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The acid hydrolysis of paper to fermentable sugars and the subsequent fermentation of the sugars to ethanol.

by

Judith Anderson

Submitted for the Degree of Master of Philosophy.

Date of submission 1·4·81
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ABSTRACT

The process of hydrolysing the cellulose content of various waste materials to sugars was first utilised in Germany prior to World War 11 to counteract the industrial ethanol shortage which existed during that period. Now as the anticipated shortage of vital energy-producing fossil sources becomes more apparent, cellulose is once again recognised as an important potential source.

A bench scale rig has been designed to effect the chemical hydrolysis of cellulose at high temperature ($230^{o}C$) and pressure ($3.4x10^{6}$ Pa) with 1-2% sulphuric acid as a catalyst. The rate of conversion to sugars is a function of temperature and pH, and a screening and optimisation exercise of these variables has been undertaken. The yields of sugars achieved ranged from 0.4% to 30% conversion (glucose equivalent).

The sugars formed were collected, neutralised and used for fermentation tests. These were carried out using Saccharomyces cerevisiae and certain bacteria in pure and two-member batch and continuous cultures to produce ethanol. Analyses of yields, specific growth and production rates and concentrations were made, with which knowledge, comparison could be made between the

performances of the various organisms on the hydrolysis sugars, under specified conditions. S. cerevisiae performed well in batch and continuous cultures and yields of ethanol near the theoretical maximum were achieved. Of the bacteria Aerobacter aerogenes gave a similar performance in continuous culture. The other two, Pseudomonas saccharophila and Bacillus polymyxa gave low yields and hindered the activities of the yeasts in two-member cultures.

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I am grateful for the technical help and advice received during the construction of the hydrolysis rig from Philip Payne and Roger Frith; and to Graham Jeffs for the chromatographical analyses.

My thanks also to David Marsland, Brunel University.

STATEMENT OF RESEARCH

This is to certify that the work described in this thesis is the result of the investigation by the candidate over the period January 1976 - September 1980 as a part-time student at the Open University. Neither the thesis or any part of it has been presented or is being concurrently submitted in candidature for any degree at any other university.

A paper consisting of a brief outline of procedure and initial results of this work was presented by the candidate and Dr. A. Porteous at the 1980 ISWA 3rd International Solid Wastes Congress, London.

Copy of this thesis may be deposited in the Open University Library and be made available for the purposes of study, and may be photocopied.

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This research programme is in two parts:

(a) the design of a bench scale rig to produce sugars by the continuous acid hydrolysis of waste cellulose at high temperatures and pressures.

(b) the biochemical utilisation of the sugars formed, in batch and continuous cultures by one or more micro-organisms for the production of ethanol.

PART I INTRODUCTION

CHAPTER 1

CELLULOSE: ITS STRUCTURE AND DEGRADATION

1.1 The Structure of Cellulose

Cellulose is our most abundant renewable resource; it is produced by trees and higher plants and is their major constitutent and chief structural element (bound up with lignin). It is a polymer with an empirical formula of $C_6H_{10}O_5$ and consists of inert, strong, insoluble fibres, which by X-ray diffraction reveal a definite crystalline structure.

At the molecular level cellulose is a linear unbranched polysaccharide consisting of long-chain molecules of D-glucopyranose (glucose) linked at the 1 and 4 carbon atoms by a β-glycosidic linkage (figure 1a). A glycosidic linkage occurs when the hydroxyl containing carbon of one monosaccharide is attached to a carbon of another monosaccharide (figure 1b).

Figure 1a Segment of the cellulose molecule

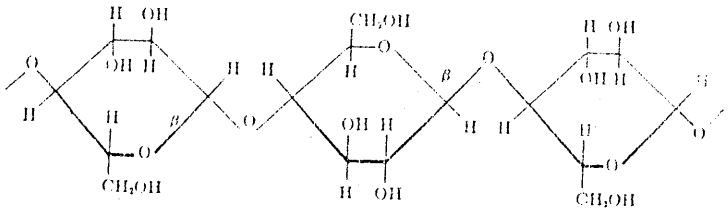
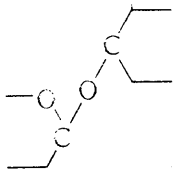


Figure 1b β-glycosidic linkage



The number of glucose units per molecule is called the degree of polymerisation (D.P.) and may range from 15 to 15,000. The molecular weight is at least 1.5×10^6. The chains of cellulose molecules are organised three-dimensionally and in the form of four distinct crystal lattice structures (micelles) designated as cellulose I, II, III and IV, which are characterised by their differing X-ray patterns.

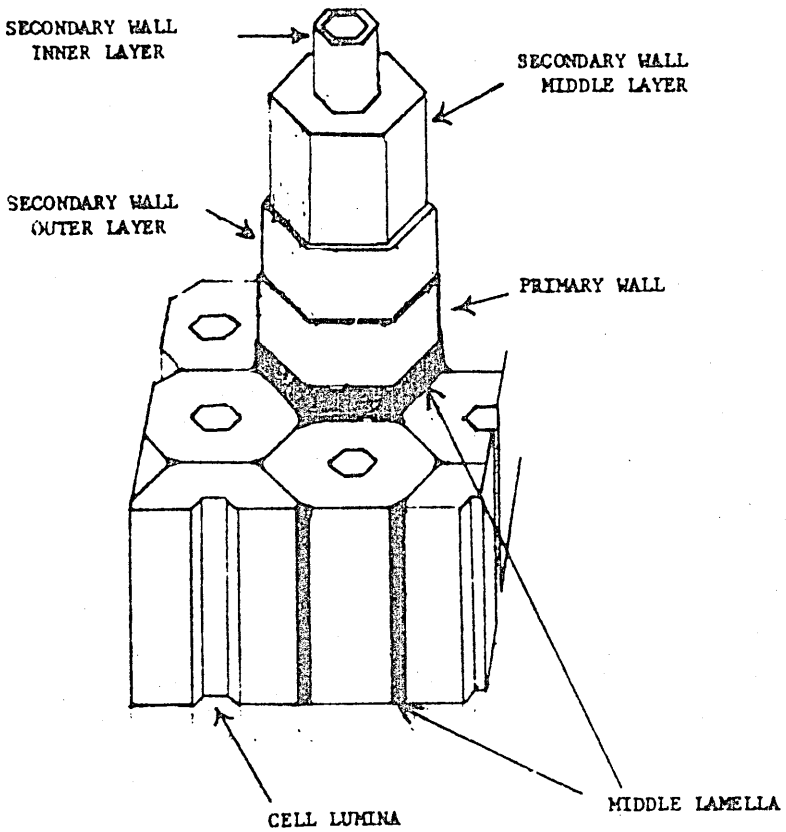
Cellulose I is present in native cellulose.
Cellulose II represents regenerated celluloses such as cellophane and mercerised cotton.
Cellulose III and IV may be produced artificially by chemical methods.

In the crystalline areas, the molecule chains are parallel and are strongly bound by intermolecular forces. However, these crystalline structures are not sufficient to account for the particular physical properties of cellulose; its strength and insolubility.

There are also non-oriented, amorphous areas in which the chains are not fully bound to one another. Figure 2 (FAO 1972) illustrates the main structural features of a mature plant cell wall.

The primary wall consists of a loose random network of cellulose microfibrils embedded in an amorphous pectin and hemicellulose matrix. The outermost layer of the secondary wall consists of cellulose fibrils arranged in a cross-hatched pattern. The middle layer is made up of parallel fibrils arranged in a steep helix which is nearly parallel to the structural cell axis. The inner layer consists of parallel fibrils arranged in a flat helix. The elementary fibrils in each wall are 3.5 nm in diameter, made up of approximately 40 cellulose chains and then grouped into larger microfibrils of varying size. Between the plant cells is the middle lamella consisting of lignin in the amorphous state.

Figure 2 Mature plant cell wall



There are two concentric walls, primary and secondary, the latter being composed of three layers.

Briefly, the other two consistuents of plant cell walls are hemiculluloses and lignin (in the middle lamella). The former is a blanket term covering polysaccharides with 5 and 6 carbon atoms in the repeating units. Their general formulae are $(C_5H_8O_4)_n$ and $(C_6H_{10}O_5)_n$, pentosans and hexosans respectively.

All plant hemicelluloses have a main chain structure based on β-(1-4) linked glycopyranosyl polymers, i.e. similar to that of cellulose. However the degrees of polymerisation are much smaller, from 50-200 units only and nearly all show some degree of molecule branching.

Lignin which is present surrounding the cellulose micro-fibrils in the later stages of a plant's life is non-carbohydrate and is a complex, three-dimensional polymer of phenylpropane residues.

1.2 Degradation by Enzymes and Acids

In nature the degradation of cellulosic biomass occurs very slowly by enzymatic means e.g. the rotting of dead wood and plants. This is essentially a process of hydrolysis, the reaction of the polysaccharides with water to simple sugars.

$(C_6H_{10}O_5)_n + nH_2O \longrightarrow nC_6H_{12}O_6$

hexosans hexoses

(present in cellulose & hemicelluloses)

and

$(C_5H_8O_4)_m + mH_2O \longrightarrow mC_5H_{10}O_5$

pentosans pentoses

(in hemicelluloses)

Since lignin is very resistant to attack by micro-organisms the presence of only 2-4% of it in wood pulp can reduce the amount of cellulose attacked by 50-60% (Olsen et al. 1973). Artificially the reaction may by catalysed by mineral acids - this is acid hydrolysis. Of the biomass constituents hemicelluloses are readily hydrolysed by acid (dilute at 100°C) but cellulose is more resistant and there is no effect until 180°C (with dilute acids). According to Baechler (1954) the resistance of wood (lignin) to mild acids is far superior to that of common steel however Sarkanen and Ludwig (1971) state that 'lignins are quite sensitive to even mild treatment with mineral acids'.

(a) <u>enzymatic degradation</u>

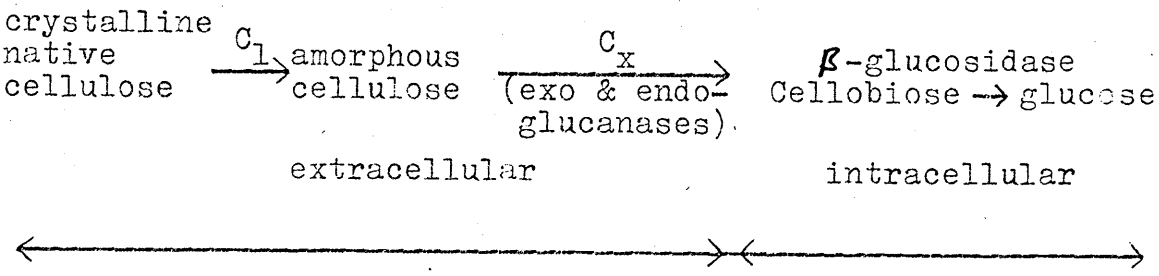
Such organisms as ruminants, termites and some snails, as well as many bacteria and fungi, are metabolically

dependent on their ability to degrade cellulose and to use the products for energy and new cell construction. Organisms that can degrade and metabolise cellulose are termed cellulolytic. The degradation is brought about mainly by the action of extracellular enzymes (cellulases) which are capable of splitting the β1-4 glycosidic bonds linking the glucose units of which cellulose is comprised. Siu and Reese (1953) have shown that the cellulase enzyme system is in fact a mixed enzyme system consisting of both endo- and exo-enzymes. An important feature of the degrading action of this mixture is an enhanced rate of glucose release. The endo-enzymatic rate is very slow, and that of the exo-system not much faster, but the combined rate is much higher than the sum of the individual rates (Sugin et al. 1975). This is due to the fact that the endo-enzyme increases the substrate concentration available for the exo-enzyme by breaking the polysaccharide links and so making shorter chains.

The Trichoderma viride enzyme complex may be used as an example of the cellulase system, as it has been well investigated by Mandels (1974). The system, being multi-enzymatic, consists of three components which are physically and enzymatically distinct. Each plays an essential role in the overall process of converting cellulose to glucose.

The enzyme complex in its total effect is capable of converting crystalline, amorphous and chemically derived cellulose to glucose.

Figure 3 Cellulase enzyme complex



As can be seen from Figure 3, the three components have been termed C_1, C_x and β-glucosidase.

C_1 is required to initiate hydrolysis of the highly crystalline components of cellulose. However, several lines of evidence (King 1966) suggest that the C_1 reaction may not be hydrolytic but more simply a cleavage of the intermolecular hydrogen bonding* system. Proof of this has been cited by King (1965) in that if D_2O is substituted for H_2O in the reaction system the reaction

* A hydrogen bond is one where an H atom bonded to atom X in one molecule makes an additional bond to atom Y in the same or in another molecule.

rate is unaffected. This event would be highly improbable for a hydrolytic reaction.

Further evidence is given by the activation energy of the C_1 reaction (3000 cals $mole^{-1}$). This is a low value more in line with H bond cleavage than with hydrolysis (Rautela and King 1968).

The C_x (glucanase) components have the capacity to degrade amorphous cellulose. They include exo- and endo-β 1-4 glucanases.

β-glucosidases in general vary in their specificities. Those involved in cellulose breakdown are highly active on cellobiose, and conversion to glucose occurs, the glucose is then assimilated by the cell. Alternatively cellobiose may be assimilated into the cell directly.

Cellulases are inhibited by the presence of cellobiose (Ghose et al. 1971), an excess of cellulose (Van Dyke 1971) and by glucose (Eriksson et al. 1974). But work by Mandels et.al. (1974) on the cellulase system of T.viride has regarded the reaction as being limited by the varying degrees of crystallinity, and only secondarily by substrate and product inhibition. However this enzyme system has not been effective on lignin-containing materials such as forest and agricultural residues.

(b) by mineral acids

This process involves the scission of the bonds betwe the monose residues and the addition of water. This would by very slow if just water were to be used but catalysed by acids (H+ ions). The cellulose macromc are bonded strongly together by H bonds, these are ruptured as well. The reaction occurs in stages (Harris 1952).

natural cellulose $\xrightarrow{i}$ hydrocellulose $\xrightarrow{ii}$ soluble polysaccharides $\xrightarrow{iii}$ si

Reaction (i) is rapid, (ii) slow (first order an determining), reaction (iii) is rapid.

For hemicelluloses $\xrightarrow{i}$ soluble polysaccharides $\xrightarrow{ii}$ sim sug

(i) is faster than (ii).

There are two theories about the reasons for inconsistencies in the rates of hydrolysis. (1939) Nickerson (1941), Philips et al. (19 and Scroggie (1945) explain this by stating amorphous cellulose is hydrolysed quicker crystalline. The process being further cc 'recrystallisation'. By studying the abs X-rays by different hydrolysed cellulose increase in the crystalline fraction was

hydrolysis, due to the dissolution of the amorphous part and the partial conversion of amorphous into crystalline.

The other theory offered by Pacsu (1947) and Schulz (1946) is that the initial weak bonds are better parted by acid than are the β-glycosidic bonds and that these were regularly distributed throughout the structure of cellulose. However experimental results were lacking.

A comparison may be made between acid- and enzyme-catalyzed reactions by considering the difference in the nature of the catalysts themselves. For example acid molecules have a much smaller molecular size (molecular weight) than protein (enzyme) molecules; HCl is 36.5 and cellulase is 63,000. Consequently acid molecules may penetrate deeper into the more resistant regions of cellulose and hydrolysis by acids is more complete. Enzymes have been found to be more efficient hydrolysing agents (Gascoigne 1960) requiring less molecules of enzyme to catalyse the same degree of hydrolysis, however their conversion of cellulose to soluble products is very incomplete. The large enzyme molecules, unable to diffuse into the cellulose, readily catalyse only the hydrolysis of the regions easily accessible to them. The reaction proceeds slowly at the surfaces of the crystalline areas with dissolution

of each chain as it becomes exposed to the surface. Extensive pre-treatment, often by milling, is needed to expose a larger area to the enzyme molecules. The smaller acid molecules are able to penetrate deeply into the structure, hydrolysing many more glycosidic bonds and giving rise to shorter chains. Acid hydr is further characterised by an increase in fluidity a solution of the fibres due to loss of strength produced by the reduction in chain length.

The specificity of the enzyme or acid catalyst is difference which affects the extent of cellulose hydrolysis. Acids will attack all glycosidic li within the molecules, while enzymes are much mo specific.

In addition to the differences between acids a themselves, when estimating hydrolysis potent are certain characteristics of naturally occ cellulose materials which will influence the susceptibility to hydrolysis. Those factor been reported as affecting susceptibility t attack have been listed by Siu and Reese (

1 degree of crystallinity
2 degree of orientation
3 degree of substitution
4 amount and nature of non-cellul

To complete this list, point 5, should be added, the degree of polymerisation; as already noted, the overrid factor in point 4 will be the degree of lignification.

Points 1 and 2 may be linked together for discussion purposes. Cellulose fibrils are laid down mainly in the secondary wall of plant fibres, those nearest the primary wall are laid down first. The degree or orientation is the angle at which the fibrils lie ir respect to the long axis of the fibre. This orient depends to a certain extent on age; during maturati most of the cellulose chains become arranged in crystalline areas. Other less oriented molecules the amorphous regions. The less crystalline and oriented the fibrils, the more susceptible they a enzyme attack. The extent of hydrolysis by acid affected too much by the crystallinity. Howeve hydrolysis occurs preferentially in amorphous r and then later the acid diffuses into, and att crystalline regions, the rate is slowed up by presence of crystalline regions.

The presence of one or more substituents in every glucose unit of a chain renders the ce immune to enzymic attack (Siu et al. 1949). rayon, which has at least 2 acetyl groups i units, is highly resistant to degradation.

of acid hydrolysis the presence of other chemical groups will have an effect on the end-products.

The non-cellulosic components of a fibre may either be resistant to enzyme degradation, e.g. lignins and waxes, or be easily broken down, e.g. pectins. Acids will deal with all these components more efficiently and easily.

In the case of lignin, hydrolysis with sulphuric acid removes the carbohydrate consituents by 'condensation reactions'* and the remainder is an insoluble residue known as Klason lignin (Sarkanen and Ludwig, 1971).

The degree of polymerisation, (the number of glucose units per molecule) affects degradation in that the larger the molecule the more resistant it will be to enzymic and to some extent, acidic attack.

It is evident that hydrolysis by acid is speedier, more effective and more complete. The only drawback is the lack of specificity, once the reaction has been started hydrolysis of most constituents of the cellulosic material will be effected. This precludes

*A condensation reaction is a combination reaction in which two or more molecules form a larger molecule with the elimination of some relatively small molecules such as water or ethanol.

an ability to control the reaction if only part of the material is needed to be broken down. However careful control can be performed to reduce the occurrence of decomposition products.

CHAPTER 2

SOURCES OF CELLULOSIC WASTES

It has been estimated by the World Health Organisatio (1971) that the total world fixation of carbon dioxi by green plants is 3.2×10^{11} tonnes $year^{-1}$ of which 26.5×10^{9} tonnes, (approximately 6%) is converted cellulose in plant material. Of this plant tissue cellulose only about 20% is readily available as p cellulose (such as cotton) which is relatively rar nature. Usually it occurs in combination with ot polymers such as lignin, pectin and hemicellulose the plant cell walls. It is the presence of lig ligno-cellulose complexes which strengthen plant material but which constitutes a major obstacle commerical breakdown and utilisation of cellulc wastes.

Sources of waste cellulosic material are wide and widely distributed so their UK availabil accordingly. (Table 1, Porteous 1976).

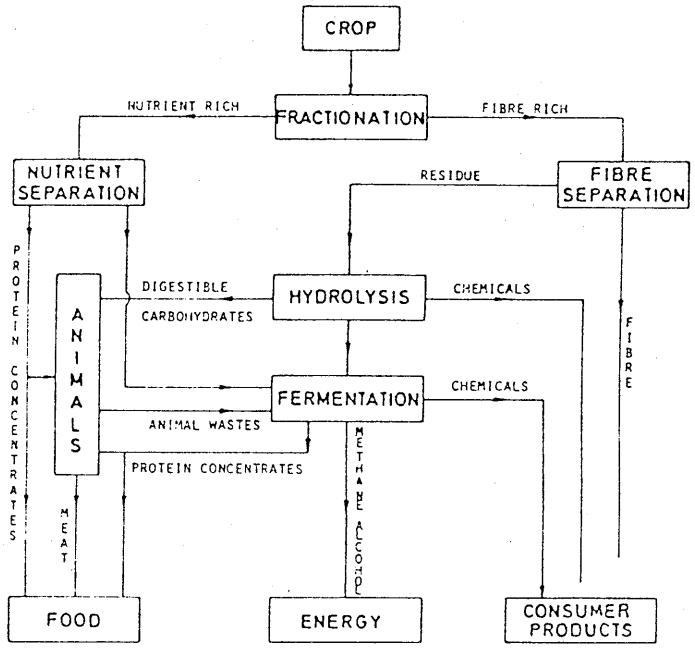
Table 1 The UK availability of cellulosic wastes (Porteous 1976)

	m tonnes Gross amount of waste	m tonnes Nett cell
Domestic refuse	18	4.0
Straw (50% cellulose)	3.5 (burned) - 9 available	1.75 -
Sawmill wastes (45% cellulose)	2.0	.9
Forestry Wastes (45%)	1.0	.45
Wood processing wastes (45%)	1.0	.45
TOTAL		7.55

The processing alternatives for using cellulos resources of renewable crops have been depict Weiss (Figure 4, 1976).

From a crop two pathways diverge, one being conventional feedstock itself such as wheat etc., the other is concerned with the 'was as stems. The main process envisaged invc hydrolysis followed by fermentation to us

Figure 4 Cellulose resource alternatives (Weiss 19



As an extension of these ideas on crop cellulo utilisation, is the availability of cellulose in urban wastes (Wiley, 1954).

Domestic solid wastes were defined as follow WHO Expert Committee (1971).

> "These wastes are a consequence of housekeeping activities, such as food preparation, sweeping and vacuum cleaning; they also comprise fuel residues; empty containers and packaging; wastes from repair and redecorating, gardening, and hobbies; old clothing; reading matter; old floor coverings; and old furnishing."

Within these categories there are many items whi comprised of cellulose.

Both, commercial wastes which are mainly the wa produced by shops and offices and consists lar fibreboard containers, wooden crates, paper pa paper, carbon paper, typewriter ribbons, punc tape, etc., and street cleaning wastes, may l with domestic wastes for the purposes of thi

The proportions of the constituents of dome collected at a disposal site are virtually for a particular town but may be subject t

and long-term changes. Table 2 updated from Skitt (1972) gives a breakdown of the quantitative and qualitative content of domestic refuse in the UK. It can be seen that the average annual paper content is 29.6% (1975). The total amount of refuse produced annually is 18 x 10^6 tonnes and 5 x 10^6 tonnes of this is paper. If the cellulose content of paper is assumed to be 50% this becomes 2.5 x 10^6 tonnes cellulose per annum available in domestic refuse.

From Table 2 it can be seen that 3.44 kg per household per week of paper is discarded, approximately 1/12 tonne per person per year.

Table 2 Quantitative and qualitative breakdown of domestic refuse (Skitt 1972, updated)

Constituents	Amount of refuse/% by weight				Average for four seasons 1969/% by weight	Average for four seasons 1968/% by weight	Average for four seasons 1967/% by weight
	Winter	Spring	Summer	Autumn			
Fine dust and small cinder (<0.5 in)	15.68	15.48	14.45	15.35	15.24	17.44	22.66
Cinder content (0.5 - 1.75 in)	2.21	2.25	1.62	1.86	1.98	4.45	8.29
Vegetable and putrescible content	20.03	19.09	19.08	19.61	19.45	17.61	15.50
Paper content	37.16	38.38	38.68	37.92	38.03	36.91	29.50
Metal content (ferrous and non-ferrous)	9.57	9.36	10.19	9.78	9.71	8.87	8.00
Rag content (including bagging and all textiles)	2.34	2.12	2.35	2.31	2.28	2.35	2.10
Glass content (bottles, jars and cullet)	10.28	10.19	10.94	10.42	10.45	9.11	8.10
Unclassified debris (combustible and non-combustible)	1.35	1.77	1.30	1.41	1.50	2.14	4.70
Plastics	1.38	1.36	1.39	1.34	1.36	1.12	1.15
	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Density of refuse lb. cu.ft.$^{-1}$	9.20	8.09	8.07	8.08	8.09	9.08	10.00
cwt. cu.yd.$^{-1}$	2.22	2.15	2.11	2.12	2.15	2.37	2.42
Yield per house per week (lb.)	28.30	28.44	26.45	29.10	28.07	29.02	28.50
Average capacity of dustbin required, assessed on average output per week (cu.ft.)	3.07	3.2	3.04	3.03	3.1	2.98	2.85

Figure 5 Domestic refuse trends 1953-1979 (updated from DOE 1980)

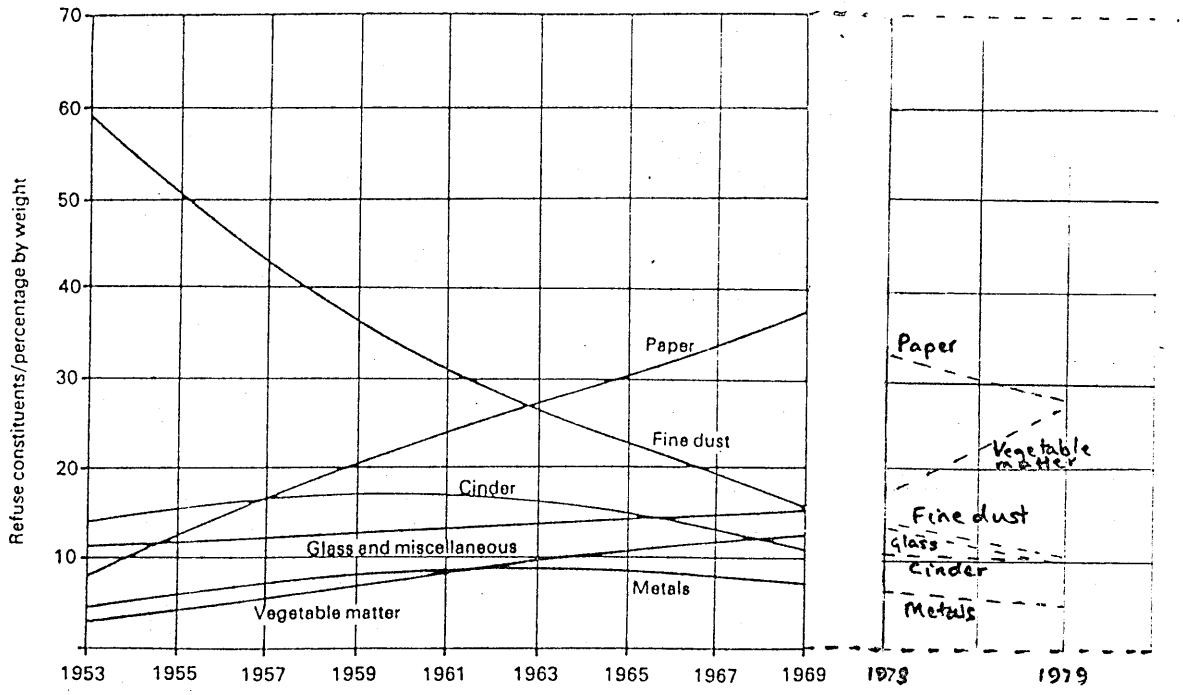


Figure 5 shows graphically the increase in paper content between 1953 and 1969 which has probably been the result of less open fires and more packaging of goods. It has been somewhat reduced in later years. Table 3 (Frost & Sullivan 1973) is a prediction of the composition of domestic refuse (%) in Europe up to 1990.

Table 3 Predictions of composition of domestic refuse in Europe (Frost and Sullivan 1973)

	1965	1970	1975	1980	1990
			%		
Ashes, dust and cinders	60	32	25	18	5
Paper and cardboard	14	40	45	50	60
Organic matter	18	17	16	15	13
Metals	3	3	3	3	3
Glass	3	4	5	7	10
Plastics	1	3	4	6	8
Textiles	1	1	1	1	1

While the volume of domestic refuse is increasing the density is decreasing because of a diminution in inorganic matter and a marked increase in total organic matter. It should therefore be easier to handle. The latter (vegetable matter 17% and rags 2%) contains a high proportion of cellulose.

Apart from agricultural wastes and domestic (urban) wastes other areas of cellulose production could be developed such as the use of fresh water reed swamps and waste marshland where plant types of high cellulose productivity could be grown and harvested (Heslop-Harrison 1975). Also cereal crops grown for maximum yield of both grain and stalk;

it has been estimated by Smith et al. (1975) that this could yield a further 5×10^6 tonnes of cellulose, over that normally available from such crops.

CHAPTER 3

THE UTILISATION OF CELLULOSIC MATERIALS AND CURRENT RESEARCH

3.1 Historical

As early as 1819, Braconnot found that water soluble sugars could be obtained by the reaction of strong sulphuric acid on sawdust. The experiment was not intended to produce wood sugar but to investigate the effects of various chemicals on wood. In 1866 Tilghman discovered that sulphurous acid would dissolve the lignin in wood, leaving the cellulose fibres. This discovery paved the way for the development of the pulp and paper industry. The acid hydrolysis of cellulose itself was reported by Calvert in 1855 and an systematic investigation was reported by Girard in 1875.

The enzymatic breakdown of cellulose was also being investigated at the end of the last century. By 1899 MacFaydyen and Blaxall had discovered a bacteria which would ferment cellulose at 60°C. Omeliansky (1904-6) pioneered much of the early work in the fermentation of cellulose by micro-organisms but in 1906 he was still taking 9-15 months to ferment a few grams of filter paper!

By 1920 large scale experiments (4550 litres) were being carried out by the Distillers Company and a paper by Langwell in 1932 cited yields of alcohol, acetic and butyric acids of 3.2%, 31.6% and 1% respectively from dry corn cobs in 7 days.

In the field of acid hydrolysis, Willstatter and Zeichmeister (1913) used hyperconcentrated hydrochloric acid on wood and obtained 15% cellulose decomposition but with only 3-4% monosaccharide. Hagglund's (1951) discovery that spent hydrochloric acid which had lost its dissolving capacity still had an effect on a fresh supply of wood, finally produced up to 30g of sugar dissolved in 100g HCl. This made possible the economical recovery of HCl by evaporation under vacuum.

On a larger commerical scale three methods were used in the years encompassing the two world wars; the 1914-1918 'war' process, the Bergius-Rheinau process and the Scholler process. The 'war' process (in Germany) which employed dilute acids in autoclaves proved uneconomical and was abandoned in 1919. Unfortunately this gave rise to the assumption that wood saccharification with dilute acid was uneconomical.

The Bergius-Rheinau process used concentrated hydrochloric acid at room temperature and pressure. Scholler (1936) and his collaborators in Germany returned to the use of dilute acid (H_2SO_4) and managed to obtain a four-fold increase in yield compared with the process used during the 1914-1918 war. Realising that sugar decomposition takes place as well as saccharification he operated a percolating process obtaining 80% of theoretical sugar yield. This was fermented to ethanol.

In all calculations of sugars from wood the following values are used as a theoretical stoichiometry:

dry wood 100kg ⟶ cellulose 45kg ⟶ glucose 50kg ⟶ ethanol 25.6kg

In the USA research was conducted by Saeman (1945) during the same period to develop a commercial process for ethanol from wood waste. It was based on the Scholler process (Madison process). In both countries these commercial practices gradually came to an end with the increased availability of relatively cheap oil and its associated products. Now as the anticipated shortages of energy producing raw materials from fossil fuels becomes a reality, cellulose is once more becoming recognised as an important potential resource, the use of which may help the conservation of scarce and costly oil reserves.

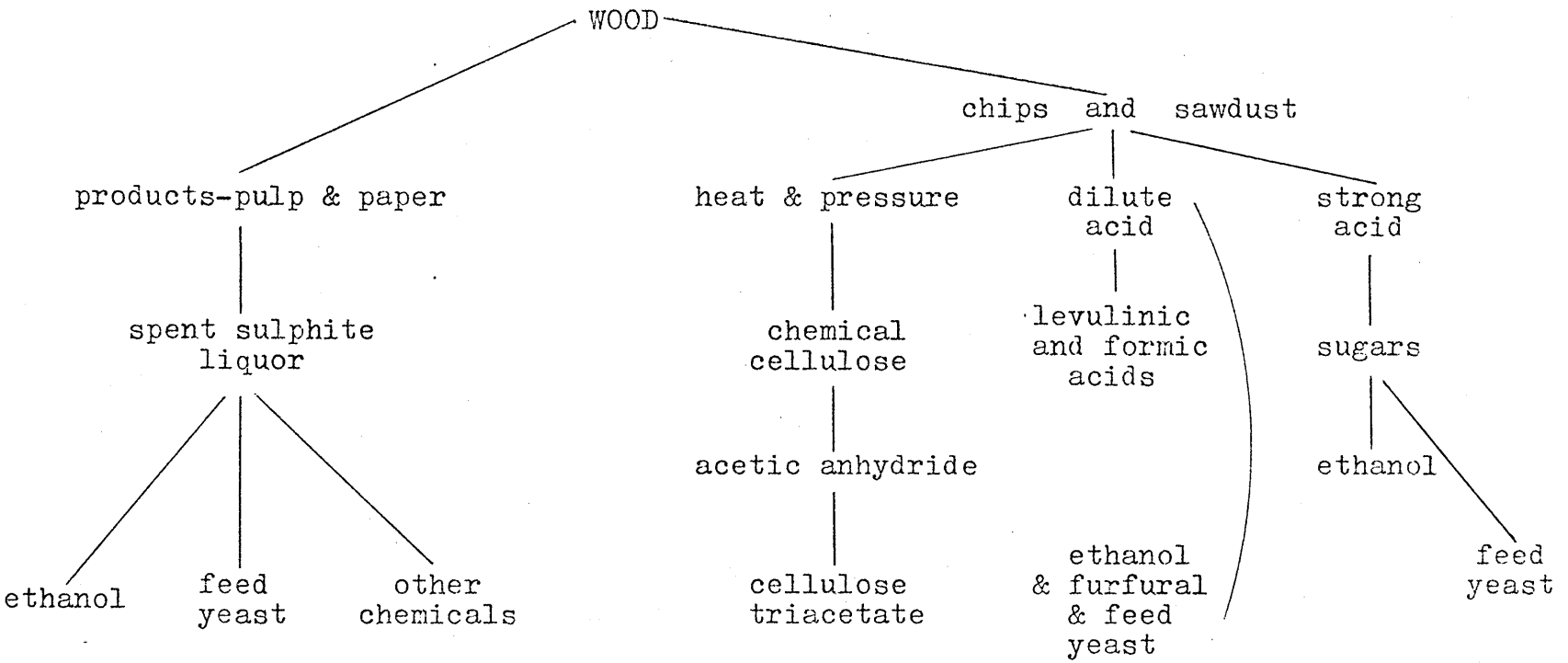
A process using concentrated sulphuric acid was used in Italy during the 2nd World War and has recently been developed in Japan as the the Hokkaido process (Rieche 1964). 80% concentrated H_2SO_4 is sprayed on ground dried wood in the reaction chamber with a contact time of 30 seconds. The resultant sugar solution is neutralised and the glucose precipitated and purified. 28 kilos of sugar from 100 kilos dry wood are produced representing a yield of 56% of the theoretical.

The key to any type of cellulose utilisation lies in the development of methods to convert the waste economically to readily useable forms of energy.

3.2 Present Day Usage

There are already some industrial chemical processes which use cellulosics directly or indirectly, these include wastes from wood processing, pulp and paper manufacture, and agriculture. Figure 6 shows an outline of such uses which are then discussed in more detail (the strong acid process is thought to be operating in the USSR at present).

Figure 6 Uses of wood products



In addition some agricultural wastes such as sugar cane stalks (bagasse) and potato starch are used.

(a) spent sulphite liquor (Inskeep 1951)

This is a weak (1.5 - 2.2%) sugar solution containing about 0.5% acetic acid and 5-6% lignin as lignosulphonic acids. The sugars are hexoses (1.1 - 1.6%) and pentoses (0.4 - 0.6%). The traditional method of utilising this waste stream has been to grow Candida utilis (Torula yeast) for feed yeast (134kg tonne^{-1}). Other products include alcohol (80 litres tonne^{-1}) by fermentation of the hexoses, vanillin (from the lignosulphonic acids), oxalic acid, tanning material and dispersing agents.

(b) chips and sawdust (Frost and Kurth 1951)

These are heated with dilute acid to form glucose and then on to levulinic and formic acids (1474kg tonne^{-1}). The levulinic acid is used as raw material for the production of diphenolic acid (DPA) which is an ingredient of resins, printing inks and coatings. Processing of the chips and sawdust by heat and pressure produces chemical cellulose which is converted to cellulose triacetate with acetic anhydride.

In the USSR two dilute acid processes are definitely in use (Nikitin 1966). One is a batch reaction based on the Simonsen method, wood is heated to 170^{o}C with 0.1N

H_2SO_4. This yields 25-26% monosaccharides in 4-6 hours. This process with its low yield is used only for the pre-hydrolysis of wood to remove the hemicelluloses. The sugars are used for fodder yeast, ethanol and furfural production. The residue from the wood is used to make a high quality viscose pulp by sulphate cooking, or crystalline glucose by hydrolysis with concentrated acids.

A continuous percolation method (0.5% acid, 180-190^{o}C) with a total of 3 hours contact time is used for the production of sugars for alcohol (170-180l tonne^{-1} oven dry wood). Recent developments have been towards counter-current percolation (Pohjola 1977). A bottom-fed 440 litre pilot percolator has given 45-52% yield sugar from dry wood, the reactor operating at 80 kg hour^{-1} substrate feed.

3.3 Present Day Research

A wide range of research has been initiated in the USA and other countries (Dunlap et al., Eriksson et al., Grethlein et al., Thayer School of Engineering, Darmouth College; Mandels et al., US Army Natick Laboratories Mass; Wilke et al., University of California at Berkeley), with the exception of the Thayer School work, this research has been concentrated on the biological breakdown

of cellulose by cellulase-producing organisms; the resultant sugar being converted to single cell protein (SCP).

(a) enzymatic research

Since the cellulase enzyme system is far more efficient when the cells themselves are used rather than a cell-free extract the most favoured method of microbial degradation has been by the culture of the micro-organisms themselves. Work has included batch and continuous cultivation.

The micro-organisms which have been used experimentally for cellulolytic breakdown fall into three distinct groups according to optimum temperature and oxygen requirement:

aerobic mesophiles anaerobic mesophiles	optimum temperature 20-45°C
aerobic thermophiles	- obligate 47-75°C facultative 40-60°C

A fourth group, anaerobic thermophiles, has been used for the production of organic acids from cellulose in laboratory studies.

1. aerobic mesophiles

The use of these organisms for the degradation of waste cellulosics has proved the most popular, both fungi and bacteria have been used. Table 4 gives some details of these experiments. The maximum utilisation of cellulose was 57% plus and also in the shortest time. In recent years the US Army Natick Laboratories have concentrated on maximising cellulase production in a two-stage continuous process (Mandels et al. 1979) since the hydrolysis system they had developed was not considered economically viable (Noyes Data Corporation 1980). Realising this Wilke (1977) continued the investigation on enzymatic hydrolysis by using dilute acid as a pretreatment to remove the hemicelluloses and open up the cellulose prior to using the enzyme.

The initial growth medium used for the cellulase-producing organisms affects their later performance. For example T.viride first grown in glucose, and then put into a cellulose substrate has an adaptation time of 30 hours (Rosenbluth and Wilke 1970) before the lag phase commences. Yamane (1969) found that both filter paper (cellulose) and sophorose (a disaccharide) enhance cellulase production but as found by other authors glucose (Eriksson and Goodall 1974) and cellobiose (Ghose and Das 1971) inhibited it.

Table 4 Degradation of cellulosic wastes by aerobic mesophiles

Organism	substrate	pre-treatment	fermentation parameters	conversion	reference
Cellulomonas species (bacteria)	sugar cane bagasse (stalks & leaves)	ground in knife mill 2% NaOH for 30 minutes	30 hours, 35°C pH 7.0 doubling time 3.2 - 3.7 hrs.	57% cellulose digested 23% CHO to protein.	Dunlap (1974) Han et al. (1971)
Cellulomonas + Alcaligenes faecalis	as above	as above	better growth modified amino acid composition.	increased protein yield	as above
Trichoderma viride (fungus) also Pestalotiopsis westerijkii	powdered cellulose, newsprint, wood	various types of milling NaOH	10-13 days 28°C pH4.8	cellulase first produced then 27-55% saccharification in 48 hrs.	Mandels (1974)
Myrothecium verrucaria (fungus) also T.lignorum and Aspergillus sp.	newsprint	ballmilled	9-24 days 30°C pH 5.3 - 6.5	34.8% cellulose utilised 10% protein yield.	Updegraff (1971)
Sporotrichum pulverulentum (rot fungus)	waste fibres from paper manufacture	---	<6 days 30°C, pH5.5	13.8% protein formed 55% fibre degradation.	Eriksson et al. (1975)
also rot fungus plus Candida utilis					
Trichoderma viride	newsprint	shredded and hammer milled	40 hours 45°C	4% sugar concentration 27% conversion	Wilke et al. (1976)

2. anaerobic mesophiles

These organisms have been used to produce methane from cellulosic wastes, with SCP as a by-product (Pfeffer 1974, Bellamy 1974).

Pfeffer (op cit.) used shredded refuse seeded with raw sewage sludge and fermented it anaerobically to produce methane. The disadvantage of this process being that lignin and large fractions of lignin-cellulose complex are not utilised by the anaerobic organisms.

The most common and effective of these organisms are those in nature, the micro-flora of the alimentary tract of ruminant herbivores. However no large scale experiments have been attempted using these organisms, probably because of the isolation and cultivation difficulties.

3. aerobic thermophiles

Some of these use lignin and because of the higher working temperature exhibit a higher rate of digestion. The most frequently used have been the thermophilic actinomycetes (Bellamy 1974, Crawford and McCoy 1973). Both pulping fines and fibre were used and utilisation of cellulose reached 60-80% with up to 30-35% protein yield.

In reviewing these methods it is apparent that they all have problems in common, viz. the necessity for pre-treatment of the cellulose, thin suspensions only (1%) can be used, the presence of lignin and lignocellulose complexes which resist degradation and the need for additional nutrients to promote adequate growth.

Klee and Rogers (1978) noted a substantial number of disadvantages, to the enzymatic hydrolysis process applied to municipal solid waste:

1 the feedstock must be milled to approximately 250 μm (60 mesh), a costly operation;

2 the feedstock concentration typically is low (5% to 10%), since higher concentrations are difficult to stir;

3 it required 48 to 64 hours to produce a dilute (2.5% to 5.0%) sugar solution (high glucose concentration interferes with the enzymatic hydrolysis);

4 there have been difficulties in recovery of the enzyme for reuse;

5 precautions must be taken to maintain sterile conditions to prevent loss of enzyme or sugar (some bacteria will consume either);

6 if conversion to ethyl alcohol is elected, the dilute sugar solution must first be concentrated (about 15% or better is required for best results).

On the positive side, the enzymatically-produced sugars are free of extraneous decomposition products, also the process operates at low temperatures (30° to $60^{\circ}C$) and does not require corrosion-resistant equipment.

(b) acid hydrolysis research

Porteous (1967) analysed the kinetics of cellulose hydrolysis by acid and predicted that under suitable processing conditions, namely temperature and acid concentration, the hydrolysis time could be cut (from hours) to minutes or even seconds. This also meant that a continuous reactor could be used and the process transformed from a slow batch method to a rapid continuous one, resulting in fermentable sugars. He suggested the application of the process to the cellulose content of domestic refuse.

Figure 7 Concentration v.time profiles

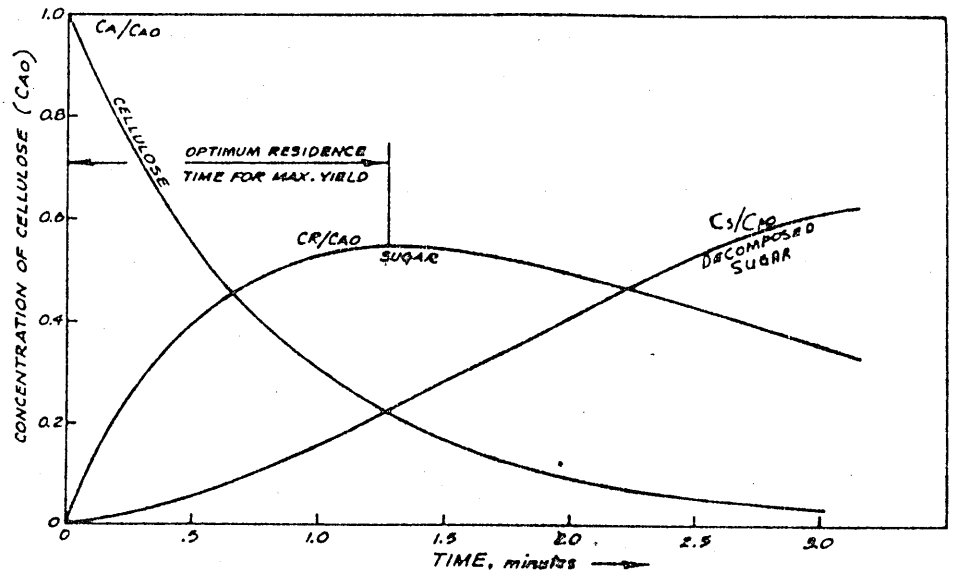


Figure 7 gives the predicted concentration in time profiles for the continuous hydrolysis of cellulose at 230°C and 0.4% H_2SO_4. It can be seen that the predicted reactor residence time for maximum yield is 1.2 minutes.

Experimental work was then carried out in two stages at the Thayer School of Engineering, Dartmouth College, New Hampshire, USA to verify: 1. the kinetic predictions of Porteous (1967) and 2. the effect of refuse contaminants on sugar fermentability. The experimental results of Fagan (1969) showed a substantial agreement between the predictions and the experimental results; yields of approximately 75% of those predicted by Porteous, with Saeman's (op cit) kinetics, were obtained. The main cause of error in the calculations for these initial

kinetic studies was the time to heat the sample from 170°C, where the reaction rate first becomes significant to the final temperature. This time was approaching 3 minutes giving a large error due to heat up.

It was also shown that a 10% yield increase was encouraged by doubling the acid concentration at 230°C, and that a 5% yield increase occurred when a 10° rise in temperature was used.

Converse et al. (1973) obtained yields of 52-54% sugar in a bench scale flow reactor while verifying the kinetics of the process, and also attempted fermentation of the sugars (see Chapter 8).

Brenner et al. (1977) at New York University also verified the kinetics, using a batch 1 litre autoclave with glucose yields of up to 50% of the original cellulose. Klee and Rogers (1978) reported on Brenner's later research in which the substrate is subjected to an ionising radiation pre-treatment before heating to 230°C in a batch reactor with 1-2% sulphuric acid. Yields of 50% were achieved in 10-20 seconds. Recent work by Grethlein (1978) has yielded 10% glucose solutions using newsprint (77% cellulose and hemicelluloses). In September 1978 Grethlein was advocating the use of acid hydrolysis as a pre-treatment of solka floc and other particulates from wood mills, followed by enzymatic

conversion with T.viride. The pre-treatment converts the hemicelluloses to mainly pentoses and the enzymes attacking the cellulose. Thompson and Grethlein (1979) obtained about 50% of the potential glucose in solka floc and in newsprint at 240°C, 1% acid and 0.22 minutes residence time in a plug flow reactor. They concluded that a continuous acid hydrolysis process would be of commercial interest if slurries greater than 10% $^{w}/_{v}$ were used.

In 1978 Guha et al. performed an engineering evaluation of the chemical conversion of wood to liquid fuel alcohol. They found that a dilute sulphuric acid process was the most attractive for present day energy application. They used kinetic data obtained from the investigation of the four reaction steps (diffusion of acid, conversion of cellulose to sugar, diffusion of sugar, decomposition of sugar) in a non-isothermal batch reactor to optimise a percolator reactor. Calculations showed that the percolation time can be reduced from 3 hours (Madison process) to 45 minutes with a higher yield than can be obtained in a tubular reactor. Also Emery (1979) has costed the hydrolysis of newsprint, sorted refuse, straw and ryegrass by enzymatic and acidic methods. He concluded that ethanol produced (by fermentation) from the sugars obtained by acid hydrolysis was cheaper than by the other route, and that sorted refuse was the only

material which appeared to be energetically and economically feasible as the substrate.

Work in Russia is being directed towards dilute acid percolation plants using dry wood. The parameters studied include hydrolysis rate constants, the effects of particle size (wood shavings), temperature gradients, liquid to solid volume ratios, contact time and coefficients of mass transfer (Belyaevskii 1973, Starostina and Belyaevskii 1973, Molchanova et al 1973 and 1974).

PART II ACID HYDROLYSIS

CHAPTER 4

THE KINETICS OF ACID HYDROLYSIS

As noted in Chapter 1, chemical hydrolysis of cellulosic wastes may be accomplished by treatment with dilute acid. The rate increases with temperature. Between 160-200°C the rate increases 2-2.5 times for each 10°C rise. The process is exothermic 36 cal.g^{-1} wood (Rieche 1964). The rate is also a function of the pH. The chemical reaction may be simply expressed as:

$$\underset{\text{cellulose}}{C_6H_{10}O_5} + H_2O \xrightarrow{H_2SO_4} \underset{\text{hexoses}}{C_6H_{12}O_6} \qquad (1)$$

The acid acts as a catalyst. Unfortunately, those conditions which favour cellulose hydrolysis also favour the decomposition of the sugars so formed. The decomposition reactions are shown in equations 2-5. If pentoses are present (from the hemicellulose fraction) they degrade as in equations 4 and 5.

$$C_6H_{12}O_6 \longrightarrow \underset{\substack{\text{Hydroxymethyl-}\\\text{furfural}}}{C_6H_6O_3} + 3H_2O \qquad (2)$$

$$C_6H_{12}O_6 \longrightarrow \underset{\text{levulinic acid}}{C_5H_8O_3} + \underset{\text{formic acid}}{HCOO\,H} + H_2O \qquad (3)$$

$$C_5H_{10}O_5 \longrightarrow \underset{\text{furfural}}{C_5H_4O_2} + 3H_2O \qquad (4)$$

$$C_6H_{12}O_6 \text{ and } C_5H_{10}O_5 \longrightarrow \text{humic substances (sludge)} \qquad (5)$$

The hydrolysis of cellulose is a heterogeneous reaction but for small particle sizes and high liquid to solid ratio it may be considered as homogeneous. The two types of reaction (production and decomposition) are consecutive and have been expressed by Saeman (1945) (for wood) and Fagan (1969) as pseudo-first order.

$$C \xrightarrow{k_1} S \xrightarrow{k_2} D$$

C = cellulose concentration

S = fermentable sugars

D = decomposition products

k_1 & k_2 are reaction rate constants

The rate of cellulose decomposition $\frac{dC}{dt} = k_1 C$ (a)

The rate of sugar accumulation $\frac{dS}{dt} = k_1 C - k_2 S$ (b)

and similarly the rate of decomposition products $\frac{dD}{dt} = k_2 S$

and $K_1 = P_1 C_a e^{-E_1/RT}$

$K_2 = P_2 C_a e^{-E_2/RT}$

where P = pre-exponential factor s^{-1}

E = activation energy $J.mol^{-1}$

C_a = acid concentration (%)

T = temperature ^oK

R = ideal gas law constant $J.mol^{-1}$

By integrating (a) and (b):

$C = C_o e^{k_1 t}$ C_o = initial cellulose concentration

and $s = \frac{C_o k_2}{k_1 - k_2} \left\{ e^{-k_1 t} - e^{-k_2 t} \right\}$

The maximum yield of sugar $= C_o (K_1/K_2)^{(1-K_1/K_2)^{-1}}$

is dependant only on k_1 and k_2 and it increases with temperature (and/or acid concentration) due to the increase in the ratio of $\frac{k_1}{k_2}$ (the selectivity).

The maximum temperature is limited by the practicality of the very short residence time which would be needed. The residence time in a plug flow reactor at which

maximum concentration would occur is:

$$t_{(max)} = \left(\frac{1}{k_1 - k_2}\right) \ln \frac{k_1}{k_2}$$

The rate of production of glucose (or the rate of conversion of cellulose) is the mass of the product (or reactant) in moles per unit time and unit volume. Since the acid hydrolysis of cellulose is a fast reaction (once started) the mixing of the reactants (cellulose, water and acid) will probably be rate-limiting. It has also been shown (Saeman 1945) that for any given hydrolysis conditions (e.g. acid concentration and temperature) there is an optimum reaction time for maximum sugar yield after which the temperature must be sharply reduced to quench the reaction and stabilise the yield. Porteous (1967) extended this work in order to produce an economic process for the disposal of municipal waste by hydrolysing the cellulosic content in a continuous tubular reactor. Conditions of 0.4% H_2SO_4 and 230°C were found to be the approximate upper limits for a controllable reaction (Porteous 1969). The predicted optimum residence time in the continuous reactor, under these conditions was 1.2 minutes with a 55% conversion to fermentable sugars. The design of such a reactor (length large with respect to diameter) is such that the fluid velocity is sufficient to retard or inhibit back-mixing and it is possible to approach plug flow performance.

The form of the rate expression and the value of the rate constant for a reaction cannot be predicted or calculated without experimental data. The most commonly used method for homogeneous reactions involves adding known quantities of the reactants to a batch reactor which is operating isothermally at constant volume. The reactants are mixed thoroughly and the change in concentration of the key component with time is measured. The data obtained are compared with various rate equations to find the one giving the best agreement. This comparison can be made by either the integration or the differential method.

The integration method involves a comparison of the predicted and observed compositions of the reaction mixture as a function of time and it is necessary to integrate rate expressions to give concentration as a function of time.

If irreversible and first order as predicted in the formation of sugar from cellulose

then $\frac{dC}{dt} = -kC$

If the initial condition of cellulose equals C_o and C_t represents the concentration at time t, then integration leads to:

$$\ln \frac{C_o}{C_t} = k\ t$$

and a plot of $\ln \frac{C_o}{C_t}$ versus t should be a straight line

with a slope equal to k_1.

The differential method involves comparison of predicted and observed rates obtained by differentiating the experimental data when the concentration of glucose is plotted against time, the slope of the curve at any point is equal to rate of reaction dS and a collection of these values (r) at different times can be obtained.

If the reaction is first order

rate (r) = $dS = k_1 C$

which may be written as log rate (r) = log k_1 + log C.

If the log rate (r) is plotted against log C, a straight line of slope 1.0 should be obtained.

CHAPTER 5

HYDROLYSIS RIG DESIGN AND CONSTRUCTION

The objective of this part of the research was to verify in outline the acid hydrolysis reaction in a laboratory bench-scale set up worked on a continuous basis at high temperature and pressure and to obtain sugars for subsequent fermentation. The planning of the process development included information of the following:

flow diagram of the process (Figure 8)

raw material available, the effect of its quality on the process and product

pre-treatment of substrate

the effects of process variables on conversion and yield

equipment

collection and cooling of product

constructional materials

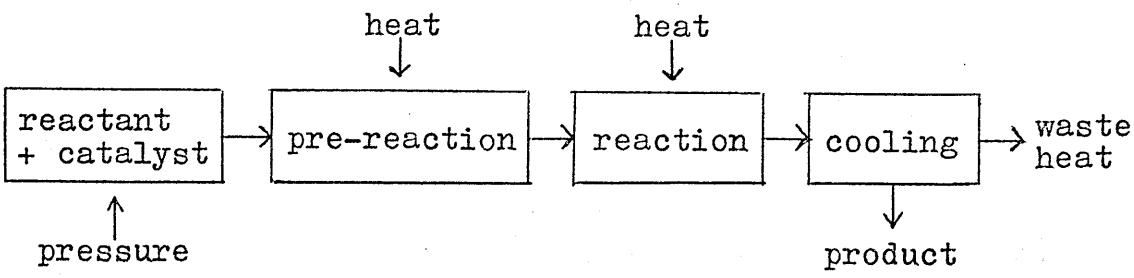
analytical methods for evaluation

safety

A short discussion of each point now follows.

5.1 Flow Diagram for the Process

Figure 8 Flow diagram



Fagan (1969) has demonstrated the desirability of a single stage continuous flow reactor (with recycle capabilities) and later Converse et al. (1973) used the same basic plant. However Fagan et al. (1971) found that the heat up time that had to be allowed for caused considerable errors in the kinetic calculations. In his process the total heating of the cellulose slurry was accomplished by direct mixing with a high temperature acid stream. No mention was made of the starting temperature or the mechanisms of maintenance of a 200°C reactor temperature.

In this present research a pre-reactor has been used, heated to approximately 180°C, and a reactor proper, in-line heated to 230°C. The reaction, cellulose to sugars does not take place appreciably below 180°C, and because of the problems of heating (no steam in the laboratory) it proved more feasible to heat the cellulose slurry in two stages.

5.2 Raw Materials

The cellulose input to the process was both computer paper and filter paper. The use of these will give an approximation of the conditions likely to prevail when the process is applied to the cellulose content of domestic refuse which includes newsprint and other types of paper.

In addition sulphuric acid and water were used.

5.3 Pre-treatment of the Substrate

The need and the methods for the pre-treatment of various cellulosic wastes has been the subject of a considerable amount of research (Rogers et al. 1972, Crawford and McCoy 1973, Han et al. 1971, Mandels et al. 1974, Updegraff 1971 and Bellamy 1974). The methods employed include both physical and chemical treatment. Of the physical treatments a variety of mills have been tested to reduce the cellulose to very small diameter (50μ) particles. The types most commonly used were ball mills, hammer mills and knife mills. Shredders were also used for preliminary work. In this research initial thoughts on the pre-treatment of computer paper produced the idea of a two stage pre-treatment, first shredding and the use of a knife mill to produce the fine particles demanded by the

very small scale experimental apparatus. It is quite possible that an industrial scale plant would not need such fine milling of the feedstock.

5.4 Process Variables

The screening and optimisation of variables according to the Plackett-Burman designs (Stowe and Mayer 1966) has been done retrospectively.

The candidate variables were:

1 acid concentration (% H_2SO_4 w/v)

2 flow rate ($l.hr^{-1}$)

3 residence time in reactor sections v_1 and/or v_2 (minutes) (see Figure 9).

4 pre-reactor temperature (^oC)

5 reactor temperature (^oC)

6 difference in temperature between the two reactors (^oC)

Three of these factors were chosen (acid concentration, flow rate and temperature difference) and an 8-trial design utilised (see Chapter 6 - results). To eliminate bias errors the trials were held in random order. Four unassigned factors were employed in computation to get

some measure of experimental error in order to decide which variables are really significant.

5.5 The Equipment

(a) knife mill

A 3 phase 1.5 kw (3HP) mill, comprising a rotating cylinder (300 rpm) with 3 knives within the body containing 4 stationary knives. Approximately 17 minutes grinding of 1 kg paper produces a fine dust of which 25% of the particles were below 150 μm.

(b) plunger pump

This is made of Hastelloy and stainless steel with a ceramic plunger and has a capacity of 0-30 $1.hr^{-1}$. The stroke control may be altered from 0-100%. The pumping speed is 135 strokes per minute at a working pressure of 3.45×10^6 Pa. It was used to handle a 1:20 w/v acid and paper slurry at 20 $1.hr^{-1}$.

(c) pre-heaters

There are two containing feedstock (including acid) and flushing water respectively, heated to 90°C. The one containing feedstock includes a 'stirrer'.

(d) pre-reaction tube

This is heated to 180°C by electrical tapes, it is 6 metres in length, 2 cm internal diameter, of a spiral form and made of stainless steel E W 58J.

(e) reactor tube

This is heated to 230°C by electrical tapes, it is 1 metre long, 2 cm internal diameter, and of stainless steel E W 58J. Using a flow rate of 17-20 $l.hr^{-1}$ gives a residence time of approximately 1 minute.

(f) electrical heating tapes

These are of varying kilowattage and have fibre or quartz fabric insulation.

(g) temperature control sensors and thermocouples

These are placed on the reactor tubing and connected to control boxes.

(h) pressure relief valves

There are two of these, one on the inlet manifold with the pressure gauge, adjustable from 2.4×10^6 Pa (350 psi) to 1×10^7 Pa (1500 psi) and set to vent at 4.1×10^6 Pa (600 psi). The second is a stainless steel holder with bursting disc at the maximum temperature end of the reactor tube and is calibrated to burst at 4.6×10^6 Pa (675 psi) at 260°C or 8.7×10^6 Pa (1255 psi) at 15°C.

(i) needle, or orifice valve

This is at the end of the reactor.

(j) cooling coils

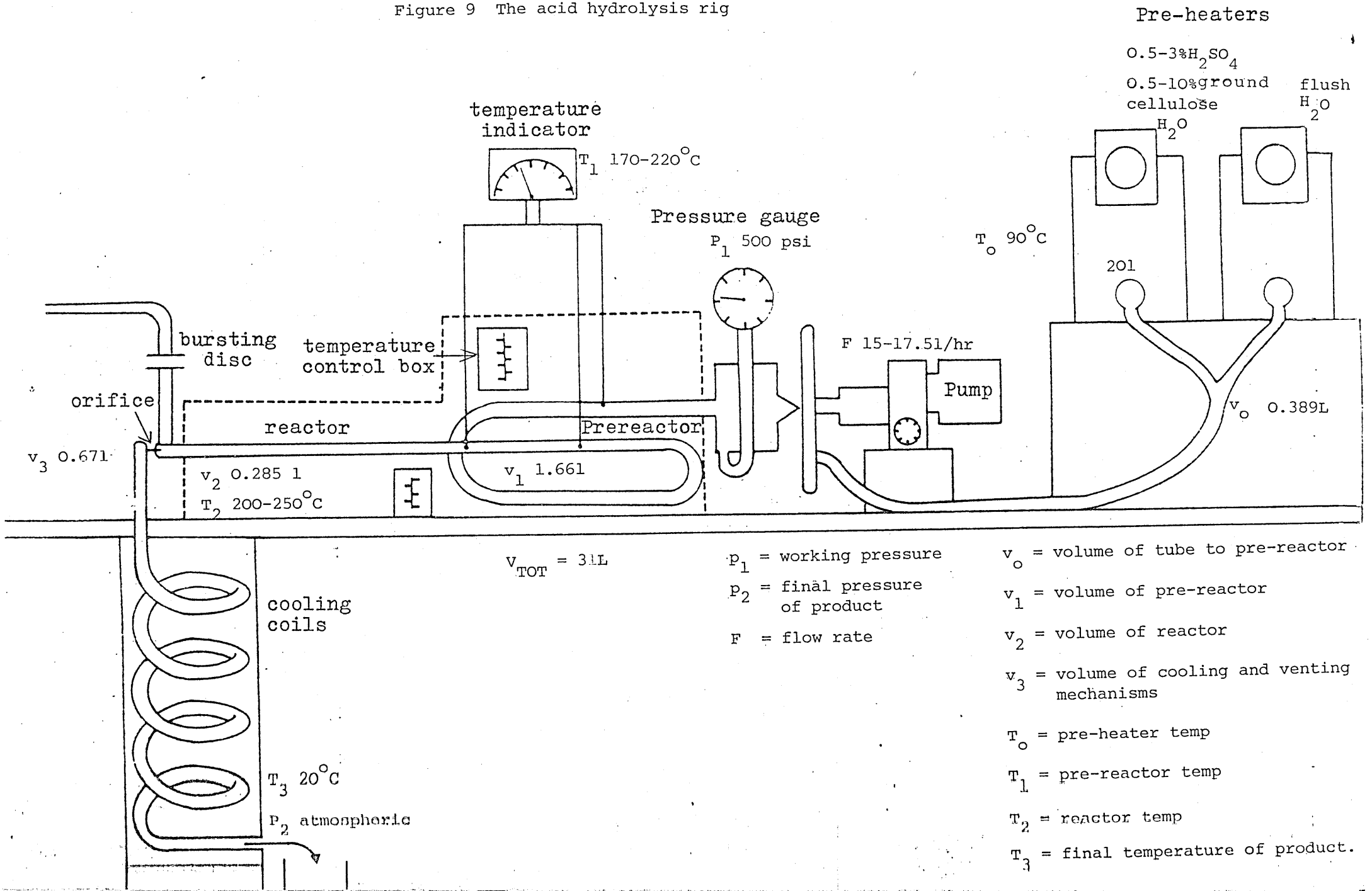
These are used to reduce acid vapour to liquid and to cool it to a manageable temperature.

Figure 9 shows the layout.

5.6 Collection and Cooling

Converse (1973) quenched his reaction by flashing to 150°C and then centrifuging to separate out the solids. At this point some of the solids (containing unreacted cellulose) were recycled to the slurry pre-heater. The liquid stream was neutralized with lime and entered a second centrifuge to removed unreacted lime and precipitated calcium sulphate. The sugar solution was concentrated in a series of long tube vertical evaporators. In the Scholler wood sugar process (Greaves 1945) the sugar solution was neutralised hot (60 - 70°), first with calcium carbonate which neutralised most of the sulphuric acid, and then with calcium oxide which neutralised the organic acids (formic, acetic, etc.). However, Harris et al. (1946) found that if the hydrolysate were neutralised hot (130° - 135°C) with lime and then flashed

Figure 9 The acid hydrolysis rig



p_1 = working pressure

p_2 = final pressure of product

F = flow rate

v_o = volume of tube to pre-reactor

v_1 = volume of pre-reactor

v_2 = volume of reactor

v_3 = volume of cooling and venting mechanisms

T_o = pre-heater temp

T_1 = pre-reactor temp

T_2 = reactor temp

T_3 = final temperature of product.

to atmospheric pressure, cooled to 30°C and then filtered, all traces of furfural and calcium sulphate could be removed.

The actual procedure followed was to flash to atmospheric, cool through a cooling coil and neutralise to pH 5.0 with sodium carbonate before use for fermentation.

5.7 Constructional Materials

It was necessary that all materials used in the rig were acid resistant, and capable of withstanding pressures and temperatures of up to 3.45×10^6 Pa and 230°C respectively. The additional chemicals needed were sulphuric acid, to be diluted to 1-2%, and sodium carbonate for neutralising the product.

5.8 Analytical Methods

During the running of the hydrolysis rig various physical and chemical tests need to be carried out. Table 5 summarises them.

Table 5 Physical and chemical tests during the running of the rig

	w/v solid/ liquid ratio	temperature	pressure	pH	cellulose content	sugar analysis	decomposition product
cellulose slurry	X	X			X		
pre-reactor constituents		X	X				
reactor constituents		X	X				
final product				X		X	X

Sugar analysis was by the Somogyi-Shaffer-Hartman method (Shaffer & Somogyi 1933) and by high pressure liquid chromatography. (Appendix 1). The former method was found suitable by Saeman et al. (1945) and was easily adapted to sugar concentrations as low as 0.2 $mg.ml^{-1}$ by increasing the boiling time to 30 minutes. The chromatographic method was far quicker and more accurate.

5.9 Safety

Safety valves and bursting discs were incorporated into the system and set to certain limits. Before each run the system was run on 90°C water, up to pressure and then the slurry was released. After each run the system was flushed with hot water.

CHAPTER 6

EXPERIMENTAL DETAILS OF THE TRIALS, THE RESULTS AND DISCUSSION

Details of the successful tests are listed below and the results in Table 7 (only successful trials have been numbered). Notes on the unsuccessful trials and problems are summarised in Table 8. Analysis of the sugars was by the SSH method and high pressure liquid chromatography (Appendix 1).

6.1 Trials

Trial 1 - Ground computer paper was the substrate. It proved difficult to grind and an evenly distributed slurry was not achieved because of the poor wetting ability of the paper. A needle valve was used (i.d. 1.19 mm); considerable blocking occurred behind this valve. The end product was a clear brownish liquid.

Trial 2 - In all trials from now on, ground filter paper was used (99%α-cellulose). The 'wetting' was good and the consistency of the slurry even. Balling occurred behind the needle valve. The end product was a clear brownish liquid. After this trial the rig was seriously blocked with unreacted paper. Following the experience

of Grethlein and Lang (1977) the needle valve was replaced by a fixed orifice valve. Valves of 0.25 mm - 0.75 mm i.d. were tested.

Trial 3 - A fixed orifice valve of 0.75 mm i.d. was used. A 10% slurry was used in error, however only a minor amount of blocking occurred. The end product was a brownish liquid containing some carbonised paper particles (identified microscopically).

Trial 4 - Fixed orifice valve blew a hole in the side.

Trial 5 - No mechanical problems.

Trial 6/7 - Blocking occurred early on in runs. Brownish-black end product containing charred paper particles. Despite the macroscopic colour, microscopically they resembled freshly ground filter paper particles.

Trial 8 - No mechanical problems.

Trial 9/10 - Pre-reactor temperatures were too high, sugars decomposed to volatile components such as furfural. Sample colour ranged from green through to brown and black, fluctuating during the 25 minutes of collection. The product was found to be contaminated with chromium, iron and nickel which could have come either from corrosion of the pre-heaters or the stainless steel tubing (Table 6).

Table 6 Metallic contamination (May 3 & 4)

Metal ion	ppm	gl^{-1}
Chromium	1400	1.4
Iron	6000	6.0
Nickel	1500	1.5
Copper	<1	---

These ions are roughly in the same proportions as their original alloy quantities in the type of stainless steel used for most of the pipe work. However on dismantling the reactor and pre-reactor no corrosion of the tubing was found to have occurred.

Trial 11 - Pre-reactor temperature kept lower, but no sugars, unidentified products were present, but not furfural.

Trial 12/13/14 - All pre-reactor temperatures were kept steady but one of the trials (13) failed to produce sugars. But no evidence of charred paper particles.

Table 7 Hydrolysis trials

Date	No.	Concentration % Paper	Concentration % Acid	Flow Rate l hr^{-1}	Pre-reactor °C Temperature	Pre-reactor Minutes Residence Time	Reactor °C Temperature	Reactor Minutes Residence Time	Sugar Concentration g l^{-1}	Yield % (dry basis)
1977										
Dec 9	1	1	1	15	170	6.84	200	1.14	0.448	6.0
15	2	0.5	1	15	170	6.84	200-230	1.14	0.096	1.2
1978										
Feb 6	3	10	1	15	170	6.84	200	1.14	0.23	3.0
9	4	1	2	15	170	6.84	200	1.14	0.304	4.0
13	5	1	2	15	170	6.84	200	1.14	0.18	2.0
March 6	6	1	2	15	170	6.84	230	1.14	0.026	0.4
14	7	1	2	16/17	170-200	6.05-6.43	230-249	1.01-1.07	0.06	0.7
1979										
April 25	8	2	2	17.5	199	5.88	240	0.98	5.0	30.0
May 3	9	1	2	17	232	6.05	232-245	1.01	<0.09	<1.0 (0.5)
4	10	1	2	17	230	6.05	232	1.01		0
17	11	1	0.5	17.5	180-220	5.88	250	0.98		0
June 5	12	1	0.5	17.5	180	5.88	230	0.98	0.1	1.2
13	13	1	3	17.5	190	5.88	250	0.98		0
18	14	1	1	17.5	200	5.88	250	0.98	0.9	11.0

Between trials 7 and 8, new valves, heating tapes, condenser, bursting discs and pre-heaters were fitted and tested.

The main problems with the failed tests were blockages in various parts of the equipment, and corrosion.

Table 8 Testing and failed runs

1977 December 7	Pressure trials, 90°C water
1978 January 23 June 21	New valve, pressure trials Fire in heating tape
1979 January 30 February 1 February 3 February 12	Pressure trials 90°C water:- 300 psi only reached 360 psi reached 500 psi reached satisfactory
April 78 - March 79	New parts because of pre-heater corrosion, valve blocking and unsatisfactory heating
1979 April 2/3 April 9/10 May 9	Bursting disc blew, small valve blocked with rusty particles. Pressure and temperature tests Bursting disc blew, pressure difficult to control.

6.2 Screening and Optimisation of the Variables

As mentioned in Chapter 5, the process variables (Table 9) may be screened and optimised by use of Plackett-Burman designs. Using an 8-trial design, flow rate, acid concentration and temperature difference between the pre-reactor and reactor were tested. (Table 10).

Table 9 Process variables and responses

		High Level −	Low Level +
X_1	flow rate $l.hr^{-1}$	16-17.5	15
X_2	acid concentration % $^{w}/_{v}$	2-3	0.5 - 1
X_3	temperature difference ^{o}C	40-60	<40

X_4-X_7 Unassigned factors to calculate standard deviation and minimum significant fact effect.

The minimum value for factor effect (MIN) to be significant is computed using the 4 unassigned factors as in Table 10. Using that value (1.71 see Table 10) as the cut off point to eliminate experimental error it can be seen that the temperature difference between reactor and pre-reactor (X_3 Table 10) is of some significance but the flow rate and acid concentration are not significant within the 90% confidence level. In Table 10 X_4 (unassigned factor), as all the others should be as near zero as possible if there

is to be minimal experimental error. However in this case an effect calculated from a dummy variable in which no change was made is appearing as a significant effect. The explanation of this could be 'confounding', in which the interaction of some of the other effects is producing an experimental error which is showing up as a significant effect. The results (responses) of the process in this case yield, are considered to be functions of the process variables and a study of them and their interactions may allow optimisation of the variables and the development of an empirical model. The following generalisations are made:

1 Over the experimental range of interest the response function is usually smooth; slopes and curves but not bumps and sharp kinks.

2 Interactions between the process variables occur and are common.

3 Experimental error occurs and is significant.

The experimental design to which the variables and responses have been fitted is Yates pattern, for 3 factors (process variables) 2^3 (8) trials are used: there is one critical response (yield). Some trials were duplicated. The results are shown in Table 11. The data from runs 8 and 14 have both been omitted because of the significant deviation of their yields from the other trials and from the mean, the $4\bar{D}$ method was used to decide this. (Appendix 2).

Table 10 Screening experiment

Trial	Rig Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Yield Y %
1	1	+	+	+	-	+	-	-	6
2	2	+	+	-	+	-	-	+	1.2
3	4	+	-	+	-	-	+	+	4.0
4	12	-	+	-	-	+	+	+	1.2
5	6	+	-	-	+	+	+	-	0.4
6	9	-	-	+	+	+	-	+	0.5
7	3	-	+	+	+	-	+	-	3.0
8	7	-	-	-	-	-	-	-	0.7
Sum +'s		11.6	11.4	13.5	5.1	8.1	8.6	6.9	
Sum -'s		5.4	5.6	3.5	11.9	8.9	8.4	10.1	
difference		+6.2	+5.8	+10.0	-6.8	-0.8	+0.2	-3.2	
effect (÷4)		+1.55	+1.45	+ 2.5	-1.7	-0.2	+0.05	-0.8	
					1.44	0.04	0.0025	0.64	$(UFE)^2$
					2.12				$\Sigma(UFE)^2$
					0.53 - variance				$\frac{\Sigma(UFE)^2}{4}$
					0.728 = S_{FE}				$\frac{\sqrt{\Sigma(UFE)^2}}{4}$

$(MIN)_{90} = 0.728 \times 2.353$

$= 1.71$

S_{FE} = significant factor effect

$(MIN)_{90}$ = minimum significant factor effect to 90% confidence level

2.353 - from Gosset distribution table of t-values at 90% confidence level, 3(n-1) degrees of freedom

at 95% confidence level $(MIN)_{95} = 0.7288 \times 3.182 = 2.3$ (still significant).

Table 11 Results of the three factor experiment

Trial	Rig run	Yield % Y_1	Y_2	$\bar{Y}$
1	7,13	0.7	0	0.35
2	6	0.4		0.4
3	11,12	0	1.2	0.6
4	2	1.2		1.2
5	9,10	0.5	0	0.25
6	4,5	4.0	2.0	3.0
7	3	3.0		3.0
8	1	6.0		6.0

Standard deviation = 2.03
Mean 1.85
Degrees of freedom $4(2-1) + 4(1-1)$
$= 4$

t value	90%	95%	99%	level of confidence
	2.132	2.776	4.604	

The computational analysis for the experiments is shown in Table 12. The design matrix has been supplemented with a computation matrix used to detect any interaction effects. The minimum significant factor effect (MIN) is derived from t - test significance criteria.

Table 12 Optimising the variables

Trial	Mean	DESIGN X_1	X_2	X_3	COMPUTATION X_1X_2	X_1X_3	X_2X_3	$X_1X_2X_3$	Y
1	+	−	−	−	+	+	+	−	0.35
2	+	+	−	−	−	−	+	+	0.4
3	+	−	+	−	−	+	−	+	0.6
4	+	+	+	−	+	−	−	−	1.2
5	+	−	−	+	+	−	−	+	0.25
6	+	+	−	+	−	+	−	−	3.0
7	+	−	+	+	−	−	+	−	3.0
8	+	+	+	+	+	+	+	+	6.0
Sum +	14.8	10.6	10.8	12.25	7.8	9.95	9.75	7.25	
Sum −		4.2	4.0	2.55	7.0	4.85	5.05	7.55	
Difference		+6.4	+6.8	+9.7	+0.8	+5.1	+4.7	-0.3	
Effect	1.85	+1.6	+1.7	+2.43	+0.2	+1.28	+1.18	-0.08	

$$(MIN) = t.s\sqrt{\frac{2}{m.k}}$$

s = standard deviation (Table 11)

t = 2.132 (90%) (Table 11)

m = number of plus signs in column = 4

k = number of replicates in each trial = 1.5

$$(MIN) = 2.132\ (2.03)\sqrt{\frac{2}{4(1.5)}}$$

$$= 4.33\sqrt{0.3333}$$

$$= 4.33\ (0.577)$$

$$= 2.498$$

6.3 Discussion

By looking at the calculated effects and using the (MIN) (Table 12) we see that the effect of the temperature difference between the two reactor stages is just under this figure and is not really significant at 90% confidence level. However, of the 3 factors it is the most significant. The rate of hydrolysis of cellulose to sugars and then to decomposition products increases 2-2.5 times for each 10°C rise in temperature between 160°C and 200°C. Looking at the trial results (Table 7), which were operated within these temperatures, the fluctuations which occurred in both the pre-reactor and the reactor for different runs indicate a possible reason for the difference in yields. A rough graphical analysis of reactor temperature, pre-reactor temperature, and temperature difference versus yield, showed that there was indeed some significance, especially for the temperature difference. The results of the Plackett-Burman analysis substantiates this claim (for this particular rig).

Apart from the temperature difference, the actual presence of a two-part reactor has been rate - limiting and error producing. The initial plan for overcoming the various problems of heat-up time, residence time at the critical temperatures, the necessity to add the acid at the beginning and the method of heating, was to pre-heat as far as possible without the hydrolysis reaction taking

place. This though has proved to be the major drawback and weakness of the rig, since the balance between the two sets of temperatures has proved to be significant. In addition, the length of the pre-reactor caused heating and pumping problems, and there was a lack of knowledge of what was going on inside, as evidence by the occasional appearance of charred particles and the two runs from which the sugars were contaminated with metal ions. However, this last problem has not occurred in any other subsequent runs.

The variation in acid contents, and the flow rate which are not obviously significant at 90% confidence level, may be shown to be so at the 75% level:

$(MIN)_{75}$ = 0.728 (Table 10) x 1.42 (t-value) = 1.03

Under the conditions of the rig the parameters proposed by Porteous (1967) were undertaken i.e. 230°C and reactor residence time of < 1.2 minutes. As seen in Table 7 the temperature varied because of control problems, but the residence time in the reactor proper was kept below 1.2 minutes (0.98 - 1.14). The acid concentration was higher than that recommended by Porteous; since the rate expression is affected by pH this added concentration probably accounted for the low yields produced especially in the later runs when the reactor temperature was at a high level too.

This method of screening the process variables and their optimisation has enabled the design of this hydrolysis rig to be analysed and assessed. The heating in two parts of the pre-reactor and reactor has been a major problem and is an area where future work could take place in order to eliminate the errors and facilitate the continuous running of this type of rig.

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PART III FERMENTATION

CHAPTER 7

CARBOHYDRATE METABOLISM AND FERMENTATION

Before discussing the fermentation and the types of organisms used, it is necessary to define various biochemical reactions concerned with carbohydrate metabolism by micro-organisms. The breakdown of carbohydrates (and of other compounds) is biochemically significant for two reasons: firstly the cell is provided with energy which is released as the carbohydrate breaks down to compounds of lower energy content, secondly, the cell is furnished with various other carbon compounds which are used in a variety of biochemical reactions.

It is now well established that there are several different metabolic pathways by which sugars can be fermented by micro-organisms, in all these pathways a key position is occupied by pyruvate. There are therefore several ways in which pyruvate is formed initially from the sugars, each pathway providing a characteristic amount of adenosine triphosphate (ATP a high energy molecule) per mole of substrate fermented.

7.1 · Hexose Diphosphate Pathway (Figure 10a)

Also known as the Embden-Mayerhof-Parnas (EMP) pathway, is the best documented glycolytic scheme, and the most commonly used by micro-organisms. The overall results of the breakdown of 1 molecule of glucose to pyruvic acid may be summarised as follows:

$$C_6H_{12}O_6 + 2\ NAD + 2\ ADP + 2P \rightarrow 2CH_3CO.\ COOH + 2\ NADH_2 + 2\ ATP$$

It is an anaerobic reaction and is the major energy-yielding pathway of many yeasts, fungi and bacteria.

7.2 Hexose Monophosphate Pathway (Figure 10b)

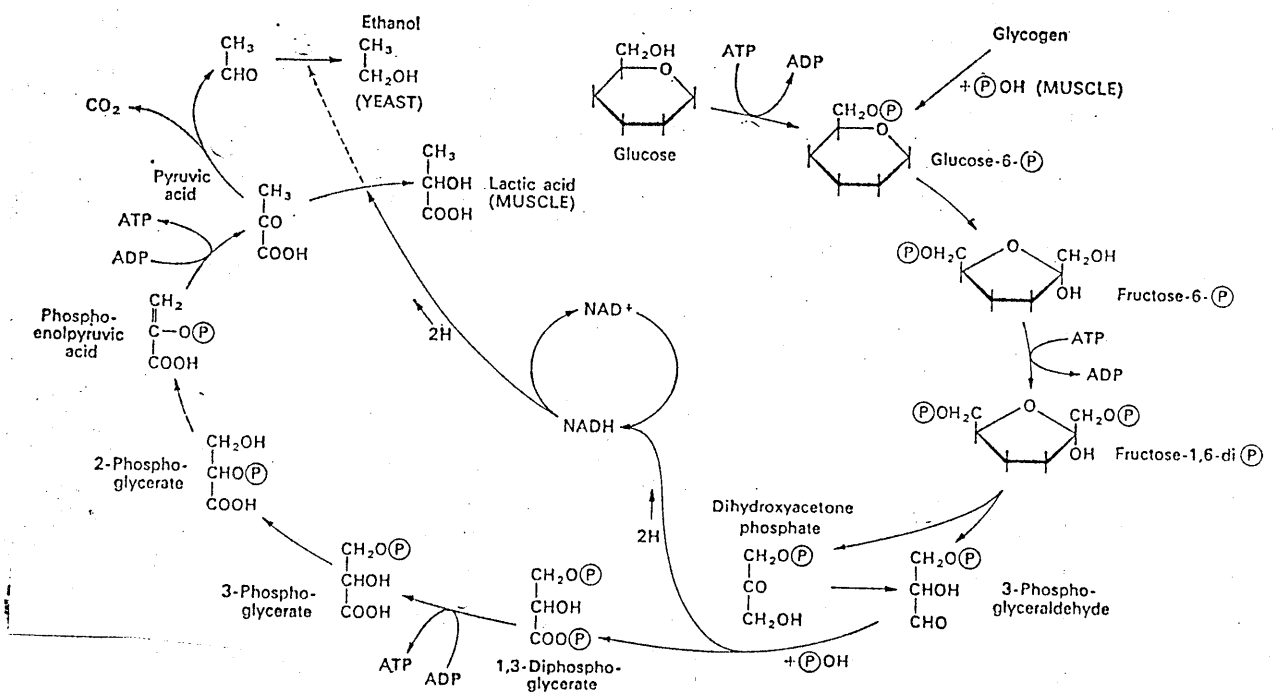
Many of the alternative schemes to EMP operate via hexose monophosphate.

(a) via pentophosphate

this is a 'shunt' mechanism occurring during the breakdown of sugars during the EMP scheme; its point of departure is the oxidation of glucose - 6 - phosphate. The scheme is variously known as the hexosemonophosphate (HMP) shunt, the Warburg-Dickens scheme and the pentose cycle. It is aerobic, and non-proliferating cells of S.cerevisiae and C.utilis are capable of utilising a substantial amount of glucose by means of this cycle. Although the cycle can provide for the anaerobic breakdown of carbohydrates, its

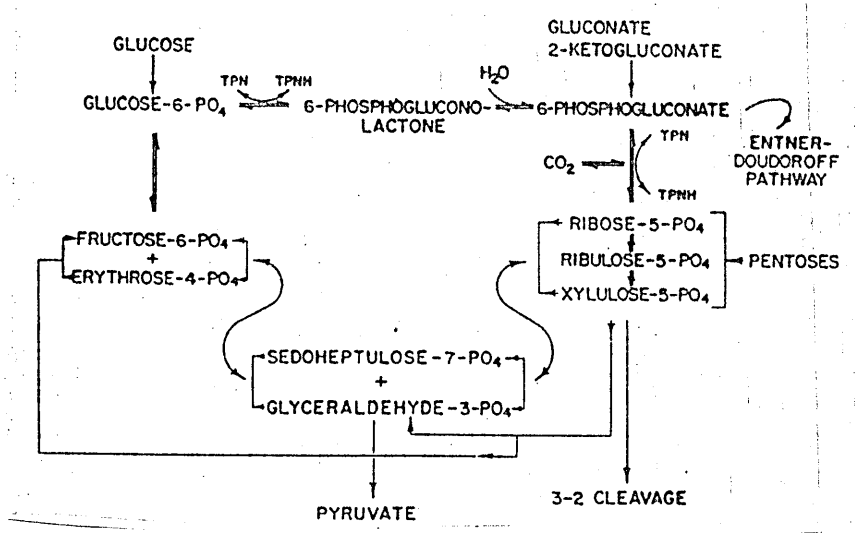
Figure 10 Carbohydrate metabolic pathways

(a) Embden-Meyerhof pathway

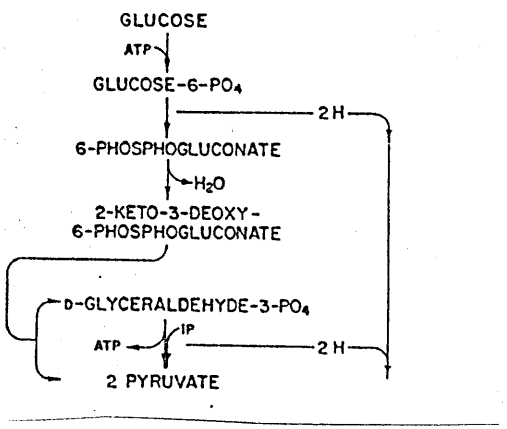


1a EM anaerobic

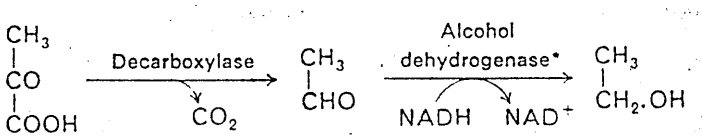
(b) Hexosomono phosphate shunt



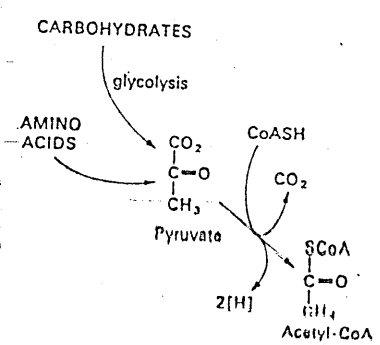
(c) Entner-Doudoroff pathway



(d) yeast alcoholic fermentation



(e) Pyruvate → Acetylco-enzyme A



significance in this respect is uncertain. It is known, however that Leuconostoc mesenteroides uses this pathway during the fermentation of glucose to lactic acid, ethanol and carbon dioxide.

(b) via 2-oxo-3-deoxy-6-phosphogluconate

Otherwise known as the Entner-Doudoroff pathway (Figure 10c). This is relatively rare, it has been reported in some bacteria including Pseudomonads and some species of Aeromonas, (Ramachandran and Gottlieb 1963) and in two fungi (Newburgh and Cheldelin 1958). It is known to be ineffective or absent in the lactic acid bacteria, Clostridia, propionic acid bacteria and the yeasts.

Once pyruvate has been formed, it may be used anaerobically or aerobically. As the formation of ethanol is of prime importance in this research below are the four possible routes for its formation from hexoses.

1. Yeasts, some other fungi and a few bacteria (e.g. Zymosarcina ventriculi) will anerobically convert pyruvate to ethanol via acetaldehyde. The complete cycle may be represented by:

$$\text{Glucose} + 2i\ P + 2\ ADP \rightarrow 2\ \text{ethanol} + 2\ ATP + 2H_2O$$

(2i P: inorganic phosphate)

Figure 10 (d)

2 moles of ethanol can be formed from 1 mole of glucose. In addition approximately 15.4 K calories of biologically useful energy are mobilised. Table 13 from Neish and Blackwood (1951) shows the main and subsidary products of this reaction.

Table 13 Ethanol production by yeast
(condensed from Neish and Blackwood 1951)

Product	mMoles 100 mmoles^{-1} of glucose fermented		
	pH 3.0	pH 6.0	pH 7.6
2, 3-Butanediol	0.75	0.39	0.33
Acetoin	Nil	Nil	0.01
Ethanol	171.5	165.9	148.0
Glycerol	6.16	10.4	25.1
Mixed acids*	2.36	7.99	11.24
Carbon dioxide	180.8	178.0	167.8
Glucose carbon assimilated	12.4	---	---
Fermentation time, hr.	29.0	16.0	32.0
Glucose fermented, %	98.5	98.5	98.1
Carbon recovered, %	93.8	94.0	94.1

*Butyric, acetic, formic, succinic and lactic

Also a mixed acid fermentation of glucose occurs via the EMP pathway yielding, lactic, acetic and butyric acids as well as ethanol by such organisms as E. coli.

2. The only bacterium which exhibits an almost pure ethanolic fermentation, as displayed by yeast is Pseudomonas lindneri (Zymomonas mobilis), but the pathway to pyruvate is the Entner-Doudoroff not the EMP (Horecker 1962). This organism is one of the principals in the mixed fermentation of cactus fruit to the Mexican drink pulque.

3. During aerobic metabolism the pyruvate formed is converted to acetyl coenzyme A (Figure 10e). Then via the TCA (tricarboxylic acid) cycle, (not shown), carbon dioxide and water are formed as the final products. Two types of bacteria, the Enterobacteriaceae and Clostridia form acetylco-A from pyruvate by a cleavage reaction involving lipoic acid. A reduction to acetaldehyde and then to ethanol follows. The maximum yield of ethanol is 1 mole per 1 mole of glucose.

4. In the last route organisims such as Leuconostoc mesenteroides ferment glucose to yield lactate, ethanol and carbon dioxide (Horecker 1961). The production of these is via pentophosphate (HMP shunt).

7.3 Pentose Fermentation

The glycolytic pathways previously discussed pertain to hexoses. The fermentation of pentoses does not occur through those pathways; there are two distinct different patterns (Gunsalus and Stanier 1961).

1. A cleavage of the pentose molecule resulting in the formation of lactate and acetate, with the end products of lactic and acetic acids. This is restricted to the lactic acid bacteria and Fusarium.

Pentose + 2iP + 2ADP → lactate + acetate + 2ATP + H_2O

2. Involves the synthesis of hexose from the pentose molecule.

3Pentose + 5iP + 2ADP → 5 pyruvate + 10H + 5 ATP + $5H_2O$

After the formation of pyruvate, ethanol formation can proceed. In virtually all organisms the enzymes for the initial steps in pentose metabolism are inducible. (Gunsalus and Stanier 1961). The production of these adaptive enzymes may be encouraged by growing the organism on a pentose prior to its use as a pentose fermenter, as demonstrated by Karström (1938), Lampen et al. (1951) and Cohen et.al. (1951).

CHAPTER 8

THE KINETICS OF BATCH AND CONTINUOUS CULTURE OF MICRO-ORGANISMS

8.1 Microbial Growth

The cells of all micro-organisms consist of carbohydrate, protein, lipids, nucleic acids and vitamins. The exact composition depends upon the species or strain. During growth each biochemical change is catalysed by a specific enzyme and within a culture medium, the components are changing all the time, being depleted of those substances which the cells need, and being enriched by excretory products.

When a micro-organism is inoculated into a nutritionally balanced medium it starts to grow and divide. The metabolism of this cell in the microbial culture falls into four well defined stages (lag, logarithmic, stationary and death) as described by Monod (1949).

The variation in metabolism through these phases is dependent on the concentration gradients of the substrate and metabolites in the immediate environment.

When microbial cells are transferred from one medium to another, a period elapses before a constant rate of growth is established. This is the lag phase. A variety of physical and physiological conditions govern this phase, such as the previous growth medium, the nutritional substances present or absent in the new medium and the method of cultivation technique. During this phase there is little or no visible increase in the number of cells in the culture, but it is a period of intense metabolic activity during which the cells become adapted to the new conditions. If the organism has already adapted to this medium (in a previous culture) then the lag phase is considerably shortened or is non-existent.

Once a constant growth rate has been achieved, growth will continue at the maximum rate possible, given that all of the factors are at a steady state. This is the log or exponential phase, when the cells are in a state of balanced growth and are dividing at a constant rate. The growth rate is proportional to the concentration of biomass; the logarithm of the number of cells plotted against time yields a straight line graph. The time taken for the population to double in size (generation time g) is given by the equation:

$$g = \frac{t_2 - t_1}{3.32\ (\log X_2 - \log X_1)}$$

where the concentration of organisms at time t_1 is X_1
the concentration of organisms at time t_2 is X_2

The generation time may be 20-30 minutes for some bacteria or several days for slower organisms.

Eventually the concentration of nutrients decreases, and growth and reproduction slow down (negative acceleration of growth). The concentration of nutrients eventually drops so low that the specific growth rate approaches zero. In addition the waste products of metabolism will have accumulated probably making the medium undesirable for growth. A stage is reached when the rate of multiplication equals the rate of death (stationary phase). At this point the number of viable cells is constant. It is not only the lack of nutrients which is responsible for this phase, but also the possible rise in temperature, alteration of pH value and the presence of inhibiting products. This phase may last for a considerable period of time.

In the death phase, the rate of death is faster than multiplication; the cells cease to reproduce and the number of viable cells (not the total number of cells) drops steadily. This phase too may extend over a long period of time.

8.2 Types of Culture

The growth phases described occur naturally in a batch culture, which is a spatially closed and constant volume of culture medium, where the concentration of the nutrients decreases and the amount of products increases until growth finally stops. The final mass and volume of cells depend on the size of the initial inoculum, the starting concentration of nutrients and the sum of conditions determining the process (O_2, pH, temperature). To operate the batch system economically the process must be stopped at the end of the log phase, and the cells or products harvested.

The basic arrangement for a batch fermenter (B.F.) is a deep tank in which motion of the liquid is induced by either mechanical stirring, or by the evolution of a gas as a biochemical product or by bubbling air through the medium. This last provides the free O_2 demanded by aerobic processes. Industrially the capacity of the vessel may range from a few hundred to several thousand gallons. The time required for a batch fermentation varies from hours to weeks. Throughout this time contamination must be avoided, the contents kept agitated and their temperature and pH controlled.

The design problems associated with these fermenters include the specification of size of vessel, the process time, the initial nutrient concentrations, the volume of microbial mass per unit volume of fermenter, the power and aeration requirements and the area of heat transfer surface.

If fresh nutrients could be added to the culture and at the same time, the waste products removed, it should then be possible to maintain the micro-organisms indefinitely in the exponential phase. Much research work has been done on continually growing cultures such as that by Abbott & Clamen (1973), Mateles (1971), and Humphrey (1968).

The logical conclusion of this has been to the continuous culture in which a continuous supply of nutrients is fed into the fermenter, while biomass and byproducts are bled out. During an ideal homogeneous continuous culture the log phase of microbial growth is extended by maintenance of a constant unchanging environment. The nutrients must enter the system, of constant volume, at the same rate as they are used up or leave the system. This type of culture can be characterized ideally as a system of constant volume in a steady dynamic state with a constant concentration of all necessary components. The reactions proceed at a constant rate but are independent of time.

Mathematically the stoichiometric relationship (Monod 1942) between organic substrate consumed and micro-organisms produced in either batch or continuous culture is usually expressed as a:

$$\text{yield } \frac{dx}{dt} = -Y\frac{ds}{dt} \qquad Y = \frac{\text{weight of organisms formed}}{\text{weight of substrate consumed}}$$

over any finite period of time during exponential growth phase. Similar relationships can be established for the formation of products. Y, the yield coefficient is a function of the species of organism, the type of substrate and the environmental conditions. Factors which affect it are the utilisation of the substrate to provide maintenance energy, the formation of storage products and changes in the concentration of viable organisms.

The rate of increase, the specific growth rate, (μ) is proportional to the concentration (s) of the limiting factor in the system.

$$\mu = \mu_m \left(\frac{s}{K_s+s}\right) \qquad (1)$$

where μ_m is the maximum value of μ at saturation level of the substrate and K_s is a saturation constant numerically equal to the substrate concentration at which $\mu = 0.5\ \mu_m$. It is a measure of the affinity of the organism for a substrate, the higher the substrate affinity the lower the K_s value. The growth rate is the actual rate of increase of concentration of organisms $\frac{dx}{dt}$. The specific

growth rate is the rate of increase per unit of organisms concentration:

$$\therefore \quad \frac{dx}{dt} = \mu x \qquad (2)$$

In batch culture, when nutrients are initially present in adequate concentrations during the exponential growth phase the growth rate is generally equal to μ max.

An important characteristic of continuous cultures is the dilution rate (D), that is the number of complete volume changes per hour ($\frac{\text{flowrate}}{\text{volume}}$). The reciprocal of this value is the mean residence time of the culture. All continuous cultures start their existance as batch cultures, however if during the log phase fresh medium is added at a rate sufficient to maintain the culture population density at a submaximal value, then growth should continue indefinitely. If the nutrient is added too quickly the culture will be washed out of the vessel, if too slowly the growth rate will be diminished. In a continuous culture with one growth limiting substrate,

increase = growth - output

$$\text{or} \quad \frac{dx}{dt} = (\mu - D)\, x \qquad (3)$$

If $\mu > D$, $\frac{dx}{dt}$ will be positive and the concentration of organisms will increase. If $\mu < D$, $\frac{dx}{dt}$ will be negative, the number of organisms will decrease and will eventually be 'washed out' of the culture vessel.

Only when $\mu = D$ will $\frac{dx}{dt} = 0$ and x the concentration of organisms will be constant. At this stage a steady state will exist. The continuous culture of organisms in a chemostat depends on providing such conditions so that μ and D can be equal and invariant with time.

One must also consider the effect of D on the concentration of organisms formed and of the substrate (Monod 1950). By substituting equation 1 into equation 3:

$$\frac{dx}{dt} = \left[\mu_m \left(\frac{s}{K_s+s}\right) - D\right] x \qquad (4)$$

The net change in substrate concentration resulting from passage through the culture vessel = input- output - consumption

$$\text{or} \quad \frac{ds}{dt} = Ds_r - Ds - \frac{\text{growth}}{\text{yield}} = D\,(s_R - s) - \frac{\mu x}{Y} \qquad (5)$$

where Y (over any finite period of growth) = $\frac{\text{weight of bacteria formed}}{\text{weight of substrate used}} \frac{x}{s_R - s}$; s_R is the substrate concentration entering the culture vessel from the reservoir and s is that concentration leaving the culture vessel.

Substituting for equation 1:

$$\frac{ds}{dt} = D\,(s_R - s) - \frac{\mu m}{Y} x \left(\frac{s}{(K_s+s)}\right) \qquad (6)$$

When equations are solved for $\frac{dx}{dt} = \frac{ds}{dt} = 0$ i.e. the steady state when unique values of x and s exist:

$$\text{then } x = Y\ (s_R - s) \tag{7}$$

$$\text{and } \quad s = K_s\ \left(\frac{D}{\mu_m - D}\right) \tag{8}$$

Substituting s from equation 8 into equation 7 then

$$x = Y\left[s_R - K_s\ \left(\frac{D}{\mu m} - D\right)\right].$$

From equations 7 and 8, if x s D and s_R can be measured, then Y, K_s and μ_m may be calculated. They will be constant for any given organism and growth medium. Then the steady-state concentrations and micro-organisms and substrate in the vessel may be predicted for any value of D and s_R. It follows that product formation also has a yield coefficient associated with substrate consumption, and since product formation cannot occur without cells, it is closely coupled with growth and/or cell mass concentration

product accumulation	=	product synthesis	-	product removal	-	product destruction

$$\frac{dP}{dt} = q_p\ x - DP - KP$$

Where q_p is the specific rate of product accumulation g g cell mass^{-1} hr^{-1} independent of cell mass concentration. It describes the effectiveness of the cells in product synthesis or material utilization. It is most useful for comparing results between fermentations.

The volumetric rate Q g.l^{-1} hr^{-1} is dependent on cell mass concentration and describes the rate of product synthesis or material demand per unit of fermenter capacity. Also in the case of primary metabolites such as ethanol from glucose

$$\frac{dP}{dt} = \frac{\mu x}{Y_{p/c}}$$

where $Y_{p/c}$ is the g product g cell^{-1}.

Productivity P (volumetric) g l^{-1} hr^{-1} in batch cultures is a measure of the overall performance of the process.

In continuous culture the fundamentals of product formation have been described by Fencl (1966). P = Dx and qp (specific rate of product accumulation) = $\frac{DP}{x}$. So as D decreases P increases and efficiency of conversion of sugar to product also improves. Bazua & Wilke (1977) calculated growth and production rates for ethanol production from glucose by S.cerevisiae using the following equations:

$$\mu_{max} = \mu_o \left(1 + \frac{\bar{P}}{P_{max}}\right)^{\frac{1}{2}}$$

and

$$V_{max} = V_o \left(1 + \frac{\bar{P}}{P_{max}}\right)^{\frac{1}{2}}$$

where u_o and V_o are the productivity rates at P = 0 and P_{max} is the highest concentration of ethanol that can be tolerated before either growth limitation or ethanol production limitation occurred. A different value in each case.

When there is more than one substrate present as a nutrient source for the growth of an organism, the nutrients will usually be utilised sequentially. This phenomenon was named 'diauxie' by Monod (1942). In batch cultures this represents a sequence in which growth takes place successively on substrates which are all present at the beginning of growth and which are consumed successively during the period.

In the case of a continuous culture containing more than one substrate the situation differs in that the organisms are always exposed to all substrates and might either utilise all simultaneously or attack one or the other depending on the growth and dilution rates.

The physiological explanation of the sequential utilization may be in two different ways:

(a) enzyme repression

The enzymes necessary for the utilisation of the various other substrates are not synthesised by the microbial culture in the presence of the repressor substrate. This was originally called the 'glucose effect' but it has since been demonstrated that it is not caused by glucose itself but by the products originating from the catabolism of the readily accepted or available carbon source.

(b) enzyme inhibition

The enzymes are formed but remain inactive, inhibited by either the other carbon sources or their intermediary catabolites.

Chian and Mateles (1968) showed that in pure cultures of an organism there was simultaneous uptake of both glucose and fructose at low dilution rates. At higher dilution rates, when substrate concentration increases, the cultures showed diauxic behaviour.

Yoon et al. (1977) described the growth of an organism on two substrates

$$\frac{dx}{dt} = x\ (\mu - D) \tag{10}$$

and

$$\frac{ds_1}{dt} = D\ (s_{R1} - s_1) - \frac{\mu_1 x}{Y_1} \tag{11}$$

$$\frac{ds_2}{dt} = D\ (s_{R2} - s_2) - \frac{\mu_2 x}{Y_2} \tag{12}$$

$$\mu = \mu_1 - \mu_2 \tag{13}$$

At a steady state the following linear relation can be obtained by eliminating μ_1 μ_2 and μ from equations 10 - 13.

$$x = Y_1\ (s_{R1} - s_1) + Y_2\ (s_{R2} - s_2) \tag{14}$$

It is difficult to find the dependency of μ_1 and μ_2 on s_1 and s_2 experimentally because a biomass measurement only represents total growth.

There are two possible steady-states:

1. the washout state where $x = 0$

$$s_1 = s_{R1}$$

$$\text{and} \quad s_2 = s_{R2}\ .$$

This can be obtained when $D > \mu_m$.

2. When the organism is present and equation 14 is true. It can be obtained when $D < u_m$ and for all possible combinations of s_1 and s_2, $u_m \quad u_{m1} + u_{m2}$.

For competition by 2 organisms on 2 substrates (Yoon & Blanch 1977).

$$\frac{dx_1}{dt} = (u_1 - D)\, x_1 \tag{15}$$

$$\frac{dx_2}{dt} = (u_2 - D)\, x_2 \tag{16}$$

$$\frac{ds_1}{dt} = D\,(s_{R1} - s_1) - \frac{u_1 x_1}{Y_{11}} - \frac{u_2 x_2}{Y_{21}} \tag{17}$$

$$\frac{ds_2}{dt} = D\,(s_{R2} - s_2) - \frac{u_1 x_1}{Y_{12}} - \frac{u_2 x_2}{Y_{22}} \tag{18}$$

where Y_{11} is the yield constant given by the effect of substrate 1 on organism 1;

Y_{21} is the yield constant given by the effect of substrate 1 on organism 2;

Y_{12} is the yield constant given by the effect of substrate 2 on organism 1;

Y_{22} is the yield constant given by the effect of substrate 2 on organism 2;

and

$$u_1 = u_{m1} \frac{s_1 s_2}{(K_{11} + s_1)(K_{12} + s_2)} \qquad (19)$$

$$u_2 = u_{m2} \frac{s_1 s_2}{(K_{21} + s_1)(K_{22} + s_2)} \qquad (20)$$

The above model equations yield four possible steady states:

1 both organisms coexist

2 organism 1 exists

3 organism 2 exists

4 both organisms are 'washed out'

For steady state 1 to occur the population densities of the two organisms, and dilution rates and the concentration of substrates must satisfy equations 15 - 18 equalling 0.

There will be a range of conditions which might support steady-state populations of organisms 1 and 2.

s_R and s

At a steady state, $D = u_1 = u_2$ and equations 17 and 18 must yield positive values of x_1 and x_2. from these equations the following may be obtained:

$$\frac{x_1}{Y_{11}} + \frac{x_2}{Y_{21}} = s_{R1} - s_1 \qquad (21)$$

$$\frac{x_1}{Y_{21}} + \frac{x_2}{Y_{22}} = s_{R2} - s_2 \qquad (22)$$

The solution of equations 21 and 22 may be obtained and from them the feed concentration of substrates 1 and 2 can be found and should be larger than the steady-state concentrations of substrates which satisfy equations 15 and 16.

D

There will be a range of dilution rates rather than a point value.

u_m

If $u_{m1} = u_{m2}$ then competition depends on the value of saturation constants.

CHAPTER 9

CHOOSING THE ORGANISMS

The types of sugars present following the acid hydrolysis of cellulose materials include both hexoses and pentoses; a mixture of glucose and smaller amounts of xylose, arabinose, galactose and mannose. Different species of micro-organisms (fungi and bacteria) and also different strains within the species are able to utilize widely varying substrates. Two common examples are: Saccharomyces cerevisiae which is able to ferment only hexoses, and Candida utilis which can ferment hexoses and be adapted to grow on pentoses.

When considering the use of these sugars for the production of ethanol, or other chemicals, rather than SCP, it has been necessary to differentiate between fermentable and non-fermentable sugars. Generally if a sugar has been labelled non-fermentable it has been thought of in terms of S. cerevisiae only. There are, however, many organisms which will ferment pentoses, as well as hexoses, to ethanol and other by-products. The fermentation tests practised by Saeman et al.(1945) and the Herich-Toth-Osztrovsky method cited by Snell and Hilton (1966) where only S. cerevisiae is used do not give an accurate analysis and must be adapted for the evaluation of the ability of other micro-organisms to ferment a wide variety of sugars

if the true fermentability of the cellulose hydrolysate is to be known. From both an economic and pollution prevention aspect it is advantageous that all sugars present in the acid hydrolysis product prove to be fermentable by some organism. (A fail-safe may be provided by using the particular sugar as a growth substrate for SCP).

Both fungi and bacteria are able to ferment a variety of carbohydrates. Fungi are commonly thought of as strictly aerobic organisms, as they cannot grow without oxygen. However many fungi can utilise carbohydrates anaerobically to produce 'typical' fermentation products. Ethanol formation is quite common, especially in the true yeasts, the Mucorales and Fusaria. In addition, certain members of the genus Aspergillus, ordinarily thought of as strongly oxidative (A. clavatus and others) have been shown to have an extremely high capacity for alcoholic fermentation, (Foster (1949) and Cochrane (1958)). In attempting to find possible micro-organisms to fully utilise the acid hydrolysis sugars consideration must be given to those already employed in the fermentation industries. These may be placed in one of three groups: Yeasts, other fungi and bacteria and it is under these headings that their possible use in relation to previous research on wood hydrolysate sugars will be discussed, (wood sugars may be used to represent the sugars that might be expected from the acid hydrolysis of cellulosic wastes).

For industrial use a micro-organism must have amongst other characteristics the ability to:

1 propagate readily on the substrate

2 reproduce itself in large quantities or to produce quantities of useful chemicals, as required

3 maintain uniform growth or fermentation rates

4 grow or ferment without the use of special treatments or the addition of extra substrates other than a few cheap inorganics to boost the nutritional levels of Nitrogen, Potassium and Phosphorus

9.1 Yeasts

Many yeast species have been identified, all varying widely in choice of habitat and nutritional requirements. The types that would be obvious to investigate are those similar to the ones in use in the beers, wines, spirits and bread making industries, Saccharomyces species; and also the 'feed and food' yeasts (i.e. edible) such as Torula species.

Often a yeast (and indeed other micro-organisms) can be persuaded to utilise a particular sugar only after a period of adaption. Johnson and Harris (1948) experimented

with various types of yeast, using Douglas Fir hydrolysate as the carbohydrate substrate. During World War II, Torula yeast species had been adapted to grow on this substrate, so they attempted to acclimatise other yeasts, both to propagate and to produce alcohol. Their results may be seen in Table 14.

Six of the eight Torula species showed good initial sugar utilisation as did C. albicans, S. anonensis and the unidentified yeast (x). S. ellipsoideus was also initially quite well adapted. These strains showed little change in utilisation at the 12th transfer but the percentage yield was increased. All yeast strains showed some improvement in sugar utilisation and yeast cell yield between the first and 12th transfers. It may be noted that only three of the eight Torula species were initially well adapted to utilise the sugar and produce a good yield of alcohol, as also were C. albicans and C. arborae, but that improvements occurred between the 1st and 12th transfers.

In this study, for both yeast growth and alcoholic fermentation, it was found necessary to transfer the cells regularly to new sugar solutions during the acclimatization procedure. If the yeasts were left in contact with a spent solution after the necessary time for the complete utilisation of the sugars, autolysis of the remaining cells and an inhibition of activity resulted. During the thirty or so consecutive transfers, the cells became discoloured and small but still remained active.

Table 14 Growth and ethanol production by various yeast strains in wood hydrolysate (Johnson and Harris 1948)

Yeast	Growth				Alcohol Production			
	% Sugar utilisation		% Yeast yield		% Sugar utilisation		% ethanol yield	
	1st transfer	12th transfer	1st	12th	1st transfer	12th transfer		
Torula utilis								
major	78	82	25	42	70	84	18	37
thermophilus	78	80	33	38	60	82	18	35
No. 2	78	82	25	38	64	75	18	31
No. 900	47	79	20	38	50	80	13	36
No. 3	50	88	22	37	47	85	10	40
No. 660	80	83	32	35	71	82	33	35
No. 793	80	85	31	38	71	80	33	33
No. 957	80	82	30	37	74	79	34	36
Candida								
albicans	81	94	32	41	72	80	32	37
arborae	45	86	21	35	74	79	34	35
arborae No. 17	30	83	15	37	64	81	24	34
arborae No. 198	25	84	12	37	66	82	25	33

/Continued .....

Table 14 Continued

Yeast	Growth				Alcohol Production			
	% Sugar utilisation		% Yeast yield		% Sugar utilisation		% ethanol yield	
	1st transfer	12th transfer	1st	12th	1st transfer	12th transfer		
Mycotorula								
lipolytica No. 1094	10	84	5	36	5	77	1	35
Unidentified X	75	83	30	35	55	80	21	36
Hansenula								
anamala	50	80	21	33	17	81	1	37
suaveoleus No. 838	25	82	11	31	5	80	1	37
Saccharomyces								
anonesis	75	84	30	36	53	78	20	36
cerevisiae No. 46	50	83	22	30	27	81	10	32
ellipsoideus	60	80	27	35	33	81	23	37

Leonard and Hajny (1945) tested Saccharomyces species as well as Torula species. They found that the Torula species and S. ellipsoideus were slower fermenters than S. cerevisiae, C. tropicalis and S. anonensis. The work by Johnson & Harris, (1948) does not support this, but considering the varying strains within the species and the unlikelihood of both sets of investigators using the same ones, this is not surprising.

Concerning the use of pentoses; Plevako reported a weak fermentation of xylose by Monilia murmanica and Kadriavzev found that some species of Hansenula, Zygowillia, Deboromyces and Schwanniomyces could use some pentose sugars (both cited in Karczewska (1959)).

Karandikar (1971) compiled a set of batch fermentations on sugars from hydrolysed ground refuse. A continuation of this work has been carried out by Converse et.al. (1973). The sugars from the hydrolysed refuse were filtered and neutralised with calcium carbonate. The precipitate of calcium sulphate is inhibiting to yeast and had to be removed by heating to 100°C for 15 minutes to eliminate it more or less completely. Two levels of temperature, 30° and 35°C and two levels of pH, 4.4 and 5.0, were used in the trials.

Also two levels of initial sugar concentration, 4% and 12% and two levels of initial micro-organism (S. cerevisiae)

concentration, 10 and 50 million ml^{-1} (0.7 and 3.4 g l^{-1}) were used. The yield of ethanol was not significantly affected by a variation in either of these last two variables. However, a higher rate of fermentation was obtained for the higher cell concentration and the higher initial sugar concentrations.

With respect to the temperature and pH, a higher rate of fermentation was obtained at 30°C and pH 4.4; since the optimum temperature for yeast activity is about 27°C, 30°C is a little too high and 35° much too high! If Table 15 (composed from information in Converse et al. op.cit) is examined it may be seen in columns 6 and 7 that at pH 5.0 and 35°C alcohol production dropped off around 4 - 6 hours compared with 30°C results but caught up again after 10. Thus with both unfavourable conditions, the yeast had to become acclimatized before a yield equivalent to that of only 1 unfavourable condition was achieved. At pH 4.4 (Table 16) the temperature effect was not so noticeable.

The conditions for fermenting S. cerevisiae in a low concentration of sugar may be likened to that of brewing beer. During that process the temperature naturally rises rapidly during fermentation from 10°C to about 22°C and then drops back to 15°C or less as fermentation ends. Similarly the pH starts about 5.6 and drops to 3.9 - 4.4 approximately. As long ago as 1892 Max Delbruck

Table 15 Fermentation tests on hydrolysed ground refuse (i) (Converse et al. 1973)

pH 5.0

Time (hours)	Cell concentration (millions ml^{-1})			Ethyl alcohol produced (g 100 ml^{-1})			Sugar concentration (g 100 ml^{-1})		
	30°C	35°C	35°C	30°C	35°C	35°C	30°C	35°C	35°C
1	2	3	4	5	6	7	8	9	10
0	48.2	50.0	49.5	0.075	0.075	0.125	4.25	4.50	4.2
2	49.2	50.2	49.75	0.2	0.2	0.25	3.75	3.90	3.7
4	51.8	50.75	50.1	0.67	0.5	0.4	2.20	3.35	2.6
6	55.9	51.8	50.75	1.3	1.25	0.95	1.30	1.94	1.75
8	58.6	52.8	52.0	1.75	1.75	1.6	0.22	1.10	1.0
10	59.7	53.8	53.1	1.95	2.1	1.92	0.06	0.24	0.22
11		54.0	53.3		2.15	2.0		0.05	0.048

Table 16 Fermentation tests on hydrolysed ground refuse (ii) (Converse et al. 1973)

pH 4.4

Time (hours)	Cell concentration (millions ml^{-1})		Ethyl alcohol produced (g 100 ml^{-1})		Sugar concentration (g 100 ml^{-1})	
	30°C	35°C	30°C	35°C	30°C	35°C
0	48.45	45.3	0.05	0.1	4.10	3.95
2	48.8	45.65	0.20	0.2	3.50	3.575
4	49.9	47.0	0.60	0.6	2.65	2.45
6	52.6	49.4	1.30	1.3	1.14	1.10
8	54.2	51.25	1.82	1.76	0.206	0.16
9	54.6	51.6	1.90		0.056	
10				1.80		0.034

investigated the possibilities of accelerating the fermentation process. His experiments with an increased amount of yeast (up to 20 times the usual, which is 2g l^{-1}) at a higher temperature showed that the yeasts did not grow, they only fermented, at 30°C. (Mitchell's results (1973) disagree with this.)

From Table 17 (Converse et al.) it may be seen that in general when there is a high concentration to start with (50 million ml^{-1}) the increase in yeast cell concentration during the runs is slight, 7.1 - 23.8%. The higher percentage (18 - 24) occurred rather erratically, run 4 probably because of the lower temperature and in run 9 because of a longer incubation time. It is significant that in runs 10 and 11 where the initial concentration was much lower, the yeast tended to grow, but that a similar yield of alcohol was achieved presumably because of the longer running time.

Mitchell (1973) used C. utilis on refuse hydrolysate but did not acclimatise the cells. Growth of yeast did not start for 18 hours on average and was slow up to 24 hours. Using Black-Clawson fibre claim refuse he obtained approximately 1.4 gm dry yeast 100 ml^{-1} hydrolysate. However, using laboratory refuse which consisted of 80% food wastes, only 0.5 gm dry yeast 100 ml^{-1} were obtained. In tests Nitrogen and Phosphorus had to be added to the hydrolysate.

Table 17 Summary of experiments (Converse et al. 1973)

Run No.	Operating Conditions Temp. (°C)	pH	Initial concentration Sugar (g 100 ml^{-1})	Yeast (millions ml^{-1})	Run Time (hours)	% ethanol yield $\frac{\text{ethanol produced}}{\text{sugar present}}$	Increase in yeast during the run (millions ml^{-1})	% Increase
1	35	5.0	4.50	50.0	11.0	47.8	4.0	8.0
2	35	5.0	4.20	49.5	11.0	47.6	3.8	7.7
3	35	5.0	3.90	46.3	10.75	48.7	3.3	7.1
4	30	5.0	4.25	48.2	10.0	45.9	11.5	23.8
5	30	4.4	4.10	48.45	9.0	46.4	6.15	12.7
6	35	4.4	3.95	45.3	10.0	45.6	6.3	13.9
7	30	4.4	4.15	48.2	10.0	47.0	11.1	23.0
8	35	5.0	4.10	49.1	12	47.6	4.5	9.2
9	35	5.0	12.6	47.4	20	46.0	8.6	18.1
10	35	5.0	11.8	9.2	32	47.5	6.6	71.7
11	35	5.0	4.05	9.8	26	45.7	4.3	43.9

Following the enzymatic hydrolysis of cellulosic wastes two sets of researchers Cysewski and Wilke (1976) and Ghose and Tyagi (1979) have used the sugars so formed to produce ethanol.

Cysewski and Wilke (1976) concentrated their sugars from 4% to 14.3% and obtained an ethanol yield of 46.5% (average) at 35°C. They also added nutrients $(NH_4)_2SO_4$, Mg SO_4, CaCl and protein nutrient to the fermentation vessel.

Ghose and Tyagi (1979) used bagasse hydrolysate containing 6-7% w/v total reducing sugars (70% glucose and 30% xylose and cellobiose), which they concentrated to 10-26% before use. They obtained 12% alcohol from 26g l^{-1} glucose (yield 46.2%), and 10% from 22g l^{-1} (45.5%). They also used $CaCl_2$, Mg SO_4 and an Nitrogen source, pH 4.00 and temperature 30°C.

Leonard and Hajny (1945) conducted research into a possible preparatory treatment which would yield a more easily fermentable sugar solution and also to find a fermentation process to give rapid sugar conversion. They investigated the effects of metals, methods of neutralization, addition of reducing substances, additional nutrients and micro-organism types. Their main conclusions were that heat treatment of a neutral solution for 15 - 30 minutes at 140°C pH 4.5 - 5.2 was favourable to fermentation (however, the favourable pH for fermentation is 5.6 - 5.8) as was

the addition of reducing substances such as $Na_2 SO_3$, sulphite waste liquor, alkali-decomposed sugar, ascorbic acid and cysteine.

The difficulty of fermentation may be enhanced by the presence of toxic constituents. Four potential sources of toxic substances have been identified:

1 metals resulting from corrosion of the equipment;
2 carbohydrate decomposition products;
3 lignin decomposition products;
4 extraneous products in the wood.

Of these it is likely that the carbohydrate decomposition products, and possibly the corrosion metal ions will be the most influential on the rate or extent of fermentation of acid-hydrolysed cellulose. Of the possible decomposition products furfural is the one most toxic to yeast. According to Luers et al. (1938) the concentration of furfural needed to produce a 25% inhibiting effect in yeast fermentations is 0.74g $litre^{-1}$ (740 ppm) and in yeast propagation 1.1g $litre^{-1}$ (1100 ppm). Hydrolysates from the Scholler process in Germany were found to contain furfural in concentrations varying from 0.15 to 0.4g $litre^{-1}$ (150-400 ppm). However, Harris et al. (1946) found that under laboratory conditions flashing the hydrolysate after neutralisation with lime removed all

but traces of furfural* and at the same time permits removal of calcium sulphate which would otherwise inhibit yeast fermentation.

A variety of authors (Leonard and Hajny (1945), Johnson and Harris (1948), Harris et al. (1946), Luers et al. (1938), Ledland et al. (1954)) have researched other treatments or conditions that are advantageous in reducing toxicity of hydrolysates such as steam distilation, acclimatization of the yeast, detoxification reactions brought about in the presence of actively fermenting yeast, use of large yeast inocula and neutralisation at high temperatures. Other treatments have been reviewed in Industrial Fermentations, Volume 1 (Leland et al).

Other problems include ethanol or end-product inhibition. When a concentrated sugar solution is fermented and the ethanol concentration increases above 7-10%, the specific ethanol production rate and the specific growth rate of the yeast is severely suppressed. This ethanol inhibition may produce economic implications when consideration of industrial ethanol fermentation is made although it is possible to use certain yeast species which will produce 14-20% ethanol, as in the production of sake.

* It is converted to furan which may be used commercially as an intermediate in the manufacture of nylon.

To circumvent the problem of ethanol inhibition, ethanol could be removed from the fermenting solution as it is formed. This may be achieved by taking advantage of the high volatility of ethanol and boiling it off as it forms. Vacuum operation is necessary to achieve a boiling of the fermentation broth at temperatures compatible with those for yeast survival and metabolism (about 30°C).

Cysewski and Wilke (1977) have investigated both semicontinuous and continuous vacuum operation using about 33% glucose feedstock. The major constraint of the semicontinuous process is the accumulation of non-volatile components in the fermenter which poison the yeast. The continuous process in which bleeding off of some broth to maintain a non-inhibitory concentration of these components solves this problem. About 4-6% (by weight) of alcohol was formed per hour.

This then seems an ideal process to use, however, it has been more or less abandoned owing to economic constraints (Cysewski (1978)). There has to be at least 11% fermentable sugars in the broth for the process to be economically viable; this concentration of sugars is not available in enzymatically hydrolysed cellulose.

9.2 Other Fungi

Leonard and Hajny (1945) noted that Fusarium lini produced alcohol from hexoses and pentoses but at a slow rate; 1.6% alcohol was formed from glucose in 7 days with a 45% sugar conversion. Prior to this date White and Willaman (1928) completed a series of studies on the metabolism of F. lini, including tests for the production of alcohol from xylose, arabinose and rhamnose. Production was greater from xylose than from either of the other two. They also found that F. lini would grow on ethyl alcohol itself if the concentration were less than 3.5%.

9.3 Bacteria

Hajny et al. (1951) while investigating the thermophilic fermentation of cellulose by bacteria, tested two types of bacteria (unnamed), which they had obtained from soil samples, for the fermentability of various wood sugars. Table 18 reports the results; the end-products were acetic, butyric and lactic acids but no ethanol. From their descriptions of the bacteria they seem to be representatives of Lactobacillus and Bacillus species.

Leonard and Peterson (1947) produced butanol and acetone from wood hydrolysate sugars, with Clostridium butylicum using from 24 - 38% of the sugars present.

Table 18 Production of organic acids by thermophilic soil bacteria on wood sugars. (Hajny et al.1951)

substrate	% concentration	products (acids) acetic	butyric	lactic	% yield acid / sugar
glucose	2.85	0.69	0.25	0.58	53.3
galactose	3.04	0.39	0.59	0.52	49.3
mannose	2.52	0.33	0.17	0.37	68.4
xylose	3.16	0.37	0.36	0.81	48.0
arabinose	3.00	0.35	0.41	0.68	48.8

Various other types of bacteria have been reported by Horecker (1962) to ferment pentoses, such as the aerobes, E. coli, Aerobacter aerogenes, Pseudomonas saccharophila, Acetobacter xylinum, and the anaerobes, Clostridia sp. and Leuconostoc mesenteroides. Table 19 shows their products. The only purely ethanolic fermentation in bacteria is by Pseudomonas lindneri.

Dr. Rosenburg (1978), University of California, Berkeley is working on the fermentation of xylose by fungi and bacteria. He is using a made up xylose solution with a view to utilising the xylose from an enzymatic hydrolysis process.

Table 19 Products of various bacteria which ferment hexoses and pentoses

Organisms	Reference	Sugars Fermented	Products	Comments
1. Aerobacter aerogenes	Gunsalus & Stanier (1961)	pentoses and hexoses	2, 3-Butanediol, ethanol, formic acid, acetic acid, CO_2, H_2	
2. Bacillus polymyxa	Bergey (1974)	glucose and xylose	2, 3-Butanediol ethanol CO_2, H_2	from glucose ratio 2, 3-But:ethanol = 1:1 from xylose shifted in favour of ethanol
3. B.stearothermophilus				
4. Clostridium cellobioparum	Bergey (1974	glucose xylose only	ethanol ethanol	
5. C.thermocellum		glucose		
6. C.oroticum				
7. Escherichia coli	Bergey (1974)	pentoses and hexoses	ethanol, acetic acid, lactic acid, CO_2, H_2	
8. Lactobacillus pentoaceticus	Gunsalus & Stanier (1961)	D-xylose and L-arabinose	lactic acid acetic acid ethanol	no ethanol formed
9. L.plantarum	Bergey (1974)	hexoses	lactic acid acetic acid	
10. Leuconostoc mesenteriodes	Bergey (1974)	arabinose xylose	lactic acid acetic acid	equimolar quantities
		glucose	D-lactic acid ethanol, CO_2	1 mole of each (approximate)
11. Pseudomonas saccharophila	Bergey (1974)	L-arabinose galactose	ethanol	fructose, mannose and D-arabionose by mutation only
12. Zymomonas mobilis	Bergey (1974)	glucose	ethanol CO_2 lactic acid	1 mole glucose = 1.8 ethanol + 1.8 CO_2 + 0.2 lactic acid

He has fully investigated Fusarium suboxydans (a fungus) which produced up to 4% ethanol by weight but which is very, very slow (weeks). He is currently using Bacillus macerans, which produces ethanol much faster but in not nearly so large quantities, in addition it produces acetone, formic and acetic acids. Its optimum temperature is 40-50°C so any fermentation vessel must be heated.

Dr. Rosenburg is interested in Bacillus thermoacidans which effects a mixed acid fermentation containing acetic, butyric etc. According to his information engineering and economic restraints mean that any organism must produce at least 5% ethanol to be useful.

9.4 Interactional Utilisation of Micro-organisms

Ideally, for the efficient use of the acid hydrolysis sugars, the conversion of both hexoses and pentoses, to useful products, should take place in the shortest possible time and with the maximum conversion of those sugars. A mixed population of two or more organisms which will utilise both hexoses and pentoses simultaneously would be advantageous as yet no attempt has been made to do this. Commercially the cultures of micro-organisms in fermentable sugars has so far been confined to the growth of one organism (Faith et al 1965) or two in series (Skogman 1976). In the former either the hexoses or the

pentoses could be utilised, in the latter example (Symba process) potato starch wastes are converted in a two stage system. Endomycopsis is grown, this hydrolyses the starch to glucose, which is then utilised by C. utilis.

In a simultaneous, or a two stage conversion of hexoses and pentoses the nutrient requirements for optimum cell growth may be very different from those which will give maximum yields of the metabolic by-products. If, for example, the fermentation of hydrolysis sugars is for the production of ethanol then the aim is to produce the maximum concentration of this and not to encourage the production of microbial cell material. In the case of two organisms being used together there is the danger that the ethanol formed by one of them may be used as a carbon source by the other.

A detailed understanding of population dynamics is necessary. In areas where mixed populations are already worked to produce a desired end result such as in sewage works (activated sludge process) and certain commerical fermentations (brine pickling and certain cheeses) this understanding still awaits elucidation. Attempted studies of mixed batch cultures have often yielded data which is difficult to interpret by virtue of the many interactional culture factors which occur simultaneously.

Naturally occurring microbial systems such as in the soil and in streams are also a complex mixture of many different kinds of organisms. To effect an ecological analysis of such a system various parameters must be examined; these have been summarised by Paynter and Bungay (1971).

1 the system boundaries must be defined

2 the major chemical changes which occur must be recognised

3 identification of organisms, qualitatively

4 identification of organisms, quantitatively

5 detection of biochemical reactions associated with the important organisms

6 kinetics and stoichiometry of significant reactions

7 population dynamics and biochemical interactions of the populating organisms

8 environmental influences on all system variables

Before attempting the setting up and simulation of a balanced culture the above points must be carefully investigated and experimented. If points 1-8 are applied to the theoretical setting up and working of a system in which 2 or more micro-organisms will ferment or use all

available hexoses and pentoses in the hydrolysate from the acid hydrolysis of cellulose, these experimental parameters may be described in more detail:-

1 The system boundaries will be defined by the results of sugar analysis which will show the amounts and proportions of hexoses and pentoses, and other products present.

2 The major chemical changes may be pre-set; they are the goal of the exercise, i.e. ethanol production/SCP/ other useful chemicals.

3 Similarly the organism identities are pre-defined to achieve points 1 and 2.

4 Once the types of organisms have been decided upon, the correct balance between them must be established. This factor may change over the period of time, or may fluctuate about a stable balance.

5 This point links intimately with all 4 above, since it will be those major biochemical reactions which will determine the success or failure of the operation.

6 For the successful system to work economically the kinetics of the reactions must be established to enable the minimum requirements of say nutrients, initial starter cultures, oxygen, time and heat to be used.

7 This could be the most important unknown factor. The effect that one organism will have on another may well alter the end-products or the population balance.

8 Hopefully, in a continuous steady-state mixed population experiment, external environmental factors will be at a minimum. The internal environment will be set by points 5 and 7.

9.5 Mixed Culture Interactions

Before making an attempt to decide on 2 or more organisms, some generalisations must be made about microbial interactions. As noted in point 7 above the population dynamics and interactions will be vital to the efficiency of the system. Little research attention has been paid to the various microbial interactions which occur in natural mixed populations. However, the various interactions which do occur have been described and may be divided into benevolent and antagonistic reactions.

Benevolence is the interaction between one or more different species during which none is harmed, and often one or more are benefited by the association. Antagonism is the interference with, or the inhibition of, growth of one kind of organism by another through the creation of unfavourable conditions e.g. exhaustion of food supply or the production of a specific inhibitory substance -

Table 20 Microbial interactions

Benevolent		Antagonistic	
Commensalism	- Close association with one partner benefiting	Parasitism	- One steals from another
Synergism	- Both benefit by co-operative metabolism	Predation	- One feeds on another
Mutualism	- Broad relationship where in some way each benefits	Antibiosis	- One excretes harmful factors
Symbiosis	- Close physical relationship with interchange of physiological functions - an extreme form of mutualism.	Competition	- A race for nutrients, etc.

(a) commensalism

One of the partners of the relationship profits by living in close association with the second species, but the latter receives neither good nor harm from the organism it favours. Various kinds of commensalism involving micro-organisms have been described by Alexander (1971).

1 One population converts a substrate unavailable to a second population into a product that is assimilated and serves as a macro-nutrient for the latter.

2 One species excretes a growth factor essential for the proliferation of the second.

3 One destroys toxins or removes inhibitory factors from the environment, thereby allowing for multiplication of its associate. Destruction of organic or inorganic toxins, lowering or raising of the pH,removal of O_2, the creation of light-shading effects, reducing the osmotic pressure by microbial metabolism of sugar or salts, and changing the inorganic nutrient level to favourable concentrations are means by which the benefit is conferred.

4 A macro- or a micro-organism provides a surface that is particularly suitable for colonization by the commensal, a surface giving the partner a marked ecological advantage over free individuals of the same or different species.

5 One individual provides nutrients, protection, or shelter to another that is living within it, the commensal doing neither harm nor good to the organism in which it resides. Such relationships occasionally verge on parasitism.

A particular example of commensalism is noted by Oates et al. (1963). The proliferation of a mesophilic bacillus species at 65°C may be made possible when it is cultured at that temperature with a thermophile. Also Chan and Johnson (1966) investigated the growth of the bacterium Arthrobacter citreus at 37°C in the presence of a variety of fungi and bacteria, but it would not grow in pure culture.

(b) synergism

This is a state of co-operative metabolism; an example being given by Castallani (1953). In his example gaseous products were evolved during the fermentation of mono- or disaccharides by the joint participation of two microbial species, neither of which could generate gas from the test sugars in pure culture. Among the gas produced in this way were CO_2, H_2 and CH_4. The transformations leading to gas formation were probably performed by a sugar-decomposing, non gas-producing culture and a gas producer that is inactive on sugars. The latter then uses the decomposition products of the former to generate the gases.

(c) mutualism and symbiosis

The two may be discussed simultaneously. Mutualism is a broad relationship in which each benefits the other. Symbiosis, an extreme form of mutualism, is a state in which two dissimilar species co-exert mutually beneficial effects. Not uncommonly one, or possibly both, of the associates is an obligate symbiont, always requiring the other to maintain an active existence in nature.

(d) antagonistics

These harmful relationships must obviously be avoided in the fermentation vat. The occurrence of the first three in Table 20 will be easily identified but the last,

competition, may occur at a slow rate the results of which will not be altogether obvious at first. It is widely believed that competition is keenest between closely related strains and species. They tend to have similar needs, nutrient requirements, biochemical functions and tolerance ranges. The essential factor is their ecological likenesses rather than their systematic position in a taxonomy textbook.

9.6 Method of Investigation

The problem of finding two or more organisms to efficiently ferment these sugars has to be approached in both a theoretical and a practical way: study of biochemical literature first, then repeated controlled fermentation and growth experiments to decide the final composition of the culture.

The first step in making the preliminary selection of micro-organisms which might possibly ferment pentoses and hexoses efficiently is by consultation with various taxonomical reference works, such as Skerman 1967, Lodder 1970 and Bergey 1974, in order to evaluate the organisms' biochemical reactions and by-products. In an industrial fermentation process the overall aim is to use a feature of the metabolism of an organism for a particular biochemical conversion. The industrialist is

usually concerned with using cheap readily available raw materials as the substrate and the efficient conversion of these to the desired end product. This latter may be achieved by selection of a particular strain of a species or by altering the process parameters to as near the optimum as possible. It is advantageous to enhance the production of the desired end products while suppressing any undesirables. The final choice of organism will be governed by the speed and efficiency of sugar fermentation, and the formation of useful by-products, such as ethanol.

Since S. cerevisiae is more than adequately competent to ferment hexoses to alcohol, it remains to find an organism which will likewise utilise the pentoses. Cysewski and Wilke (1976) found that 70% of their enzymatic cellulose hydrolysis product was fermentable by Saccharomyces cerevisiae and Saeman et al. (1945) estimated that 73 - 81% of wood sugars from spruce and Douglas fir was fermentable by that yeast.

In order to establish that the theoretically chosen organisms will ferment the sugars, it has been necessary to run initial tests using: (1) solutions of pure sugars corresponding to those found in the hydrolysate, and (2) a mixture of these sugars in the similar proportions in which they occur in the hydrolysate in small laboratory scale fermentations (500 ml or 1 litre). The by-products and the degree of utilisation of the sugars have been

evaluated and the hydrolysate from the reactor used. This latter test evaluates the effects of other, extraneous, substances on growth and ethanol production of the organisms.

In moving from laboratory bench-scale fermentations to a larger pilot plant it is more economical to mix together the two organisms - S. cerevisiae and the pentose fermenter in the fermentation vessel instead of an in-line plant. The main problem with this layout could be the occurrence of antagonistic or inhibitory effects between the two organisms. In natural ecosystems the complete dominance of one organism over any others does not usually occur. In a fermenter, under relatively artificial conditions, if two organisms are competing for a common growth-limiting substance then the dominance of one species might happen in a relatively short time.

The behaviour of mixed organisms on mixed substrates (e.g. the hydrolysate sugars) depends on the similarities in the organisms' affinities for the contents of the mixed substrate. If the two organisms have closely overlapping preferences for the substrate they will compete severely for the common nutrient source. This may result in the exclusion of the 'weaker' one from the system. If however they have different preferences they may co-exist, occupying different ecological niches. Ideally a symbiotic state should occur - each one growing or

fermenting better, than if it were alone, or at least commensalism - where each will derive some benefit from the other. In the latter, the one organism could be dependent upon the production of an essential nutrient by the other organism, rather than the growth-limiting substance being in the medium. Only a few experiments have been carried out with mixed cultures showing commensalism. It has been observed in continuous cultures by Contois and Yango (1964), Shindala et al. (1965), and Mateles and Chian (1969). Also mixed culture studies in multi-stage continuous systems have received little attention, Fencl and Berger (1958) have described the microbial transformation of hexoses and pentoses from sulphite waste liquor, using 2 or 3 reactors connected in series.

Two other methods of investigation are of interest:

1 the use of a naturally occurring mixed population e.g. from sulphite wastes (Spencer et al.) (1974) or activated sludge (Painter et al.) (1968).

2 the use of cell-free enzymes (Fullbrook & Vabø) (1977).

The use of the latter would reduce the problems of inhibition caused by the by-products of microbial cells. However the scope of these two alternatives is too wide to be encompassed within the present research restraints.

CHAPTER 10

FERMENTATION TRIALS, THE RESULTS AND DISCUSSION

10.1 Introduction

The parameters of Paynter & Bungay noted in Chapter 9 are those which maybe needed to effect an analysis of a microbial system and its workings. The important points that are unknown and which are to be investigated are 4-7, namely quantitative identifications of organisms, detection of biochemical reactions, kinetics of significant reactions, and population dynamics and interactions.

The qualitative identification of the organisms has been undertaken in the light of their taxonomic classification. Saccharomyces cerevisiae is the most commonly used species of yeast for ethanol production. Many strains have been described which exhibit varying capabilities and tolerances for the environments encountered. It is possible to adapt and breed yeast types for a given set of conditions. Quantitatively, tests with high and low inocula have been undertaken to determine their effect on ethanol production. Heavy inocula may be needed to overcome initially unfavourable growth conditions. In addition various bacteria were chosen for testing.

The detection of specific biochemical reactions, other than the production of ethanol and cells, was not undertaken because of analytical difficulties.

A study of the kinetics produced values for yields, production and growth rates, and productivity and fermentation rates. Graphical representation of these values gave a picture of the population and product formation interactions.

10.2 Organisms

1 Saccharomyces cerevisiae National Collection of Yeast Cultures, NCYC 1060.

2 Bacillus polymyxa National Collection of Type Cultures. NCTC 10343.

3 Pseudomonas saccharophila National Collection of Industrial Bacteria, NCIB 8570.

4 Aerobacter aerogenes National Collection of Type Cultures, NCTC 10006.

5 Mixed soil coliforms (isolated by the researcher).

Organisms 2 - 4 were obtained freeze dried, see Appendix 3 for details of resuscitation media.

10.3 Media

A medium for microbial growth should contain similar elements (except the carbon) to those making up the cell and in the correct proportions. In the case of hydrolysis sugars a minimal amount of extra constituents is desired to be added because of cost. A basal synthetic medium was devised (Appendix 3) according to elemental cell composition. The sugars were added according to the test, using up to 5 g l^{-1} hydrolysis rig sugars.

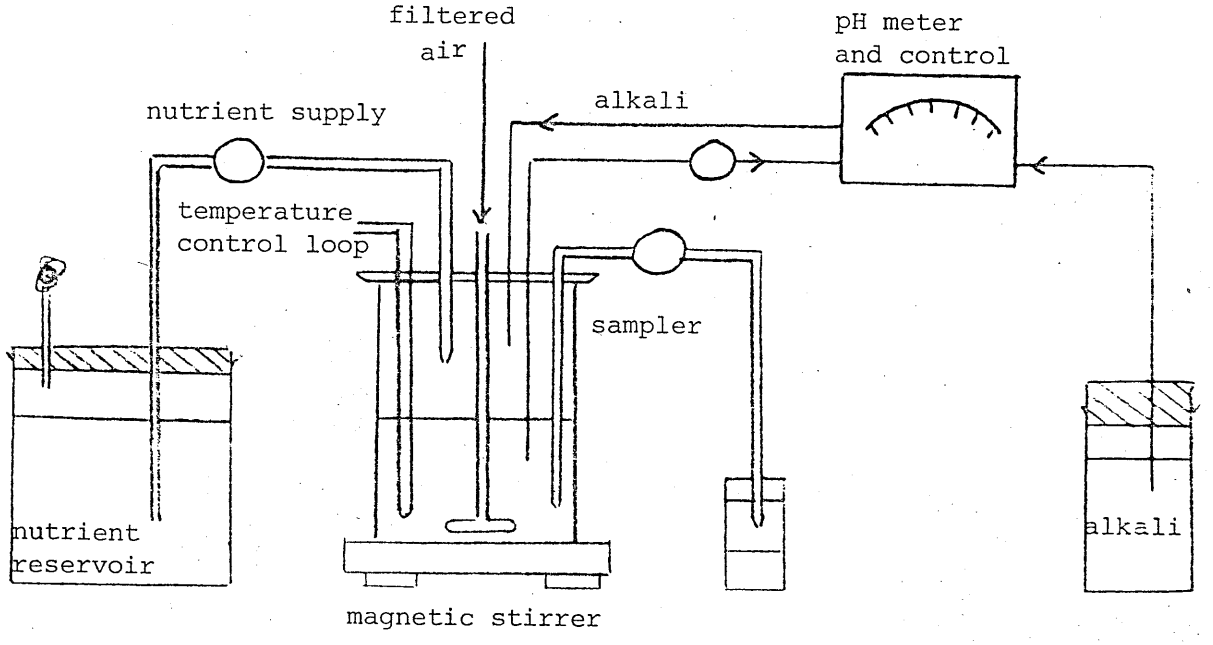
10.4 Analytical Methods (Appendix 1)

Both sugars and ethanol analyses were carried out by chemical 'wet' methods and by chromatography. Cell counts were by haemocytometer and Coulter counter. The Coulter method was used so that separate organisms could be identified by size distribution; this would have been impossible using an absorbance method. Calibration graphs were prepared relating numbers of organisms to dry weight values.

10.5 Apparatus

The bench scale apparatus consisted of a 1 litre capacity Gallenkamp modular fermenter (Figure 11), working volume about 600 mls and included accessory pH and temperature controls, magnetic stirrer, and nutrient supply and harvesting pump.

Figure 11 Modular fermenter for batch and continuous cultures



The fermenter was sterilised empty in an autoclave at 1.4×10^5 Pa for 20 minutes. The medium was sterilised separately by membrane filtration and was added aseptically to the fermenter. For batch cultures the apparatus consisted of a magnetic stirrer and temperature

and pH control. For continuous runs a peristaltic pump controlling nutrient supply and sampling was added.

10.6 Tests Performed

1 Mixed soil coliforms were used to practice the 'wet' sugar analysis methods and to gain expertise using the fermentation apparatus.

2 Part way through the hydrolysis trials a clear greenish-black sugar solution was found to be contaminated with chromium, nickel and iron. In order to evaluate the effect of this anomalous product of the hydrolysis rig, a series of Saccharomyces cerevisiae glucose fermentations was set up. Since the solution from the rig was low in sugars (glucose) the medium was made up to 5% concentration with glucose. (Appendix 3.)

3-11 acid hydrolysis sugars augmented with glucose.

3 S.cerevisiae, batch - low inoculum < 1.0 g l^{-1}.

4 S.cerevisiae, batch - high inoculum 2-3 g l^{-1}.

5 S.cerevisiae, continuous.

6 Ps.saccharophila, batch.

7 Ps.saccharophila, continuous.

8 Ps.saccharophila + S.cerevisiae, batch and continuous.

9 B.polymyxa, batch.

10 B.polymyxa + S.cerevisiae, batch and continuous.

11 A.aerogenes, batch and continuous.

10.7 Growth Conditions

(a) temperature

since all organisms are mesophilic the temperature was kept at 30°C.

(b) pH

the pH value of 5.0 was set at a compromise between the optima of bacteria and that of yeasts.

(c) aeration

the apparatus was not artificially aerated; there was an air vent into the fermenter and filtered air was allowed to diffuse in by the action of mixing. The production of ethanol from glucose by S.cerevisiae is essentially an anaerobic process but is stimulated by small amounts of oxygen; in fact, there is a rapid loss of cell viability at high cell concentrations if the process is completely anaerobic and, therefore, a drop in ethanol production. The other organisms used also required aerobic conditions.

(d) initial inocula

all taken from 24 hour cultures and of varying rates according to the test (<0.1 g l^{-1} - 4.5 g l^{-1}).

(e) sugars

for the earlier experiments Analar sugars were used (glucose and xylose). Later hydrolysis sugars (neutralised with $Na_2 CO_3$) up to 5% were used.

(f) sampling

samples were taken from the fermenter at regular intervals and tested for sugar and alcohol concentration and cell growth. Ideally trials were terminated when the sugar concentration dropped to below 10% of its original value. Continuous cultures were allowed to grow in batch conditions, for about 10-20 hours to establish logarithmic growth before starting the pump. Then samples were removed regularly to confirm the establishment of steady state conditions; this was assumed when cell densities and sugar concentrations in the outgoing medium became steady.

(g) dilution rate in continuous cultures

using a peristaltic pump a range of dilution rates (D = 0.1 - 0.4) were tested corresponding to a flow rate of 60 - 240 mls hr^{-1}.

Theoretically, steady state operation may be achieved over a range of dilution rates. The culture is self-adjusting over this stable range so that each time a new

dilution rate was used the substrate and organisms concentrations would adjust to a new level (after a period of stabilisation; about 20 hours).

10.8 Methods of Analysis Applicable to the Results

Various graphical methods were used. In the test cultures, after a variable lag period depending on size of original inoculum and adaptation of the organism to the environment, growth proceeded exponentially until in batch conditions almost all the substrate has been exhausted. From each batch run, log-lin plots of biomass versus time were drawn and the value of μ_{max} was found from the slope of the straight line section. This value varied, according to the initial inoculum value and the affinity of the organisms for the substrate. In certain cases μ_{max} was not measureable as there did not appear to be a logarithmic phase.

To illustrate the phases of substrate utilisation, and cell and product formation in batch culture lin-lin plots of biomass, substrate and product versus time were drawn from the results of trials. From this type of graph may be estimated the optimum time for the culture to continue for maximum product formation.

The graph also serves to pinpoint the efficiency of the culture in dealing with the substrate, or producing its by-products.

Various yield values may be calculated:

$Y_{product}$ (Y_p) $\frac{\text{ethanol g } l^{-1}}{\text{sugar used g } l^{-1}}$ represents the efficiency of the conversion

$Y_{effective}$ (Y_e) $\frac{\text{ethanol g } l^{-1}}{\text{initial sugar g } l^{-1}}$ represents the effectiveness of the organisms to produce ethanol from the substrate

In continuous cultures:

Volumetric productivity rate Q (DP) in g l^{-1} hr^{-1} - describes the rate of product synthesis per unit of fermenter capacity and is dependent on the cell mass concentration.

Specific fermentation rate $qp = \frac{DP}{X}$ g g $cell^{-1}$ hr^{-1} - independent of cell mass concentration and describes the effectiveness of the cells in product synthesis (or substrate utilisation). Useful for comparing results between fermentations.

While the value of D (dilution rate) is set by the experimenter, values of D_{max} and Dcrit (the value at which washout of the organisms occurs) may be obtained from plots of DX and Y_p against D.

The determination of K_S (glucose) (a measure of the affinity of the organism for the nutrient involved) from continuous culture results uses the formula:

$K_S = S_{out} \left(\frac{\mu_m - D}{D}\right)$ where μ_m is determined in batch culture and when $D = \frac{\mu_m}{2}$ $S_{out} = K_S$

The values obtained from these experiments are those of apparent K_S, a value very much higher than normal for S.cerevisiae in a glucose medium. This is an indication of the lack of affinity that this test yeast had for the hydrolysis sugar medium.

10.9 Results

Tables 21-29 following, show the various fermentation trials, and their results.

Table 21 S.cerevisiae-medium contaminated with chromium, iron and nickel ions.

Batch 30°C pH5 (allowed to drop to 4), 50 g l^{-1} initial glucose, time 4-6 days.

Trial	Initial inoculum g l^{-1}	μ_{max} hr^{-1}	Final yeast conc. g l^{-1}	Final glucose g l^{-1}	ethanol g l^{-1}	Y_P g g^{-1}	Y_E g g^{-1}	$\frac{Y_E}{Y_P}$	Comment
1	<0.1	0.1	<0.1	45	4.0	0.8	0.08	0.1	
2	<0.1	0.3	0.3	30	9.0	0.45	0.18	0.4	
3	0.1	0.18	<0.1	25	12.0	0.48	0.24	0.5	
4	<0.1	0.3	0.1	25	9.0	0.36	0.18	0.5	cells from 3
5	<0.1	0.3	0.1	20	10.0	0.33	0.20	0.61	cells from 4
control	<0.1	0.6	0.4	9	20.0	0.49	0.40	0.8	

Table 22 S.cerevisiae - batch, temperature 30°C, pH regulated at 5.0

Initial sugar conc. g l^{-1}	Initial inoculum g l^{-1}	u_{max} hr^{-1}	Final yeast conc. g l^{-1}	Final sugar g l^{-1}	ethanol g l^{-1}	Y_p g g^{-1}	Y_E g g^{-1}	$\frac{Y_E}{Y_p}$
10	0.06	0.19	1.1	0.8	3.8	0.4	0.38	0.93
10	0.1	0.2	0.4	0.6	4.0	0.4	0.4	1.0
10	0.14	0.23	1.7	0.8	3.9	0.42	0.39	0.93
10	0.1	0.17	1.0	0.8	3.9	0.42	0.39	0.93
10	0.3	0.3	3.4	0.8	5.0	0.5	0.5	1.0
10	0.65	0.15	2.6	1.0	6.0	0.66	0.6	0.9
50	0.06	0.4	1.0	13.0	9.0	0.24	0.18	0.8
5	2.3	nm	2.7	0.2	2.0	0.42	0.40	0.95
5	2.4	nm	2.7	0.2	1.9	0.4	0.38	0.95
50	1.3	nm	1.6	5.0	20.0	0.44	0.4	0.91
41	3.3	nm	3.8	0.6	19.0	0.47	0.46	0.98
45	3.4	nm	3.7	0.5	21.0	0.48	0.47	0.98
42.5	3.3	0.02	4.1	0.6	19.5	0.46	0.46	1.0
100	3.2	nm	3.8	1.0	58.0	0.59	0.58	0.98

Table 23 S.cerevisiae - continuous, temperature 30°C pH 5.0

Initial sugar concentration $g\ l^{-1}$	Yeast concentration at beginning of continuous run $g\ l^{-1}$	Final sugar $g\ l^{-1}$	Ethanol $g\ l^{-1}$	Y_P $g\ g^{-1}$	Y_E $g\ g^{-1}$	DP $g\ l^{-1}hr^{-1}$	$\frac{DP}{X} = qp$ $g\ g\ cell^{-1}hr^{-1}$	Apparent k_s (glucose) $g\ l^{-1}$
D = 0.1								
10	1.2	0.1	4.9	0.49	0.49	0.49	0.48	0.1
10	1.2	0.1	4.9	0.49	0.49	0.49	0.48	0.1
10	1.4	0.05	5.0	0.50	0.50	0.50	0.36	---
10	1.3	0.05	4.75	0.53	0.48	0.48	0.37	0.05
150	3.2	0.0	75.0	0.50	0.50	7.5	2.3	0
160	4.5	50.0	40.0	0.36	0.25	4.0	0.89	50
D = 0.15								
160	4.5	90.0	30.0	0.43	0.2	4.5	1.1	36
D = 0.2								
10	1.3	0.2	4.6	0.46	0.46	0.92	0.71	0.01
150	3.2	5.0	75.0	0.51	0.50	15.0	4.69	0.25
160	3.0	120.0	20.0	0.50	0.13	4.0	1.3	6.0
D = 0.25								
160	1.5	140.0	6.0	0.3	0.04	1.5	1.0	washout
D = 0.3								
10	1.3	0.4	4.75	0.49	0.48	1.43	1.1	2
150	2.8	25.0	60.0	0.48	0.40	18.0	6.4	---
D = 0.4								
150	1.0	100.0	25.0	0.50	0.13	10.0	10.0	---

Table 24 Pseudomonas saccharophila - batch, temperature 30°C pH 5.0

Initial sugar concentration g l^{-1} G	X	Initial inoculum g l^{-1}	u_{max} hr^{-1}	Final bacterial concentration g l^{-1}	Final sugar g l^{-1}	Ethanol g l^{-1}	Y_P g g^{-1}	Y_E g g^{-1}	$\frac{Y_E}{Y_P}$
50	50	0.01	0.05	0.18	40	6	0.1	0.06	0.6
50	50	0.01	0.04	0.14	40	14	0.23	0.14	0.6
50	50	0.01	0.04	0.16	50	5	0.1	0.05	0.5
50	50	0.05	0.2	0.18	29	5	0.07	0.05	0.7
50		0.1	nm	1.5	10	10	0.25	0.2	0.8
100		0.05	0.12	1.2	30	14	0.2	0.14	0.7
100		0.05	0.06	1.1	36	12	0.19	0.12	0.6
100		0.01	0.04	0.14	40	14	0.23	0.14	0.6

Table 25 Ps.saccharophila - continuous, temperature 30°C pH 5.0 D = 0.1

Initial sugar g l^{-1} G	X	Bacterial concentration g l^{-1}	Final sugar g l^{-1}	Ethanol g l^{-1}	Y_P g g^{-1}	Y_E g g^{-1}	$\frac{Y_E}{Y_P}$	DP g $l^{-1}hr^{-1}$	$\frac{DP}{X}$ g $g^{-1}hr^{-1}$	Apparent k_s (glucose) g l^{-1}
50	50	0.01	60	2.5	0.06	0.025	0.4	0.25	25	60
50		0.01	5	9.0	0.2	0.18	0.9	0.9	90	5
50		0.01	10	10.0	0.25	0.2	0.8	1.0	100	10
50		0.045	40	10.0	1.0	0.2	0.2	1.0	22	nm
50		0.01	0.5	0.9	0.2	0.018	0.9	0.09	9	nm

Table 26 Ps.saccharophila + S.cerevisiae - batch

Initial sugar 50 g l^{-1}; temperature 30°C; pH 5.0

Initial inoculum g l^{-1}		μ_{max} hr^{-1}		Final concentration g l^{-1}		Ethanol g l^{-1}	Y_P	Y_E	$\frac{Y_E}{Y_P}$
Y	B	Y	B	Y	B		g g^{-1}		
0.1	0.02	0.16	0.15	1.1	0.2	10	0.25	0.2	0.8
0.1	0.05	0.15	0.03	1.0	0.2	6	0.15	0.12	0.8
0.1	0.1	0.1	0.04	0.6	0.19	7	0.18	0.14	0.78
0.1	0.2	0.15	0.04	1.2	0.29	10	1.0	0.2	0.2
0.1	0.02	0.2	0.06	1.1	0.22	10	0.22	0.2	0.9

Table 27 Ps.saccharophila + S.cerevisiae - continuous

temperature 30°C; pH 5.0; D = 0.1; Initial sugar 50 g l^{-1}

Initial concentration g l^{-1}		Final sugar g l^{-1}	Ethanol g l^{-1}	Y_P	Y_E	$\frac{Y_E}{Y_P}$	DP	$\frac{DP}{X}$
Y	B			g g^{-1}			g $l^{-1}hr^{-1}$	g $g^{-1}hr^{-1}$
0.05	0.05	41	9	0.22	0.18	0.8	0.9	9
0.1	0.01	40	10	0.25	0.2	0.8	1.0	9
0.1	0.05	40	6	0.15	0.12	0.8	0.6	4
0.1	0.3	41	4.5	0.11	0.09	0.8	0.45	1.1
1.0	0.35	45	10.0	0.22	0.2	0.9	0.1	0.7

Table 28 B.polymyxa

(a) A - on its own B - with S.cerevisiae - batch

Initial sugar	Initial inoculum	μ_{max}	Final concentration	Final sugar	Ethanol	Y_P	Y_E	$\frac{Y_E}{Y_P}$
g l^{-1} GX	g l^{-1}	hr^{-1}	g l^{-1}	g l^{-1}	g l^{-1}	g g^{-1}		
A 100	0.1	0.15	0.9	2	18	0.18	0.18	1.0
B 50	Y 0.15 B 0.1	Y 0.06 B 0.06	Y 1.7 B 0.9	0.4	9.9	0.2	0.2	1.0

(b) with S.cerevisiae - continuous D = 0.1

Initial sugar 50 g l^{-1} (G & X)

Initial inoculum	Ethanol	Final sugar	Y_P	Y_E	$\frac{Y_E}{Y_P}$	DP	$\frac{DP}{X}$	Apparent K_s (glucose)
g l^{-1}	g l^{-1}	g l^{-1}	g g^{-1}			g l^{-1} hr^{-1}	g g^{-1} hr^{-1}	g l^{-1}
1.85	4.6	5.0	0.1	0.09	0.9	0.46	0.34	2.5

Table 29 A.aerogenes

(a) batch, initial sugar 50 g l^{-1} (G).

Initial inoculum g l^{-1}	μ_{max} hr^{-1}	Final bacterial concentration g l^{-1}	Final sugar g l^{-1}	Ethanol g l^{-1}	Y_P g g^{-1}	Y_E	$\frac{Y_E}{YP}$
0.2	0.18	1.5	1.2	14	0.29	0.28	0.96

(b) continuous, D = 0.2 initial sugar 50 g l^{-1}

Initial inoculum g l^{-1}	Ethanol g l^{-1}	Final sugar g l^{-1}	Y_P g g^{-1}	Y_E	$\frac{Y_E}{Y_P}$	DP g $l^{-1}hr^{-1}$	$\frac{DP}{X}$ g $g^{-1}hr^{-1}$
D = 0.2							
1.33	21	0.6	0.43	0.42	0.97	4.2	3.2
1.39	23	0.6	0.46	0.46	1.0	4.6	3.3
D = 0.3							
1.3	20	0.6	0.41	0.40	0.97	6	4.6
D = 0.6							
1.29	16	0.6	0.32	0.32	1.0	9.6	7.4

10.10 Discussion of Results

A fermentation process is evaluated by the conversion yields and the overall productivity. Theoretically the maximum effective yield of ethanol from glucose is 0.51 g g^{-1} sugar with a possibility of obtaining 90-95% of this. i.e. 0.46 - 0.48 g g^{-1}. In this study $Y_E \left(\frac{\text{ethanol produced}}{\text{initial sugar}}\right)$ represents the effectiveness of the chosen organism to produce ethanol from the substrate, this should approach the theoretical.

$Y_P \left(\frac{\text{ethanol produced}}{\text{sugar used}}\right)$ gives the efficiency of the conversion. Since the two values should be as near equal as possible, their ideal ratio should be 1.

The value k_s also represents the affinity of the organism for the substrate; a low value representing a high affinity.

In continuous culture volumetric productivity (Q) and specific fermentation rate (qp) are both important.

Various authors working on the fermentability of enzyme hydrolysed cellulose by S.cerevisiae and S.uvarum have published figures for some of these parameters. These are annotated in Table 30.

Table 30 Yields and productivities of Saccharomyces sp. on cellulase hydrolysed sugars

Type of fermentation	Y_E	Y_P	μ	k_s	Q (DP)	qp $(\frac{DP}{X})$	Reference
	$g\ g^{-1}$	$g\ g^{-1}$	hr^{-1}	$g\ l^{-1}$	$g\ l^{-1} hr^{-1}$	$g\ g^{-1} hr^{-1}$	
1. pH 4.0 temp. 35°C 100 g l^{-1} glucose feed		0.465			7.0		Cysewski & Wilke (1977) (S.cerevisiae)
2. as above with cell recycle					34.0		ditto
3. with vacuum					40.0		ditto
4. with vacuum & cell recycle					82.0		ditto
5. Glucose 120 g l^{-1} with cell recycle	0.45	0.35	0.4	0.22	26.4 @ D = 0.3	1.0	Rogers & Rosario (S.uvarum) (1978)
6. Glucose 10-15 g l^{-1}			0.24	0.476			Ghose & Tyagi (1979) (S.cerevisiae)
7. 160 g l^{-1}					4.1 @ D = 0.13		ditto
8. with cell recycle					18.3 @ D = 0.3		ditto

(a) S.cerevisiae - metal ion contaminated medium

The slow growth and relative inactivity of the yeast cells suggests a state of inhibition presumably by the metal ions. The higher the initial inoculum the slightly better the alcohol production performance. The use of cells grown in experiment (3) in test 4 and then in test 5 does indicate facility for adaptation by the yeast cells to this adverse environment. By test 5 the ratio of Y_E and Y_p is the highest and approaching the control, indicating the effectiveness of S.cerevisiae to produce ethanol from the sugars in this substrate.

On inspection, the cells present at the end of each run seemed healthy, and not shrivelled. Compared with the control cells there was more chain formation and clumping, and a slight tendency towards gigantism. This slightly irregular type of growth was probably due to the metal ion concentration but does not seem significant since the cells will adapt to the adverse conditions as seen in Table 21. Johnson & Harris (1948) similarly found that in Douglas Fir hydrolysate the yeast cells became discoloured and abnormally small but still remained active.

(b) S.cerevisiae - batch cultures

The mean of several determinations of ethanol yield Y_E was 0.44 g g^{-1} sugar for initial sugar concentrations of 10 g l^{-1} and a low inoculum <1.0 g l^{-1}. If the inoculum is higher >1.0 g l^{-1} Y_E was slightly lower (0.39) for the lower initial sugar concentrations but 0.47 g g^{-1} for sugar of 50-100 g l^{-1}, which is nearing the maximum possible. In all cases the ratio $\frac{Y_E}{Y_P}$ approximated to 1.0.

If the ratio of initial yeast inoculum to initial sugar concentration is plotted against Y_P (log-lin), Y_P is seen to increase proportionally with an increase in the ratio (Appendix 4). The more initial yeast there is compared with initial sugar, the greater the yield of ethanol. The reverse is rougly true of μ_{max}. The results of Converse et al. (1973) cited in Chapter 9 show no such proportionality.

(c) S.cerevisiae - continuous cultures

The productivity g $l^{-1}hr^{-1}$ describes the rate of product synthesis (or substrate demand) per unit of fermenter capacity and is dependent on the cell mass concentration. During these runs the most effective use of substrate within the fermenter was at 150 g l^{-1} sugar, yeast 2.8 g l^{-1} giving a DP of 18 g ethanol per litre of sugar solution per hour when D = 0.3. The ethanol concentration

produced (6%) is about at the limit before serious inhibition of the yeast occurs (7-10%); over 11% the yeast will stop producing ethanol.

The specific fermentation rate describes the effectiveness of the cells in product synthesis or substrate utilisation and is independent of cell mass concentration.

Again this was at its highest under the above conditions.

Experimentally D_{max} varied between 0.2 and 0.3 and D_{crit} (the value at which washout of the organisms occur) between 0.3 and 0.4.

Judging by the results in Table 23 D_{max} may be set at 0.3 hr^{-1}.

By regrouping some of the results Table 31 may be drawn up.

Table 31 S.cerevisiae - continuous cultures at varying dilution rates and initial sugar concentrations

(a) S_R = 10 g l^{-1} X = 1.3 g l^{-1}

D	qp	P	Y_P	Y_E	$\frac{Y_E}{Y_P}$	DP	
hr^{-1}	g g^{-1} hr^{-1}	g l^{-1}	g g^{-1} sugar			g l^{-1} hr^{-1}	
0.1	0.37	4.75	0.53	0.48	0.90	0.48	
0.2	0.71	4.6	0.46	0.46	1.09	0.92	
0.3	1.1	4.75	0.49	0.48	0.98	1.43	
(b) S_R = 150 g l^{-1} X = 2.8 - 3.2 g l^{-1}							
0.1	2.3	75	0.5	0.5	1.0	7.5	
0.2	4.7	75	0.51	0.5	0.98	15.0	
0.3	6.4	60	0.48	0.4	0.83	18.0	
							washout probably occurring
0.4	10.0	25	0.5	0.13	0.26	10.0	
(c) S_R = 160 g l^{-1} X = 4.0 - 4.5 g l^{-1}							
0.1	0.89	40	0.36	0.25	0.69	4.0	
0.15	1.1	30	0.43	0.2	0.46	4.5	
0.2	1.3	20	0.5	0.13	0.26	4.0	
							washout probably occurring
0.25	1.0	6	0.3	0.04	0.1	1.5	

Taking each of the measured qualities separately and attempting to define the optimum culture conditions:

1 Specific productivity increases with an increase in dilution rate i.e. the cells work better at producing ethanol than doing anything else such as growing or maintaining themselves. The most spectacular increase being linked also with a concentration of 15% sugar.

2 Actual product formation (g l^{-1}) varies in its response to dilution rate but when compared with productivity rates (DP) at 1 & 15% sugar concentration, the productivity increases with D up to 0.3. At 16% sugar (probably an inhibiting concentration) productivity is more or less independent of D. Ghose & Tyagi (1979) found that at 100 g l^{-1} glucose initial feed into a continuous culture, the ethanol production concentration and the amount of sugar concerned decreased with increased dilution rates.

3 Y_E is more or less independent of D, but Y_P varies according to the initial sugar concentration. The ratio $\frac{Y_E}{Y_P}$ represents the efficiency of utilisation of the substrate and should be as near 1 as possible i.e: at D = 0.2 S_R = 10 g l^{-1} or D = 0.1 at S_R = 150 g l^{-1}.

Cysewski & Wilke (1976) found that fermenter ethanol productivity ($g\ l^{-1}hr^{-1}$) was highest at high dilution rates, but a large percent of the glucose remained unfermented at these conditions, that is Y_P is high and very different from Y_E and therefore ratio is well below 1 (low dilution rate = 0.17 hr^{-1} for efficient running).

Typical K_S values of S.cerevisiae on glucose are quoted as 25 mg l^{-1}. Since the K_S (glucose) values from these experiments are much higher (0.01-50g l^{-1}) it is to be assumed that S.cerevisiae has less affinity for this substrate, with its extraneous hydrolysis products than for just glucose. From table 30 K_S = 0.476 g l^{-1} on 10-15 g l^{-1} sugar (Ghose & Tyagi 1979) and 0.22 g l^{-1} on 120 g l^{-1} glucose (Rogers & Rosario 1978).

(d) Pseudomonas saccharophila - batch

By itself Y_P values were 0.07 - 0.25 (Y_E 0.05 - 0.2) and μ_{max} 0.04 to 0.2. When associated with S.cerevisiae Y_P ranged 0.15-1.0 Y_E 0.12-0.2 and μ_{max} 0.04-0.15. Judging by the ethanol produced the presence of this organism seems to have prevented the yeast from achieving its maximum potential.

Continuous - (D = 0.1) all values of Y_P 0.02-1.0 Y_E 0.018-0.2 DP 0.09-1.0 and $\frac{DP}{X}$ 9-100 were erratic and inconsistent between the separate fermentations. With S.cerevisiae values of Y_P 0.11-0.25, Y_E 0.09-0.2 DP 0.45-1.0 and $\frac{DP}{X}$ 0.7-9 were just as erratic.

(e) B.polymyxa

Batch

Fermented by itself there was a range of Y_P values 0.1-0.2 and μ_{max} 0.06-0.15. When associated with S.cerevisiae the overall yield of the fermentation was similar, the bacterium having apparently an adverse effect on the performance of S.cerevisiae.

Continuous

Problems with getting a low enough dilution rate so as not to cause washout, prevented intelligible results.

(f) A.aerogenes

Only one batch experiment was performed, in view of the low yield, this type of culture was abandoned in favour of continuous culture, the results of which were a substantial improvement.

10.11 Interpretation

There are difficulties in comparing the results of batch and continuous cultures because of the difference in developmental history of the organisms. A batch culture develops in time so that all observations, including the product, are functions of time and the culture age is important. In continuous culture, the culture develops in time but is time independent however the age of the individual cells is important and therefore so is the generation time or growth rate. This rate is a function of the nutrients in the culture. If plots of growth rate (μ) versus concentration (s) are made, both K_s and μ_{max} may be obtained from them. If there is competition between organisms then $K_sA < K_sB < K_sC$ and $\mu_mA > \mu_mB > \mu_mC$. Under these circumstances the left hand organisms will grow faster than the right hand ones at any value of s and D. Only if $K_sA < K_sB < K_sC$ and $\mu_mA < \mu_mB < \mu_mC$, would A, B and C be dependent on D (dilution rate) at some concentration of s, and the growth rates would be equal. If this concentration could be maintained in the chemostat, the concentration of organisms and presumably their activities can be made to remain constant. In the 2-member cultures used here, no instance of example two occurred.

The formation of a product may be either growth associated or non-growth associated. The production of ethanol from

glucose solutions is growth-associated and therefore dependent on the development of the population but in this research the maximum synthesis did not always coincide with the same phase of growth of the organisms used.

In batch cultures, two patterns emerged; either the maximum occurred during the exponential phase (starting from early on in the phase) or, at the end of the exponential phase and into the stationary phase. This can be seen from the graphical representation of population and product versus time. For S.cerevisiae and A.aerogenes the first condition applies, but for Ps.saccharophila and B.polymyxa, the second is applicable. When S.cerevisiae and Ps.saccharophila were grown together, the product formation coincided with the second condition, that is at the end of the log phase. Or, it more rarely paralled the growth of S.cerevisiae, cutting across that is, not dependent on the growth of Ps.saccharophila.

Since continuous cultures artificially create a permanent log phase it is advantageous that the product is produced then rather than at any other batch stage. The use of two organisms with varying growth-association patterns of product formation is of distinct disadvantage since, it seems in this form of competition the ability of the one to produce ethanol from early on in its log phase is suppressed by the one whose norm is to produce it later in its growth phase.

In all 2-member cultures experimented with, a diauxic effect occurred, with the yeast growing preferentially on the substrate before the other organism. However the competition within the culture vessel encouraged the yeast to use the sugar for growth (and presumably maintenance) rather than product formation, especially in the earlier stages. This can be verified by examining the lower performance yields in 2-member cultures.

CHAPTER 11

SUMMARY AND RECOMMENDATIONS FOR FUTURE WORK

The results of these experiments have shown that the production of sugars from cellulose (paper) at high temperature and pressure by the process of acid hydrolysis is feasible on a continuous basis, even though the yields were lower than expected from the kinetic predictions of Saeman (1945) and Porteous (1967). However they confirm the work of Converse et al. and Grethlein. The series of experiments however, has established a basis for a more detailed engineering consideration of this rig structure. Reactor optimisation studies are now taking place in the slightly modified rig in which all the heating tapes are wound on the pre-reactor, although the reactor itself is still in place and in use. The heating of the slurry from 90°C to approximately 230°C takes place in the pre-reactor and the maximum temperature is more or less maintained as the mixture passes through the reactor tube.

Sugar concentrations of up to 30% conversion are being produced from newsprint, although the repeatability of results has not yet been established. The use of a single stage heating system has eliminated the problems (previously identified) of temperature difference between the two reactors and its affect on the production of sugars.

It has also made the rig easier to run, with far fewer fluctuations in temperature and pressure than were previously experienced.

The origin of the metal ion contamination, which occurred in two of the runs, is probably the original pre-heaters which were replaced part way through the experimentation time. No evidence of corrosion of the stainless steel tubing which makes up the pre-reactor and reactor has been found and other runs have not been contaminated. The colour of the product from the rig was usually some shade of brown, which was probably due to the formation of humic substances which will occur at the breakdown of the sugars when the optimal temperature for sugar production has been exceeded. The colour during the contaminated runs varied from green through brown to black, which was the first indication of possible metal ion contamination. The sugars now being formed in the modified rig are a pale straw colour which darkens on standing. This discolouration is probably due to the acid continuing to attack the sugars and forming humic substances.

Fagan (1969) had metal contamination (iron mainly) which interfered with his sugar analysis giving unrepresentative low yields. In his opinion the ions were affecting the test only and not the hydrolysis process. The contaminated trials of the present research (9 and 10 Table 7) show

low or non-existent conversion, this was probably due to temperature fluctuations which, however, were not exclusive to these two runs. Providing the construction of the parts of the rig continues to be of resistant stainless steel and other non-corrosive materials it is unlikely that metal ion contamination will be a problem for future work. It has been shown that yeast cells will adapt to this type of medium and achieve near theoretical yields.

It will be necessary to do liquid:solid ratio studies and feedstock impurity analysis before making any economic considerations or scale up designs. The maximum solid concentrations which can be pumped and handled in the reactor must be known.

Future fermentation work will include the use of the rig sugars as the only carbon source. The presence of any impurities or by-products of the feedstock, such as inks from newsprint and materials other than cellulose contained in sorted refuse could adversely affect the fermentation yields and growth of the organisms. For this reason it will be necessary to analyse the rig product for chemicals other than the sugars; If inhibition occurs, screening tests will have to be devised in order to find out if one or all of the impurities is at fault.

In the two member cultures much more experimentation is needed. The establishment of whether enzyme inhibition or repression is taking place to prevent higher yields and differences in growth association production is necessary. Also the exploration of the particular growth phases with which production formation is associated and the effect of using continuous culture versus batch.

This range of experimental work has shown that sugar may be successfully produced from cellulose and fermented to ethanol, and has laid down a basis for future experimentation.

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APPENDIX 1 CHEMICAL ANALYSES

(a) Somogyi-Shaffer-Hartman Determination of Sugar in Micro-biological Cultures

Method

1 Pipette 5 mls reagent into 50 ml boiling tube.

2 Add 1 ml sample (up to 5 mls possible depending on concentration).

3 Make volume up to 10 mls with distilled water.

4 Blank = 5 ml H_2O and 5 ml reagent.

5 Mix, stopper with a loose-fitting glass stopper and place in a boiling water bath for 30 mins - avoid agitation.

6 Remove and cool for 3 minutes in cold water to below 40°C - avoid agitation and do not cool below 30°C.

7 Add 1 ml 5N sulphuric acid, mix and allow to stand for 2 minutes.

8 Titrate with 0.005N sodium thiosulphate until most of the iodine has reacted, then add a few drops of boiled starch solution and titrate until blue colour just disappears.

Result

Calculation: Blank - unknown = cuprous oxide formed by the reducing action of the sugar.

Use a table of prepared standards to convert to milligrams of glucose.

Reagent	g l^{-1}
$CuSO_4 5H_2O$	100.0 - 75 mls of 10% solution
$NaHCO_3$	20.0
$Na_2 CO_3$ (anhydrous)	25.0
$C_4H_4KNaO_6$	25.0
KI	5.0
KIO_3	3.567 (0.1N)

Dissolve 25 g anhydrous sodium carbonate and 25 g Rochelle salt (potassium sodium tartrate) in 500 ml water. Do not heat to dissolve. Add 75 ml 10% copper sulphate using pipette, and keeping tip under solution to avoid losing carbon dioxide. Then add 20 g sodium bicarbonate and 5 g potassium iodide. Add 0.1N potassium iodate (3.567 g l^{-1}) according to the quantity of sugar to be analysed, usually 200 mls will do. Dilute to 1 litre and mix.

Sodium thiosulphate must be prepared daily - dilute 25 ml 0.1N sodium thiosulphate to 500 mls. Starch solution - boil 1 g reagent grade soluble starch with 50 ml water.

Milligrams of Glucose Corresponding to the Difference Between the Titrations Values for the Blank and the Unknown

Ml. of 0.005 N Sodium thiosulphate	Tenths of ml of 0.005N Sodium thiosulphate									
	0	1	2	3	4	5	6	7	8	9
0			0.05	0.06	0.08	0.09	0.10	0.11	0.13	0.14
1	0.15	0.16	0.17	0.19	0.20	0.21	0.22	0.23	0.24	0.25
2	0.27	0.28	0.29	0.30	0.31	0.32	0.33	0.35	0.36	0.37
3	0.38	0.39	0.40	0.41	0.42	0.43	0.45	0.46	0.47	0.48
4	0.49	0.50	0.51	0.53	0.54	0.55	0.56	0.57	0.58	0.59
5	0.60	0.61	0.63	0.64	0.65	0.66	0.67	0.68	0.69	0.70
6	0.72	0.73	0.74	0.75	0.76	0.77	0.78	0.79	0.80	0.82
7	0.83	0.84	0.85	0.86	0.87	0.88	0.89	0.90	0.91	0.93
8	0.94	0.95	0.96	0.97	0.98	0.99	1.00	1.01	1.02	1.04
9	1.05	1.06	1.07	1.08	1.09	1.10	1.11	1.12	1.13	1.15
10	1.16	1.17	1.18	1.19	1.20	1.21	1.22	1.23	1.24	1.26
11	1.27	1.28	1.29	1.30	1.32	1.33	1.34	1.35	1.36	1.37
12	1.38	1.39	1.40	1.42	1.43	1.44	1.45	1.46	1.47	1.48
13	1.49	1.50	1.52	1.53	1.54	1.55	1.56	1.57	1.58	1.59
14	1.60	1.61	1.63	1.64	1.65	1.66	1.67	1.68	1.69	1.70
15	1.71	1.72	1.74	1.75	1.76	1.77	1.78	1.79	1.80	1.81
16	1.82	1.83	1.85	1.86	1.87	1.88	1.89	1.90	1.91	1.92
17	1.93	1.94	1.95	1.96	1.97	1.98	1.99	2.01	2.02	2.03

(b) Somogyi-Shaffer Method for Ethanol

Method

1 Distill 20 mls of culture, collect 10 mls.

2 Take 1 ml - 250 ml flask.

3 Add 10 mls oxidising reagent, stopper, mix.

4 Do blank of 1 ml distilled water.

5 Let stand 30 mins.

6 Add 100 mls water + 0.5 gm KI.

7 Titrate with N sodium thiosulphate and starch indicator.

8 Result. test-blank = amount of dichromate reduced by the alcohol present.

9 $2Cr_2O_7^{2-} + 3\ C_2H_5OH + 16H^+ = 4Cr^{3-} + 3CH_3COOH + 11\ H_2O$

From this equation 1 molecule of ethanol consumes 4 equivalents of dichromate, 1 mg ethanol = 4 mg dichromate.

Oxidising reagent

Dissolve 3.333 g potassium dichromate $K_2Cr_2O_7$ in 500 mls water.

Add to this, 421 mls 36N H_2SO_4

Makeup 1 l with water.

(c) High Pressure Liquid Chromatography (HPLC)

By this method virtually any combination of sugars may be separated from each other. The instrument used was a DuPont 830 HPLC.

The eluent was a mixture of acetonitrile and water. By manipulating the ratio of one to the other and/or the flow rate, any desired separation of sugars may be achieved. During experimentation it has been found that 80:20 acetonitrile:water is about the optimum, and at 525 psi the flow rate was 1.1 mls $minute^{-1}$.

The concentrations of the various sugars may be determined from peak-height measurements relative to peak-height standard curves prepared from pure individual sugars, examples are shown below.

(d) Chromatography Parameters

1 sugar analysis

Polygosil NH_2 R1 0 4 x 10^{-5} R1UFSD

Differential refractometer detector - sensitivity - attenuation - recorder, chart speed - 5mm min^{-1}

columns - size 22.5cm x 5mm i.d.

temperature - ambient

eluent - acetonitrile : water mixtures - 80:20

filtered through 3 millipore filter before use

flow rate - 2.0 ml min^{-1}

pressures - 1000 psi

sugar standards - 10 1µl mixture of 7 carbohydrates

2 ethanol analysis - gas chromatography

column length - 9' i.d. 2 mm

support Chrom G Awens mesh size - 80-100

liquid phase Ucon 50 HB - 280 x-3%

Carrier gas N_2 flow - 30 ml min^{-1}

Attenuation - 1 x 10^3

chart speed - 5 mm min^{-1}

temperatures - oven 90^o, detector 250^o, injector $2[illegible]^o$

(e) Coulter Counter

micro-organism concentration

19 μ aperture

dilution made with physiological saline

counts of >3 μ represent yeasts

counts of >0.7 μ minus the yeast count represent bacteria

calibration curves were produced to link dry weight with micro-organism counts.

APPENDIX 2 REJECTION OF DATA

Since 2 measurements in the set appeared to deviate significantly from the others, a decision had to be made whether they should be rejected. The $4\bar{D}$ method was used:

1 Disregarding the 2 questionable measurements the mean and new average deviation were calculated with the remaining measurements.

disregarding 30:

average deviation = $\frac{29.55}{13}$

$\bar{D} = 2.27 \quad 4\bar{D} = 9.08$

mean = 2.31 30 - 2.31 = 27.69 so 30 differs from the mean by more than 4D.

2 for disregarding 30 & 11

$\bar{D} = \frac{20.86}{12} = 1.74 \quad 4\bar{D} = 6.96$

mean = 1.58, 11 - 1.58 = 9.42 which is more than $4\bar{D}$.

APPENDIX 3 MEDIA

Since the chemical constituents of the medium must meet all elemental requirements for cell growth and product formation, as well as energy and trace minerals, an examination of generalised cell composition is useful, as in the table below from Wang et.al. (1979).

Typical elemental composition of micro-organisms

	% of cell dry weight
Carbon	50
Nitrogen	7-12
Phosphorus	1-3
Sulphur	0.5 - 1.0
Magnesium	0.5

(a) Tests 1 & 2

	$g\ l^{-1}$
KH_2PO_4	1.0
NH_4Cl	0.2
$MgSO_4.7H_2O$	0.2
glucose	10
xylose	10

(b) Tests 3-10

	g l^{-1}
KH_2PO_4	4.4
Na_2HPO_4	4.8
NH_4Cl	1.0
$MgSO_4.7H_2O$	0.5
glucose	50
xylose	50

(c) Ps.saccharophila resuscitation medium

Solution A

	g l^{-1}
KH_2PO_4	4.4
Na_2HPO_4	4.8
NH_4Cl	1.0
$MgSO_4.7H_2O$	0.5
(Agar	2%)

Solution B

$FeCl_3$	10.0
$CaCl_2$	1.0

Solution C

sucrose	20. w/v

To 100 ml solution A, add 0.5 ml solution B and 1 ml solution C. Ps.sacc. maintained also on agar slopes of this inorganic medium.

(d) Basal synthetic

	g l^{-1}
KH_2PO_4	1.0
NH_4Cl	0.2
$MgSO_4 7H_2O$	0.2

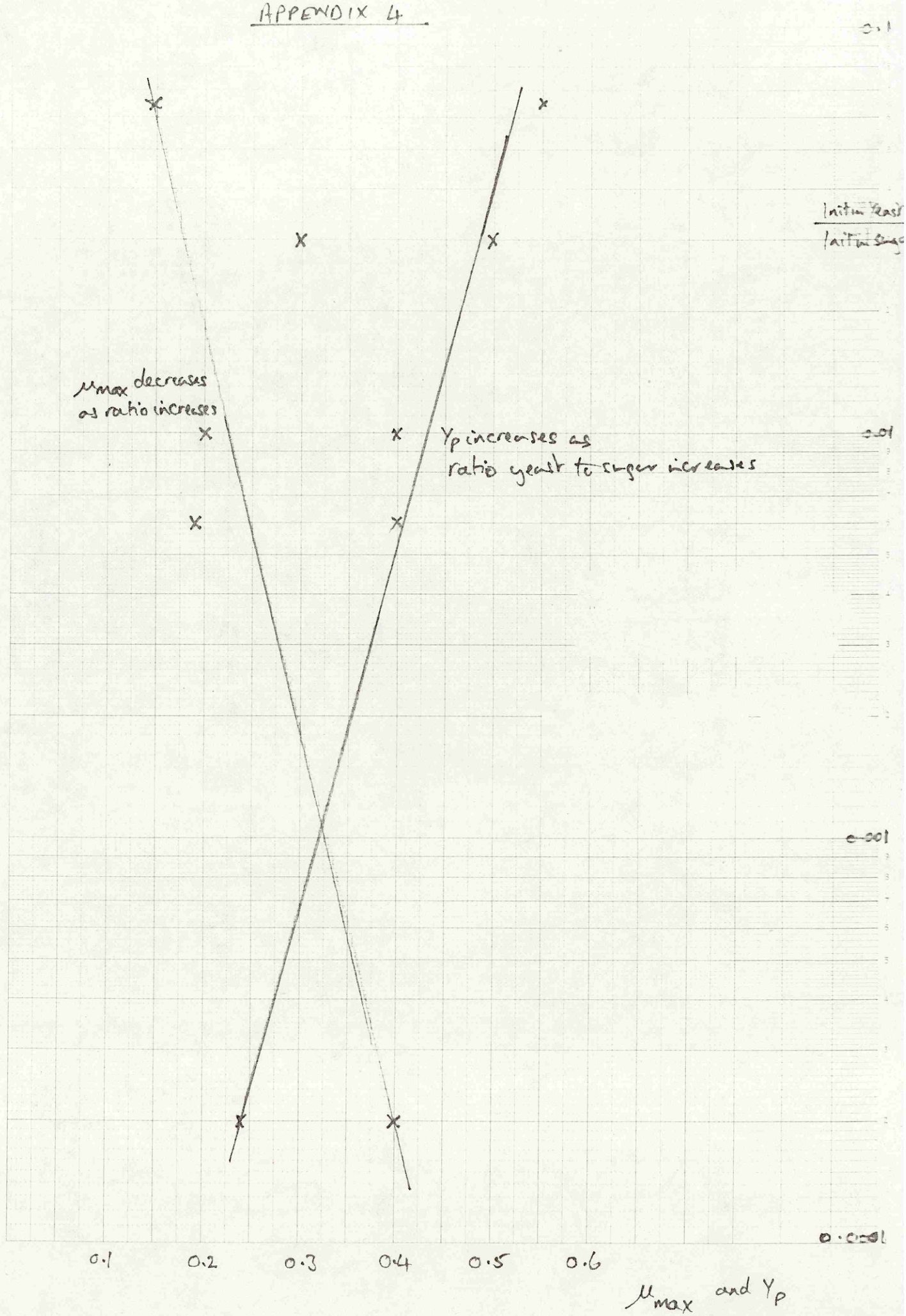
acid hydrolysis sugars up to 5.0 g l^{-1} from hydrolysis run number 8 and 14

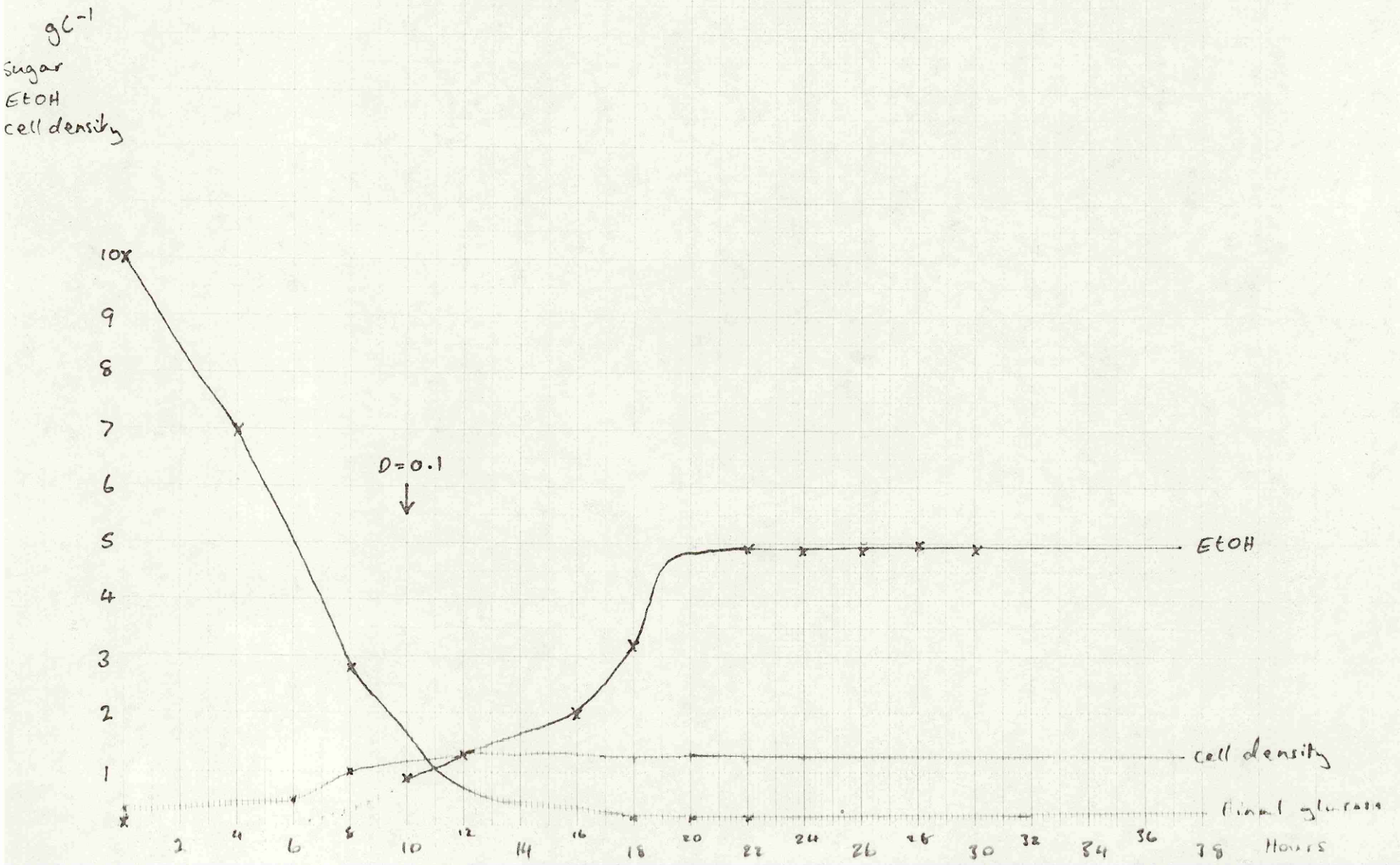
made up to varying concentrations with Analar Glucose

(e) Additions to contaminated hydrolysis sugars

	g l^{-1}
KH_2PO_4	1
NH_4Cl	0.2
$MgSO_4 7H_2O$	0.2

APPENDIX 4


0.1
Initial Yeast
Initial Sugar
μmax decreases as ratio increases
Yp increases as ratio yeast to sugar increases
0.01
0.001
0.0001
0.1
0.2
0.3
0.4
0.5
0.6
μmax and Yp

S.cerevisiae - continuous culture after 18hrs 30°C pH 5.0

initial glucose 10 gl^{-1} D = 0.1