate. The latter was prepared by the dropwise addition of ammonia solution (0.880 specific gravity) to a 5% (w/v) silver nitrate solution until the brown precipitate just dissolved. The sprayed plate was heated in an oven at approximately 100°C for 5 to 10 minutes: the sugars were revealed as brown spots on a light brown background. The experimentally determined R_f values for cellobiose, D-glucose and D-xylose were 0.29, 0.43 and 0.65 respectively.

8.2.4 Determination of Carboxymethl Cellulase (CMCase) Activity

The method adopted for the determination of CMCase activity was that described by Cooney et al. (1978).

2.0 g of carboxymethylcellulose (BDH Chemicals Ltd., Poole, Dorset) were added gradually to 100 ml of 0.05 M sodium citrate buffer, pH 4.8, whilst agitating with a laboratory-scale homogeniser (Silverson Ltd., London SE1). The solution was stored at 4°C. 0.2 ml of culture supernatant was added to 1 ml of buffered carboxymethyl-cellulose solution. A control was prepared by adding 0.2 ml of culture supernatant to 1 ml of sodium citrate buffer solution. The tubes were then incubated at 60°C for 1 hour. Total reducing sugars were determined by the Nelson Somogyi procedure (see 2.4.3). The reducing sugar content of the control was subtracted from the reducing sugar content of each sample and the CMCase activity of each sample was then expressed as μmoles of glucose equivalents formed from carboxymethyl-cellulose per ml of culture supernatant per minute. All CMCcase determinations were performed within one hour of sampling.

8.3 D-xylose Fermentation

8.3.1 Results

The concentrations of fermentation products versus time for B. stearothermophilus 11d-15 (experiment 165) are shown in Figure 8.1. The concentrations were corrected for ethanol losses and for the diluting effects of potassium hydroxide as described in Appendix 2. Biomass concentrations, calculated as described in Sub-section 5.3.3 are also shown in Figure 8.1. No pyruvate was detected in culture supernatants and assays for succinate or D-xylose were not performed. The specific ethanol productivity over the period 5.6 to 9.6 hours was calculated as described in Sub-section 5.3.3 and found to be 0.39 g ethanol/g cell dry weight-hr. A plot of cell density against time is shown in Figure 8.2, an initial relatively rapid phase of growth is followed by growth at a reduced rate until the stationary phase is reached. The specific growth rate during the initial growth phase was calculated to be 0.87 hr^{-1}.

8.3.2 Discussion

The growth and fermentation characteristics of B. stearothermophilus 11d-15 on D-xylose may be compared with results for sucrose experiments 162 and 172. The initial sucrose concentrations in these

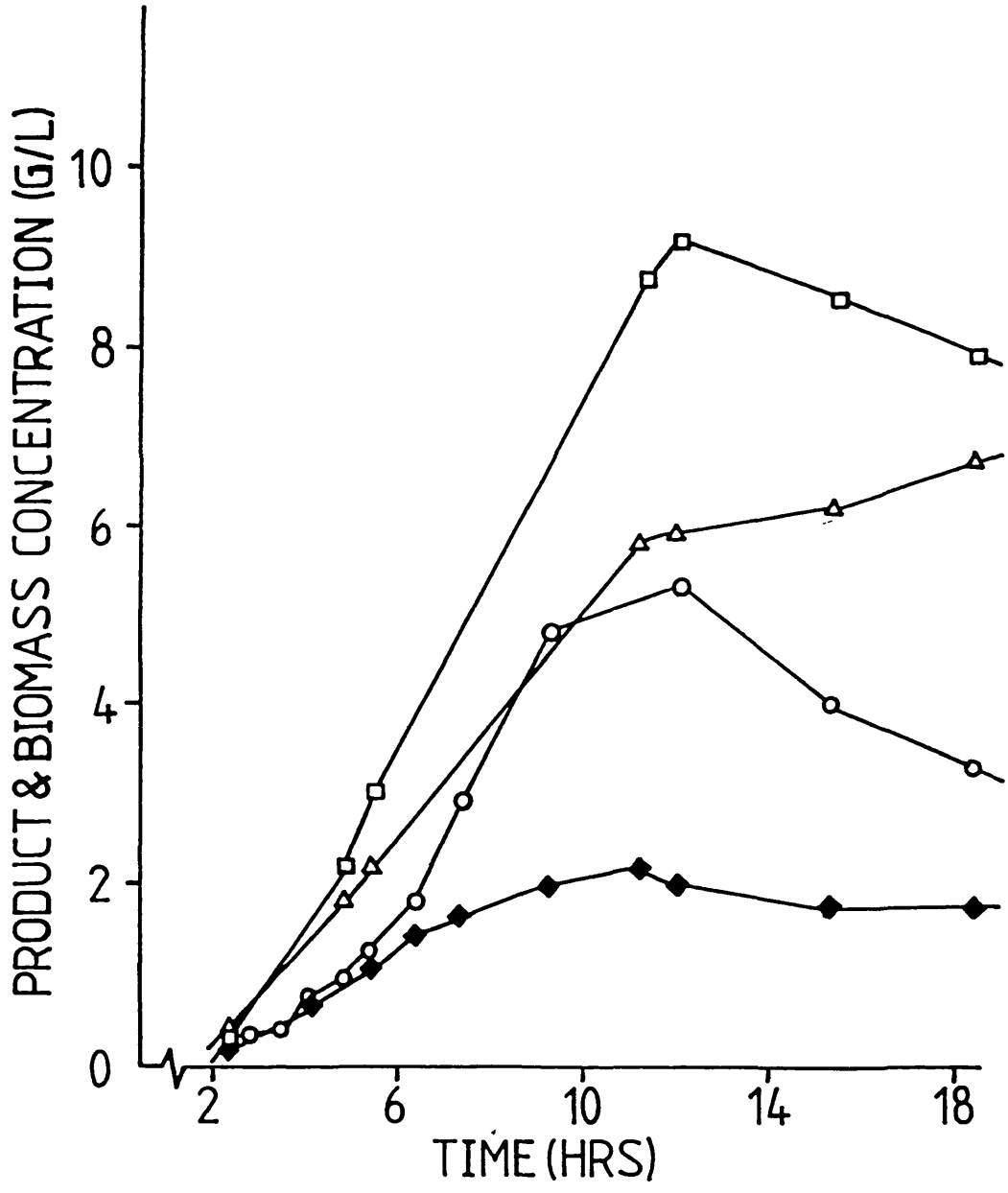


Figure 8.1 Product and Biomass Concentrations against Time for the Fermentation of D-xylosewith B. stearothermophilus 11d-115

(○) Ethanol, (□) Formate, (△) Acetate, (◆) Biomass.

Fermenter:- 5 litre New Brunswick, Working Volume: 3.4 litres. Operating Conditions: Batch, Anaerobic, Nitrogen flowrate to head : 0.2 litre/min. Impeller speed: 200 rpm. pH controlled by addition of 30% KOH soln. Medium: BST. Inoculum: 3% (v/v) of Fermenter. Working Volume. Initial xylose concentration: 23.5g/litre, T: 60°C pH.7.0

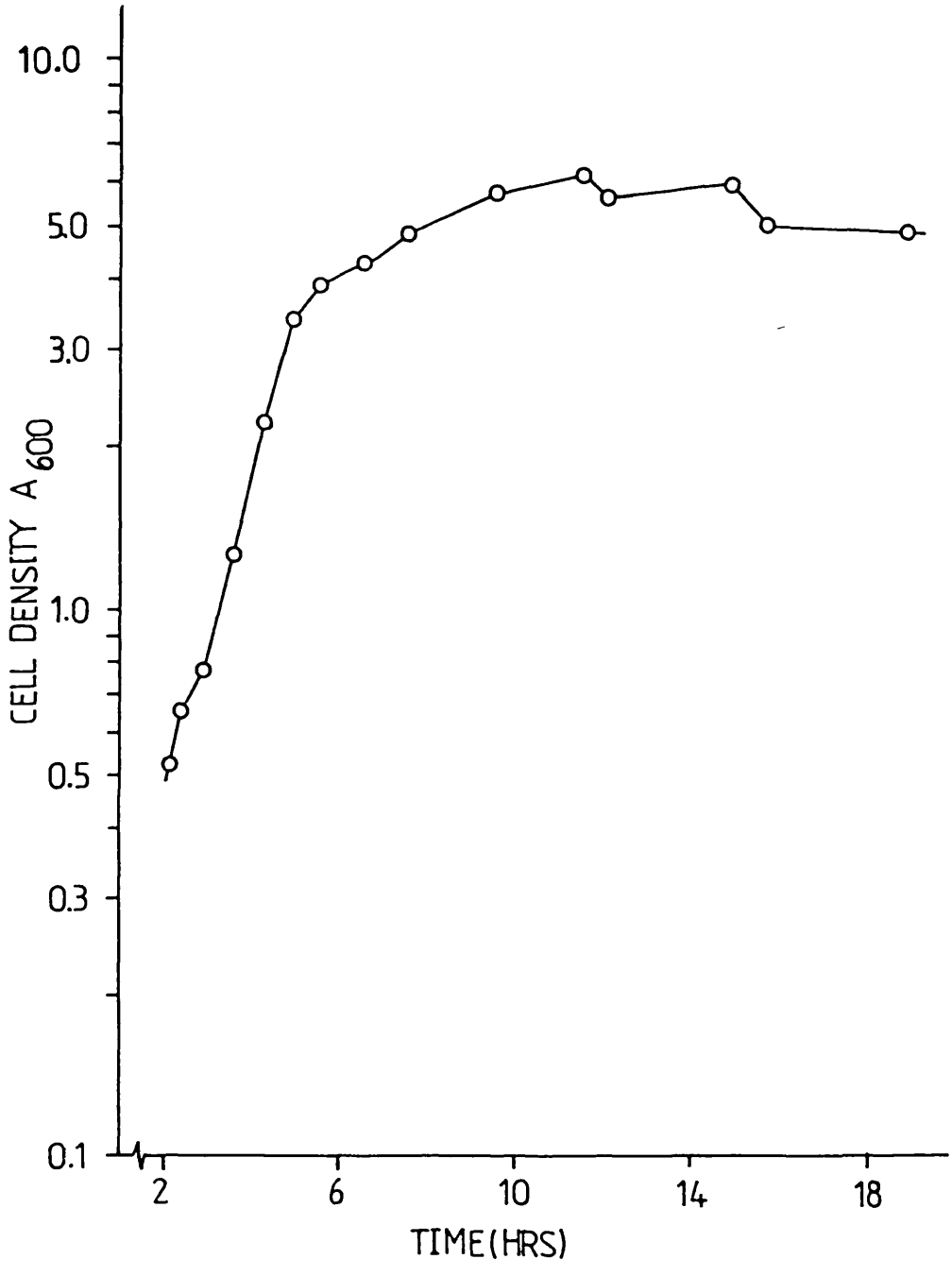


Figure 8.2 Cell Density Against Time

(see Footnote to Figure 8.1 for growth conditions)

experiments (5% (w/v) ≡ 146 mM) corresponds closely to the initial molar concentration of D-xylose in experiment 165 (157 mM). All three experiments were performed at pH7 and 60°C. The growth curve (Figure 8.2) shows a pattern similar to those obtained with sucrose as carbon source (see Figures A2.15-28). The growth rate of B. stearothermophilus 11d-15 on D-xylose - 0.87 hr^{-1} - can be compared with growth rates on sucrose of 1.87 hr^{-1} and 0.91 hr^{-1} (experiments 162 and 172 respectively). However, the wide variations in growth rate displayed by the organism on sucrose make it difficult to draw firm conclusions.

The molar ratios of products with time in the D-xylose experiment (Table 8.1) suggest that pyruvate metabolism was solely via the PFL pathway and contrasts to experiments 162 and 172 (Table 8.2 and 8.3) where the ratios suggest that pyruvate was metabolized by an additional pathway to PFL.

Further experiments would have to be conducted with D-xylose as substrate to determine whether the manipulation of environmental conditions could result in higher ethanol concentrations, as was the case for sucrose.

The ethanol productivity for strain 11d-15 with D-xylose as substrate compares favourably with the productivity data of Table 5.7 for the fermentation of sucrose. The productivity of strain 11d-15 is compared with that of other D-xylose fermenters in Chapter 10.

8.4 Co-Culture Experiments

8.4.1 Results

The medium employed in growing C. thermocellum in mono-culture and C. thermocellum and B. stearothermophilus 11d-15 in co-culture was CM3(M) medium. This medium contains cellulose as sole carbon and energy source. CM3 medium contains cellobiose in addition to cellulose. Table 8.4 shows ethanol production for mono- and co-cultures. Approximately 50% more ethanol was produced by the co-culture. Acid production is compared in Table 8.5: the quantities of acetate produced towards the end of the experiment were approximately equal for both mono- and co-cultures but considerably more formate was produced by the co-culture.

Reducing sugar accumulation (Table 8.6) is approximately equal

Time hrs	Ethanol / Acetate	Ethanol / Formate
2.38	1.39	1.32
4.92	0.86	0.55
5.59	0.84	0.47
11.59	0.94	0.48
12.17	1.18	0.59
15.75	0.88	0.49
19.00	0.67	0.44

Table 8.1 D-Xylose Energy Substrate (Experiment 165)

Time hrs	Ethanol / Acetate	Ethanol / Formate
6.58	4.89	2.30
9.52	4.31	1.51
14.25	6.21	1.80
18.08	4.78	1.72
20.37	5.29	1.80
22.68	6.50	2.34
24.70	7.39	2.29

Table 8.2 Sucrose as Energy Substate (Experiment 162)

Time hrs	Ethanol / Acetate	Ethanol / Formate
6.33	1.99	1.49
7.18	1.58	0.84
10.97	3.27	1.34
15.67	3.15	1.07
17.63	3.16	1.17
21.33	3.27	1.21
23.37	2.79	1.02

Table 8.3 Sucrose as Energy Substrate (Experiment 172)

Tables 8.1 to 8.3 Molar Ratios of Ethanol to Acetate and Ethanol to Formate in Batch Fermentation Experiments Conducted at 60°C and pH 7.0 with D-xylose or Sucrose as Energy Substrate.

Time Days	Ethanol Production		Ratio $\frac{1}{2}$
	Co-Culture 1	Mono-Culture 2	
1	8.70	3.4	2.56
2	11.30	10.2	1.11
3	19.57	15.0	1.30
4	24.13	16.74	1.44
5	25.87	17.39	1.49
6	24.80	17.58	1.41

Table 8.4 Ethanol Production (μmoles/ml) by C. thermocellum Mono-culture and B. stearothermophilus 11d-15 and C. thermocellum Co-Culture.

Time Days	Acetate		Formate	
	Co-Culture	Mono-Culture	Co-Culture	Mono-Culture
1	15.5	8.0	21.0	3.6
2	20.8	15.2	25.8	7.8
3	28.3	18.8	31.1	7.0
5	29.0	25.0	30.4	8.9
6	29.5	25.8	30.2	9.6

Table 8.5 Acid Production (μmoles/ml) by C. thermocellum Mono-culture and B. stearothermophilus 11d-15 and C. thermocellum Co-culture

Fermentation Conditions (Mono- and Co-culture):- Anaerobic Static Incubation at 60°C. Medium: CM3(M) - cellulose as carbon and energy source.

Time Days	mM Glucose Equivalents Produced/ml of Culture	
	Co-Culture	Mono-Culture
1	0.97	1.07
2	1.11	1.18
3	1.21	1.33
4	1.67	1.47
5	1.99	1.69
6	4.56	6.51
7	4.42	7.08

Table 8.6 Reducing Sugar Production by Mono- and Co-cultures

Time Days	pH	
	Co-Culture	Mono-Culture
1	6.47	6.76
2	6.30	6.56
3	6.01	6.48
4	5.73	6.40
5	5.52	6.09
6	5.48	5.84
7	5.55	5.72

Table 8.7 pHs of Mono- and Co-Cultures

Time Days	CMCase Activity*		Ratio $\frac{1}{2}$
	Co-Culture 1	Mono-Culture 2	
1	40.6	23.3	1.74
2	205.6	176.6	1.16
3	376.1	290.0	1.30
4	495.6	330.6	1.50
5	608.9	350.5	1.74
6	532.2	363.4	1.46
7	462.7	378.8	1.22

Table 8.8 CMCase Activity of Mono- and Co-Cultures

*μMoles of Glucose Equivalents Liberated per ml of Culture Broth per Minute.

Table 8.6 to 8.8 Characteristics of C. thermocellum Mono-culture and B. stearothermophilus 11d-15 and C. thermocellum Co-cultures

Fermentation Conditions: (Mono-culture and Co-culture) Anaerobic, Static Incubation at 60°C. Medium: CM3(M) - cellulose as energy and carbon substrate

initially but a higher level of reducing sugars accumulates towards the end of fermentation in the mono-culture. Thin layer chromatography revealed the presence of both cellobiose and glucose in mono-culture supernatants but only cellobiose was detected in co-culture supernatants.

It is not known whether B. stearothermophilus 11d-15 possesses cellobiose fermenting ability; the detection of cellobiose in the presence of B. stearothermophilus 11d-15 may simply be an indication that glucose is the preferred substrate. Culture pHs are shown in Table 8.7: the final pH of the co-culture was lower than that of the mono-culture. CMCase activities are compared in Table 8.8. The activity of the co-culture is seen to be greater than that of the mono-culture at all stages of fermentation. CMCase activity in co-cultures reached a maximum on day 5 and declined during days 6 and 7; approximately 77% of the maximum activity was achieved by day 3 for the mono-culture, after which activity increased at a relatively low rate. No measure of the numbers of each species of micro-organism was made owing to the considerable experimental difficulties which this would have entailed.

8.4.2 Discussion

The methods adopted in this work for the cultivation of pure and mixed cultures of C. thermocellum (see Sub-section 8.2) were not ideally suited for the measurement of volatile fermentation products such as ethanol, therefore the most reliable figures for ethanol produced are the ratios shown in Table 8.4. These figures indicate that towards the end of the experiment between 40 and 50% more ethanol was produced by the mixed culture than by the C. thermocellum culture alone. The final pH attained was below 6.0 (Table 8.7) and would certainly have inhibited B. stearothermophilus. Indeed, the glucose accumulated in the mixed culture supernatant more than doubled between days 5 and 6, Table 8.6. Under pH controlled conditions one would expect higher ethanol yields from the mixed culture. Both Wang et al. (1979) and Ng et al. (1981) (who co-cultured C. thermocellum with C. thermosaccharolyticum and C. thermohydrosulfuricum respectively), employed pH-controlled culture; the former group claimed an increased ethanol yield of 30% and the latter of 100% by employing mixed cultures.

Low concentrations of formate were detected in the C. thermocellum mono-culture supernatants (see Table 8.5). This is unexpected since formate production has not previously been reported with this organism. (Weimer and Zeikus, 1977; Lamed and Zeikus, 1980 and Ng et al., 1981). Lamed and Zeikus (1980) assayed the activities of a number of enzymes but did not suspect the presence of PFL, the enzyme most likely to be responsible for the presence of formate. All of these workers reported lactate as a minor fermentation product and traces of the acid were detected in this study.

Table 8.8 reveals that the CMCase activity of the mixtured culture was greater than that of the mono-culture. The enzymes that hydrolyze cellulose are subject to catabolite repression. Thus when the final products of the reaction sequence, glucose in this case, accumulate to levels higher than that required for optimum growth, the production of certain enzymes of the cellulase complex is repressed. The presence of B. stearothermophilus 'derepressed' cellulase production by removing glucose from the environment. The quantity of cellulose hydrolyzed was not measured in this work, however the higher CMCase activity of the co-culture does not necessarily imply more complete substrate consumption by the co-culture than the C. thermocellum mono-culture as the amount of cellulase produced by the latter may have represented an excess. Weimer and Zeikus (1977) co-cultured C. thermocellum with Methanobacterium thermoautotrophicum and found that whilst the initial rate of cellulose hydrolysis was greater for the co-culture than the C. thermocellum mono-culture, the cellulase activity of the co-culture declined more rapidly than that of the mono-culture. Furthermore, the total amount of cellulose degraded by the co-culture was less than that degraded by the mono-culture. Ng et al. (1981) found that the CMCase activity of C. thermocellum - C. thermohydrosulfuricum co-cultures was less than that of the mono-culture, but surprisingly, that more cellulose was degraded by the co-culture. They suggested that this was due to the rapid removal of hydrolytic end products by C. thermohydrosulfuricum which in some way facilitated the enzymatic degradation of cellulose.

8.5 Conclusion

These limited experiments have shown that B. stearothermophilus 11d-15 has D-xylose fermenting ability and is compatible with the cellulolytic thermophile C. thermocellum. It is clear however that

more work would have to be conducted to determine the full range of substrates which B. stearothermophilus is able to ferment before an evaluation could be made of its potential for fermenting to ethanol some of the less traditional raw materials available. However in one regard co-culturing B. stearothermophilus 11d-15 with clostridia fails to take advantage of the high growth rate and ethanol productivity (see Chapter 10) which the former possesses. C. thermocellum is a slow growing organism and in the mixed culture fermentation of cellulose with B. stearothermophilus the rate limiting step is the hydrolysis of cellulose by the former. This restriction might be overcome if the synthesis and function of the cellulose degrading enzymes were placed under the genetic control of B. stearothermophilus. There are however considerable practical difficulties in achieving this objective. Hydrolysis of cellulose to glucose usually requires at least three types of enzymes: endo-β1, 4 glucanase, exo-β1, 4 glucanase and β-glucosidase. No assay system exists specifically for any of these enzymes in isolation. The commonly used 'cellulase assays' such as CMCase, filter paper (FP) cellulase, etc., measure the combined activities of these three enzymes.

Moreover, as the cellulose degrading enzymes are exported by celluloytic organisms into the environment it may be necessary to transfer the entire transport system. However, Gilkes et al. (1984) have succeeded in cloning at least one of the genes specifying these enzymes from Cellulomonas fimi into E. coli and have detected CMCase activity in the latter. C. fimi was chosen as the source of cellulose gene(s) as its cellulase system has the ability to attack native cellulose. There is no reason why a similar exercise could not be carried out with B. stearothermophilus as recipient organism.

CHAPTER 9

ENZYME STUDIES ON B. stearothermophilus

9.1 Introduction

The proteins of thermophiles are generally more stable than the corresponding proteins from mesophiles and as B. stearothermophilus is one of the thermophiles relatively easy to culture, its proteins have attracted considerable interest. Some studies are aimed at elucidating the molecular nature of thermophily and typically in such studies one or a small number of enzymes from B. stearothermophilus are compared with corresponding enzymes from mesophiles. One example is the work of Schar and Zuber (1979) who compared the characteristics of lactate dehydrogenases from B. stearothermophilus and B. subtilis. Also, the generally good stability of B. stearothermophilus proteins is made use of in structural and related studies. Examples are studies on the structure of the pyruvate dehydrogenase complex from B. stearothermophilus (Perham and Wilkie, 1980) and the selection of tyrosyl-tRNA synthetase from B. stearothermophilus as the subject of experiments on site-directed mutagenesis (Carter et al. 1984). A third category of studies concerns the evaluation of the thermotolerant B. stearothermophilus enzymes for potential industrial applications. One example is glycerokinase (Atkinson and Comer, 1983). There are few studies on the enzymes of B. stearothermophilus concerned in the metabolism of simple sugars. McKray and Vaughn (1957) in their important work on the fermentation of glucose by B. stearothermophilus did not conduct enzyme assays. Rowe et al. (1973) however, who obtained mutants of B. stearothermophilus blocked in certain catabolic functions conducted assays for alcohol dehydrogenase and succinate dehydrogenase in aerobically grown cells.

9.2 Objectives

The strategy outlined in Chapter 1 for obtaining high ethanol producing mutants of B. stearothermophilus was based on a metabolic scheme derived from the work of McKray and Vaughn (1957). According to this scheme, mutants in lactate dehydrogenase should ferment sugars via the PFL pathway resulting in the formation of formate, acetate and

ethanol as sole fermentation products in the ratio 2:1:1. Moreover, the maximum ethanol yield should be 2 moles per mole of sucrose fermented. The ethanol yields achieved in some batch experiments with B. stearothermophilus 11d-15 exceeded this value and departures from the expected ratio of fermentation products were obtained (see Chapter 5). These results are incompatible with the operation in strain 11d-15 of the PFL pathway alone. One or more of three enzymes other than pyruvate formate lyase must also have participated in the anaerobic metabolism of sucrose. These are pyruvate dehydrogenase (PDH), pyruvate decarboxylase (PDC) and formate dehydrogenase (FDH). It is not possible to ascertain from product yields or distributions which of these three enzymes was active, so enzyme assays were conducted on crude cell extracts and permeabilized cells of strains 11d-15, NCA 1503 and a revertant isolated during the course of experiment 241 (see Chapter 7). Although the PDH complex of B. stearothermophilus had been extensively investigated (Perham and Wilkie, 1980), its presence in anaerobically grown cells has never been reported. There are no references in the literature to B. stearothermophilus possessing either PDC or FDH activities. Assays were conducted for three forms of FDH. The first is NAD-linked FDH. Detection of this enzyme in strain 11d-15 would imply that NADH produced from formate could participate in the reduction of acetyl-CoA to ethanol. The other two forms of FDH are referred to by the initial letters of the artificial electron acceptor used in assaying the enzyme. The first is methylene blue which is used in assaying for FDH(MB) and the second is benzylviologen used to detect FDH(BV). There is evidence to suggest that FDH(MB) and FDH(BV) activities are not identical (Shum and Murphy, 1972). The implications of the detection of either FDH(MB) or FDH(BV) in B. stearothermophilus would need cautious interpretation. B. stearothermophilus may possess forms of acetaldehyde dehydrogenase (AcDH) and alcohol dehydrogenase (ADH) which are linked to the same electron carrier(s) for which MB and BV can substitute. If so these forms of FDH activity could be directly responsible for increased ethanol yields, however to date only NAD linked AcDH and ADH have been reported in B. stearothermophilus. Alternatively there may exist intermediate electron carriers which transfer electrons from these unidentified

electron carriers to NAD. Another possibility is that these forms of FDH participate in reactions the products of which have not been detected.

In order to characterize further the differences between revertant, wild-type (NCA 1503) and 11d-15 strains assays for lactate dehydrogenase were also conducted.

9.3 Methods

9.3.1 Materials and General Techniques

(a) Reagents

Unless stated otherwise all reagents were obtained from the Sigma Chemical Co. Ltd., Poole, Dorset.

(b) Cell Pastes

Culture broths from batch and continuous experiments were centrifuged at 15,000 g for 20 minutes at 4°C. The cell pastes were washed by suspending them in an equal volume of saline and were then centrifuged at 15,000g for 20 minutes at 4°C. Details of the experiments which provided the material for enzyme assays are given in Table 9.1.

(c) Sonication

Between 0.3 and 0.4g of frozen cell paste were weighed into a plastic bijou bottle to which was added 4 ml 0.1M tris (hydroxymethyl) aminomethane (TRIS) buffer (pH7.6) containing 0.1M KCL and 10 mM $MgCl_2$. The cells were suspended in the buffer by vigorous agitation on a vortex mixer. Resuspended cells were stored on ice. Prior to sonication the cell sample was suspended in an ice-salt mixture for approximately 5 minutes and with the sample suspended thus the tip of the micro-sonicator probe was lowered into the suspension to within 3-4 mm of the base of the sample bottle. The sonicator used was a Model W-375 (Heat Systems - Ultrasonics Inc., Farningdale, N.Y., U.S.A.) and when fitted with a micro-probe had a maximim power output of 100 Watts. Sonication was achieved in 6 30 second bursts at full power with 30 seconds cooling in between each burst. The sonicated suspensions were then centrifuged at 20,000g for 40 minutes at 4°C and the crude cell extracts were stored on ice until required

Strain	Expt.	Growth Conditions				Date of Experiment
		Aerobic/ Anaerobic	Temp °C	pH	Batch/ Continuous	
NCA 1503	195	Aerobic	60	7.0	Batch	Oct. 1981
NCA 1503	231	Anaerobic	60	7.0	Batch	Nov. 1983
11d-15	233	Aerobic	60	7.0	Batch	Nov. 1983
11d-15	234	Anaerobic	60	7.0	Batch	Nov. 1983
11d-15	245	Anaerobic	60	7.0	Continuous	Oct. 1984
NCA 1503	249	Aerobic	60	7.0	Batch	Nov. 1984
11d-15	250	Anaerobic	60	7.0	Batch	Oct. 1984
NCA 1503	251	Anaerobic	60	7.0	Batch	Oct. 1984
Revert-ant	256	Anaerobic	60	7.0	Batch	Nov. 1984
11d-15	263	Anaerobic	60	6.5	Batch	Dec. 1984

Table 9.1 Cultural Conditions of Experiments with B. stearothermophilus strains on which Enzyme Studies were Conducted

but no extract was stored in this way for more than 4 hours.

(d) Permeabilization

As an alternative to sonication, whole cells were also permeabilized for assay. Cell pastes were suspended in buffer solution in glass bijou bottles as described above. The technique used to permeabilize the cells was that used by Kornberg and Reeves (1972) for E. coli: 40 µl of toluene-ethanol mixture (1:9) were added to 4 mls of resuspended cells in buffer. The mixture was then vigorously agitated for 60 seconds and then stored on ice. No permeabilized cell mixture older than 4 hours was assayed.

(e) Anaerobic Enzyme Assays

Anaerobic assays were performed using 1 ml disposable plastic cuvettes of 1 cm light-path sparged with argon gas. The design of the gas sparging system was based on that of Enoch and Lester (1972). The opening of the 1 ml cuvettes used was circular and could be sealed with a standard sized silicone bung. Argon gas was introduced into the cuvette via narrow bore stainless steel tubing which was inserted through the bung. To sparge the reaction mixture in the cuvette the stainless steel tube was pushed downwards to within 3 mm of the base of the cuvette. When conducting absorbance measurements this tube was withdrawn above the level of liquid in the cuvette so that a blanket of argon gas was continuously maintained above the reaction mixture. A hypodermic needle was inserted through the bung to act as gas exit line. As well as serving as a gas exit line additions were made to the cuvette through the hypodermic needle with a microsyringe without interrupting the flow of argon.

(f) Spectrophotometer

A model 8610 Kinetics Spectrophotometer (Pye Unicam Ltd., Cambridge, Cambs.) was used. The spectrophotometer was equipped with a thermostatically controlled cuvette heater and was connected to a Model 8251 (Pye Unicam Ltd.) single pen chart recorder.

(g) Protein Determinations

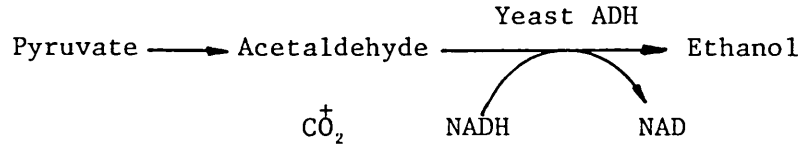
Protein determinations were made with a commercially available reagent (Biorad Laboratories GmbH, Munich, W. Germany). 5 mls of reagent (diluted 1 in 5) were pipetted into test tubes to which were added 0.1 ml of suitably diluted samples, standards and blanks (buffer

solution). The tubes were gently agitated with a vortex mixer and the absorbance was measured at 595 nm after allowing the mixtures to stand for 5 minutes. The measuring range of the assay was 0 to 1.5 mg protein/ml. Bovine γ globulins (Cohn Fraction II) were used as standards.

9.3.2 Enzyme Assays

(a) Pyruvate Decarboxylase Assay with Alcohol Dehydrogenase from S. cerevisiae

The method employed was that of Ullrich et al. (1966). The principle of the assay is as follows: pyruvate decarboxylase (PDC) catalyses the conversion of pyruvate to acetaldehyde and carbon dioxide, whilst the added alcohol dehydrogenase (ADH) catalyzes the conversion of acetaldehyde to ethanol with the concomitant oxidation of NADH to NAD which can be measured.



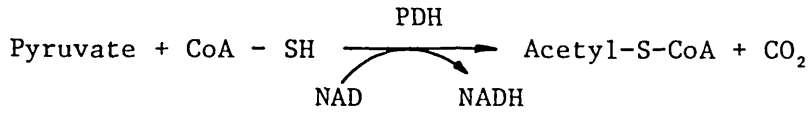
Permeabilized cells (0.15 ml) or crude cell extract were mixed with 0.15 ml 0.1 M potassium phosphate buffer (pH 6.8) containing 20 mM $MgSO_4$ and 5 mM co-carboxylase and stored on ice for 3 minutes. To a 1 ml cuvette were added 0.05 ml of this mixture, 0.375 ml 70 mM sodium succinate buffer (pH 6.0), 0.125 ml 70 mM sodium pyruvate, 0.125 ml 1.1 mM NADH and 0.125 ml aqueous ADH solution (0.4 mg per ml). The cuvette was incubated at 30°C and the progress of the reaction was monitored by measuring the rate of decrease of absorbance at 340 nm.

(b) Pyruvate Decarboxylase Assay with Alcohol Dehydrogenase from Thermoanaerobium brockii

The ADH from T. brockii is NADP-linked. The procedure followed was identical to method (a) above except that, instead of NADH, NADPH (of the same molarity) was used. The ADH solution was prepared by dissolving 0.1 mg ADH in 1 ml of distilled water. Cuvettes were incubated at 50°C.

(c) Pyruvate Dehydrogenase Assay

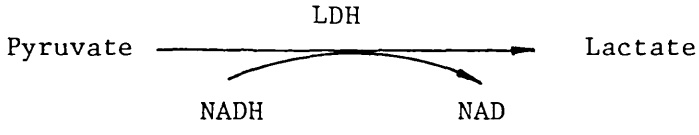
The method employed was that of Visser et al. (1982). The pyruvate dehydrogenase complex is a multienzyme complex catalyzing the oxidative decarboxylation of pyruvate to acetyl CoA



Stock solution (10 ml) of the reaction mixture was prepared by mixing 16 mg dithiothreitol, 4 mg coenzyme A, 20 mg NAD, 7.4 mg thiamine pyrophosphate, 0.08 ml 1 M magnesium chloride solution, 2.4 ml 1M potassium phosphate buffer (pH 7.0) and 7.6 ml distilled water. The reaction mixture (0.25 ml) was pipetted into a 1 ml cuvette to which was added 0.25 ml 20 mM sodium pyruvate and 0.4 ml distilled water. The mixture was incubated at 50°C for 5 minutes and the reaction was started by adding 0.025 ml of crude cell extract. The reaction rate was measured by following the increase in absorbance at 340 nm.

(d) Lactate Dehydrogenase - Assay by Pyruvate Reduction

The technique used was that of Schar and Zuber (1979). Lactate dehydrogenase catalyzes the reduction of pyruvate to lactate with the concomitant oxidation of NADH to NAD



The standard assay mixture contained 0.92 ml 0.1M triethanolamine hydrochloride/sodium hydroxide buffer (pH 6.0) 0.02 ml 10 mM NADH and 0.05 ml 0.6M sodium pyruvate. The mixture was incubated for 5 minutes at 50°C and the reaction initiated by adding 0.2 ml crude cell extract. The reaction rate was measured by following the decrease in absorbance at 340 nm.

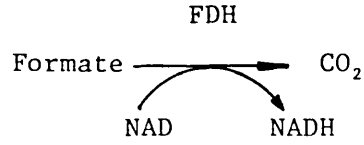
(e) Lactate Dehydrogenase - Assay by Lactate Oxidation

Schar and Zuber also published details of the oxidation of lactate by LDH. The standard assay mixture contained 0.4 ml 0.4M hydrazine sulphate and 1M glycine/sodium hydroxide buffer (pH 8.5), 0.05 ml 50mM NAD, 0.29 ml distilled water and 0.25 ml 2M L-lactate. The mixture was incubated for 5 minutes at 50°C and the reaction

initiated by adding 0.2 ml crude cell extract. The reaction rate was measured by following the increase in absorbance at 340 nm.

(f) Formate Dehydrogenase Assay by NAD Reduction

The technique of Egorov et al. (1979) was employed. NAD linked formate dehydrogenase catalyzes the conversion of formate to carbon dioxide and NADH.



The assay mixture contained 0.9 ml 0.05M potassium phosphate buffer (pH 7.0), 0.05 ml 32 mM NAD and 0.05 ml M sodium formate. The mixture was incubated for 5 minutes at 50°C and the reaction initiated by adding 0.1 ml crude cell extract or permeabilized cells. The reaction rate was measured by following the increase in absorbance at 340 nm.

(g) Formate Dehydrogenase Assay with Benzyl Viologen as Electron Acceptor

In this assay benzyl viologen acts as electron carrier thereby becoming reduced. The technique employed was that of Lester and De Moss (1971). The reaction mixture was composed of 0.67 ml 50 mM potassium phosphate buffer (pH 7.2). 0.04 ml 5 mM ethylenediaminetetra acetic acid disodium salt and 0.04 ml 20 mM benzyl viologen. When crude cell extract was used in the assay 3 µl of polyglycol P2000 antifoam were added. This mixture was gassed for 3 minutes with argon and 1.0 µl of 0.25 M $Na_2S_2O_4$-0.52M $NaHCO_3$ was added followed by 0.1 ml of crude cell extract or permeabilized cells. After gassing with argon for a further 3 minutes the reaction was initiated by adding 10 µl 7.2M sodium formate. The reaction rate was measured by following the increase in absorbance at 600 nm.

(h) Formate Dehydrogenase Assay with Methylene Blue as Electron Acceptor

The electron carrier in this assay is methylene blue. The method used was that of Kroger et al. (1979). The reaction mixture contained 0.9 ml 50 mM tris(hydroxymethyl)aminomethane (TRIS) buffer (pH 7.9), 0.035 ml 3 mM methylene blue and 0.1 ml crude cell extract or permeabilized cells. When the former was used in the assay 3 µl

of polyglycol P2000 antifoam were also added. The mixture was gassed for 3 minutes with argon and the reaction was initiated by adding 10 µl 7.2 M sodium formate. The reaction rate was measured by following the decrease in absorbance at 578 nm.

9.4 Results

Table 9.2 shows the PDH activities of crude cell extracts prepared from *lld*-15, NCA 1503 and revertant strains. The activities were calculated from the initial slopes obtained from recorder traces of increases in absorbance at 340 nm against time. Duplicate assays performed on certain samples indicated that the results of Table 9.2 are accurate to within ± 5%. The activities of extracts of anaerobically grown *lld*-15 cells are considerably greater than those of anaerobically grown NCA 1503 or revertant cells. The latter have similar anaerobic PDH activities. It is also apparent that the PDH activity in extracts of anaerobically grown *lld*-15 at pH 6.5 (Expt. 263) is higher than at pH 7.0 (Expts. 234, 245 and 250). The anaerobic activities of *lld*-15 at pH 7.0 whether grown in batch or continuous culture (Expt. 245) are remarkably similar. Some loss of activity due to storage at -20°C is noticeable, as can be seen by comparing the results of experiment 195 (0.33 units/mg crude protein) conducted in October 1981, with that of experiment 249 (0.699 units/mg of crude protein) which was conducted in November 1984. However valid comparisons may still be made, experiments 250 (with *lld*-15) 251 (with NCA 1503) and 256 with the revertant were conducted within 2 weeks of one another and loss of activity over this time period would be negligable. The PDH activities of aerobically grown cells of strains *lld*-15 and NCA 1503 are comparable.

Neither NAD linked, MB nor BV FDH activity was detected in any of the cell extracts or permeabilized cell samples. The latter 2 forms of FDH can only be detected under strictly anaerobic conditions and in order to ascertain that such conditions were being achieved and maintained in the cuvettes described in Sub-section 9.3.1(e), a crude cell extract of *E. coli* (NCIB 10115) was assayed for both of these activities. Both have been reported to exist in *E. coli* (Lester and De Moss, 1971; Shum and Murphy, 1972) and were successfully detected at 30°C using the techniques described in Section 9.3.

Strain	Experiment	PDH Activities[1] (x 1000) of Anaerobically Grown Cells	Experiment	PDH Activities[1] (x 1000) of Aerobically Grown Cells
11d-15	234	397	233	261
	245	399		
	250	448		
	263	831		
NCA 1503	231	42	195	333
	251	95	249	669
Revert-ant	256	80	-	NA

Table 9.2 PDH Activities of Cell-Free Extracts of B. stearothermophilus strains

[1]Expressed as units/mg total protein. 1 unit is defined as the amount of enzyme which catalyzes the formation of 1 μM NADH per minute at pH 7.0 and 50°C.

NA Not Assayed.

Precipitates formed when either cell extracts or permeabilized cells were assayed for FDH (all forms). The formation of a precipitate was not temperature dependent; precipitates were also formed when samples were incubated at 30°C as well as at 50°C. In the case of NAD linked FDH the increase in absorbance due to the formation of the precipitate would have masked any increase in absorbance due to NAD reduction. The MB and BV assays rely on colour changes and were less susceptible to interference of this kind.

No PDC activity was detected in any of the crude extracts or permeabilized cells prepared from strain _lld_-15 samples. Decreases in absorbances at 340 nm were detected but further investigation revealed that identical decreases in absorbance were obtained even in the absence of pyruvate from the reaction mixture. It is likely that this decrease in absorbance was due to numerous by-reactions of which the most significant was probably due to the presence of NADH oxidase in the crude cell extracts.

The PDC assay used here was developed by Ullrich _et al_. (1966) specifically for yeasts. It was reasoned that _B. stearothermophilus_ might possess a PDC with a Michaelis constant (K_m) for pyruvate considerably greater than that of yeast and that the pyruvate concentration advocated by Ullrich _et al_. may have been too low to induce measurable activity. The assay was therefore repeated with a pyruvate concentration 5 times that listed in Sub-section 9.3.2a. No PDC activity was detected. The PDC assays described above were conducted at 30°C. Attempts to conduct the assays at a higher temperature (all the other assays were conducted at 50°C) would almost certainly have resulted in the denaturation of the added yeast alcohol dehydrogenase (ADH). In order to ascertain whether PDC could be detected at 50°C, the yeast ADH was replaced by ADH from the thermophilic bacterium _Thermoanaerobium brockii_. However even at 50°C no PDC activity was detected. No attempts were made to assay for PDC in extracts from strain NCA 1503 or the revertant as the lactate dehydrogenase possessed by these strains (see below) would have caused the oxidation of NADH.

In assaying crude cell extracts for lactate dehydrogenase (LDH) by the oxidation of lactate a precipitate formed which interfered

with absorbance measurements. The cause of precipitate formation was not determined. A similar precipitate was also formed when attempts were made to assay FDH. However, no precipitate formation occurred when LDH was assayed by following the reduction of pyruvate. As in the case of PDC assays, NADH was oxidized to NAD in the absence of pyruvate. This was presumably due to NADH oxidase activity. This activity had to be taken into account in calculating the LDH activities. The reaction mixture quoted in Sub-section 9.3.2(d) was incubated with crude cell extract for approximately 4-5 minutes in the absence of pyruvate in order that an accurate estimate could be made of the decrease in absorbance due to NADH oxidase. Pyruvate was then added to the cuvette and the decrease in absorbance at 340 nm was monitored for a further 4-5 minutes. The decrease in absorbance at 340 nm due to LDH activity is then the rate of decrease of absorbance in the presence of pyruvate less the rate of decrease in absorbance in the absence of pyruvate. Extracts prepared from all cell pastes were assayed for LDH but activity was detected only in the extracts from anaerobically grown cells of NCA 1503 (0.0020 units/mg total protein) in experiment 251 and anaerobically grown cells of the revertant (0.0014 units/mg total protein) in experiment 256. One unit of enzyme activity being the amount of enzyme that catalyzes the formation of 1 μmole of NAD per minutes at pH 6.0 and 50°C. Extracts prepared from cells of anaerobically grown NCA 1503 from experiment 231 showed no LDH activity at all.

9.5 Discussion

PDH activity was markedly higher in anaerobically cultured cells of *lld*-15 than in either anaerobically cultured wild-type (strain NCA 1503) or revertant strains. Had activities been similar for all three strains it would not have been possible to argue the significance of PDH in the anaerobic metabolism of strain *lld*-15. Herbert and Guest (1975) found that although PDH did not function in *E. coli* under anaerobic conditions its synthesis was not greatly repressed. Further evidence to support the anaerobic role of PDH in *B. stearothermophilus* comes from the observation that activity in cells grown at pH 6.5 was higher than in cells grown at pH 7.0. This correlates well with the results of Chapter 5 in which it was shown that higher ethanol yields were achieved under acid conditions. Hansen and Henning (1963) showed

that in E. coli the absence of PDH activity under anaerobic conditions was due to inhibition of the enzyme by the high levels of NADH which prevailed under anaerobic conditions. If the PDH in wild-type B. stearothermophilus is subject to similar inhibition then it is likely that the mutation in lld-15 has resulted in the derepression of PDH under anaerobic conditions.

It was stated in Section 9.2 that in addition to PDH, the presence in lld-15 of PDC or FDH activities might explain the unexpectedly high ethanol yields obtained in batch fermenters under certain cultural conditions. PDC activity was not detected nor were FDH(MB) and FDH(BV) activities. Assays for NAD linked FDH were inconclusive as under the assay conditions employed a precipitate formed which interfered with absorbance measurements. Although it is possible that wild-type and lld-15 strains of B. stearothermophilus may possess NAD linked FDH it is unlikely, in view of the large differences in anaerobic PDH activities of wild-type and strain lld-15 that this enzyme participates significantly in anaerobic metabolism.

S. Amartey (personal communication) conducted LDH assays according to the method shown in Sub-section 9.3.2(d) on freshly grown cells of strain NCA 1503. The activity of anaerobically grown cells of NCA 1503 were approximately 10 times greater than the activities reported here. It is clear that the LDH enzyme of B. stearothermophilus is considerably more labile than the PDH complex.

9.6 Conclusion

Enzyme assays were conducted in an attempt to account for ethanol yields in excess of 2 moles per mole of sucrose utilized, achieved under certain cultural conditions in batch fermenters. There was a marked difference between the PDH activities of extracts of anaerobically grown wild-type and lld-15 strains of B. stearothermophilus. It is suggested that the high levels of PDH in anaerobically grown cells of strain lld-15 (particularly in cells grown under acid conditions) is an indication that pyruvate metabolism via the PDH pathway occurs in B. stearothermophilus lld-15.

CHAPTER 10

GENERAL DISCUSSION

10.1 Suggestions for Further Work

The detection of the previously unreported anaerobic fermentation products carbon dioxide and succinate indicate that B. stearothermophilus 11d-15 utilizes additional pathways to PFL during the fermentation of sucrose. However, whilst strong evidence was obtained which indicated that the PDH enzyme complex functioned under anaerobic conditions no information was obtained on the metabolic pathways leading to the minor fermentation products. The elucidation of these pathways might enable future mutant selection strategies with B. stearothermophilus to be planned with greater confidence. One difficulty encountered in using growth media containing undefined constituents such as tryptone was that of determining whether the fermentation products were derived from sucrose or from the undefined medium constituents. Although fermentation balances enabled the quantities of carbon dioxide derived from sucrose and the undefined medium constituents to be estimated, the origin of the minor fermentation products acetoin (or diacetyl) and those acids detected but not identified by the gas chromatographic techniques used remains uncertain. One method commonly used to resolve such issues is to conduct fermentations with cells suspended in a buffered solution of substrate. Difficulties were however encountered in this approach and although further experimentation might resolve these difficulties, resting cell suspension experiments may be criticized on the grounds that the metabolism of resting cells may be different to that of growing cells.

The advantages of being able to use a defined medium in fermentation studies were stated in Chapter 4 and the elucidation of the nutritional requirements of either wild-type B. stearothermophilus or strain 11d-15 would facilitate further studies with this organism. It should however be borne in mind that the nutritional requirements of high ethanol yielding mutants of B. stearothermophilus may differ slightly from that of the wild type or strain 11d-15. Certain biosynthetic pathways might be subject to forms of regulation which depended both on the nature of the fermentation end products and on

their environmental concentrations. For example, increased ethanol production by a mutant might result in the inhibition of a particular biosynthetic pathway which in wild-type B. stearothermophilus functions free from such inibition. Alternatively, the production of fewer acidic end products by a high ethanol yielding mutant might result in the derepression of a different biosynthetic pathway.

Perhaps the most convenient way of determining the origins of the minor fermentation products and of confirming the assumptions made here regarding carbon dioxide produced from amino acids or peptides would be to conduct fermentations in the presence of radioactively labelled sucrose and then to determine the specific radioactivity of individual products of fermentation. High performance liquid chromatographic (HPLC) techniques permitting the resolution of complex mixtures of fermentation products are now commonly available (I. W. Drummond, personal communication); individual components emerging from the HPLC column could be 'trapped' in a suitable scintillation mixture and subsequently counted. Collection of the components of the culture broth could be greatly facilitated by using an automatic fraction collector.

Enzyme experiments (Chapter 9) strongly implicated PDH as functioning anaerobically in strain 11d-15 although the possible involvement of FDH could not entirely be ruled out. Confirmation of the role of PDH would be most convincingly demonstrated by examining the fermentation characteristics of a PDH^- mutant derived from strain 11d-15. One way of obtaining such a mutant would be to grow mutagenized cells on agar plates with glucose as energy source. PDH^- mutants could be isolated by replica plating onto plates with pyruvate as energy source: PDH^- mutants would be able to grow aerobically on glucose but not on pyruvate.

Evidence of shifts in metabolism with time during the course of batch fermentations was presented in Chapter 5, however no investigations were conducted into the mechanisms by which this shift may have been brought about. One possible clue to the mechanism comes from observations reported in Chapter 7 where it was shown that the ratio of ethanol to acetate was proportional to the concentration of pyruvate in the culture broth. Ratios of ethanol to acetate greater than 1.0 are an indication that the PDH pathway is being utilized.

The presence of pyruvate in the culture broth implies its intracellular accumulation, this could induce PDH activity if the Michaelis constant (K_m) of the PDH enzyme complex for pyruvate were greater than the K_m for pyruvate of the competing enzyme, PFL. Metabolism via the PDH pathway would therefore only become significant when the intracellular pyruvate concentration rose above a certain threshold value. The validity of this hypothesis would ordinarily be tested by purifying the two enzymes from crude cell extracts and measuring their K_ms for pyruvate. However, although established techniques permitting the purification and assaying of PDH from B. stearothermophilus exist (Perham and Wilkie, 1980; Visser et al., 1982), PFL would present difficulties. As a class of enzymes PFLs appear to be extremely susceptible to inactivation by oxidation (Knappe and Blaschkowski, 1975). The presence of PFL in crude cell extracts from B. stearothermophilus has not been experimentally demonstrated (M. A. Payton, personal communication). Knappe and Blaschkowski successfully isolated and assayed PFL from E. coli in the presence of a 'reducing system' which comprised an iron-containing protein ('Enzyme II'), metal ion thiol complexes, pyruvate and adenosyl methionine and claimed that PFLs from Streptococcus faecalis and clostridia are also reliant on adenosyl methionine for activation. No information is available on the activation of PFL from B. stearothermophilus and it appears from the work of Knappe and Blaschkowski that its unravelling would be a formidable task.

Mutations in cultures of lld-15 led to variability in product yields in batch culture experiments performed under identical conditions and to the replacement of lld-15 by mutants of phenotype similar to that of wild-type B. stearothermophilus in continuous culture. The possibility of reversion would impose restrictions on further work with lld-15. However recent research offers hope of avoiding this problem. S. Amartey (personal communication) has shown that wild-type B. stearothermophilus is unable to grow at 70°C whereas lld-15 grew at temperatures as high as 74°C. It would be most unlikely that in addition to the mutation in the gene coding for LDH which lld-15 - a spontaneous mutant - carries it also possesses further mutations which bestow upon it a greater tolerance to elevated temperatures than the wild-type. So that the inability of the wild-type to grow at 70°C might be caused by inhibition of

growth by lactate accumulation at this temperature. If this is so then revertants which are phenotypically similar to the wild-type would also be unable to grow at 70°C.

An alternative approach to the reliance on high temperatures to prevent reversion would be to obtain a more stable LDH^- mutant of B. stearothermophilus. Although the nature of the mutation in strain 11d-15 is unknown, more stable mutants might be obtained by subjecting a wild-type population of B. stearothermophilus to mutagenic agents known to induce the deletion of several bases from the genome; one such agent is ultra-violet light.

Whichever alternative is followed, LDH^- mutants of B. stearothermophilus will almost certainly be susceptible to growth inhibition by toxic accumulations of acetate and formate. It may be possible to minimize these effects by operating in continuous culture at a lower dilution rate than was used here (0.25 hr^{-1}) on medium containing a low concentration of sucrose (circa 0.5% w/v) and by adapting the culture to acid pHs or high temperature or a combination of both. These conditions would result in a reduction of pyruvate flux through the PFL pathway and a correspondingly higher flux through the PDH pathway. With the culture adapted in this way the concentration of sucrose in the medium could be steadily increased. Growth inhibition resulting from the production of ethanol could be prevented by vigorously sparging the culture with an inert gas.

An alternative approach would be to isolate mutants which were genetically unable to make use of the PFL pathway. A method for achieving this with strain 11d-15 was devised by M. A. Payton (personal communication) who reasoned that a PFL^- strain would be able to grow on pyruvate only under aerobic conditions. The reason for the failure of this approach is evident from the results obtained here; 11d-15 is able to make use of PDH anaerobically even in the presence of PFL. A less direct strategy exists, however the results would not be as predictable. PFL catalyses the conversion of 2 moles of pyruvate to 2 moles of acetyl-CoA and 2 moles of formate. One mole of acetyl-CoA is reduced to ethanol whilst the second mole of acetyl-CoA is first converted by the enzyme phosphotransacetylase (PTA) to acetyl phosphate and then by the enzyme acetate kinase (ACK) to acetate. Mutants in these enzymes have been obtained in Aerobacter

aerogenes (Brown et al., 1972), E. coli (Brown et al., 1977) and Salmonella typhimurum (Levine, 1980) on the basis of resistance to fluoroacetate. Mutants in these enzymes would not be able to channel pyruvate along the PFL pathway without causing an accumulation of acetyl-CoA. The reasoning behind restricting pyruvate flux through the PFL pathway in this way rests on hopes that the accumulation of intermediates (i.e. pyruvate and acetyl-CoA) might induce pyruvate flux via the PDH pathway. Finally, a very promising approach is that of Winkelman and Clark (1984); termed 'proton-suicide' theirs is a positive selection method for mutants unable to produce acids from sugars by fermentation and relies on the production of the highly toxic elemental bromine in acid conditions from agar medium supplemented with bromide and bromate.

The strategies outlined above suggest methods of increasing the yields of ethanol produced by B. stearothermophilus. The remainder of this chapter is devoted to a comparison of the properties (actual and potential) of B. stearothermophilus both with those of other thermophilic ethanologens and also with mesophilic ethanologens. Also included is more general consideration of some of the claims made for thermophiles.

10.2 Industrial Ethanol Production with Thermophiles

10.2.1 Ethanol Yield and Substrate Spectrum

The data of Table 1.2 indicated that in the production of ethanol by the fermentation of traditional substrates, i.e. glucose, sucrose and hydrolyzed starches, raw material costs comprise by far the largest input. This implies that in evaluating micro-organisms for potential industrial application, the yield of ethanol which they produce from these substrates is of critical importance. Mention has already been made that the mesophiles S. cerevisiae and Z. mobilis are able to ferment traditional substrates to ethanol in high yields. However the yields displayed by some recently isolated thermophiles, Thermoanaerobacter ethanolicus and Clostridium thermohydrosulfuricum in particular (see Table 1.4), are on a par with those of efficient mesophilic ethanologens.

Earlier mention was also made that S. cerevisiae and Z. mobilis are only able to ferment a relatively narrow range of substrates. In this respect however thermophiles show considerable advantage over these mesophilic ethanologens as Table 10.1 shows. The clostridial

Organism	Substrates Fermented	Substrates not Fermented	References
Clostridium thermocellum LQR1	Cellulose Cellodextrins Cellobiose Glucose	Xylose Sucrose Mannose Xylan	Zeikus et al. (1981) Ng et al. (1981)
Clostridium sp. M7	Glucose Maltose Mannose Fructose Cellobiose Sucrose Galactose Cellulose Xylose Lactose Cellodextrins	-	Lee & Blackburn (1975)
Clostridium thermohydrosulfuricum JW 102	Glucose Galactose Sucrose Maltose Xylose Starch Arabinose Cellobiose	Fructose	Wiegel et al. (1979) Wiegel (1980)
Clostridium thermohydrosulfuricum 39E	Glucose Xylose Mannose Cellobiose Sucrose Starch	Cellodextrins Cellulose Xylan Mannan	Zeikus et al. (1981) Ng et al. (1981)
Thermoanaerobacter ethanolicus JW200	Glucose Lactose Mannose Maltose Starch Sucrose Cellobiose Xylose Fructose	Cellulose	Zeikus et al. (1981) Wiegel & Ljungdahl (1981)
Thermoanaerobium brockii HTD4	Glucose Maltose Sucrose Cellobiose Lactose Starch	Cellodextrins Cellulose Mannan Xylose Arabinose	Zeikus et al. (1981) Lamed & Zeikus (1980)
Thermobacteroides acetoethylicus HTB2	Glucose Maltose Mannose Cellobiose Sucrose Starch Lactose	Cellodextrins Cellulose Xylan Xylose Mannan Arabinose	Zeikus et al. (1981) Ben-Bassat and Zeikus (1981)
Bacillus stearothermophilus NCA 1503	Glucose Maltose Sucrose Starch	Cellulose Lactose Xylose	Sharp et al. (1980)

Table 10.1 Range of Substrates Fermented by Various Thermophiles

isolate of Lee and Blackburn (1975) and T. ethanolicus stand out as having extremely wide ranging fermentation abilities. The fermentative abilities of B. stearothermophilus have not been fully investigated. Sharp et al. (1980) reported that B. stearothermophilus NCA 1503 was unable to ferment xylose. This finding is difficult to explain since strain 11d-15, which was derived from strain NCA 1503, ferments xylose.

10.2.2 Fermentation Rate

A frequent claim made of thermophiles is that they are able to ferment substrates to products at higher rates than similar mesophilic organisms (Wiegel, 1980; Zeikus et al., 1981; Payton, 1984). An examination of this claim is made below and the significance of rate in industrial fermentative ethanol production is considered.

Specific ethanol productivities for B. stearothermophilus 11d-15 with sucrose as substrate were shown in Table 5.6. The highest productivity achieved (1.01g ethanol/g cell dry wt-hr) was at 70°C and pH 7.0. Cultural conditions were shown in Chapter 5 to influence the distribution of pyruvate via either the PDH or PFL pathways, however in no experiment was the flux of pyruvate via the PFL pathway totally eliminated. Data does however exist which permits estimates of productivity to be made under the latter conditions. Table 10.2 shows the specific sucrose uptake rates (calculated similarly to specific ethanol productivities by the method of least squares, see Sub-section 5.3.3) for 6 experiments conducted at pH 7.0 and 60°C and at a variety of initial sucrose concentrations. Uptake rates at identical initial sucrose concentration show considerable scatter and as this parameter was not shown to influence metabolism, the mean of all 6 values (3.6g sucrose/g cell dry wt-hr) can be taken as a representative figure for fermentations at pH 7.0 and 60°C. If it is assumed that 11.35% of sucrose taken up is utilized for biosynthesis (see Sub-section 5.3.2) then the flux through the PDH pathway is 3.19g sucrose/g cell dry wt-hr. If it is further assumed that each mole of sucrose is fermented to yield 4 moles of ethanol (the theoretical maximum) then this value for sucrose uptake translates to a specific ethanol productivity of 1.72 g ethanol/g cell dry wt-hr. Although Table 5.6 indicates that productivity is higher at 70°C than at 60°C, caution should be exercised in interpreting this increase. The transition from 60 to 70°C in B. stearothermophilus 11d-15

Experiment	Initial Sucrose Concentration (g/l)	Specific Sucrose Uptake Rate
151	23.5	3.88
176	23.5	2.77
203	75.0	2.50
206	75.0	5.56
216	75.0	2.76
220	75.0	4.15

Table 10.2 Specific Sucrose Uptake Rates (g sucrose/g dry wt cells-hr) in Batch Fermenters at 60°C and pH 7.0

resulted in metabolic shifts from the PFL to the PDH pathway. To what extent the productivity increase is due to shifts in metabolism rather than to reaction rate increases can not be determined.

The specific ethanol productivities of a number of mesophilic and thermophilic ethanologens with either glucose or sucrose as substrate are compared in Table 10.3. A survey of the literature revealed no data on the fermentation rates of sucrose by yeast in a form that could readily be compared with the experimental data for B. stearothermophilus (i.e. having the units g ethanol/g cell dry weight-hr). The ethanol productivities displayed by yeast with glucose as substrate covered a wide range of values (see Table A4.1, Appendix 4) and the productivity for S. carlsbergensis represents the maximum value reported. Rather more rate data was available for Z. mobilis (Table A4.2, Appendix 4) and the values shown in Table 10.3 are the maximum displayed for each substrate. There is a paucity of data on thermophilic ethanologens in the literature. Because of the commercial potential of these organisms, advances in strain selection and improvement are rarely announced or are published as patents (see for example Ljungdahl and Carriera, 1983). Two widely differing values for the specific ethanol productivity of T. ethanolicus are shown in Table 10.3: the value quoted by Sonnleitner and Fiechter (1983) represents a theoretical maximum and was derived by them from growth rate and ethanol yield data; the second value, that of Kannan and Mutharasan (1985), was calculated from experimental data. In neither case were the strains used stated. These data illustrate the sensitivity of T. ethanolicus to cultural conditions.

Some caution should be exercised in comparing the productivities of organisms fermenting glucose with those fermenting sucrose. Z. mobilis displays similar productivities on either substrate and although this is generally the case for substrates such as glucose and sucrose, exceptions do occasionally arise. All the organisms in Table 10.3 are reported to ferment glucose and sucrose and in the absence of data that indicates otherwise, it is assumed that productivities with either glucose or sucrose as substrate if not identical are at least similar.

Organism	Specific Ethanol Productivity	Substrate	Fermentation Temperature °C	Reference
Zymomonas mobilis ZM4	5.40	Glucose	30	Rogers et al.(1982)
*Thermoanaerobacter ethanolicus[1]	1.40	Glucose	69-78[2]	Sonnleitner and Fiechter (1983)
Saccharomyces carlsbergensis ATCC 26602	0.87	Glucose	30	Rogers et al. (1979)
*Thermoanaerobacter ethanolicus	0.97	Glucose	69	Kannan and Matharasan (1985)
Zymomonas mobilis ZM4	4.60	Sucrose	30	Rogers et al. (1982)
Bacillus stearothermophilus[1]	1.72	Sucrose	60	This work
Bacillus stearothermophilus 11d-15	1.01	Sucrose	70	This work

Table 10.3 Specific Ethanol Productivies (g ethanol/g dry cell wt-hr) for Various Glucose and Sucrose Fermenting Organisms

* Strain not specified [1] Theoretical Prediction [2] Temperature not specified. The Range shown is from the Optimum Growth Temperature to the Maximim Growth Temperature (Wiegel and Ljungdahl, 1981)

Table 10.3 shows that for either sucrose or glucose as substrate the mesophilic bacterium Z. mobilis has the highest ethanol productivity. The experimentally obtained productivity for B. sterothermophilus is in excess of that for S. carlsbergensis. However, the theoretical values for both thermophiles are below the productivities for Z. mobilis.

The specific ethanol productivities of a number of organisms fermenting D-xylose are shown in Table 10.4. In all cases the organisms of Table 10.4 produced ethanol in addition to other fermentation products. B. stearothermophilus 11d-15 displays the highest productivity, that of Candida shehatae is slightly lower whilst those of the remainder of the organisms are considerably below the productivities of B. stearothermophilus 11d-15 and C. shehatae.

Two observations can be made from these comparisons of ethanol productivity: first, the productivities of thermophiles are not always greater than those of mesophiles and secondly, that the productivities of organisms within a particular classification (i.e. 'mesophile' or 'thermophile') show wide variations. Why do organisms display different productivities? Fermentation of a substrate to product(s) by a micro-organism requires (i) entry of the substrate into the cell (ii) reaction of substrate to product(s) (iii) exit of product(s) from the cell. Rate limitations may exist at any one of three stages. The rate of most enzymatic reactions doubles for a 10°C rise in temperature (Lehninger, 1977) and therefore increases in temperature may - as long as cellular integrity is maintained and unfavourable changes in metabolism do not occur - offer the prospect of increasing the rate of fermentation at the rate limiting step. This does not necessarily mean that the fermentation rate of thermophiles is greater than those of mesophiles: fermentation rate may be limited by factors which are not influenced by temperature, such as shortage of a particular enzyme or to the presence of an enzyme with an unfavourable Km, pH optimum, specificity for substrate or to the insensitivity to a regulator (Wood, 1981). Realization of the potential which thermophiles have for increased fermentation rate (by comparison with mesophiles) may therefore require intervention at the genetic level, such as for example the substitution of one enzyme by another having a more favourable Km. The generalized claim made by Wiegel (1980),

Organism	Specific Ethanol Product-ivity	Fermentation Temperature °C	Reference
Bacillus stear-othermophilus 11d-15	0.39	60	This work
Candida shehatae CSIR Y49	0.28	30	Du Preez and Van der Walt (1983)
Clostridium thermohydro-sulfuricum HG-3	0.07	60	Wang et al. (1979)
Pachysolen tannophilus NO3U2	0.04	30	Jeffries (1984)
Pachysolen tannophilus Y-2460	0.03	32	Jeffries (1984)
Pichia stipitis 5776	0.03	30	Dellweg et al. (1984)
Fusarium oxysporum ATCC 10960	0.03	30	Rosenberg et al. (1981)

Table 10.4 Specific Ethanol Productivites (g ethanol/g dry wt. cells-hr) of Various D-xylose Fermenting Organisms

Zeikus et al. (1981) and Pyton (1984) that thermophiles display higher productivites than mesophiles is therefore a misleading one.

In batch processes the fermentation rate of an organism determines the number of fermentations that can be conducted in a given time, in continuous culture fermentation rate determines the throughput of a particular fermenter. As mentioned earlier such factors will have relatively little impact on reducing the cost of ethanol produced from traditional resources. However if a choice has to be made between two organisms of similar characteristics except that one possesses a higher fermentation rate, then it is obvious that the one with the highest fermentation rate will be selected. This typifies the situation regarding yeast and Z. mobilis: both organisms produce high ethanol yields, ferment the same substrates, have similar ethanol tolerances and exist as flocculating varieties (flocculence facilitates the separation of cells from spent culture broth, see below), however Z. mobilis has a higher fermentation rate than do yeasts. Although yeasts have conventionally been used to produce ethanol it is likely that in the future processes based on Z. mobilis will become widespread.

10.2.3 Contamination

According to Wiegel (1980) and Zeikus et al. (1981) a further advantage of using thermophiles in industrial processes is that such processes would be less prone to contamination.' This is a realistic claim, and to be more optimistic about the possible risks of contamination would be unjustified. Evidence exists to show (see below) that when measures normally taken to prevent asepsis are totally set aside contamination is inevitable. Although not nearly as widespread in nature as temperature habitats, a variety of high temperature natural habitats exist. These include numerous types of volcanic and geothermal features with temperatures greater than boiling, solar heated environments such as litter and soil or sediments with temperatures reaching 70°C and biological self-heated environments such as compost or hay where temperatures rise high enough for spontaneous combustion to occur. However, thermophiles enjoy a much wider distribution throughout the ecosphere: for instance, strains of B. stearothermophilus have been isolated from mud, soil and water (Ljungdahl, 1979) and their spores are virtually ubiquitous. Certain other thermophilic spore-formers, such as clostridia and thermoactinomyces are also widely distributed in nature (Zeikus, 1979). The maximum growth temperature of B. stearothermophilus

is claimed by Ljungdahl (1979) to be 'slightly above 70°C', so that all processes operating below this temperature would be potentially subject to B. stearothermophilus contamination unless the normal precautions against contamination were taken. Zeikus (1979) reported that failure to adequately sterilize complex media for the growth of Thermus aquaticus resulted in higher cell densities of B. stearothermophilus even when large inocula of the former had been used. Zeikus and Wolfe (1972) found that the use of unsterilized medium for the large scale cultivation of the prolific obligate anaerobe and extreme thermophile M. thermoautotrophicum resulted in persistant contamination by various species of clostridia.

Operation of industrial fermenters without sterilization may necessitate utilization of species with either very high metabolic activities or species that can tolerate an additional environmental extreme such as low pH in order to avoid contamination.

10.2.4 Solid-Liquid Separation

Weigel (1980) and Zeikus et al. (1981) both claimed that operation at thermophilic temperatures would facilitate the separation of microbial cells from spent culture broths due to the reduction of broth viscosity at the higher operating temperature. This claim is examined below.

A parameter of central importance in the design of solid-liquid separation equipment such as centrifuges and settling tanks is the particle settling velocity. Whilst the latter is influenced by liquid viscosity in that a decrease in liquid viscosity - caused by an increase in temperature, for example - results in an increase in the settling velocity, a further factor to be taken account of is particle size. The significance of particle size is demonstrated below by using Stokes Law to calculate the ratio of settling velocities in water of single cells of B. stearothermophilus at 70°C and the mesophilic ethanol producer S. carlsbergensis at 30°C. It was stated in Chapter 1 that the micro-organisms most tolerant of high temperatures are prokaryotes which are generally smaller in size than eukaryotes. For example, rod-shaped B. stearothermophilus cells are approximately 2.8 μm long and have diameters of 0.8 μm (Buchanan and Gibbons, 1974). By equating the volume of a cylinder of the dimensions given above to that of a sphere, an equivalent diameter of 1.4 μm is obtained. S. carlsbergensis cells occur in a wide variety of sizes

(Van der Watt, 1974), however those in the intermediate size range have major diameters of approximately 11.0 μm and minor diameters of approximately 5.8 μm. By assuming cells to be oblate ellipsoids an equivalent cell diameter of 8.1 μm is obtained. For equal cell densities, the ratio of settling velocities of S. carlsbergensis and B. stearothermophilus is 17.3, i.e. the larger cell size of the eukaryote more than compensates for the effect on settling velocity of operating at mesophilic temperatures. This illustrates the importance of cell size in addition to liquid temperature. It should be stressed that in practice modifications of Stokes Law are used to account for the concentration of the settling suspensions.

Flocculence of microbial cells greatly facilitates their separation from culture broths and in this respect yeast possess another advantage over most ethanologens in that highly flocculating species are known. Moreover, the genetic determinants of flocculence have been identified and transferred from one yeast species to another (Barney et al., 1980). The advantages of using flocculent organisms in industrial processes has been appreciated and Rogers et al. (1982) applied mutagenesis to obtain flocculent strains of Z. mobilis.

10.3 Conclusion

An ideal micro-organism for use in industrial ethanol production might be described as one capable of converting a broad range of substrates (including cellulose) to ethanol in high yields and at high rates and which was highly ethanol tolerant; no such organism has yet been isolated or constructed in the laboratory.

The organisms currently receiving evaluation may possess one or (more rarely) two of these desirable characteristics and the strategies being adopted in reaching the ultimate objective obviously depend on the organism under investigation. For example, Saccharomyces spp. generally produce high ethanol yields from a limited number of substrates and some strains are highly ethanol tolerant. Efforts - described in Chapters 1 and 8 - are now underway to increase the range of substrates which yeast can ferment. These strategies are clearly different from that being taken with B. stearothermophilus, an organism which displays a relatively high specific ethanol productivity, has a broader substrate spectrum than Saccharomyces but which initially converted substrates to ethanol in low yields.

If yeasts are to be displaced from their monopoly position in industrial fermentative ethanol production, then in the short term at least this will probably be by Z. mobilis. In the longer term developments will depend on whether strategies to improve the fermentable range of substrates by Z. mobilis are successful, it is probably in the longer term that thermophilic ethanologens will see industrial service. However it must be borne in mind that Z. mobilis has not yet proved itself industrially - the vast majority of studies conducted with this organism have been under laboratory conditions. This of course also holds true for thermophiles and already severe growth inhibition of thermophiles has been reported to have been caused by the leaching out of trace elements from steels used in the construction of fermenters and by plasticizers from ancilliary plant due to operation at 'thermophilic' temperatures (Sonnleitner and Fiechter, 1983). It must also be stated that no convincing process plant studies have been conducted which permit direct comparisons to be made between a process for ethanol production based on Z. mobilis with its high rates of fermentation and a thermophilic-based 'vacuferm' process where ethanol is continuously removed from the fermenter as it is produced.

The relatively low ethanol yields produced by B. stearothermophilus 11d-15 rule it out as a contender for industrial ethanol production from sucrose. The isolation of improved mutants may alter this. The situation with regards to D-xylose is rather more encouraging as the ethanol productivity displayed by 11d-15 is relatively high, but here again higher ethanol yields would further increase its acceptability.

Appendix 1 Origins of Bacterial Strains Used in this Work

B. stearothermophilus 11d-15

A TYE agar plate culture of B. stearothermophilus 11d-15 was donated by Dr. M. A. Payton of the Department of Biochemistry, Imperial College. The purity of the culture was established by the isolation and culture of single colonies. GC tests were made to ascertain that the colony selected for stock culture was LDH^-.

B. stearothermophilus NCA 1503 (NClB 8924) and C. thermocellum (NCIB 10682)

These strains were purchased from the National Collection of Industrial Bacteria (Aberdeen, Scotland).

Appendix 2 Batch Fermentation Data

A2.1 Mathematical Treatment of Results from Radioisotope Experiment (238)

Bacterial samples were prepared and solubilized as described in 5.3.2. Two samples were prepared with cell paste from experiment 238. Cell paste from experiment 234 served as a control. Each sample was counted twice for 4 minutes. The counting procedure was repeated a further 2 times; first after 10 µl of ^{14}C labelled cyclohexanol standard had been added and secondly after the addition of 10 µl of ^{3}H labelled cyclohexanol standard. The figures below represent mean counts per minute in each of the 2 counting channels, 'A' and 'B'.

	I		II		III	
	UNSPIKED		^{14}C-SPIKED		^{14}C + ^{3}H SPIKED	
SAMPLE	Channel A	Channel B	Channel A	Channel B	Channel A	Channel B
238i	8,000	1,277	10,812	3,505	19,303	3,657
238ii	6,870	1,180	9,759	3,290	17,547	3,366
234	237	19	3,147	2,151	11,471	2,272

Table A2.1 Summary of Channel Counts for Unspiked and Spiked Samples

The ^{14}C counting efficiency in channels A and B, c_A and c_B are determined by subtracting the counts for each channel in column I from those of column II. The ^{14}C counting efficiency in each channel is the ratio of counts in that channel to the total number of counts. For example for sample 238i,

	II - I	
	ChA	ChB
	2812	2228

Total C counts = 2812 + 2228 = 5040

c_A = 2812/5040 = 0.558

c_B = 2228/5040 = 0.442.

Similarly the ^{3}H counting efficiency in channels A and B, h_A and h_B, are determined by subtracting the counts for each channel in II from those of column III.

For sample 238i,

III - II

ChA	ChA
8491	152

Total ^{3}H counts = 8491 + 152 = 8643

$h_A = 8491/8643 = 0.982$

$h_B = 152/8643 = 0.018$

The total counts (N_A) in channel A is equal to the sum of the ^{14}C counts (C) times the ^{14}C counting efficiency in channel A and the ^{3}H counts (H) times the ^{3}H counting efficiency in channel A, that is,

$$N_A = C(c_A) + H(h_A)$$

Similarly for channel B

$$N_B = C(c_B) + H(h_B)$$

Solving these two equations for C and H it will be found that

$$C = \frac{N_A - N_B\ (h_A/h_B)}{c_A - c_B\ (h_A/h_B)}$$

$$H = \frac{N_B - N_A\ (c_B/c_A)}{h_B - h_A\ (c_B/c_A)}$$

^{14}C and ^{3}H counts are shown below.

SAMPLE	^{14}C CPM	^{3}H CPM
238i	2617	6667
238ii	2669	5382
234	37	219

Table A2.2 ^{14}C and ^{3}H Counts per minute

Specific Activities of Standards

^{3}H cyclohexanol	4.81×10^{6} disintegrations per minute per gram on 1/3/76
^{14}C cyclohexanol	1.168×10^{6} disintegrations per minute per gram on 1/1/77
^{3}H half-life	12.35 years
^{14}C half-life	5730 years.

Knowledge of the activities of the cyclohexanol standards permits the effects of quenching to be accounted for and the sample counts to be corrected.

For example 10 µl of ^{3}H cyclohexanol should give an increase in counts in both channels of:-

$$= 4.81 \times 10^{6} \times 0.629 \times 7.73 \times 10^{-3}$$

$$= 23{,}4000 \text{ dpm}$$

For sample 238i the actual increase was 8643 cpm.

The correction factor is then 23,400/8643 = 2.707 .

The sample counts were corrected for quenching effects and are shown below. Also tabulated are the corrected specific counts

Sample	^{14}C DPM	^{3}H DPM	^{14}C DPMG*	^{3}H DPMG*
238i	4.687×10^{3}	1.805×10^{4}	3.024×10^{5}	1.164×10^{6}
238ii	4.820×10^{3}	1.602×10^{4}	3.638×10^{5}	1.209×10^{6}
234	6.6×10^{1}	6.07×10^{2}	7.752×10^{2}	7.129×10^{3}

Table A2.3 ^{14}C and ^{3}H Disintegrations per minute

*DPMG Disintegration per minute per gram of cell wet weight.

The counts for the control were subtracted from the sample counts and are tabulated below:

Sample	^{14}C DPMG	^{3}H DPMG	$^{3}H/^{14}C$
238i	3.016×10^{5}	1.157×10^{6}	3.84
238ii	3.630×10^{5}	1.202×10^{6}	3.31

Table A2.4 Corrected Sample Activities

Calculation of Proportion of Cell Carbon Dervied from Sucrose

Sample	238i	238ii
^{14}C DPM	4675	4810

Quantity of ^{14}C sucrose added to the fermenter = 0.453 μmoles

Total Quantity of Sucrose = 138 mM

Ratio of Unlabelled Sucrose/Labelled Sucrose = 3.046×10^{5}

Total Counts added = 5.55×10^{8} DPM

1DPM $\equiv 8.162 \times 10^{-16}$ moles of sucrose

Quantity of labelled Sucrose Incorporated into biomass (moles)	3.816×10^{-12}	3.926×10^{-12}
Total quantity of sucrose Incorporated into biomass (moles)	1.162×10^{-6}	1.196×10^{-6}
Carbon equivalent of incorporated sucrose (g)	1.675×10^{-4}	1.723×10^{-4}
Dry weight of cells (g)	3.30×10^{-3}	2.87×10^{-3}

Experimentally determined carbon content of cells (see 5.3.3) = 48.8%

Cell carbon content (g)	1.61×10^{-3}	1.40×10^{-3}
% Cell Carbon derived from sucrose	10.40	12.31

A2.2 Estimation of Ethanol Losses During Batch Fermentations

Gas chromatographic monitoring of the exit gases from a 5 litre New Brunswick fermenter during a batch fermentation experiment indicated that the precautions (see 2.3.2(d)), taken to prevent the volatilization of ethanol from the culture broth to the fermenter headspace were not entirely adequate.

An experimental investigation of the ethanol losses from a fermenter was conducted in order to correct previously obtained culture ethanol concentrations. The experiment involved adding a known quantity of ethanol to complex medium in a 5 litre fermenter, passing nitrogen gas through the headspace (at the flowrate used in all batch experiments) and monitoring the decrease in ethanol concentration with time. Greater importance was placed on obtaining accurate correction factors for the values of ethanol concentration obtained typically towards the end of fermentation and therefore the composition of the medium used was formulated so as to model, as closely as possible, conditions prevailing towards the end of a fermentation experiment (i.e. relatively low sucrose concentration and high product concentrations).

Method

Medium

BST medium was used but with the following modifications.

	% (w/v)
Sucrose	0.5
Ethanol	0.75
Acetate	0.30
Formate	0.35

The volume of medium dispensed into the fermenter, 3.25 litres, was selected to represent as closely as possible the volume of culture medium remaining in the fermenter at the end of the logarithmic period of cell growth in a 'typical' batch fermentation experiment, accounting for sample volume and alkali addition.

Medium pH was adjusted to 7 by the addition of potassium hydroxide. The molar ratio of acetate and formate to ethanol was 0.31 and 0.47 respectively. The ethanol was added to the medium after the latter had been autoclaved. To further prevent microbial contamination of the

medium, mercuric chloride (0.003% (w/v)) was added.

Operating Conditions

Impeller speed 200 rpm. Nitrogen gas flowrate to fermenter head-space 200 ml/hr. Temperature 60°C.

Sampling

Temperature, gas flowrate and impeller speed were monitored at 2 hourly intervals. In addition to an initial sample, two samples were taken from the fermenter over the 21 hour period of operation for ethanol determination by gas chromatography (Method B, Table 3.2). The sample bottles were cooled in ice before sampling to reduce ethanol losses.

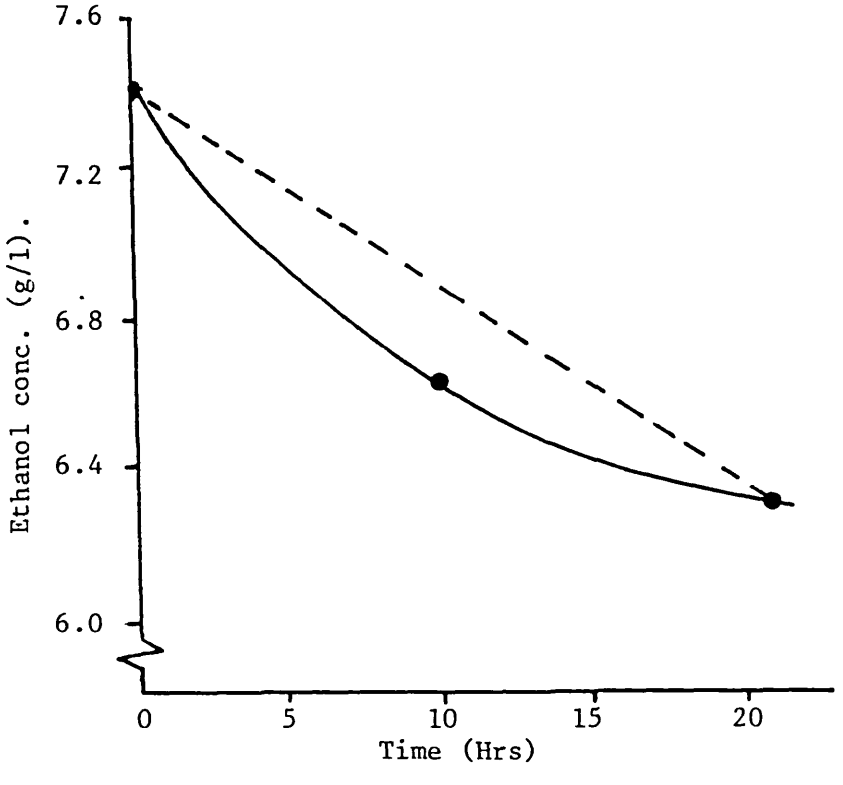


Figure A2.1 Ethanol Concentration against Time.

Fig. A2.1 is a plot of ethanol concentration against time. To facilitate analysis it was assumed that the loss of ethanol from the fermenter followed a linear pattern with time, (dotted line in Fig. A2.1). The implication of this is that the transfer rate of ethanol from the broth is not a function of its concentration in the broth. The slope of the dotted line gave the ethanol loss per unit time.

Data obtained at 50 and 70°C had also to be corrected. Nitrogen flow rate and impeller speed were equal at all temperatures, consequently as the rate of ethanol loss was assumed to be independent of its

concentration in the broth, the vapour phase concentration of ethanol at a particular temperature will be proportional to the pure solution vapour pressure of ethanol at that temperature. Table A2.5 shows the ethanol losses at 50, 60 and 70°C.

Temperature °C	Vapour Pressure mmHg	Vapour Pressure relative to vapour pressure at 60°C.	Ethanol Loss g/min.
50	215	0.642	0.0277
60	335	1	0.0432
70	535	1.597	0.0689

Table A2.5 Ethanol Losses at Various Temperatures

The procedure used to correct the ethanol data is best explained by a graphical example:

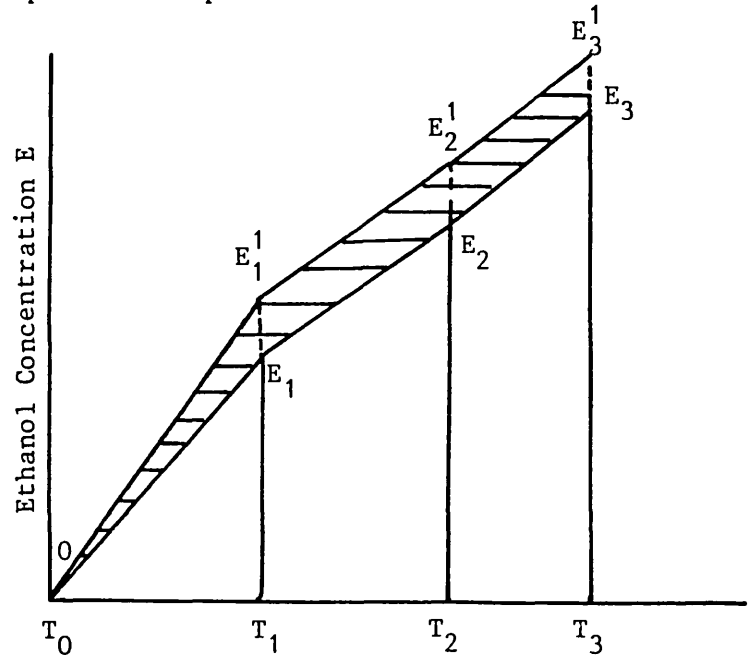


Figure A2.2 Graphical Calculation of Ethanol Losses

Points E_1 , E_2 , E_3 , ... represent experimentally determined ethanol concentrations at times T_1, T_2, T_3, respectively. The ethanol loss over the period $T_1 - T_0$ is calculated and assumed to take the form of the shaded area T_0 E_1' E_1 in Fig. A2.2. This permits calculation of the corrected ethanol concentration E_1 , at time T_1 to be made.

Similarly, the ethanol loss over period $T_2 - T_1$ is calculated and is assumed to take the parallelopiped form $E_1' E_1 E_2 E_2'$. Using the previously corrected ethanol value E_1', E_2' can be computed. This procedure is then repeated for all data points. In experiments where a long initial log phase was encountered, the concentration of ethanol was taken to be zero at a period of 3½ hours before the first experimentally determined ethanol valve. Corrected ethanol valves were of the order of 4% greater than the uncorrected valves.

An error allowance of ± 10% was made in using the corrected ethanol valves in carbon recovery and O/R index calculations.

A2.3 Estimation of the Diluting Effect of Potassium Hydroxide Solution

The addition of potassium hydroxide solution to the fermenters during batch fermentation experiments resulted in a dilution effect. The magnitude of this effect was estimated and used to adjust the batch data.

The assumptions made in calculating the 'dilution factors' ((vol. of potassium hydroxide + volume of culture medium)/volume of culture medium) are considered below. An example is then presented of the use of dilution factors in correcting the acetate data of experiment 152. The volume of potassium hydroxide solution added per fermenter was determined only at the end of the experiment, consequently, the rate of addition was assumed to be proportional to the measured cell density. (The calculated value was assumed to be subject to an error of ± 10%). The total volume of all samples taken was measured by bulking together the remainder of the samples after aliquots for cell density (circa 4 mls) and fermentation product and sucrose (10 mls) determinations had been removed. Individual sample volume was taken as the total sample volume divided by the total number of samples taken; a 10% error was assumed in the estimation of sample volumes.

The process of adding sterile distilled water to bring the fermenter contents up to the working volume following the evaporative losses incurred during the cooling down of the fermenters on removal from the autoclaves was estimated to be subject to an error of ± 3%. Using these error estimates the maximum error in the dilution factor

itself was calculated to be ± 1%: this value was accounted for in the computation of carbon recoveries and O/R indices in addition to the corresponding measurement errors.

Example

In experiment 152 the total volume of potassium hydroxide solution added to the fermenter was 250 ml.

Total sample volume was 228.0 ml.

Computed dilution factors are shown in Table A2.6.

The maximum dilution factor is 1.08: the maximum dilution factor for all experiments was 1.16.

A2.4 Sucrose Consumption

Initial and final sucrose concentrations for experiments 151 to 176 are presented in Table A2.7. Also shown are dilution factors which were calculated as explained earlier in A2.3.

The amount of sucrose consumed during a particular experiment was calculated in the following way:-

Initial Sucrose Concentration - (Final Sucrose Concentration x Dilution Factor).

A2.5 Potassium Hydroxide Consumption

Table A2.8 shows the quantities of potassium hydroxide added during the course of batch fermentation experiments. Also shown are the quantities of potassium hydroxide required to neutralize all the acidic products detected by G.C.

Time Hrs.	Cumulative Samp.Vol.ml.	Fraction of Max. Cell Density	Cumulative Alkali Add.ml.	Medium Vol.ml.	Dilution Factor	Uncorrected Acetate conc. g/l.	Corrected Acetate conc. g/l.
2.30	19.0	0.04	10.0	3381	1.00	-	-
2.62	38.0	0.08	20.0	3362	1.01	0.20	0.20
2.92	57.0	0.15	37.5	3343	1.01	-	-
3.25	76.0	0.31	77.5	3324	1.02	-	-
3.50	95.0	0.47	117.5	3305	1.04	-	-
3.75	114.0	0.63	157.5	3286	1.05	0.63	0.66
4.00	133.0	0.66	165.0	3267	1.05	-	-
4.50	152.0	0.77	192.0	3248	1.06	1.14	1.21
5.50	170.0	0.83	207.0	3230	1.06	1.27	1.35
8.50	190.0	1.0	250	3210	1.08	1.54	1.67
11.50	209.0	1.0	250	3191	1.08	1.92	2.07
16.00	228.0	1.0	250	3172	1.08	-	-

Table A2.6 Dilution Factors for Experiment 152

Experiment	Initial Sucrose Concentration g/l	Final Sucrose Concentration g/l	Maximum Dilution Factor*
151	23.9	3.28	1.11
152	22.9	1.64	1.08
153	26.6	1.58	1.11
154	26.0	8.25	1.09
156	23.5	2.90	1.10
159	24.4	1.50	1.09
160	21.9	6.33	1.09
161	109.5	65.6	1.07
162	50.0	5.5	1.07
171	26.3	0.96	1.08
172	50.0	0.70	1.16
174	24.5	0.58	1.06
176	22.1	0.54	1.08
239	45.9	5.7	1.03
240	46.2	3.9	1.09
263	50.0	39.6	ND
264	50.0	39.2	ND

Table A2.7 Sucrose Concentration Data and Dilution Factors

ND Not Determined

* See Section A2.3

Experiment	KOH Requirement (Moles)		Ratio $\frac{2}{1}$
	1 Calculated	2 Observed	
151	0.25	1.56	6.2
152	0.25	1.11	4.5
153	0.30	1.56	5.2
154	0.20	1.29	6.5
156	0.22	1.45	6.7
159	0.31	1.36	4.5
160	0.10	0.54	5.4
161	0.45	1.03	2.3
162	0.28	1.11	4.0
171	0.29	1.11	3.9
172	0.56	2.27	4.1
174	0.30	0.89	3.0
176	0.28	1.11	4.0
239	0.23	0.59	2.6
240	0.53	1.47	2.8

Table A2.8 Predicted and Observed Potassium Hydroxide Requirements

A2.6 Product Concentrations against Time (Figures A2.3 to A2.14) and Growth Curves (Figures A2.15 to A2.28) for Experiments 151-176.

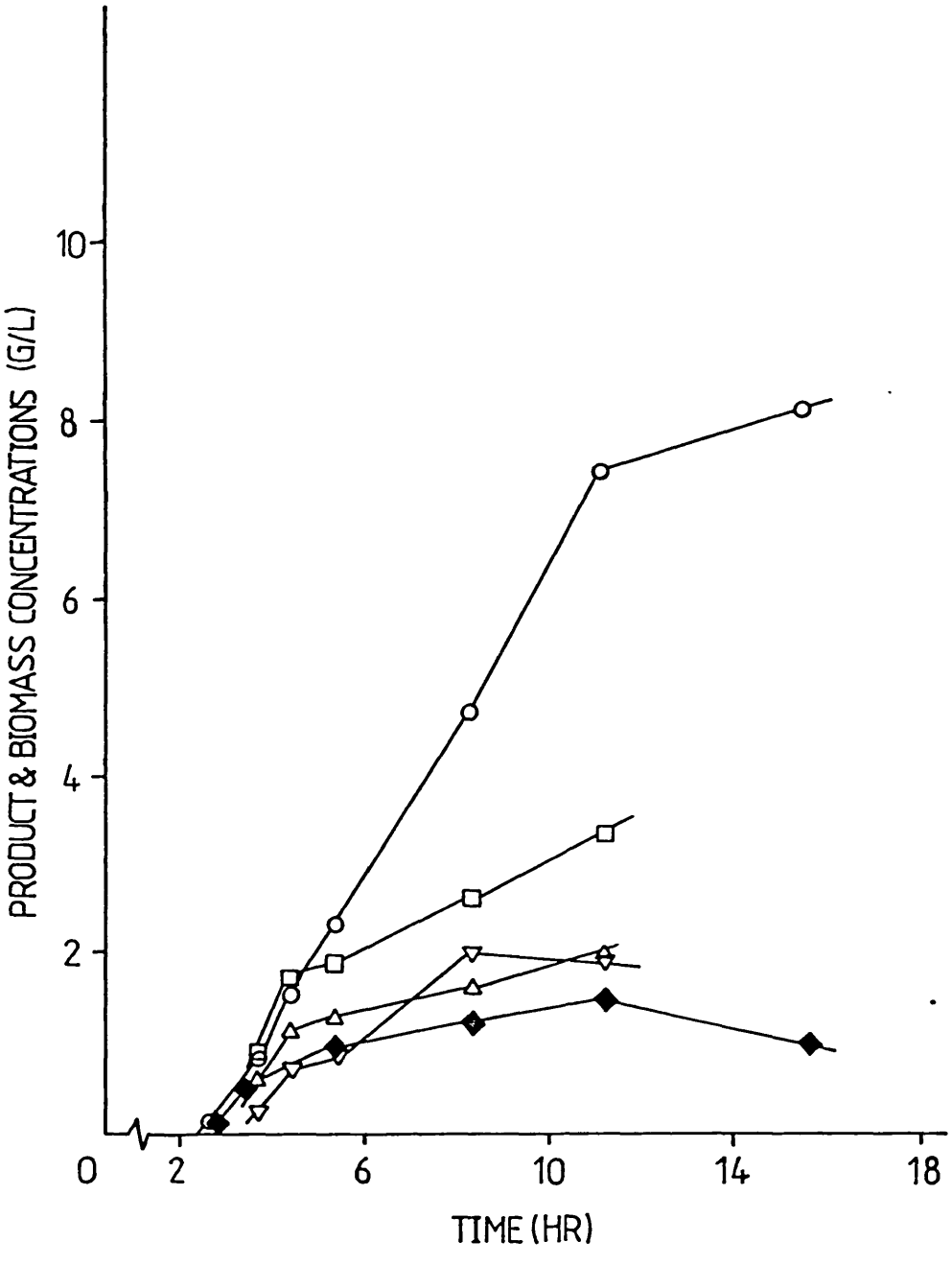


Figure A2.3 Product & Biomass Concentrations against Time

Experiment 152 (So = 23.5 g/l, T = 60°C, pH 6.2)

Symbols: (○) Ethanol, (□) Formate, (△) Acetate, (▽) Pyruvate, (◆) BIomass.

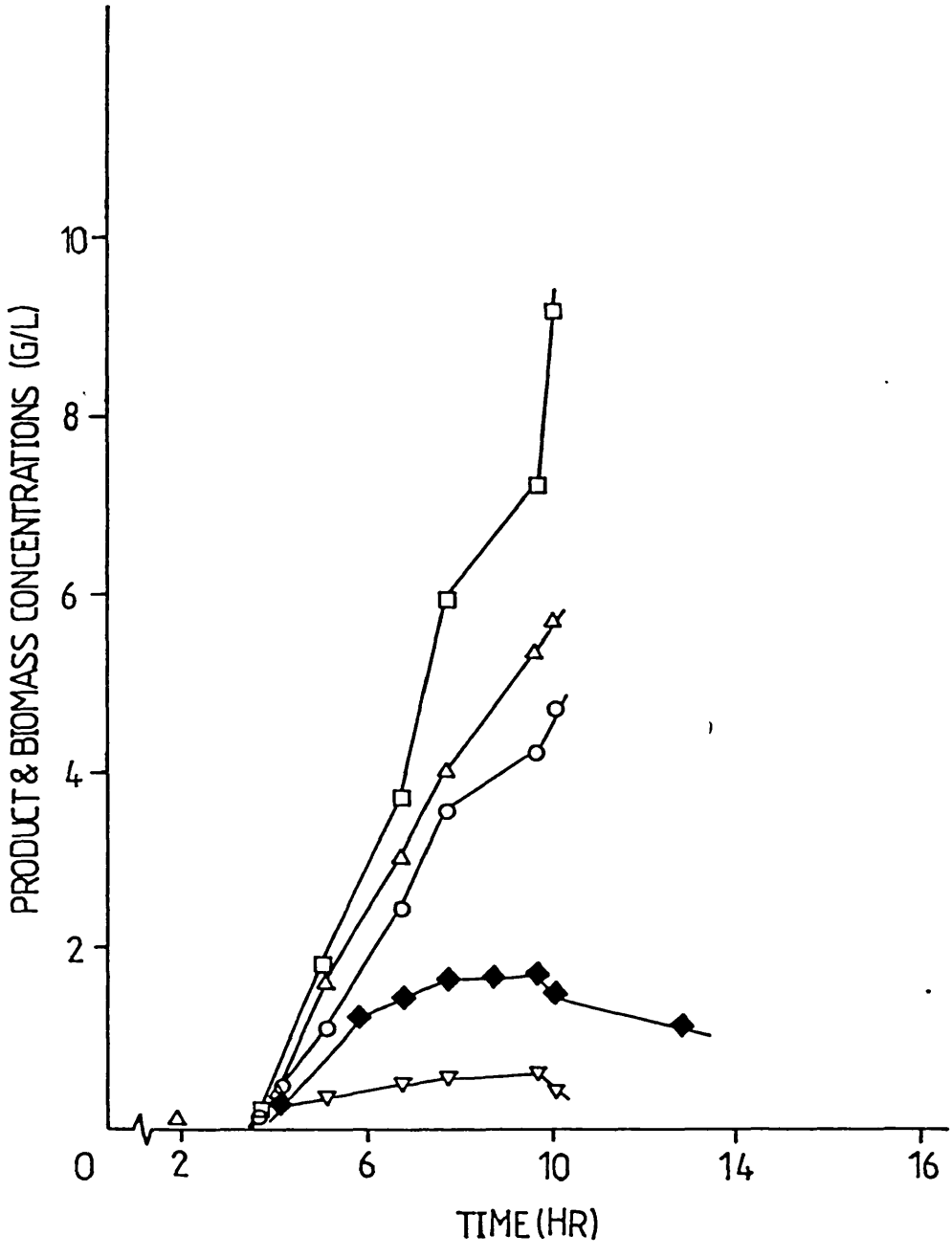


Figure A2.4 Product & Biomass Concentrations against Time
Experiment 153 (So = 23.5 g/l, T = 60°C, pH 8.0)

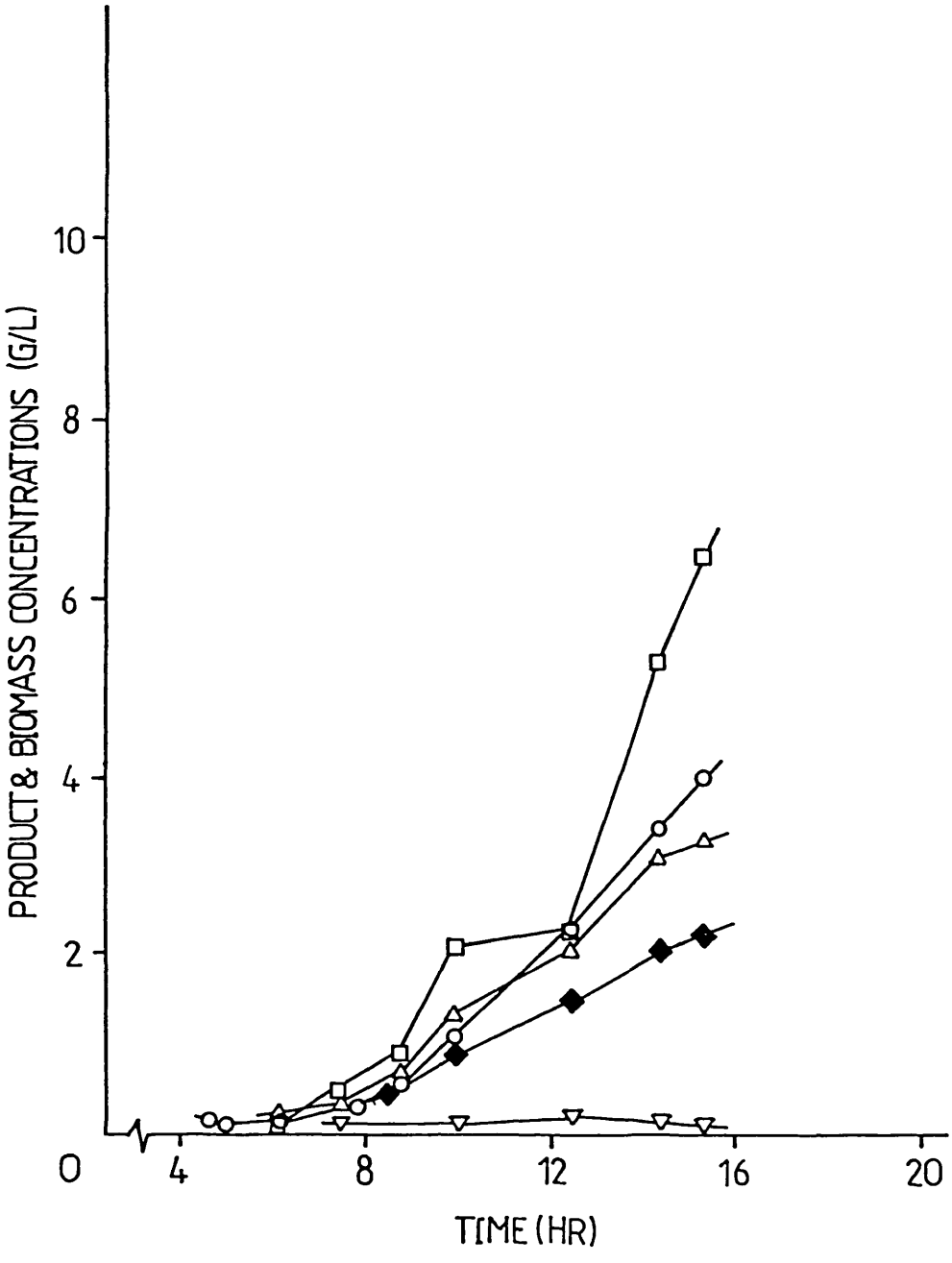


Figure A2.5 Product & Biomass Concentrations against Time
Experiment 154 (So = 23.5 g/l, T = 50°C, pH 8.0)

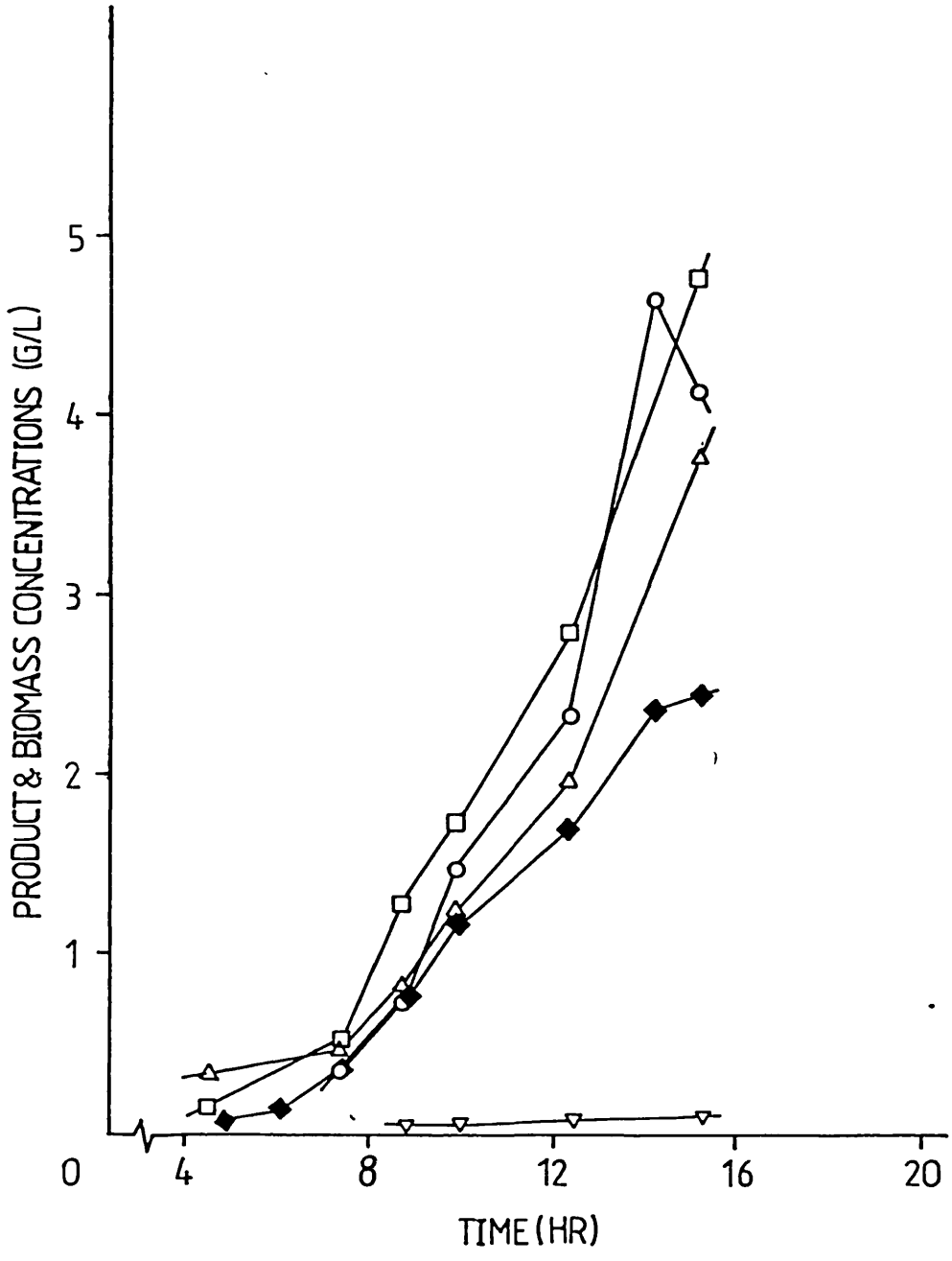


Figure A2.6 Product & Biomass Concentrations against Time
Experiment 156 (So = 23.5 g/1, T = 50°C, pH 7.0)

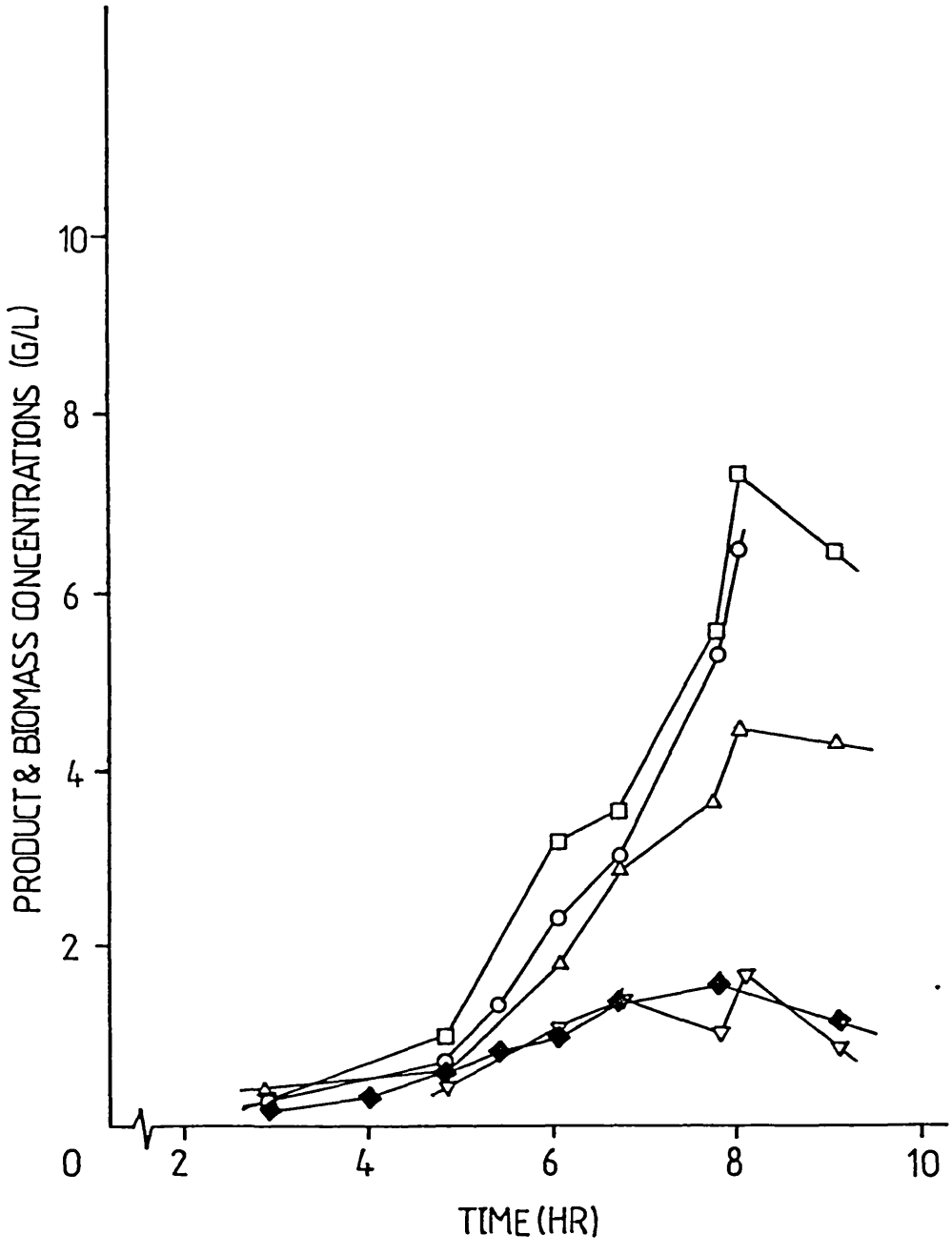


Figure A2.7 Product & Biomass Concentrations against Time
Experiment 159 (So = 23.5 g/l, T = 70°C, pH 7.7)

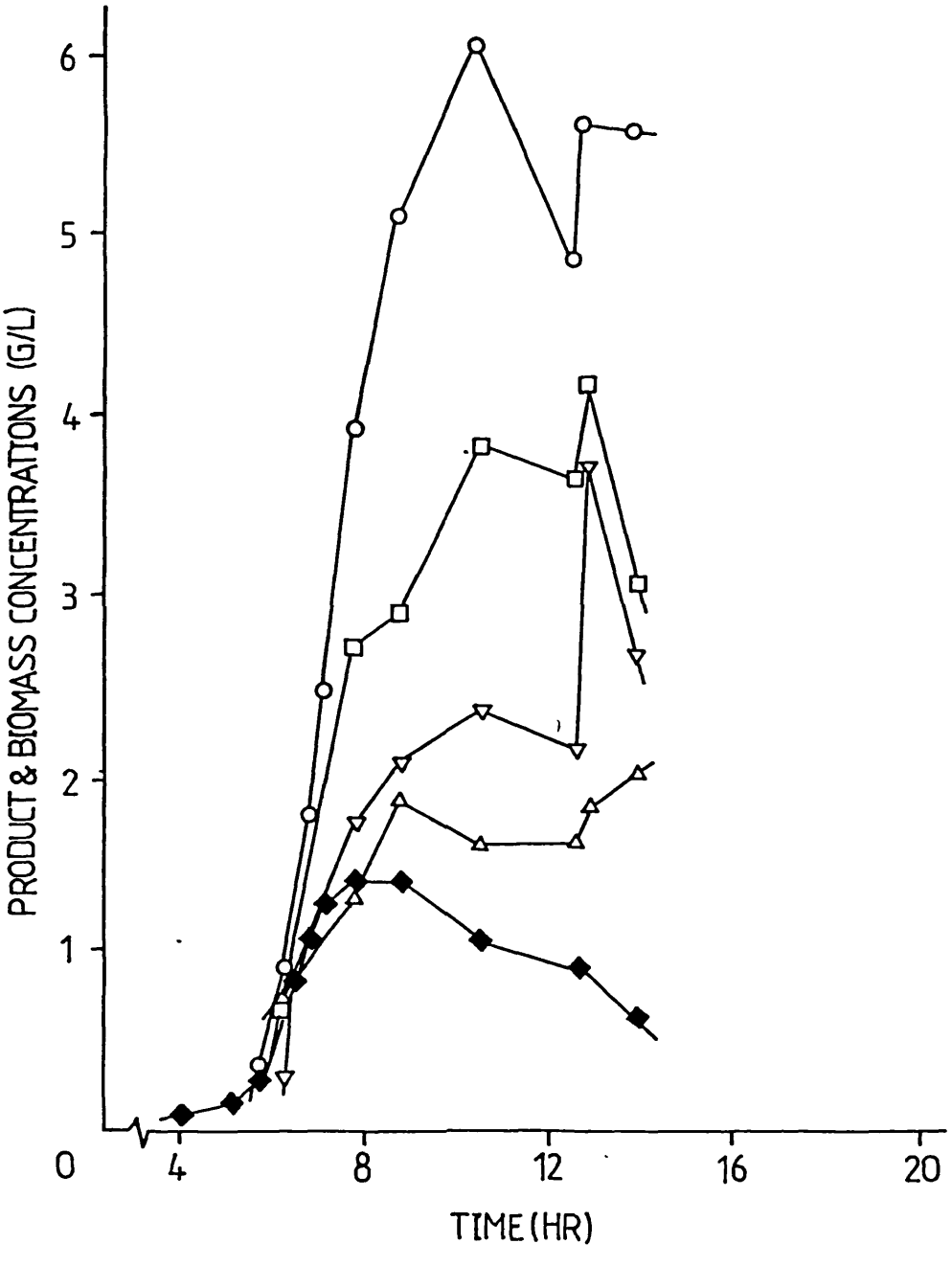


Figure A2.8 Product & Biomass Concentrations against Time

Experiment 160 (So = 23.5 g/l, T = 70°C, pH 7.0)

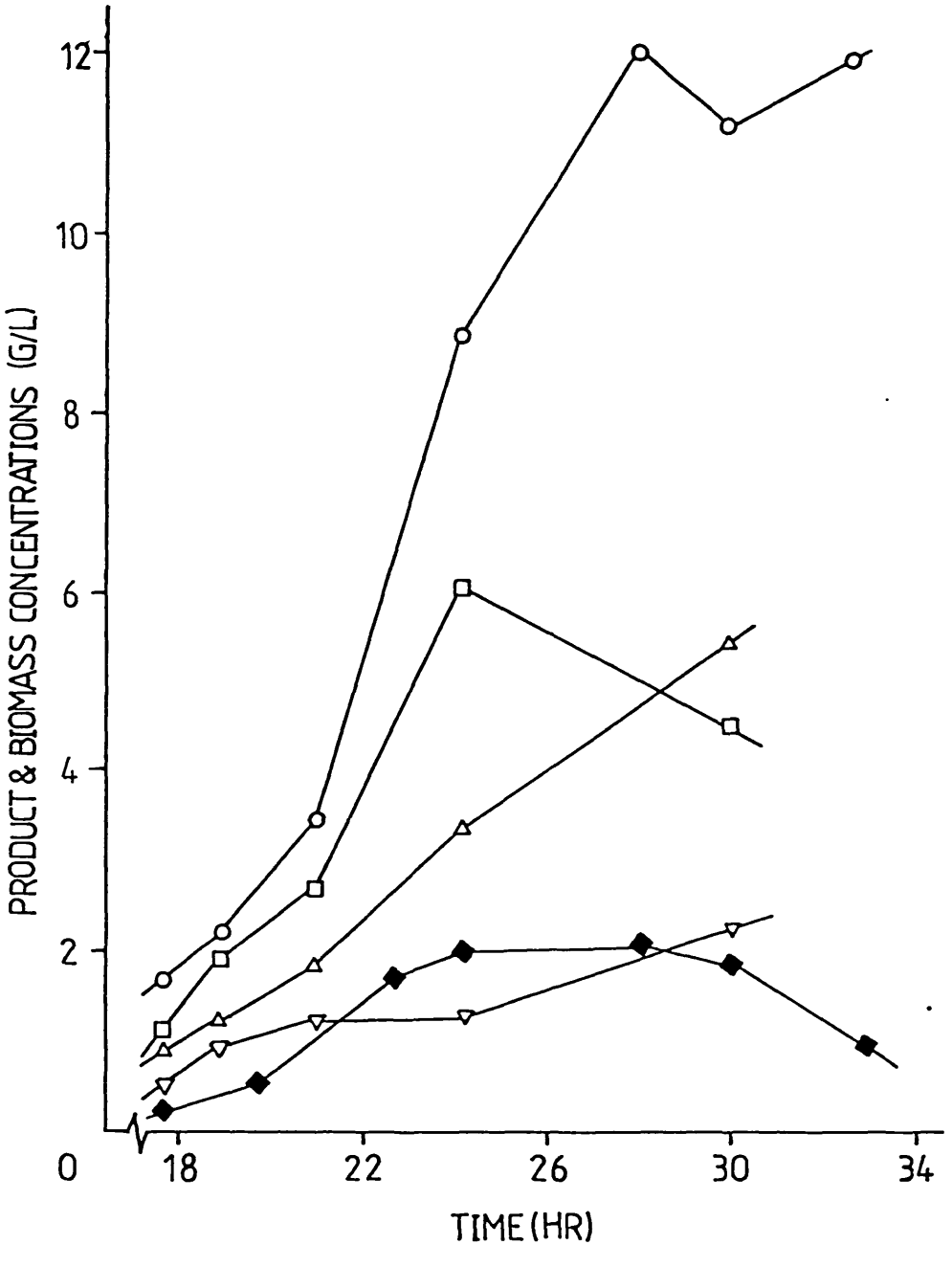


Figure A2.9 Product & Biomass Concentrations against Time
Experiment 161 (So = 100 g/l, T = 60°C, pH 7.0)

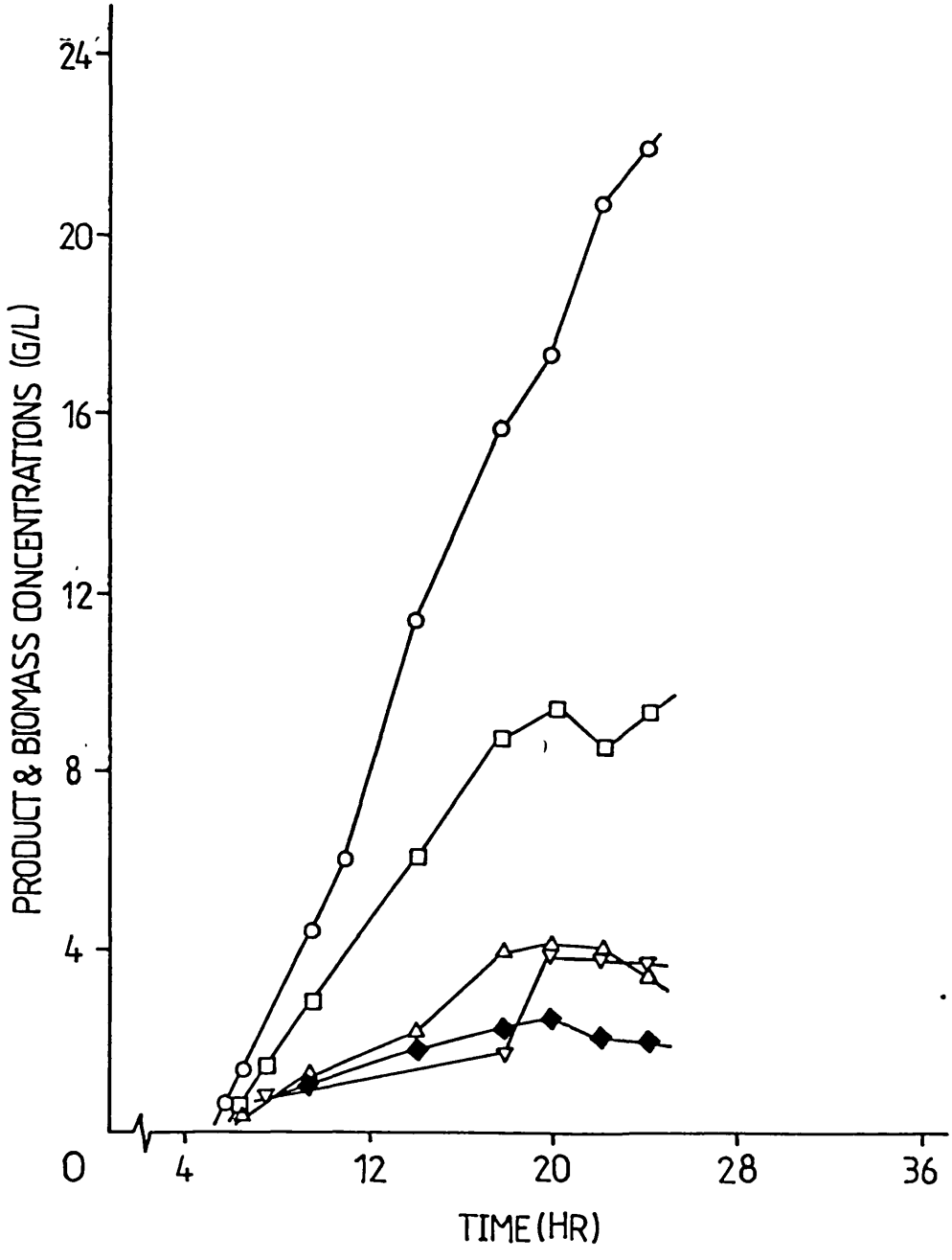


Figure A2.10 Product & Biomass Concentrations against Time
Experiment 162 (So = 50 g/l, T = 60°C, pH 7.0)

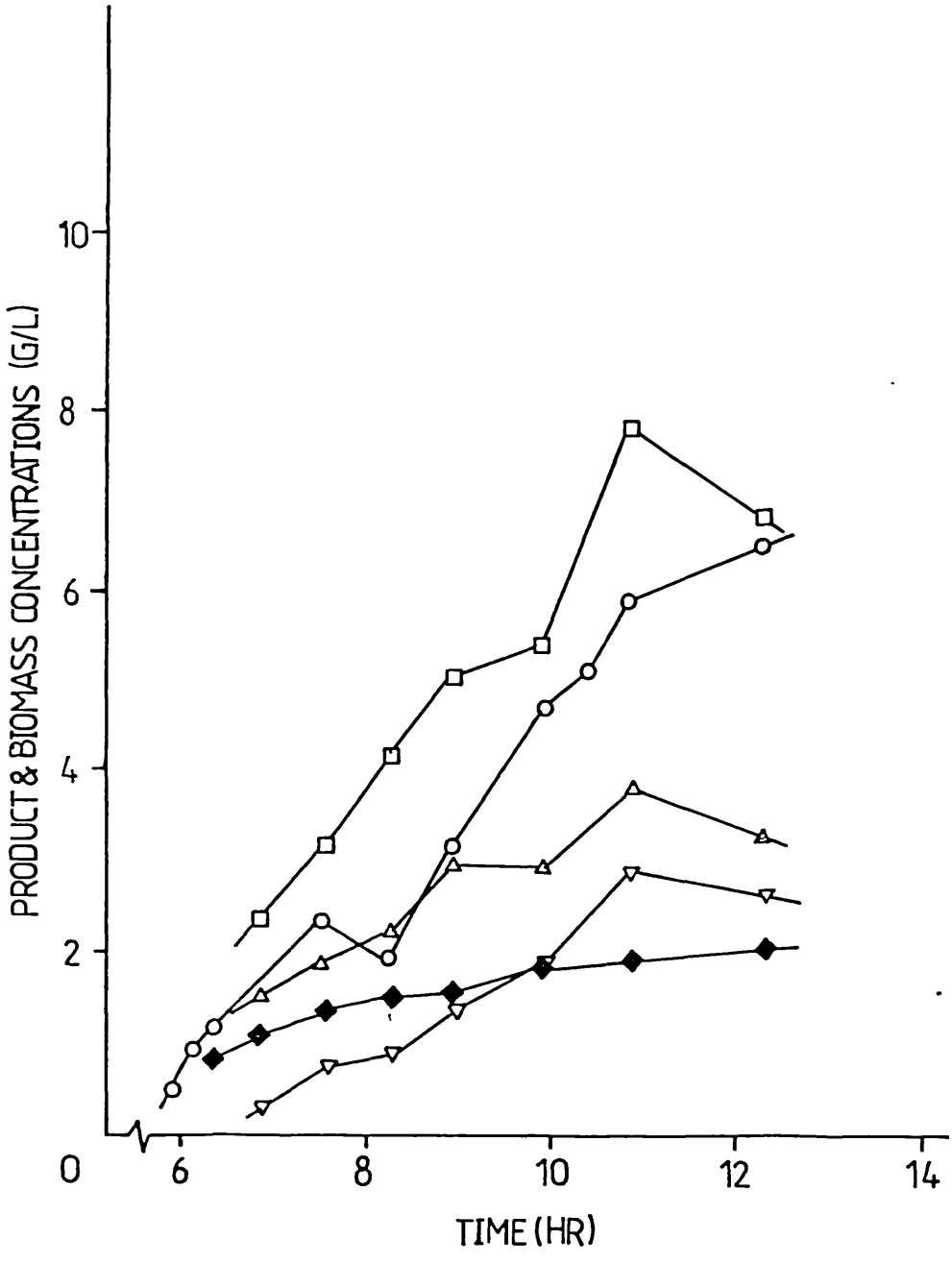


Figure A2.11 Product & Biomass Concentrations against Time

Experiment 171 (So = 23.5 g/1, T = 60°C, pH 7.0)

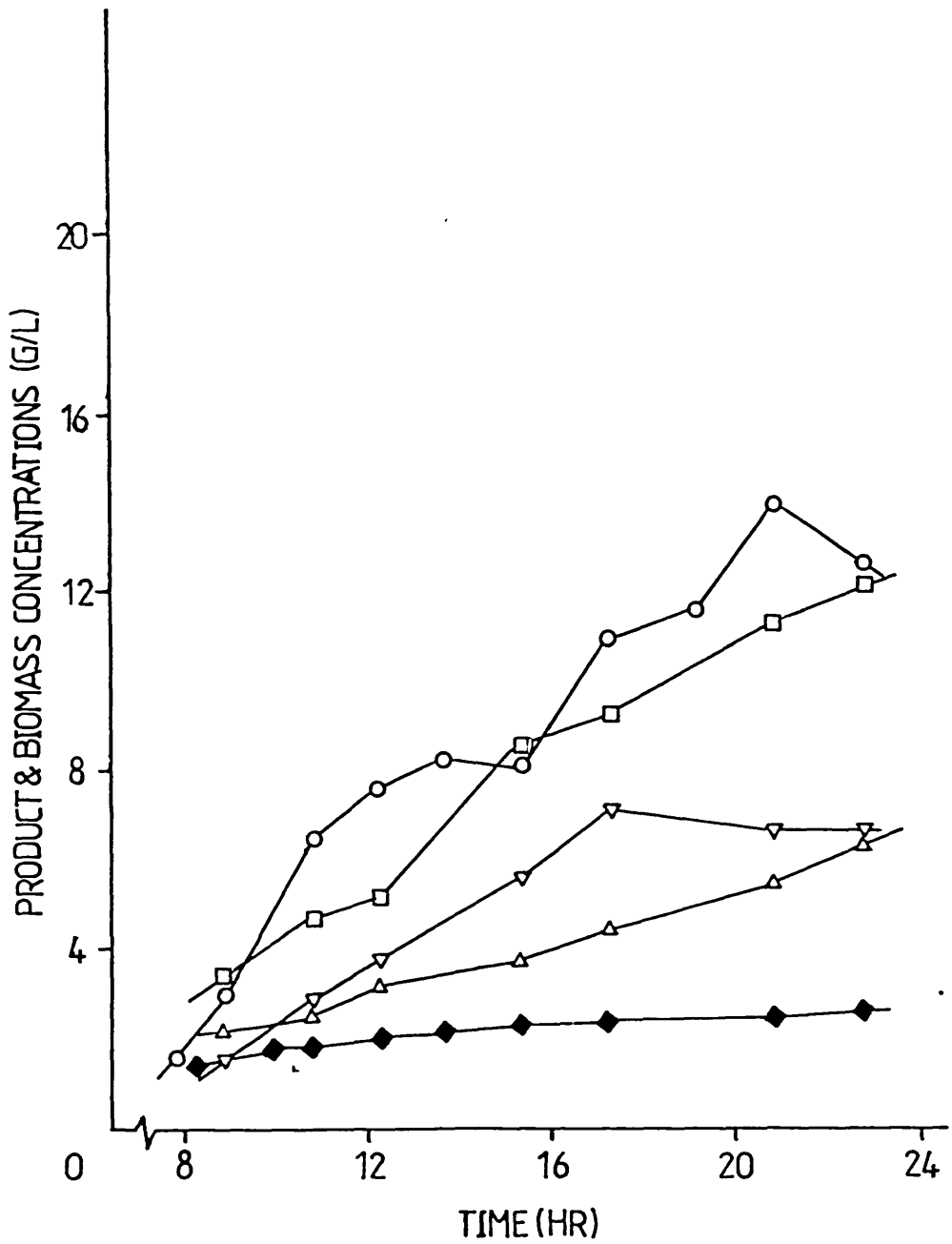


Figure A2.12 Product & Biomass Concentrations against Time
Experiment 172 (So = 50.0 g/l, T = 60°C, pH 7.0)

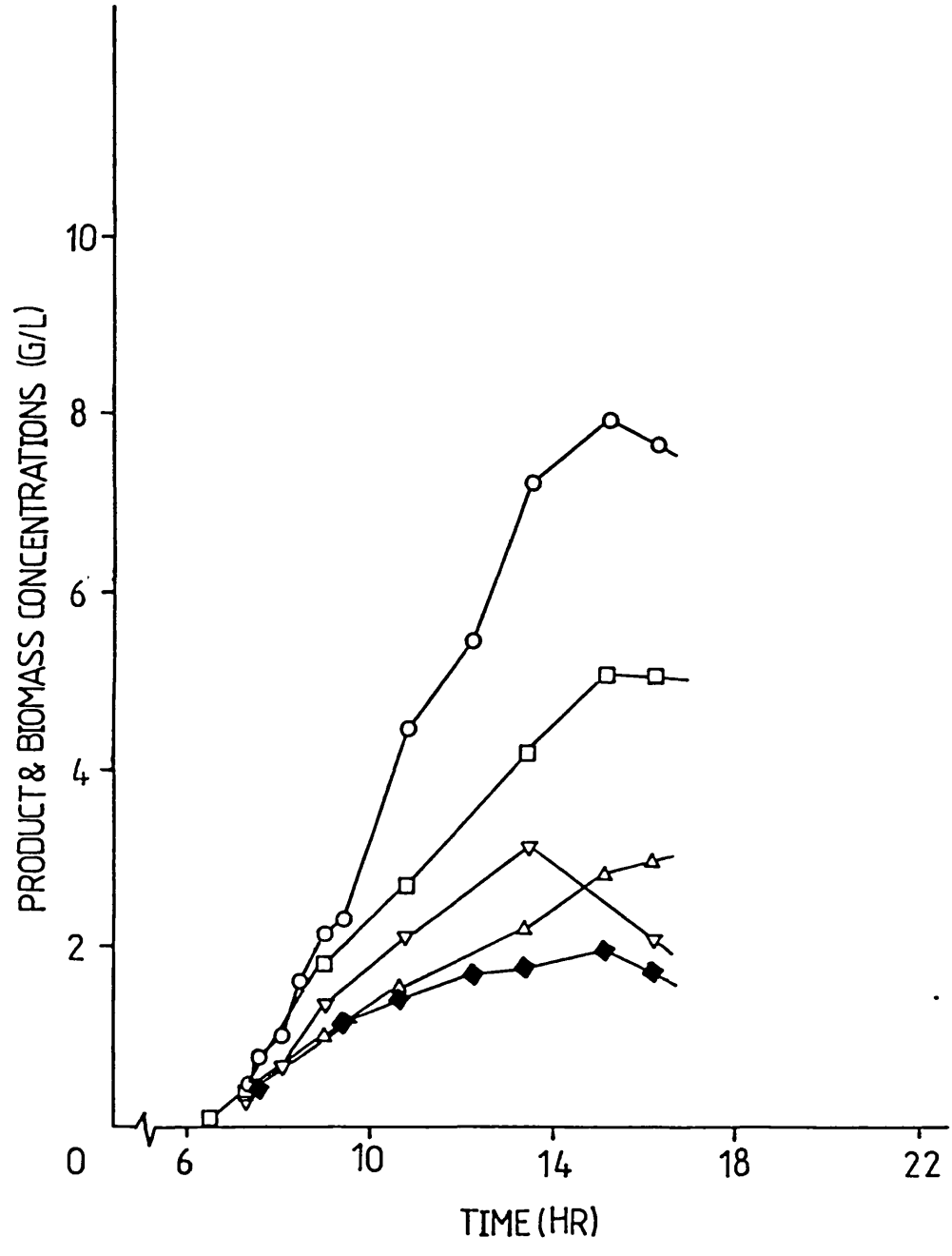


Figure A2.13 Product & Biomass Concentrations against Time
Experiment 174 (So = 23.5 g/l, T = 60°C, pH 6.6)

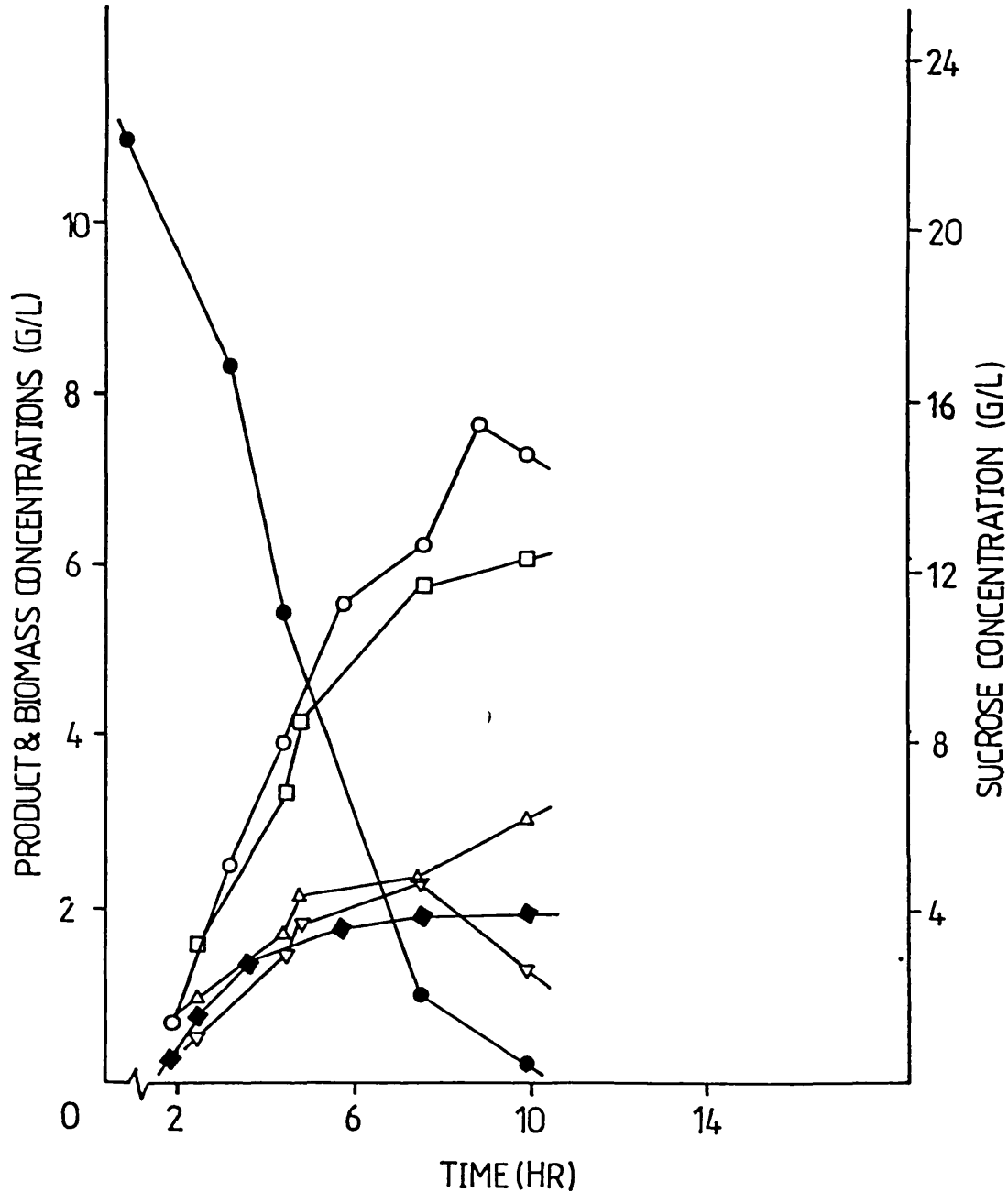


Figure A2.14 Product & Biomass Concentrations against Time
Experiment 176 (So = 23.5 g/l, T = 60°C, pH 7.0)

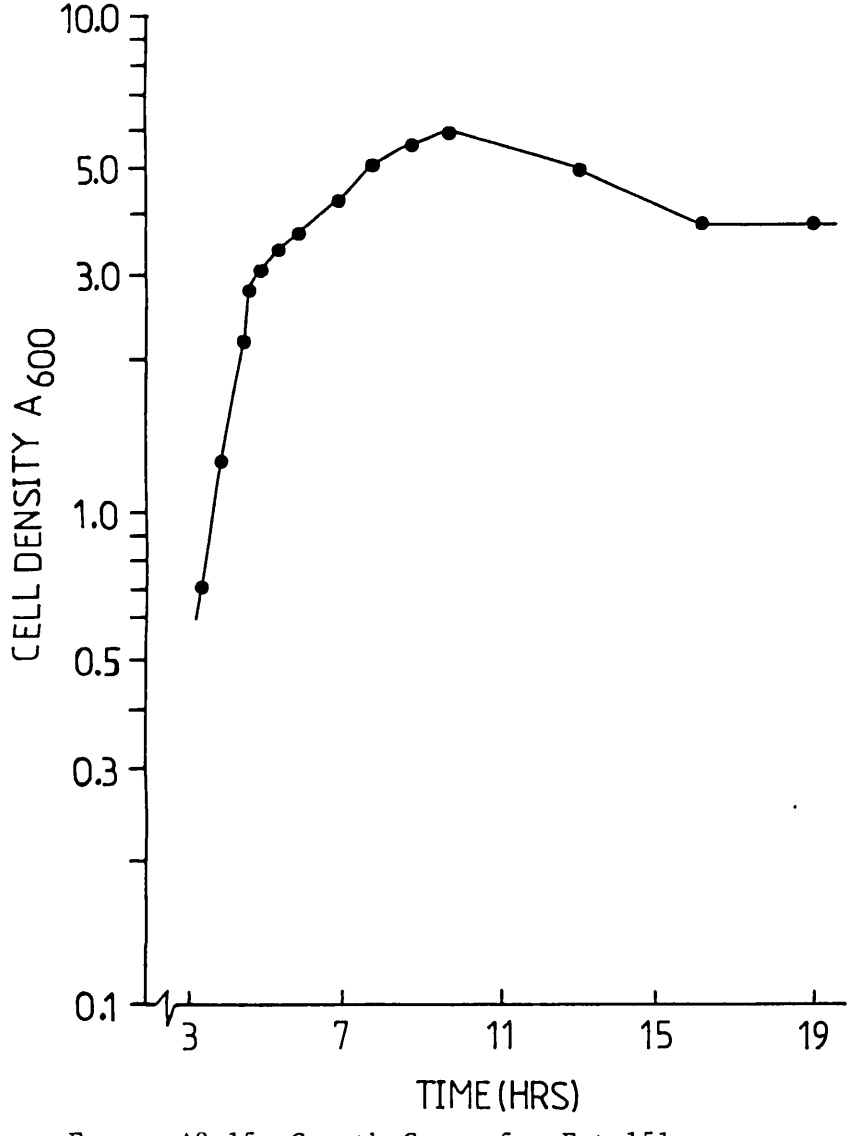


Figure A2.15 Growth Curve for Ext.151
(So = 23.5 g/1, T = 60°C, pH 7.0)

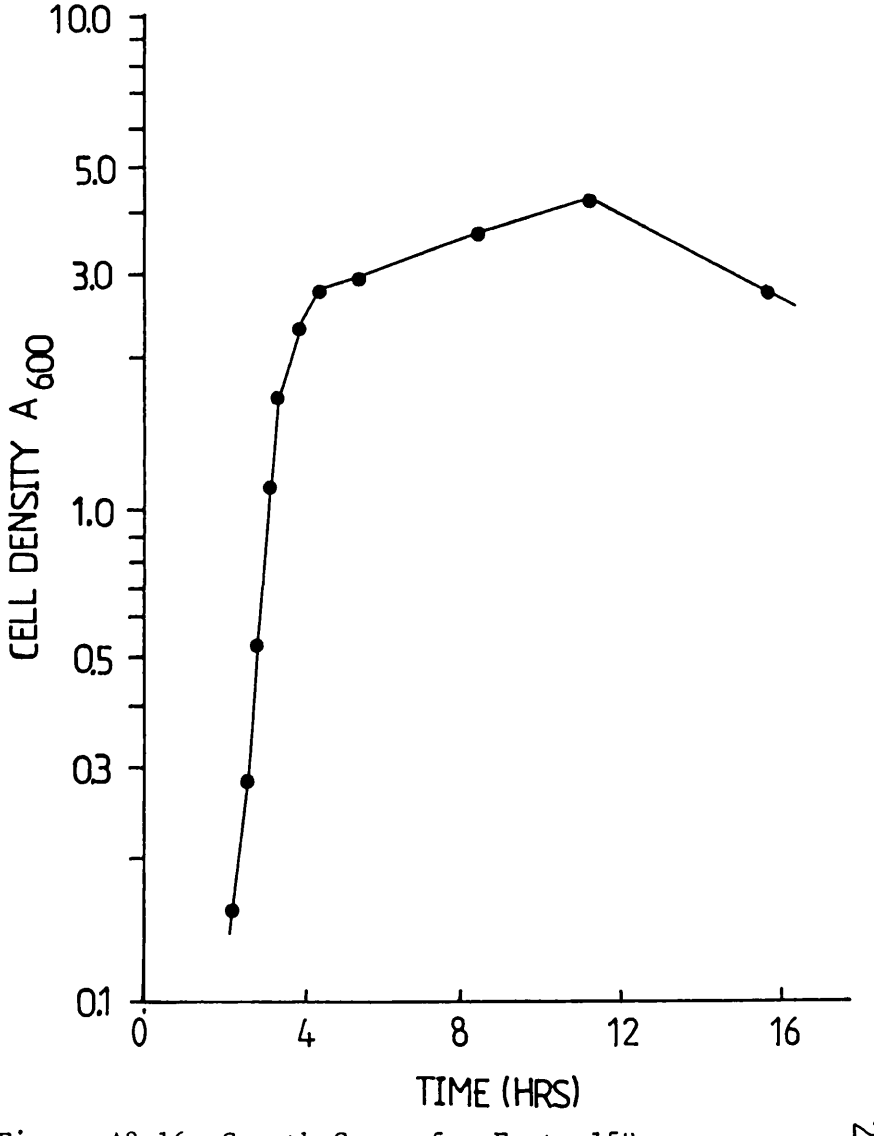


Figure A2.16 Growth Curve for Expt. 152
(So = 23.5 g/1, T = 60°C, pH 6.2)

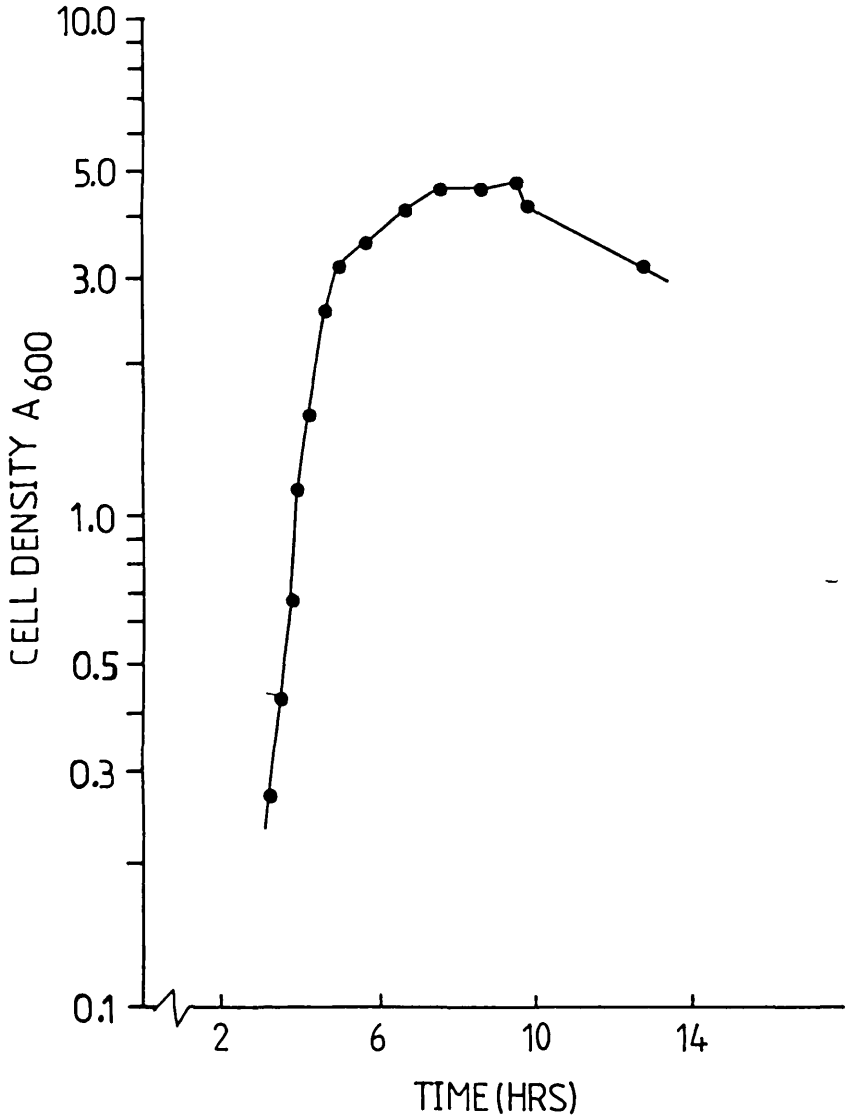


Figure A2.17 Growth Curve for Expt. 153
(So = 23.5g/l, T = 60°C, pH 8.0)

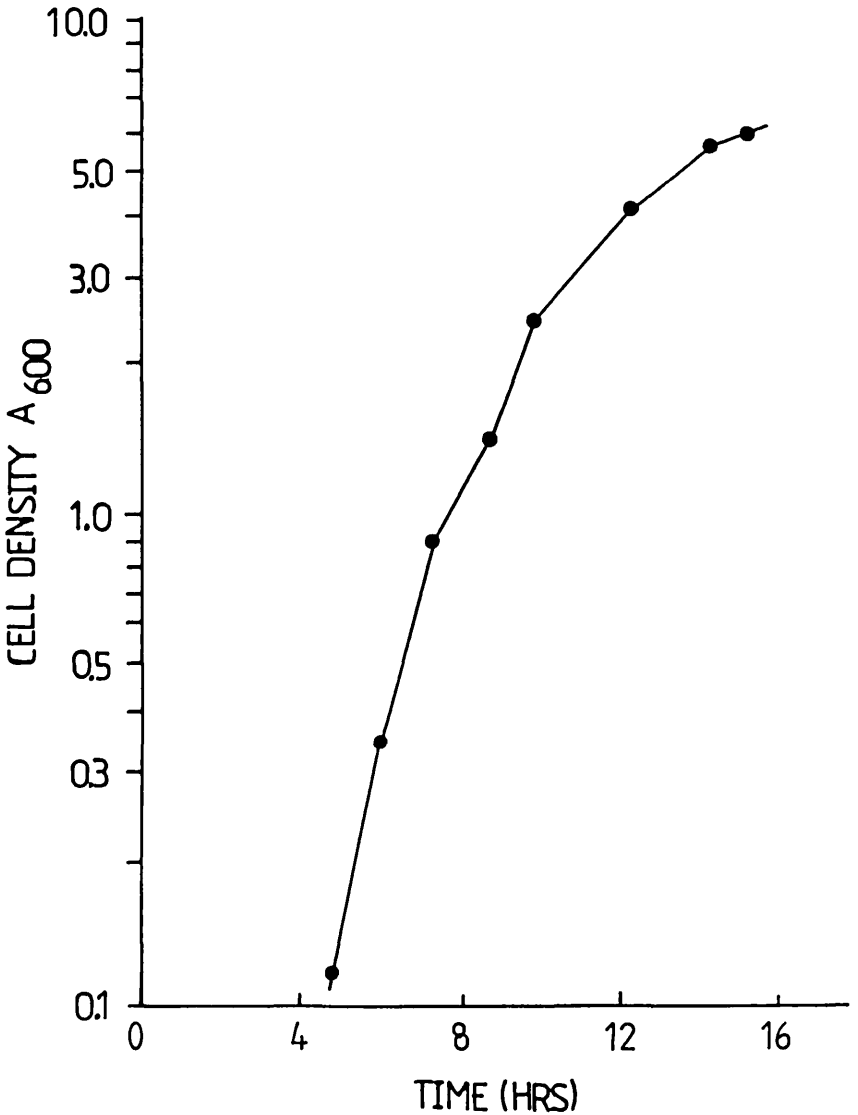


Figure A2.18 Growth Curve for Expt.154
(So = 23.5g/l, T = 50°C, pH 8.0).

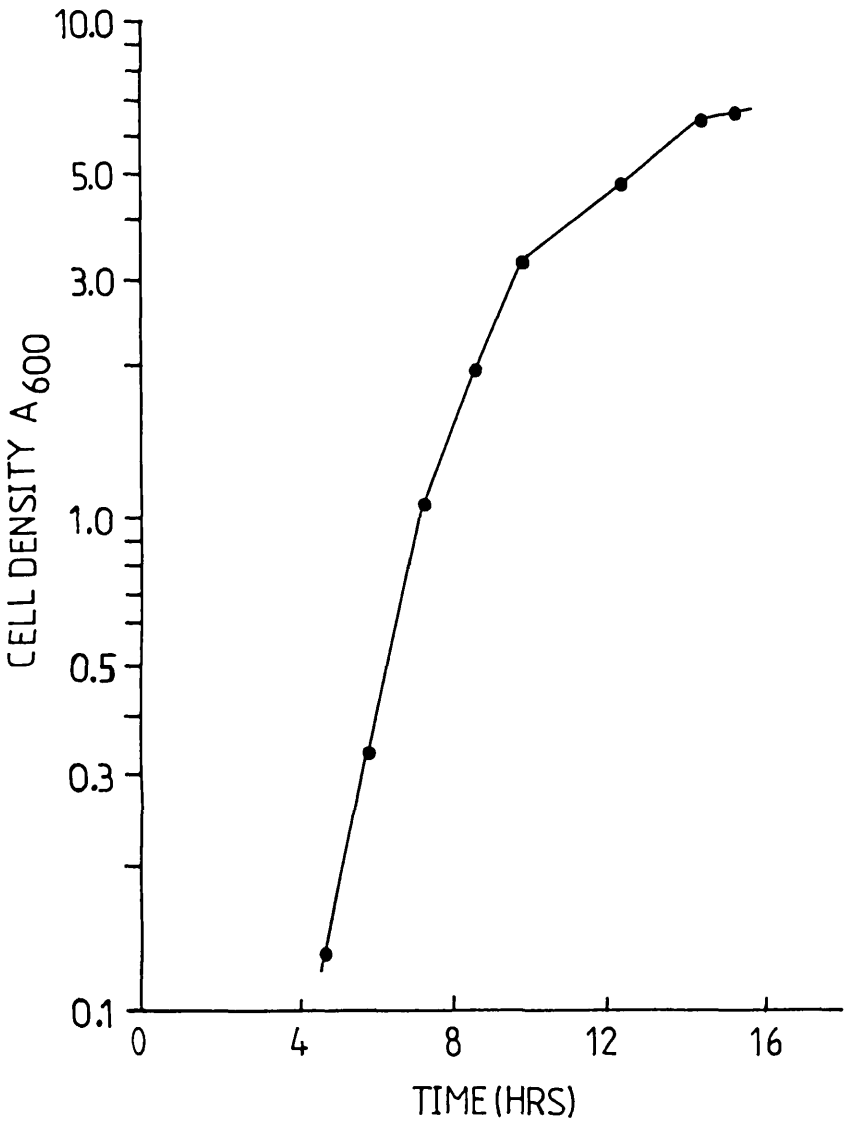


Figure A2.19 Growth Curve for Expt. 156
(So = 23.5g/1, T = 50°C, pH 7.0)

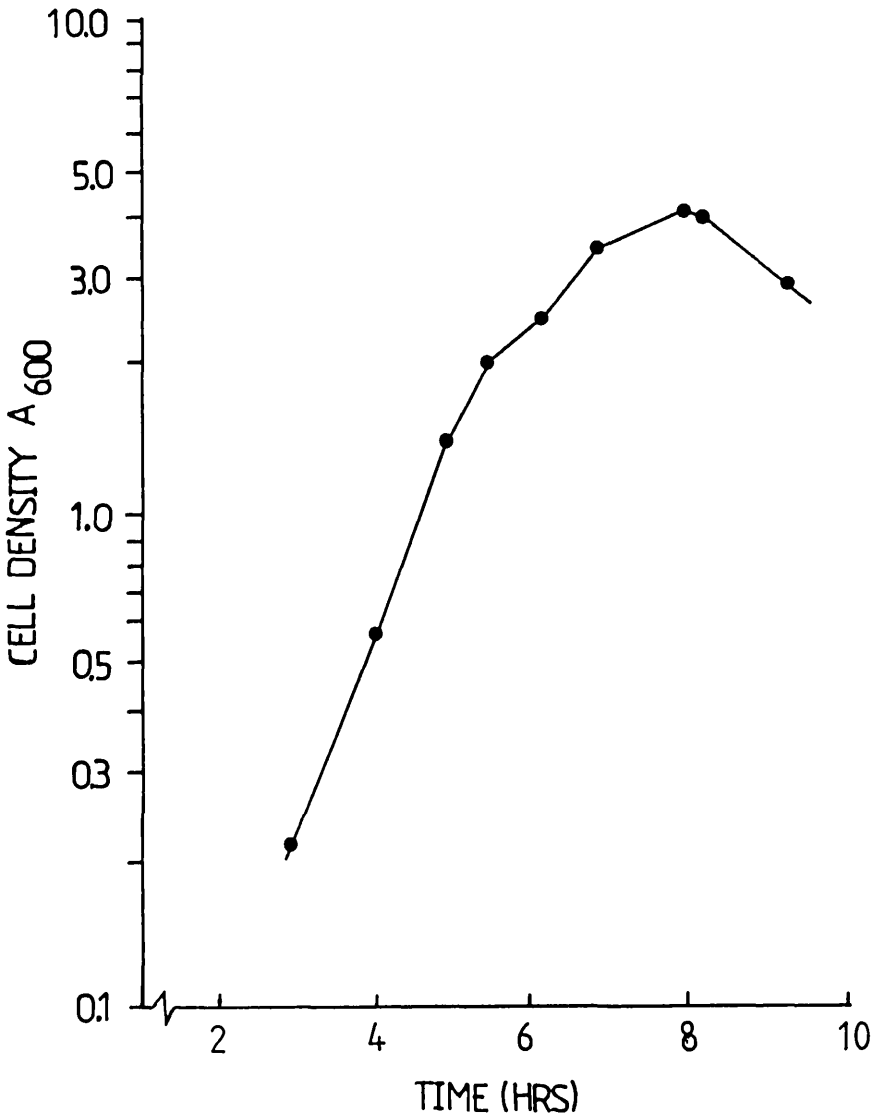


Figure A2.20 Growth Curve for Expt. 159
(So = 23.5g/1, T = 70°C, pH 7.7)

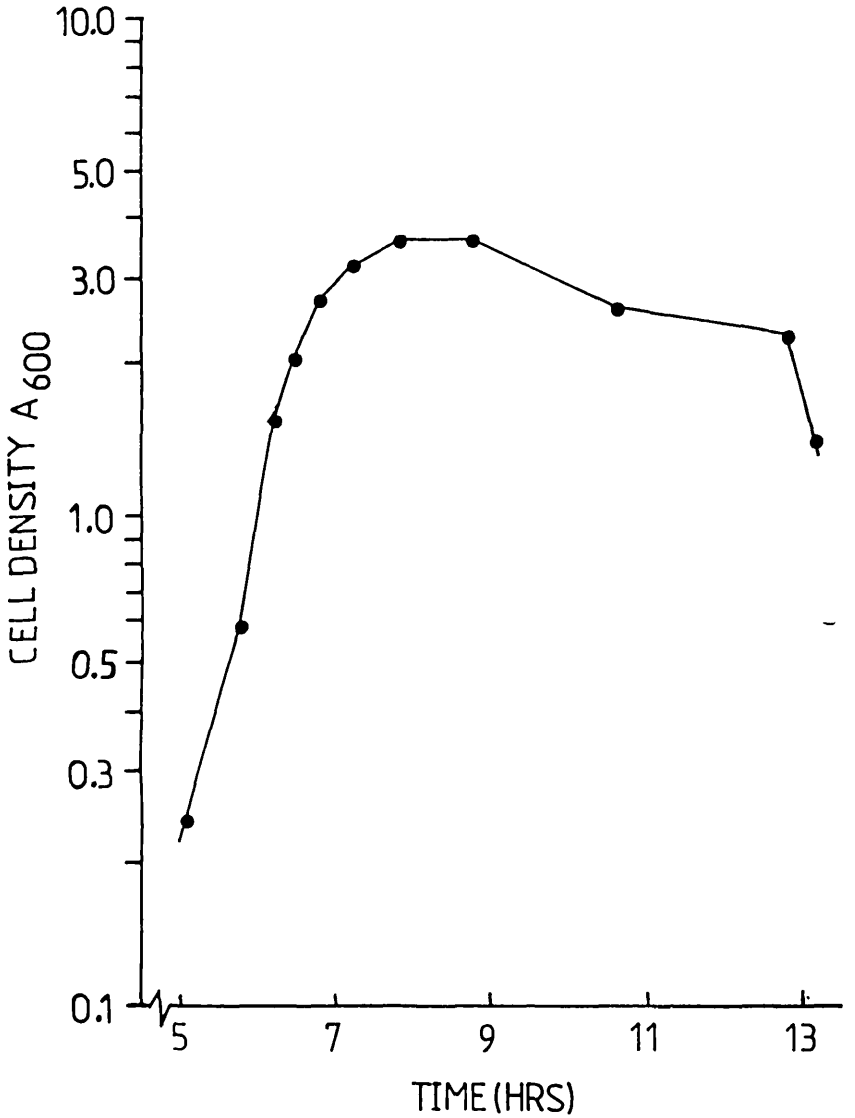


Figure A2.21 Growth Curve for Expt. 160
(So = 23.5g/l, T = 70°C, pH 7.0)

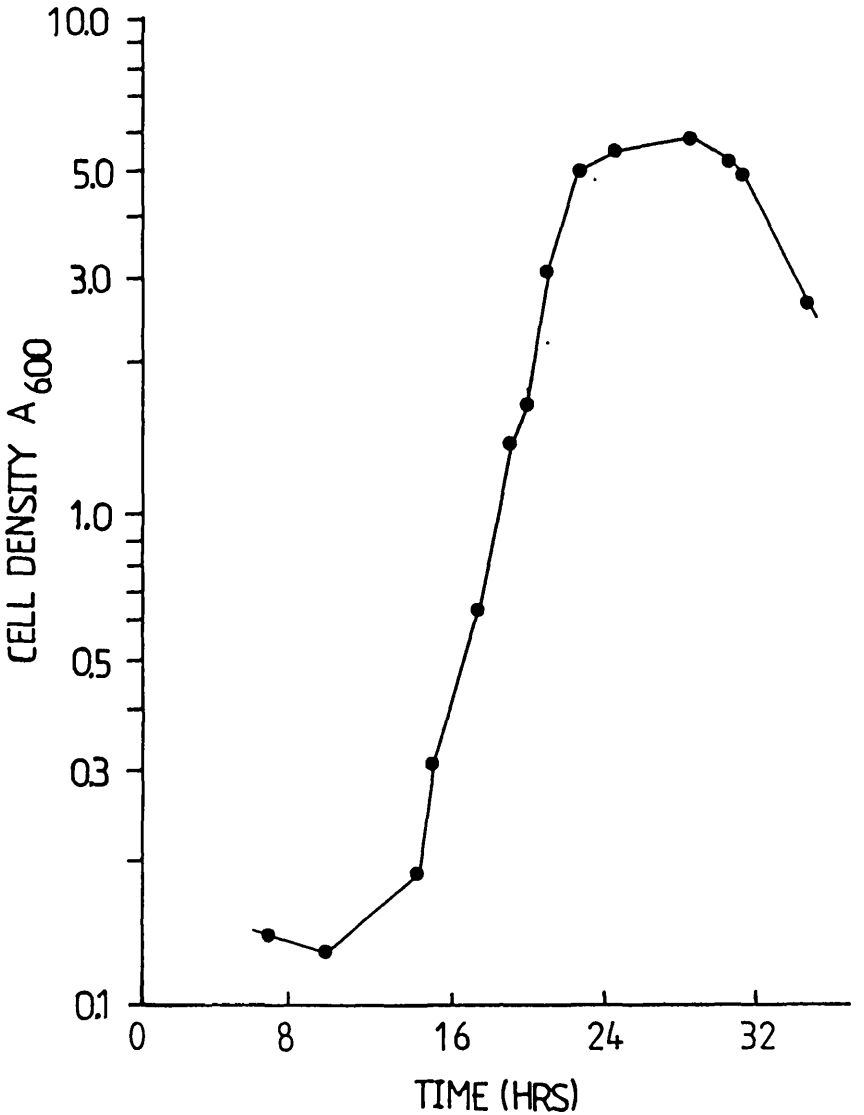


Figure A2.22 Growth Curve for Expt. 161
(So = 100g/l, T = 60°C, pH 7.0)

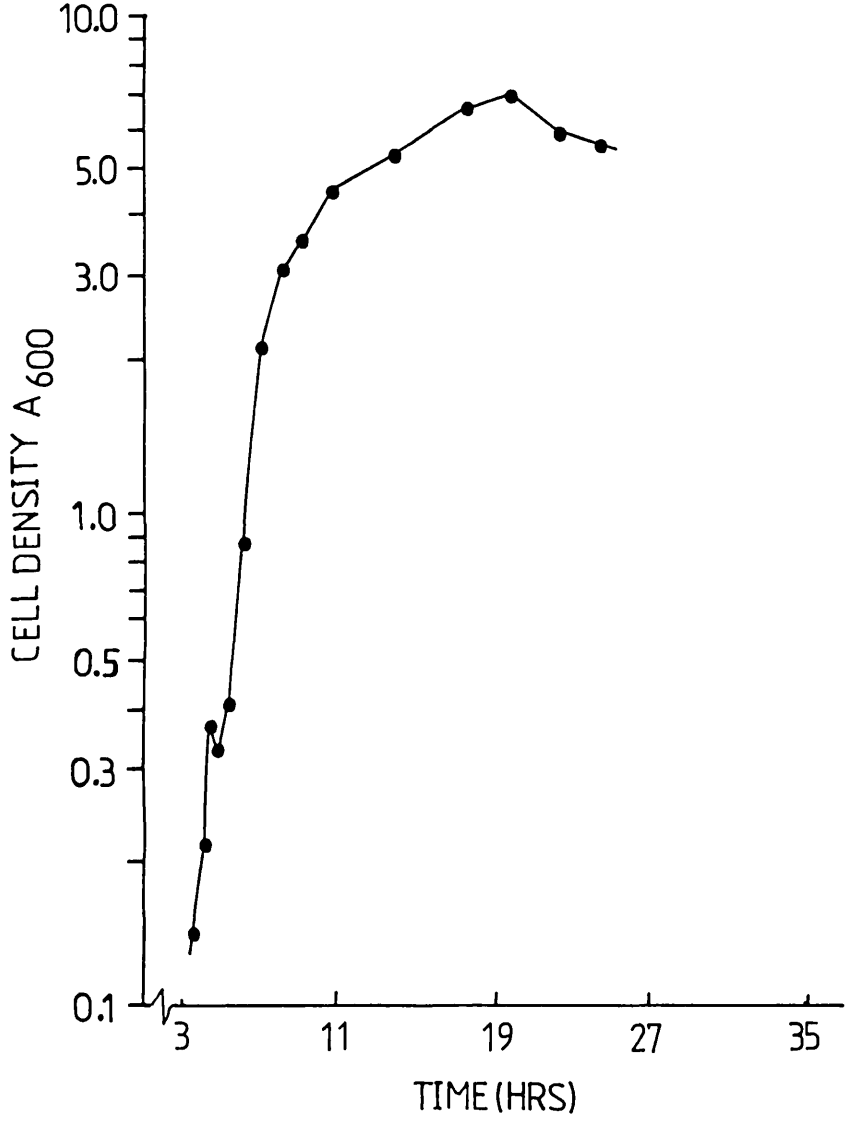


Figure A2.23 Growth Curve for Expt. 162
(So = 50g/1, T = 60°C, pH 7.0)

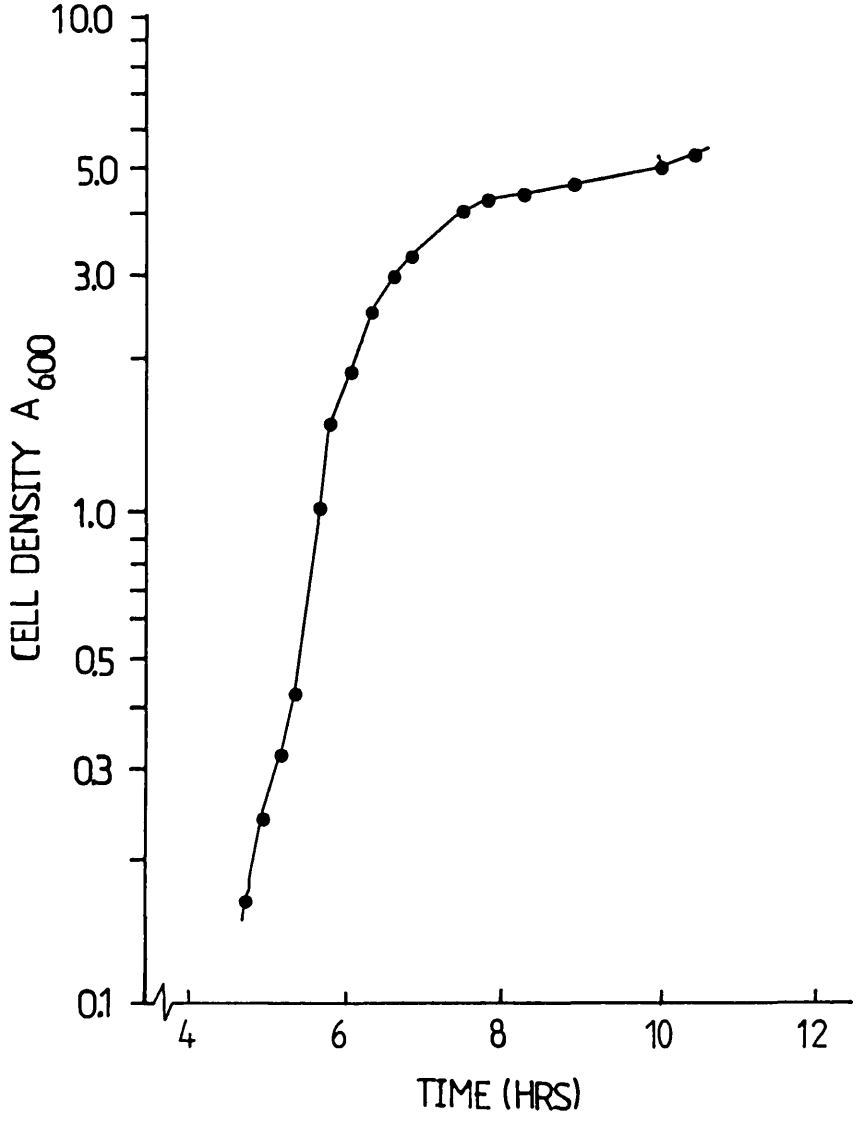


Figure A2.24 Growth Curve for Expt. 171
(So = 23.5g/1, T = 60°C, pH 7.0)

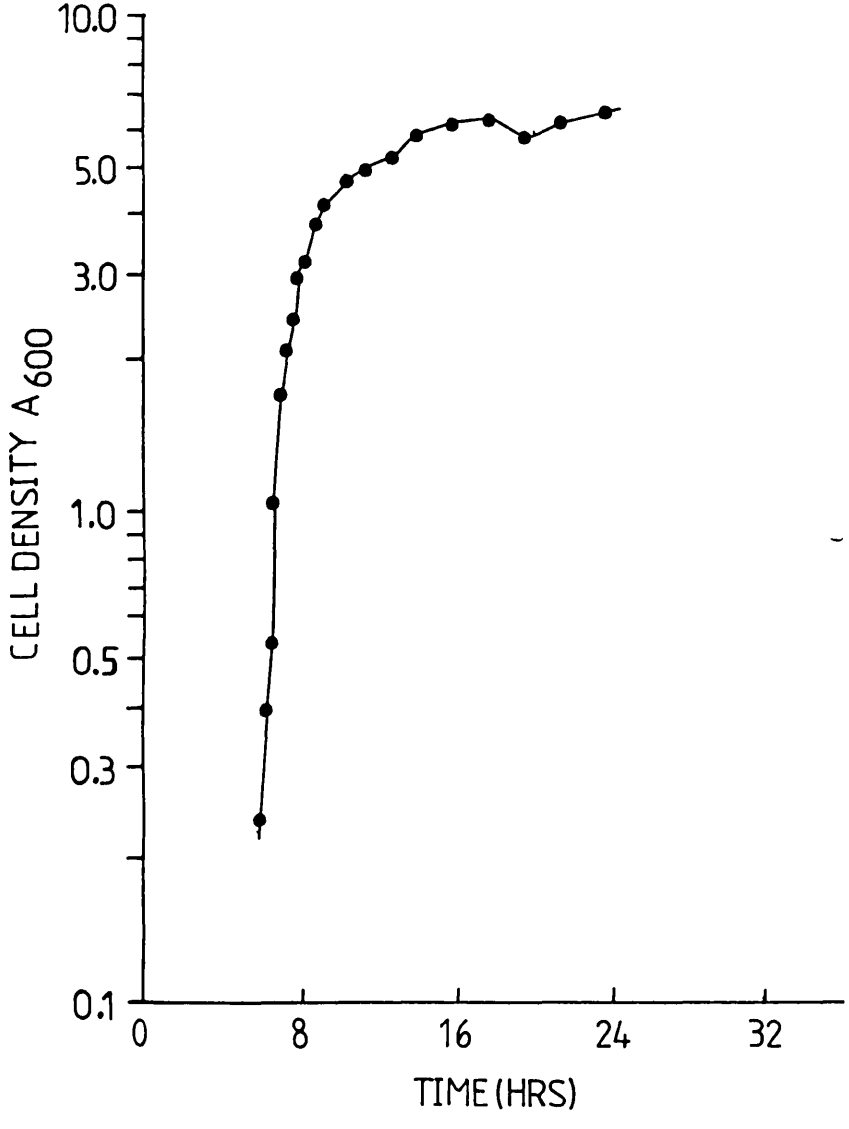


Figure A2.25 Growth Curve for Expt. 172
(So = 50g/1, T = 60°C, pH 7.0)

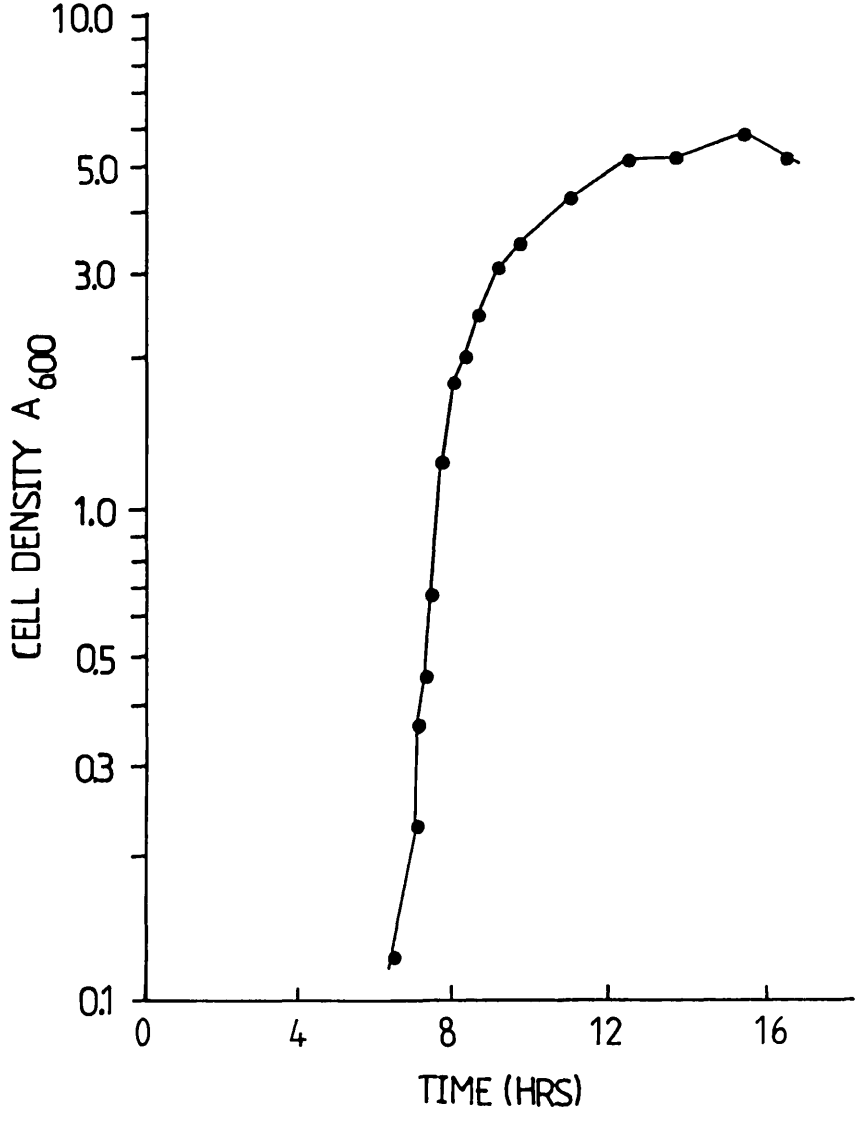


Figure A2.26 Growth Curve for Expt. 174
(So = 23.5g/1, T = 60°C, pH 6.6)

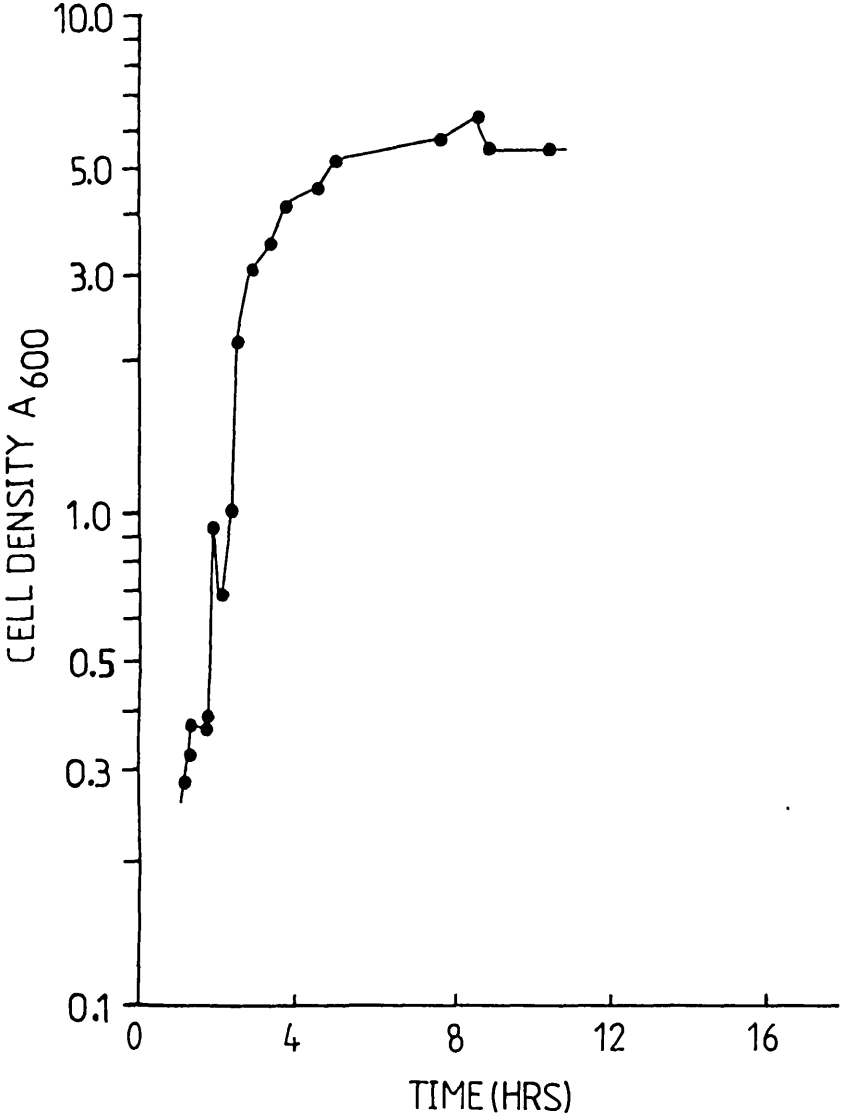


Figure A2.27 Growth Curve for Expt. 176
(So = 23.5g/1, T = 60°C, pH 7.0)

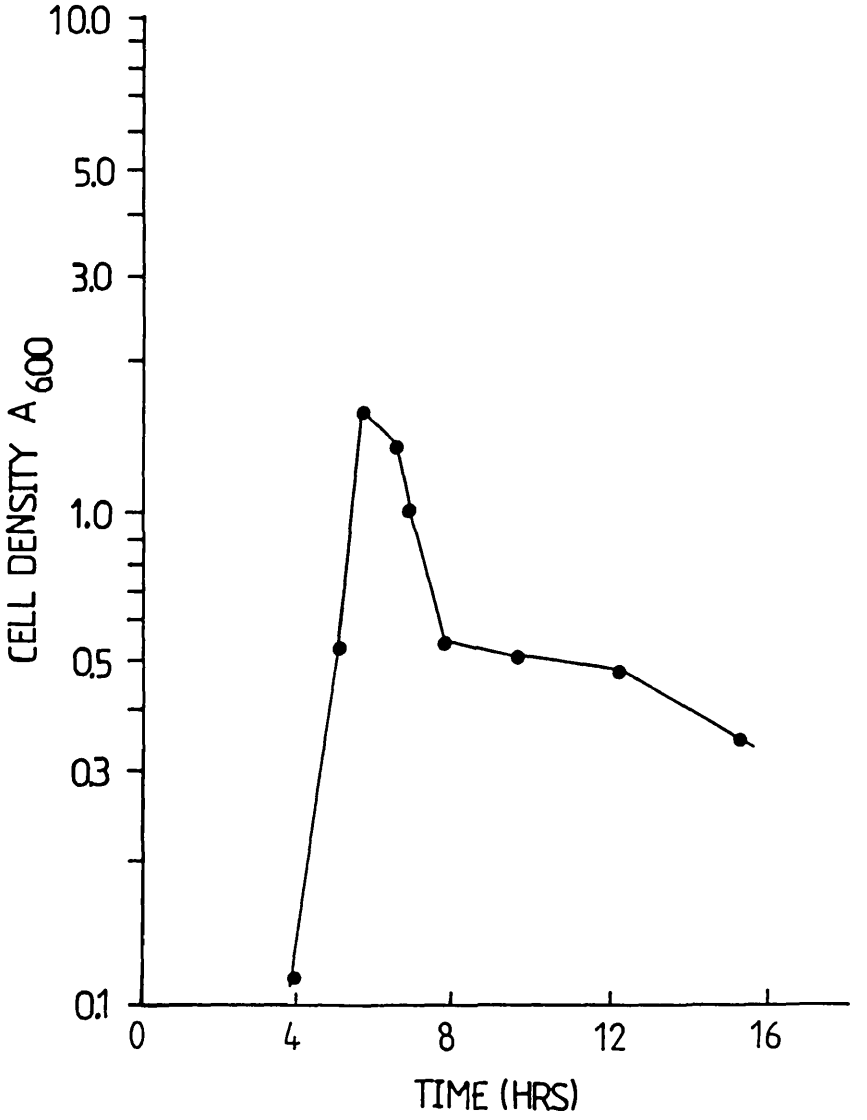


Figure A2.28 Growth Curve for Expt. 169
(So = 23.5g/1, T = 60°C, pH 5.0)

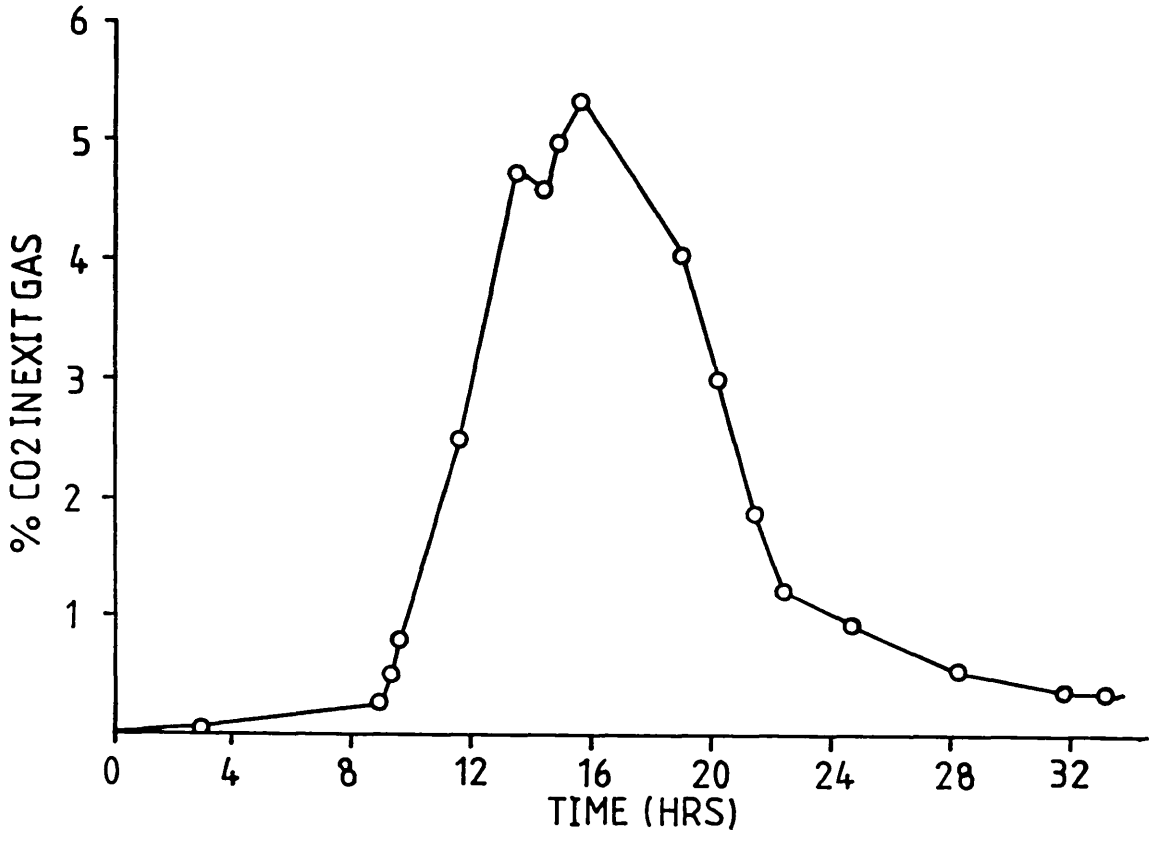


Figure A2.29 CO_2 Evolution against Time in Experiment 239

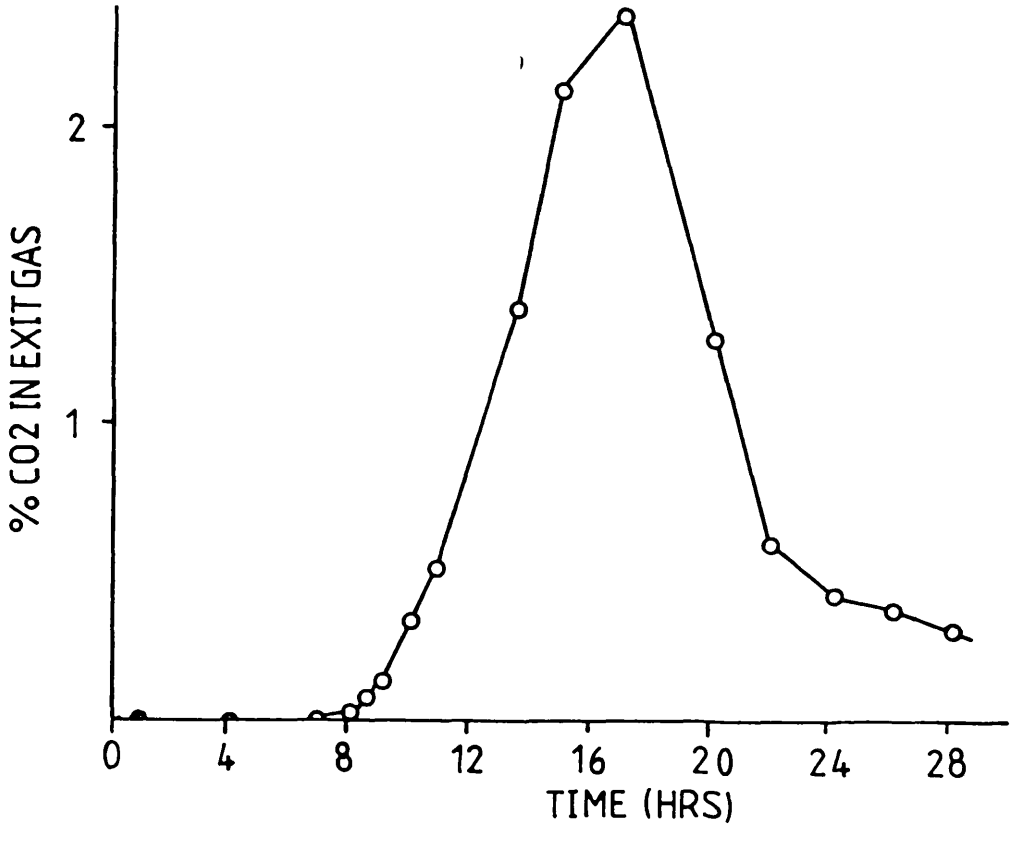


Figure A2.30 CO_2 Evolution against Time in Experiment 240

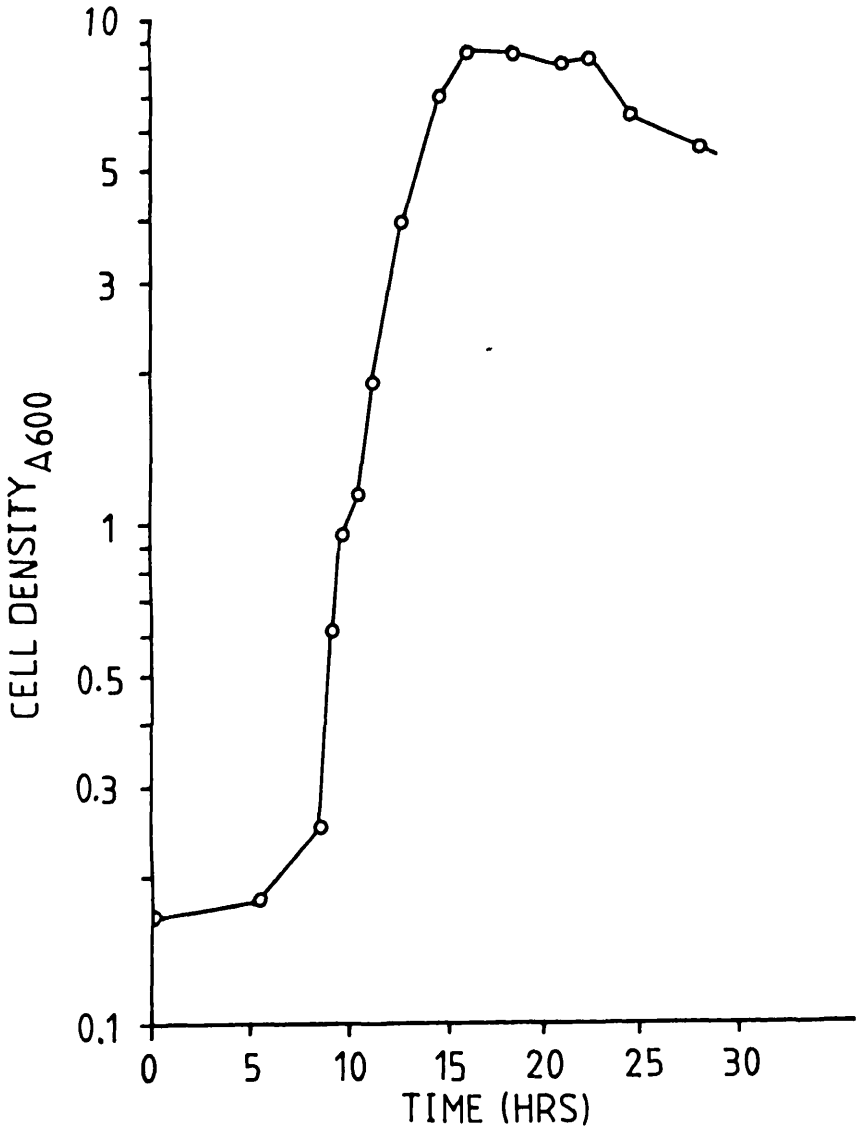


Figure A2.31 Growth Curve for Experiment 239

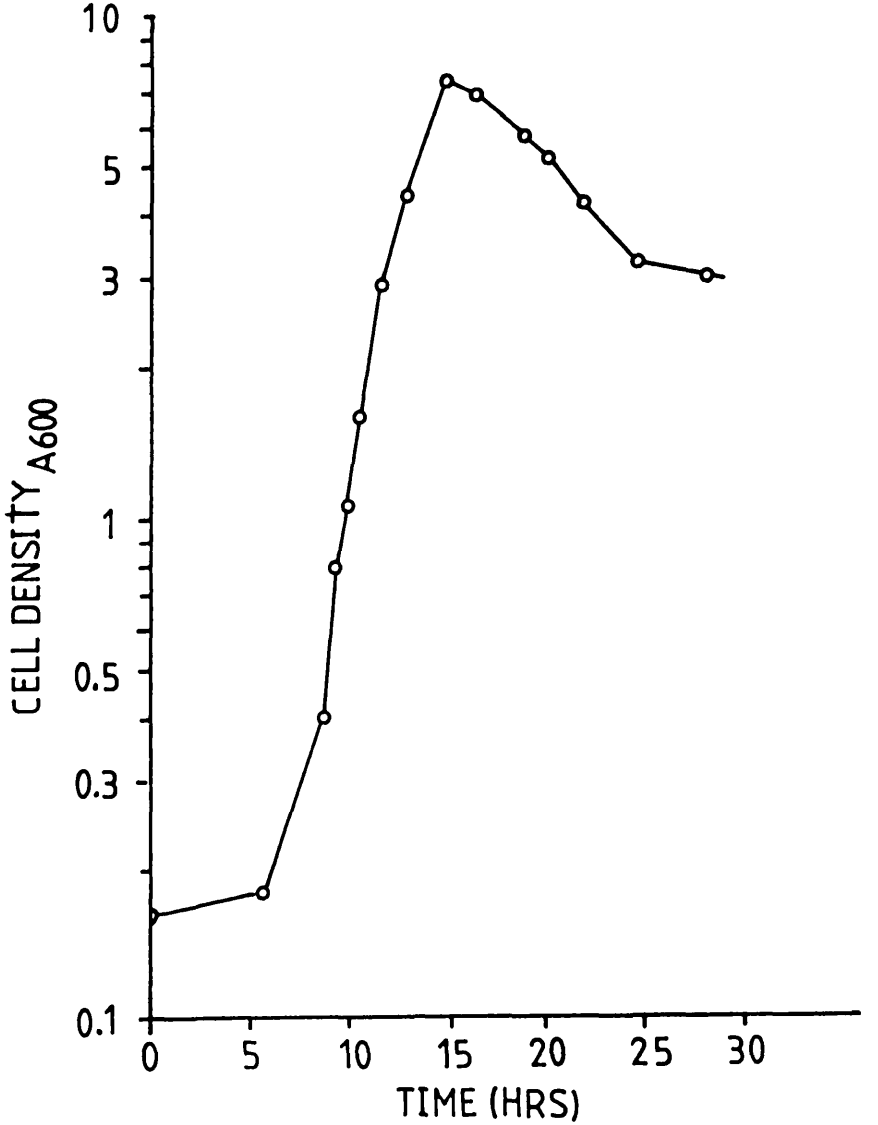


Figure A2.32 Growth Curve for Experiment 240

Appendix 3 Continuous Culture Data

A3.1 Potassium Hydroxide Consumption in Experiment 183 (with strain NCA 1503)

Actual and predicted potassium hydroxide consumption during experiment 183 is shown in Table A3.1. The latter values are the sums of the quantities of potassium hydroxide required to neutralise the acidic fermentation products detected by GC plus the quantities of potassium hydroxide required to alter the pH of the medium from its storage value (6.0) to the values shown in the Table. These were obtained by titrating potassium hydroxide (10% w/v) against a known volume of fresh BST medium.

pH	KOH Requirement		Ratio 2/1
	1 Predicted	2 Predicted	
7.0	67.1	62.8	0.94
6.6	45.3	39.9	0.88
6.35	35.9	24.7	0.69
8.0	84.1	59.4	0.71

Table A3.1 Predicted and Observed Potassium Hydroxide Requirements (mM/hr)

A3.2 Data for Experiments 241 (Figures A3.1 and A3.2), 242 (Figures A3.3 and A3.4) and 244 (Figures A3.5 to A3.7)

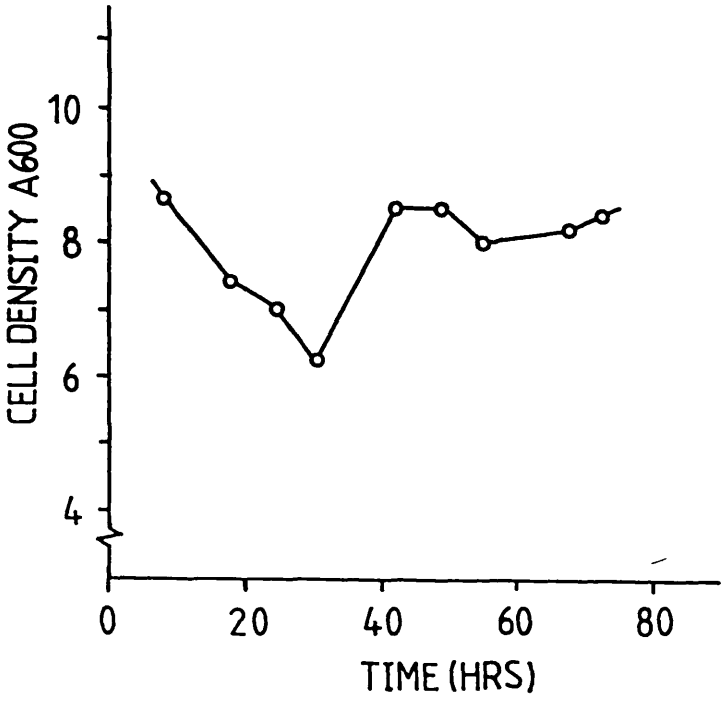


Figure A3.1 Cell Density against Time for Experiment 241

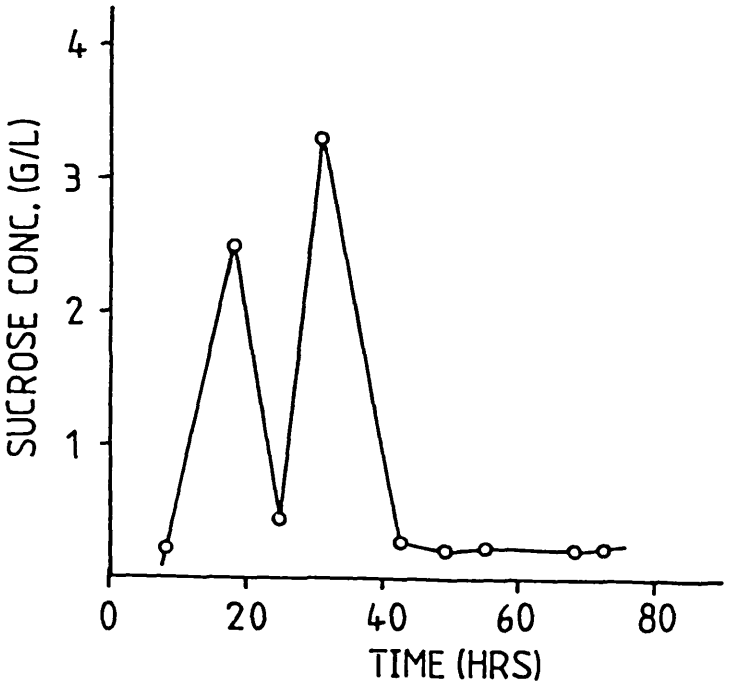


Figure A3.2 Sucrose Concentration against Time for Experiment 241

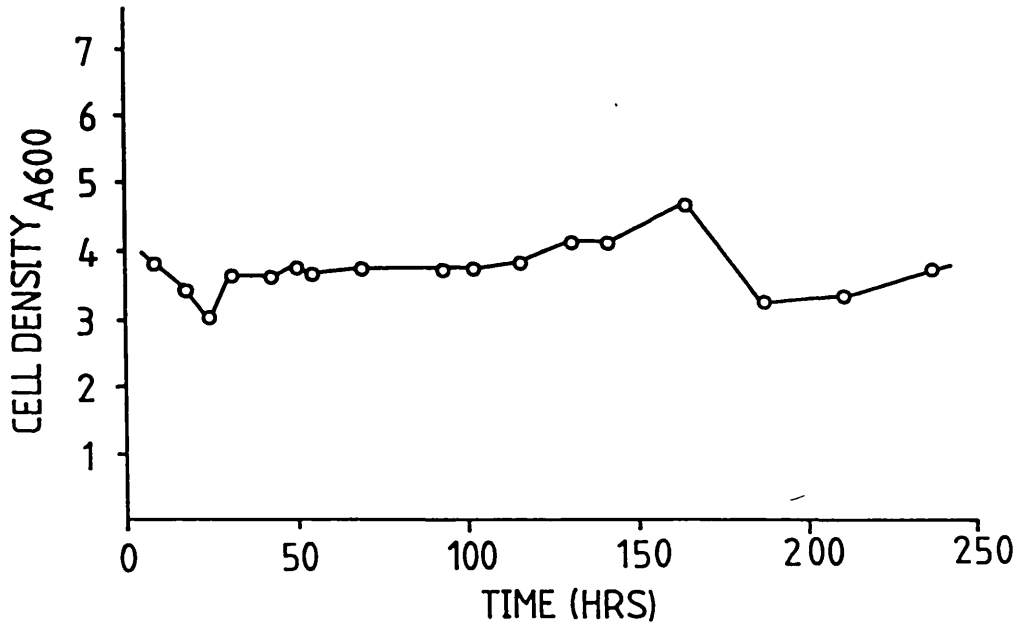


Figure A3.3 Cell Density against Time for Experiment 242

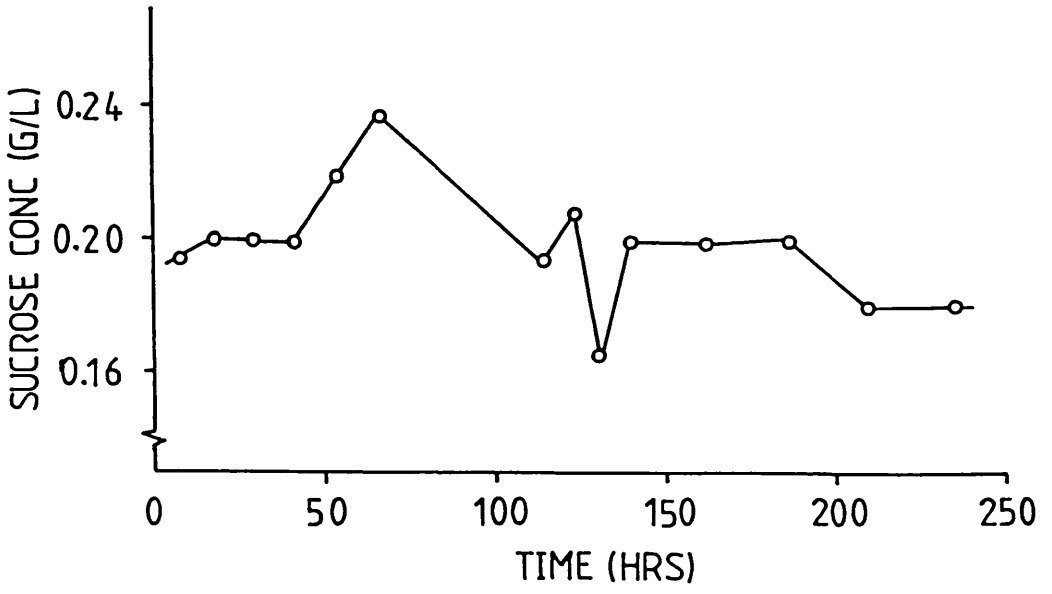


Figure A3.4 Sucrose Concentration against Time for Experiment 242

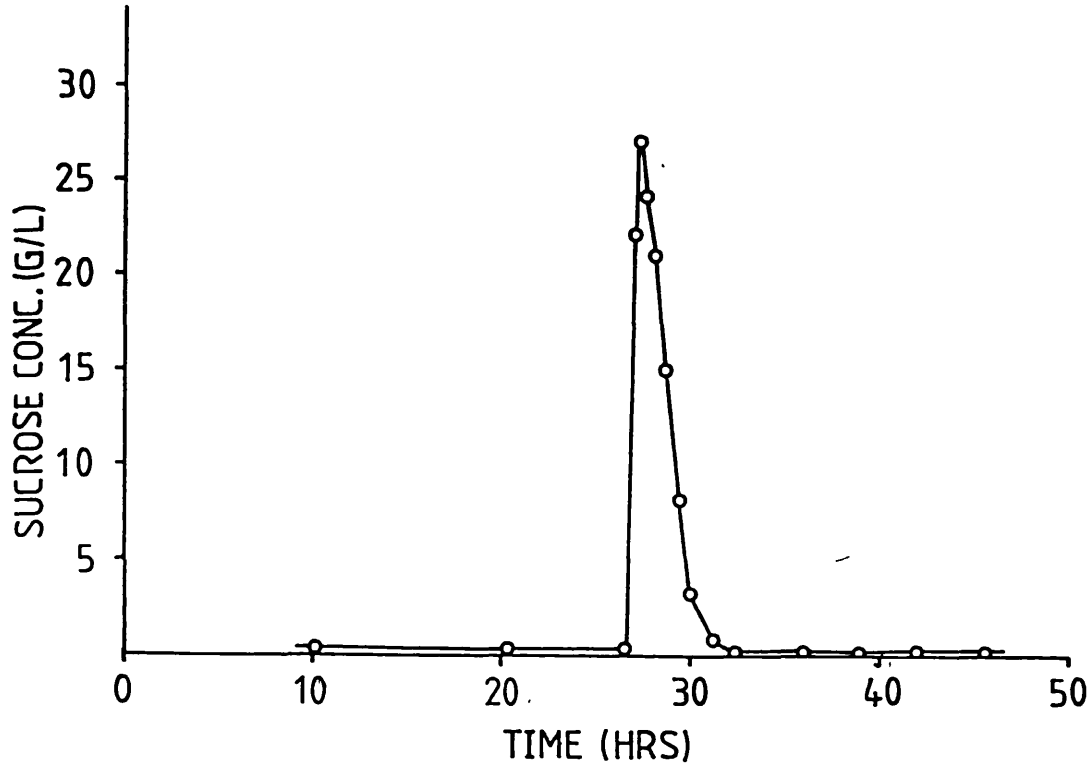


Figure A3.5 Sucrose Concentration against Time for Experiment 244

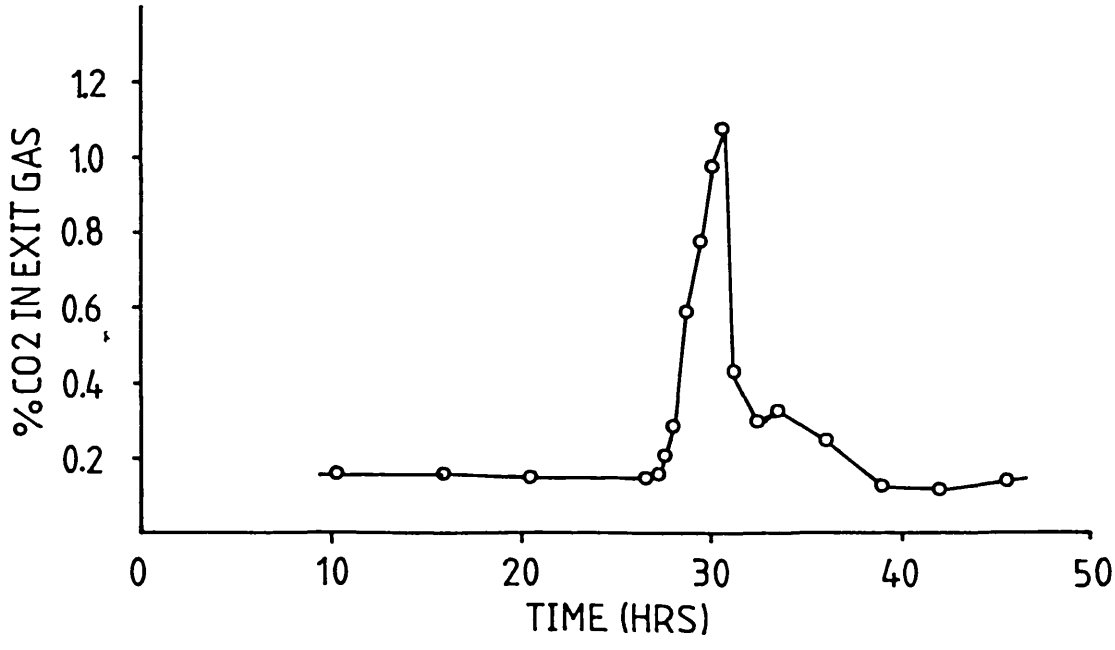


Figure A3.6 Gas Phase Carbon Dioxide Level against Time for Experiment 244

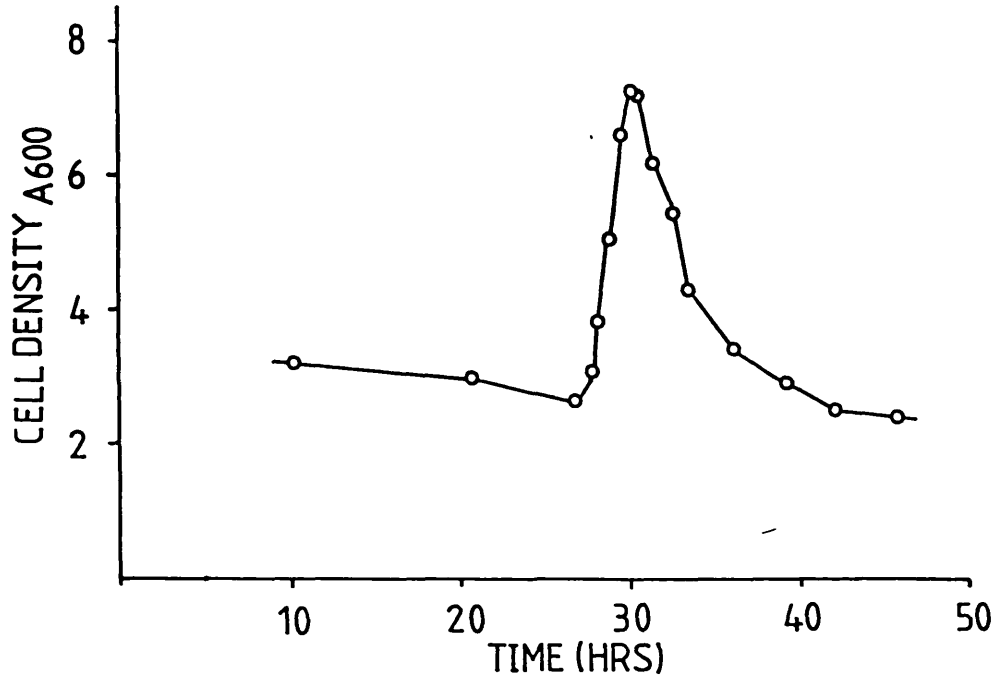


Figure A3.7 Cell Density against Time for Experiment 244

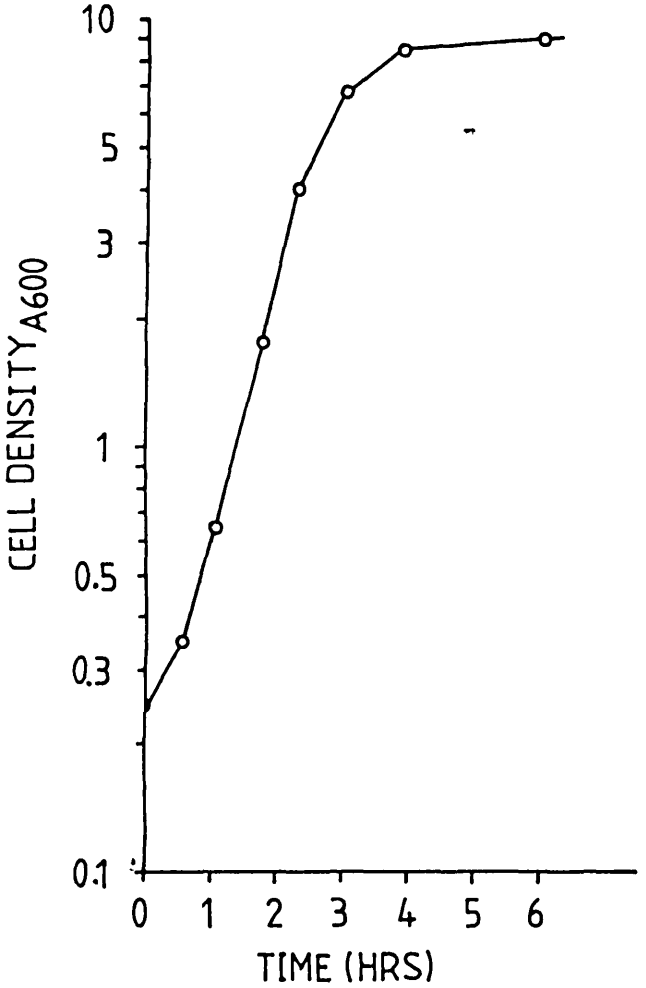


Figure A3.8 Growth Curve for Strain 11d-15 in Expt. 250

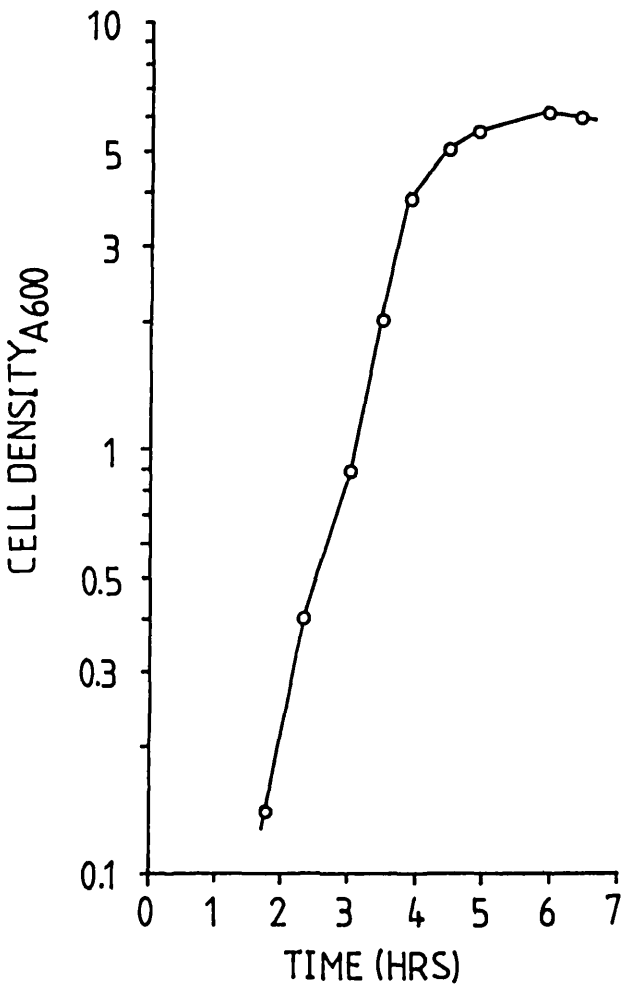


Figure A3.9 Growth Curve for Strain NCA 1503 in Expt. 251

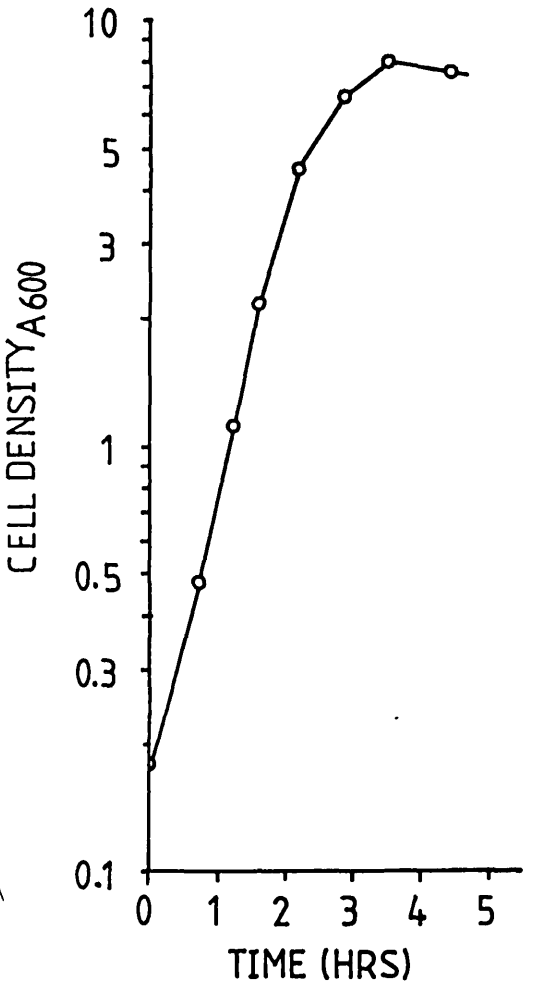


Figure A3.10 Growth Curve for Revertant in Expt. 256

A3.3 Growth Curves for 11d-15, NCA 1503 and Revertant Strains

Organism	Operation	Substrate Initial Conc. (g/l)	Temp. C	Specific Ethanol Productivity	Reference
S. carlsbergensis ATCC 26602	Batch	Glucose 250	30	0.87	Rogers et al. (1979)
S. cerevisiae ATCC 4126	Batch	Glucose 10.0	30	0.06	Bazua and Wilke (1977)
S. cerevisiae ATCC 4126	Continuous	Glucose 89.0	35	0.42	Cysewski and Wilke (1977)
S. cerevisiae H-1	Continuous	Glucose 108	30	0.87	Aiba et al. (1968)
S. cerevisiae NRRL Y-132	Continuous + Recycle	Glucose 150	30	0.49	Ghose and Tyagi (1979)
S. carlsbergensis ATCC 26602	Continuous + Recycle	Glucose 200	30	0.31	Del Rosario et al. (1979)
S. carlsbergensis	Batch	Sucrose 120	30	0.06	Navarro and Durand (1978)

Table A4.1 Specific Ethanol Productivities (g Ethanol/g dry wt cells/hr) of various Saccharomyces spp.

Strain	Operation	Substrate Initial Conc.(g/l)	Temp. °C	Specific Ethanol Productivity	Reference
ATCC 10988	Batch	Glucose 250	30	2.53	Lee et al. (1979a)
Z1	Batch	Sucrose 120	30	2.28	Lyness and Doelle (1981)
ZM4	Batch	Glucose 250	30	5.4	Rogers et al. (1982)
ZM4	Batch	Sucrose 250	30	4.6	Rogers et al. (1982)
ZM4	Continuous	Glucose 250	30	5.4	Lee et al. (1981)
ZM4	Continous + Recycle	Glucose 150	30	2.82	Rogers et al. (1982)

Table A4.2 Specific Ethanol Productivities (g ethanol/g dry wt cells/hr) of Various Z. mobilis strains.

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