THE BIOLOGICAL UTILIZATION OF BAGASSE, A LIGNOCELLULOSE WASTE

Dr J C Paterson-Jones (Editor)

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Renewable Feedstocks

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SUMMARY

1. This report describes the results of the Biological Utilization of Bagasse Programme, a programme of the CSIR's Foundation for Research Development, aimed at developing and evaluating the expertise and technology for hydrolyzing the hemicelluloses and cellulose in sugarcane bagasse, using enzymic hydrolysis of the cellulose, to provide fermentation substrates for ethanol, single cell protein or other industrial products.

2. The following processes were successfully developed:

- the extraction and hydrolysis to xylose and other monomeric sugars of the hemicelluloses using dilute sulphuric acid;
- the direct fermentation to ethanol of the sugars in the hemicelluloses hydrolysate;
- the production of single cell protein from the hemicelluloses hydrolysate;
- the pretreatment of acid-extracted bagasse by attritor milling to permit successful enzymic hydrolysis of the cellulose;
- the simultaneous enzymic hydrolysis of the cellulose in acid-extracted, attritor-milled bagasse and its fermentation to ethanol;
- the simultaneous enzymic hydrolysis of the cellulose in furfural factory residue and its fermentation to ethanol;
- the production of very high activity cellulase.

3. The processes were developed to a scale large enough to allow an evaluation by an independant engineering consultant of the commercial feasibility of their industrial adoption:

- The production of ethanol from furfural factory residue with molasses as supplementary feed during the milling off-season showed a potential internal rate of return of 24 percent over a 10 year period at a selling price of R0,70 l^{-1} for ethanol with no allowance for inflation. Allowance for a 10 percent annual inflation rate increased the internal rate of return to 34 percent.

- The production of ethanol by enzymic hydrolysis of acid-extracted bagasse cellulose is not economic at present. Even greater enzyme productivities and efficiencies would be required to make this technology currently economically viable.

- The production of single cell protein from bagasse hemicelluloses hydrolysate with molasses as supplementary feed during the milling off-season showed a potential internal rate of return of 22 percent over a 10 year period at a selling price of R730 t^{-1} crude product (R1 000 t^{-1} pure product adjusted for moisture and ash). Allowance for an annual 10 percent inflation rate increased the internal rate of return to 32 percent.

- The economic feasibility of the production of ethanol from bagasse hemicellulose hydrolysate was marginal.
- Attritor-milled acid-extracted bagasse has very high digestibility for ruminants and could provide a component of ruminant feeds.
- The utilization of the residual lignin in, for example, adhesives manufacture could improve the economic viability of these processes.

4. The technologies developed could be applied with minor modification to other lignocellulosic resources.

5. The programme provided a means by which expertise in fermentation research and technology and in associated fields was developed. During the course of the programme, funding was provided for research which resulted in the award of thirteen MSc and five PhD degrees.

6. Major advances in the state of the art of the field of lignocellulose degradation and utilization were made by researchers in this programme. This work was published in international journals.

ABSTRACT

The technology to produce ethanol and single cell protein from sugarcane bagasse has been developed. Hydrolysis of the hemicelluloses by dilute sulphuric acid produces a solution of xylose and other sugars and embrittles the cellulose/lignin residue to enable attritor milling to be an effective, low energy consuming pretreatment for the enzymic hydrolysis of the cellulose. Very high activity cellulase has been produced with high productivity at pilot plant scale. Processes have been developed for the production of single cell protein from the hemicelluloses and cellulose hydrolysates and the production of ethanol from the the cellulose by simultaneous saccharification and fermentation and from the hemicelluloses hydrolysate by direct fermentation. The economic viability in South Africa of industrial processes based on these technologies was assessed by an independent consultant.

SAMEVATTING

Die tegnologie is ontwikkel om etanol en enkelselproteïen van suikerrietbagasse te produseer. Hidrolise van die hemisellluloses deur swak swaelsuur lewer 'n oplossing van xilose en ander suikers en verbros die residu sellulose/lignien om attritormaling 'n effektiewe en energiegoedkoop voorbehandeling vir die ensiemhidrolise van die sellulose te maak. Besonder hoë aktiwiteit sellulose is op loodskaal teen hoë produktiwiteit gemaak. Prosesse is ontwikkel vir die produksie van enkelselproteïen van die hemisellulose en sellulose hidrolisaat en die produksie van etanol deur gelyktydige saggarifikasie en fermentasie van die sellulose en deur direkte fermentasie van die hemisellulose hidrolisaat. Die ekonomiese lewensvatbaarheid in Suid-Afrika van industriële prosesse gebasseer op hierdie tegnologië is deur 'n onafhanklike konsultant geëvalueer.

ACKNOWLEDGEMENTS

The research leaders who took part are listed with their reports at the end of this document.

Thanks are due to the following who served the programme as advisers or as members of the steering committee:

Mr J P de Wit, (chairman 1979 - 1981), Dr A B Ravnö, Mr M A Buchalter, Mr J L Buzzard, Mr D du Toit, Professor D E Eveleigh, Dr A Kistner, Dr B K Loveday, Dr F G Neytzell-de Wilde, Dr R G Noble, Dr L Novellie, Professor H J Potgieter, Dr D Schuler, Mr I A Smith, Dr B Strydom, Professor J P van der Walt.

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INTRODUCTION

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THE BIOLOGICAL UTILIZATION OF BAGASSE PROGRAMME

Lignocellulose, a complex natural material comprising cellulose, hemicelluloses and lignin, is produced in vast quantities every year through photosynthesis in higher plants. It is a renewable resource, the result of the growth of wild plants and also of the cultivation of agricultural crops and forests. In forestry, trees are grown specifically to produce lignocellulose as timber or for the production of paper pulp. In the production of other agricultural crops, the lignocellulose is produced as a byproduct which may or may not be utilized.

During the seventies, the CSIR's Cooperative Scientific Programmes (CSP) began funding research into the utilization of lignocellulose. In 1979 this research was consolidated into a goal oriented, cooperative programme to focus the effort on a single lignocellulosic substrate (bagasse), a single product of its conversion (ethanol) and a single conversion process (enzymic saccharification of its cellulose).

The goal of the programme was the development of a technically and commercially viable process to convert bagasse to ethanol to realize the potential for liquid fuel production from bagasse which was perceived at that time (for instance Woodburn 1977). Subsequently the goal was expanded to include single cell protein.

Of the several lignocellulosic resources potentially available in South Africa, bagasse was chosen because:

- it is produced at some sugar factories in excess of its usage as a fuel and could form a large lignocellulosic resource;
- it is available already collected and in a partially processed form at the sugar mills throughout most of the year;
- sugar mills could provide an established industrial infrastructure for a bagasse processing plant.

Ethanol was chosen as the preferred product of the process because it had potential both as a liquid fuel and fuel additive and as a chemical feedstock.

The acid saccharification of cellulose has been well studied and commercially developed and practised (Ladisch 1979; Brown 1982). Although elsewhere in the world efforts were being made to improve the process (Longo and Neto 1980; Rugg and Brenner 1980) for the acid saccharification of lignocellulose, the enzymic conversion of bagasse cellulose was chosen as the preferred route in the programme because:

- it held the promise of providing a high yield process carried out under mild conditions;
- unlike the acid process, it was a new process with potential for considerable improvement through research.

It was accepted that the research programme was a high risk effort. The successful outcome, however, would provide a process of considerable benefit to South African industry and agriculture, applicable with minor modifications to a wide range of available lignocellulosic wastes to produce a wide range of possible products including ethanol.

BAGASSE IN SOUTH AFRICA

In South Africa approximately 3 000 000 t bagasse are produced yearly by 16 factories located mainly along the Natal coast. Of this, about 90 percent is used as the boiler fuel in mills and sugar factories and a further 9 percent is also used for making furfural, animal feeds, paper and boards.

How much surplus bagasse could be generated would depend on the type of equipment installed at individual factories and on whether or not they had sugar refineries attached to them. Surplus bagasse can be generated, but at the expense of installing energy efficient equipment such as high pressure boilers. Some indication of the potential is available from the Taiwan Sugar Corporation (Sang and Yen 1981) where financial incentives caused the company steadily to convert from zero surplus in 1950 to a surplus equivalent to 35 percent of the bagasse by 1972. The newest South African factory (Felixton) was designed for high thermal economy because an associated paper factory created a demand for bagasse. Ultimately this factory could run with a bagasse surplus of 45 percent, but this will be achieved only by the installation of expensive vapour recompression equipment (Reid and Rein 1983).

The generation of surplus bagasse is therefore possible but would involve costs which would vary from factory to factory. These costs are generally less than that of burning coal as a replacement for the bagasse. In this programme, the value of bagasse has been taken as its coal replacement value (R26 t^{-1} dry in the Durban area). The bulkiness of bagasse precludes its economic transport to a central site so that in order to provide adequate bagasse for a reasonably sized hydrolysis factory it would be necessary to use all of the bagasse from a single factory and replace it with coal. Table 1 gives details of bagasse production in South Africa and of its usage in byproducts.

Table 1. Bagasse production in South Africa for the 1985-1986 season (Lamusse 1986) and its usage in byproducts.

Factory	Bagasse production (t, dry)	Byproduct usage (t, dry)	Nature of byproduct
Malelane	224 800	4 000	Board and feed
Pongola	133 526	-	
Umfolosi	163 733	-	
Entumeni	43 970	Say 5 000 [a]	Feed
Felixton	380 739	110 000	Paper
Amatikulu	250 318	2 000	Board
Darnall	207 121	-	
Maidstone	272 123	Say 5 000 [a]	Feed
Mount Edgecombe	137 985	-	
Glendale	55 821	-	
Gledhow	215 148	80 000	Paper
Noodsberg	169 869	-	
Union Co-Op	73 043	-	
Illovo	125 981	-	
Sezela	285 237	50 000 [b]	Furfural
Umzimkulu	151 572	-	
TOTALS	2 891 026	256 000	(9 percent of total)

a Actual figures are confidential.

b This excludes the residue which is returned to the sugar factory.

After hydrolysis of bagasse to remove the cellulose and hemicelluloses, a residue remains which is lignin rich and contains about 30 percent of the original calorific value of the bagasse. This residue may have value as a boiler fuel and offset some of the cost of the bagasse. It dries to coal-like lumps but no studies have been made on the economics of drying and burning it. Alternatively, the residual lignin may have potential as a raw material for wood adhesives. The National Timber Research Institute of the CSIR has developed the technology to produce adhesives from the byproducts of the pulping of bagasse.

ECONOMICS

South Africa is unique in that its heavy chemicals industry and a substantial part of its liquid fuels industry is based on coal as feedstock. During the course of this programme, industry, organized agriculture and government perceptions of the potential of ethanol as a liquid fuel and chemical feedstock changed greatly. The large increase in the price of crude oil in the late 1970's and early 1980's resulted in the establishment of the SASOL II and SASOL III fuel-from-coal plants. Simultaneously, several efforts were made to obtain Government approval to establish fuel ethanol production from agricultural products and fuel methanol production from coal. Until now, only the SASOL fuel from coal processes have become established industries. The later collapse in the crude oil price, the development of the Mossel Bay gas fields for liquid fuel production, the recent drought and the fact that SASOL II and SASOL III produce large quantities of ethanol as a byproduct have affected the potential development of a fuel ethanol industry. Despite this, but because of the impact of recent low world prices, the South African sugar industry recently commissioned a detailed study of the potential for fuel ethanol production from sugar. Internally, the economic environment is further complicated by agreements between SASOL and the oil companies and Government on the marketing of fuel. At present the potential for fuel ethanol production in the short term is uncertain.

The economic potential of ethanol and other products was investigated at different stages against this continually shifting economic background. In 1980 the economic potential of ethanol, acetone, n-butanol, acetic acid, butane-2,3-diol and xylose was investigated. In 1983, this information was updated and expanded and ethanol, furfural, acetone, butanol, butadiene, itaconic and fumaric acids, glycerol, mannitol, xylitol, erythritol, arabinitol, citric acid, lactic acid, gluconic acid, iso-ascorbic acid, tartaric acid, industrial gums and thickeners, amino acids and proteinaceous products compared (Kamper et al 1983). It was concluded that only ethanol and single cell protein produced from bagasse had any substantial market potential in South Africa.

Research progress was evaluated (Flach 1982; Ramsay 1983a; Ramsay 1983b) by cost analyses at industrial scale of the process developed by the programme to convert bagasse cellulose to ethanol. As a result, the process development unit was built at the Sugar Milling Research Institute in 1985 and was used to integrate and evaluate at a single site and at a scale large enough to allow accurate costing, the processes developed by the separate research groups.

The process development unit studies, of two years duration, were the culmination of the cooperative efforts of all the research groups and industrial advisers involved in the programme. An essential part of the process development unit studies was the final economic evaluation by an independent engineering consultant of the economic viability of the processes developed to produce either ethanol or single cell protein from bagasse hemicelluloses and cellulose.

The most economically promising technologies developed in the programme were the production of ethanol from furfural factory residue and single cell protein from bagasse hemicellulose hydrolysate. In the first case, the costing indicated a 10 year internal rate of return of 15,4 percent assuming a sales price of R0,70 l^{-1} for the ethanol. The internal rate of return increased to 24,4 percent if molasses were used as a supplemental feedstock during the milling off-season. In the second case, the costing indicated an internal rate of return of 19 percent assuming a sales price for the single cell protein of R1 000 t^{-1} crude product, increasing to 24,7 percent if molasses were used as supplement in the off-season. Both cases were based on zero inflation rate. An inflation rate of 10 percent increased the internal rates of return greatly in both cases.

MAJOR ACHIEVEMENTS

A feature of this programme has been the high level of cooperation and interaction between different research groups and between these and interested industries, both formally and informally. This was fostered by the programme management and resulted in rapid scientific and technical progress being made.

A major achievement was the successful design, erection and operation of the process development unit. Of all the advances made, three particularly stand out:

- the development of a pretreatment procedure, involving dilute acid hydrolysis of the bagasse hemicelluloses and the attritor milling of the resulting embrittled residue, for the enzymic hydrolysis of the bagasse cellulose;
- the development of technology to produce very high activity cellulase;
- the development of technology for the direct fermentation of the hemicellulose hydrolysate to ethanol.

The processes developed are summarized in Figure 1. The major part of the hemicelluloses can be removed by the production of furfural (the commercial process operated by SmithChem at Sezela) or by a dilute acid prehydrolysis. The prehydrolysis yields a xylose rich solution which, after suitable treatment, can be fermented to ethanol and/or used as the substrate for single cell protein production, and a residue which, because it is embrittled, can be readily milled as an effective pretreatment for enzymic hydrolysis of the cellulose. The residue can also be used as a boiler fuel with the added advantage that it can be readily compressed into briquettes. The cellulose in the residue from either furfural production or dilute acid prehydrolysis and attritor milling can be hydrolyzed to form a glucose solution for fermentation to ethanol or single cell protein, or for utilization as such, and a lignin rich residue. The lignin rich residue may have potential as a boiler fuel or as a source of lignin for adhesives.

The programme funded research resulting in the granting of thirteen MSc and five PhD degrees and has contributed to the establishment of a research infrastructure for the fermentation industry. In addition, the programme provided a means for communication between researchers in different disciplines and successfully mobilized a scientific community.

The programme was managed as a goal oriented cooperative programme with a coordinator active both at scientific and technical as well as at administrative level and a steering committee. The Sugar Milling Research Institute played a pivotal role in the programme at the technical level and as a contact point between the sugar industry in general and the programme. The process development unit was successfully set up and operated by the Sugar Milling Research Institute with advice from the different research groups involved in the programme.

REPORT FORMAT

The structure of this report follows the steps in the process (Figure 1). This introduction is followed by chapters on the dilute acid prehydrolysis process, the conversion of the xylose solution so formed to ethanol or other products, attritor milling as a pretreatment for enzymic hydrolysis of the cellulose in the residue, the production of the enzyme cellulase, the enzymic hydrolysis process and fermentation of the glucose so formed to ethanol and the production of single cell protein from both the hemicellulose and cellulose hydrolysates. The final chapter summarizes the various process options and the costings of industrial processes based on those options.

The research groups, advisers and consultants involved in the programme are listed, as are the unpublished reports and the publications which arose from the programme.

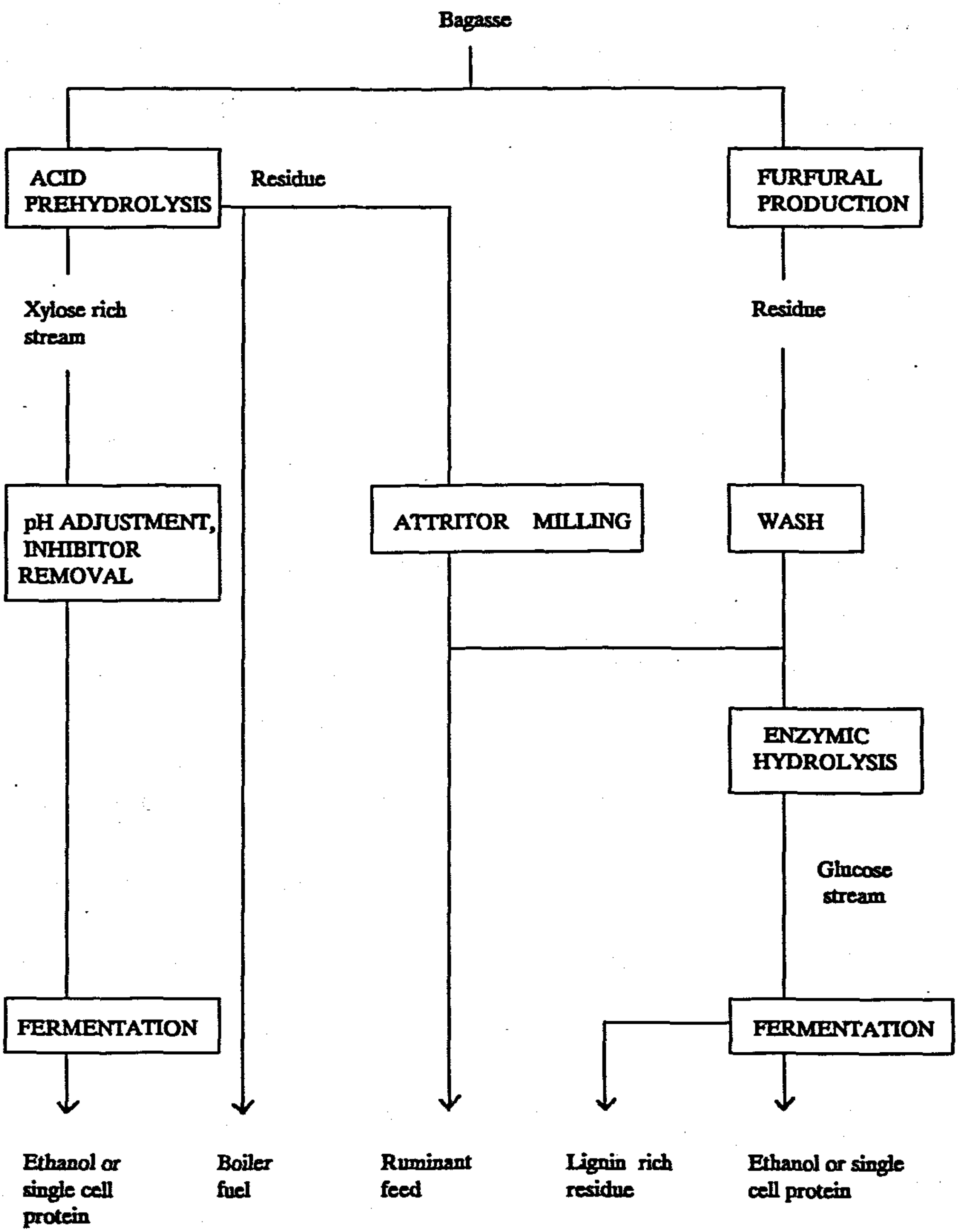

Figure 1. Process options.

The work carried out has provided an effective technology for producing pentoses and glucose from sugarcane bagasse. The technology is applicable with minor modifications to other lignocellulosic substrates of which there are several which are produced in substantial amounts in South Africa by conventional agriculture and forestry. The economics of processes to produce industrial materials from biomass will change with a changing economic and sociopolitical environment. Processes which are not currently economic may become economic in the future. The economics of the overall process to produce single cell protein or ethanol from bagasse may be improved by the utilization of the residual lignin. The possible use in adhesives of the lignin from enzymically hydrolyzed furfural factory residue is currently being examined.

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Italics indicate work carried out in the programme

CHAPTER 1. PREHYDROLYSIS OF BAGASSE

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CHARACTERISTICS OF BAGASSE

CHEMICAL CHARACTERISTICS

Fresh bagasse contains 46-52 percent moisture. The major components of dry bagasse are shown in Table 2.

Table 2. Major components of dry bagasse (Paturau 1982; Trickett and Neytzell-de Wilde 1982).

Cellulose	35-42 percent (usually 38 percent)
Hemicellulose	31-35 percent (usually 33 percent)
Lignin	19-23 percent (usually 22 percent)
Ash	1-4 percent (usually 3 percent)

The cellulose is a high molecular-mass compound of ß-1,4-linked glucose units. The chains are unbranched but hydrogen bonding holds neighbouring chains together to form a fibrous crystalline material insoluble in water and resistant to hydrolysis. At intervals along the microfibrils there are non-crystalline, disordered zones which are more susceptible to hydrolysis (Cowling and Kirk 1976).

The hemicelluloses of bagasse are xylose polymers with substituents such as arabinose, acetate and methylglucuronic acid groups (Table 3). They bind to the cellulose via hydrogen bonding but are hydrophilic and non-crystalline and are therefore more susceptible to hydrolysis than is cellulose.

Table 3. The chemical composition of bagasse hemicellulose hydrolysates (Trickett and Neytzell-de Wilde 1982; Du Toit et al 1984).

Component in hydrolysate	Quantity (percentage of original bagasse) Trickett et al	Du Toit et al
Arabinose	4,4 (6,6)*	4,9
Galactose	-	1,5
Xylose	24,0 (27,0)*	25,2
Glucose	4,0	7,6
Acetic acid	5,5	-

* () = calculated figure corrected for degradation occurring during extraction

Lignin is a complex three dimensional polymer with phenylpropane repeat units. It is closely associated with cellulose and hemicellulose, giving the lignocellulose its mechanical rigidity and part of its protection against rapid microbial decomposition.

The amino acid composition of bagasse is only 0,97 percent, mostly aspartic acid (0,12 percent), glutamic acid (0,13 percent) and leucine (0,10 percent) (Du Toit et al 1984).

PHYSICAL PROPERTIES

The cellular composition of bagasse is shown in Table 4.

Table 4. The cellular composition of bagasse. (Paturau 1982).

Cellular component	Mass (percent)	Length (mm)	Length/width
True fibres (rind and vascular tissues)	55	1,5	70
Vessel segments	20	1,0	9
Pith (parenchyma cells)	20	0,3	5

The chemical compositions of the fibre and the pith are almost identical but their physical behaviours are different in that the fibres tend to bond to one another whereas the pith separates out (Paturau 1982).

Unbaled bagasse is expensive to transport because of its low bulk density. On a dry mass basis the bulk density varies between 80 and 180 kg m^{-3} depending on compression. When piled freely in a truck it has a dry bulk density of 97 kg m^{-3} which could be increased to 148 kg m^{-3} by careful packing and compression (Wu et al 1973). The bulk density of baled bagasse is about 300 kg m^{-3} (dry basis).

PREHYDROLYSIS OF BAGASSE

In this report the term prehydrolysis is used to designate any process whereby hemicellulose is selectively hydrolysed. For reasons such as the following the need for this selective removal is often stressed:

- it ensures that the hemicellulose is exposed to the mildest possible conditions thus minimizing the formation of decomposition products such as furfural;

- it enhances the susceptibility of the residual cellulose to enzymic or acid hydrolysis (Magee and Kosaric 1985).

Prehydrolysis is particularly relevant as a first step in the conversion of bagasse to fermentable sugars for reasons such as the following:

- bagasse has a high (33 percent) hemicellulose content;

- it is appropriate to separate the hydrolysis products of hemicellulose from those of cellulose because they are different and therefore generally require different processing routes;

- the removal of hemicellulose reduces the mass of bagasse which passes to subsequent processing stages;

- prehydrolyzed bagasse is brittle and therefore requires considerably less energy for fine grinding than does whole bagasse (Purchase 1981). This is relevant because fine grinding is an effective pretreatment for cellulose prior to hydrolysis.

Prehydrolysis can thus either form part of a pretreatment process or can stand alone as a process for producing a xylose rich solution from bagasse.

Literature on prehydrolysis of lignocelluloses is plentiful, but relates mainly to substrates other than bagasse and is therefore of limited value for this programme because different substrates behave differently. Softwood, for example, generally has less than 7 percent potential xylose. An extensive general review of hemicellose extraction and utilization is given by Magee and Kosaric (1985), but bagasse is hardly mentioned. Trickett (1982) and Trickett and Neytzell-de Wilde (1982) therefore make a major contribution to the literature on prehydrolysis of bagasse.

THE INFLUENCE OF TEMPERATURE, TIME AND ACID CONCENTRATION

Trickett (1982) reviewed the process and reaction kinetics of hemicellulose hydrolysis in dilute acid solutions. He observed two first order hydrolysis rates and concluded that two fractions were present in hemicellulose, one easily hydrolyzed, the other more resistant. Trickett also reviewed the kinetics of conversion of xylose to furfural and the subsequent fate of furfural. The overall reaction sequence may be summarized as follows:

- transformation of pentosans to pentoses (hydrolysis)
  $(C_5H_8O_4)_n + nH_2O \longrightarrow nC_5H_{10}O_5$

- transformation of pentoses to furfural (dehydration)
  $nC_5H_{10}O_5 \longrightarrow nC_5H_4O_2 + 3nH_2O$

- possible furfural condensation and/or destruction.

Using the evidence that bagasse hemicellulose consists of two fractions, both hydrolyzing simultaneously but at different first order reaction rates, Trickett showed that the easily hydrolyzable fraction contained 165 mg xylose g^{-1} of bagasse while the more resistant fraction contained 105 mg g^{-1}. Based on these values the following model was proposed to express the *total xylose yield as a function of the hydrolysis time, temperature and acid concentration:*

$$\text{Total xylose yield (mg g}^{-1}) = \frac{165\,k_1}{(k_3-k_1)}(\exp(-k_1t) - \exp(-k_3t)) + \frac{105\,k_2}{(k_3-k_2)}(\exp(-k_2t) - \exp(-k_3t))$$

where

$$k_1 = 0{,}1224 \times 10^{-2} \times C \exp\left[\frac{-24{,}680 \times 10^3}{R}\left(\frac{1}{T} - \frac{1}{348{,}15}\right)\right]$$

$$k_2 = 0{,}1078 \times 10^{-3} \times C \exp\left[\frac{-22{,}343 \times 10^3}{R}\left(\frac{1}{T} - \frac{1}{348{,}15}\right)\right]$$

$$k_3 = 0{,}2793 \times 10^{-1} \times C \exp\left[\frac{-33{,}56 \times 10^3}{R}\left(\frac{1}{T} - \frac{1}{348{,}15}\right)\right]$$

R = 1,987 cal (g mole)$^{-1}$ T^{-1}

C = concentration of H_2SO_4 (g (100 ml)$^{-1}$ of H_2O)

T = temperature in K

This model applies where liquid:solid ratios are 3,6:1 or greater. Due to the effect of temperature on k_3 (xylose destruction rate), hydrolysis temperatures should not exceed 130°C unless the reaction time can be limited to a few minutes by using a continuous reactor (Taylor 1987).

Based on this model, xylose yields as a function of time at different temperatures for a constant acid concentration has been plotted in Figure 2. The effect of temperature on the time required to produce a given yield is evident. Increasing the temperature from 100°C to 110°C results in a saving of over 50 percent in time. The presence of the two xylan fractions causes the rate of hydrolysis to be relatively fast initially and then slow. The implications of this are important when considering hydrolysis time and yield because to increase the yield from 165 mg xylose g^{-1} bagasse to 180 mg g^{-1} (a 9 percent increase), a 50 percent increase in reaction time is required.

The effect of acid concentration on the time required to achieve a constant yield at various temperatures is shown in Figure 3. The curves show the strong influence of acid concentration.

The effect of liquid : solid ratios on xylose yields was also investigated by Trickett (1982) using hammer milled bagasse at ratios of 5, 10, 15 and 20:1. The two lower ratios gave appreciably lower yields, possibly due to difficulties with stirring and heat transfer when liquid levels were low.

The existence of two hemicellulose fractions in bagasse was also investigated by Du Toit et al (1984) who used 5 percent HCl at 96°C or 4 percent NaOH at room temperature for the hydrolysis. The portion of easily hydrolyzable hemicellulose was slightly higher than that reported by Trickett (1982), but otherwise the results were in good agreement.

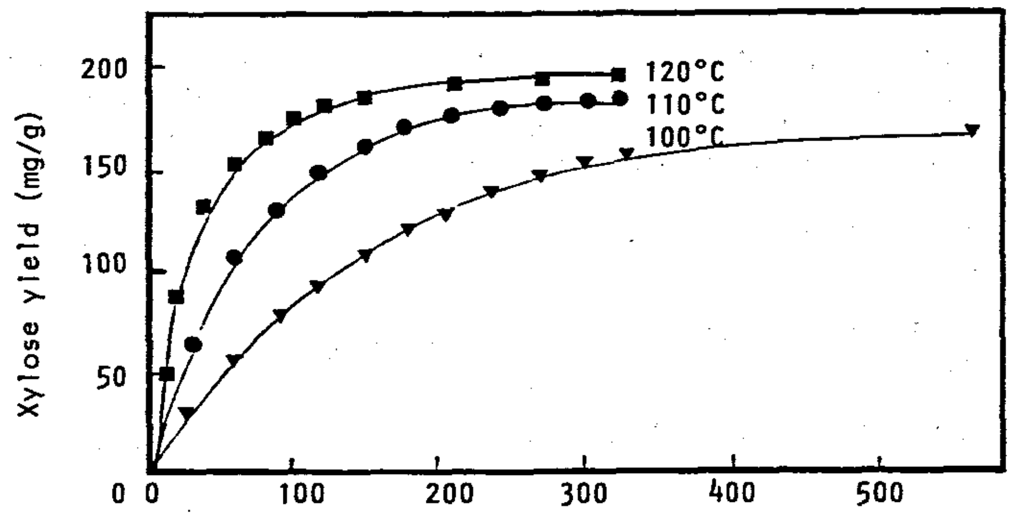

Figure 2. Yield of xylose as a function of time in 0,5 percent H_2SO_4 at different temperatures (Trickett 1982).

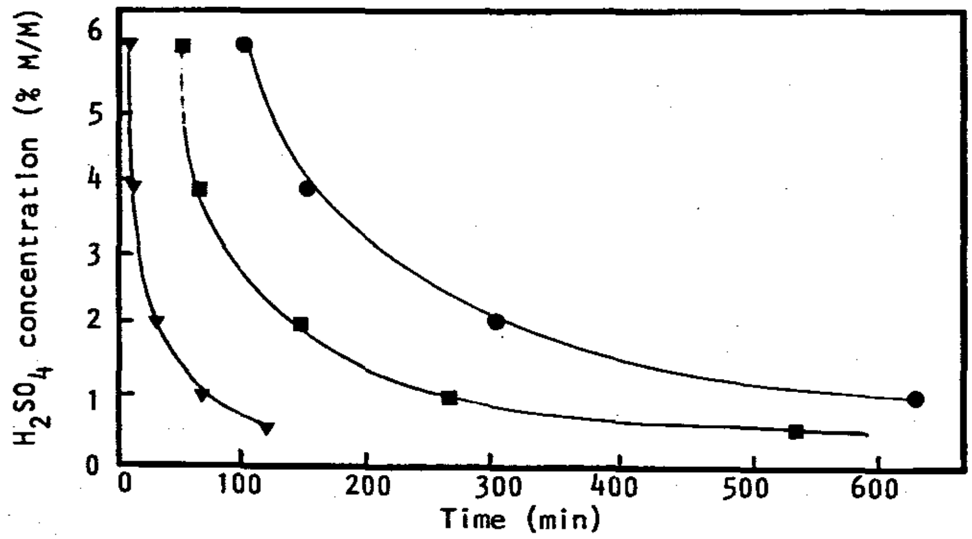

Figure 3. Acid concentrations required to produce 180 mg xylose g^{-1} bagasse at various times and temperatures. (Trickett 1982).

AUTOHYDROLYSIS

It is possible to acidify bagasse by steaming it at temperatures high enough to hydrolyze the acetyl groups on the hemicellulose thereby generating acetic acid. The hemicellulose can then be hydrolyzed without addition of further acid and the process is called autohydrolysis.

Trickett and Neytzell-de Wilde (1982) reported on trials of autohydrolysis of bagasse at temperatures between 100 and 160°C. At 100 and 120°C negligible hydrolysis took place. At higher temperatures appreciable hydrolysis took place but produced only small quantities of free xylose. Xylans accumulated in the liquid and there was substantial xylose loss due to furfural formation.

Autohydrolysis has been studied at higher temperatures and higher acetic acid concentrations (lower liquid : solid ratios), but endproducts were mainly xylans not xylose (Puls and Dietrichs 1981; Taylor 1987). Autohydrolysis is not therefore suitable for producing xylose as the endproduct unless an additional hydrolysis step using H_2SO_4 or xylanase enzymes is included.

PROPOSALS FOR FACTORY SCALE PREHYDROLYSIS

The data presented by Trickett (1982) related to smallscale batch and continuous experiments using hammer milled bagasse. To address the problems associated with a large scale unit, a reactor for 5 kg batches (dry mass) was constructed at the Sugar Milling Research Institute as part of a process development unit (Walford et al 1983). It consisted of two 100 l polyethylene tanks arranged so that acid could be heated in the one and pumped through bagasse in the other. It operated at 92-96°C so as to minimize the production of furfural which was believed to be a problematic toxin produced at higher temperatures (Trickett 1982 and Watson 1984). This relatively low temperature necessitated acid concentrations of between 1 and 2 percent in order to keep hydrolysis times within reasonable limits. Recycling of the prehydrolysate through sequential batches of fresh bagasse was necessary so as to achieve reasonable xylose concentrations (4-5 percent) and acid

R = 1,987 cal (g mole)$^{-1}$ T^{-1}

C = concentration of H_2SO_4 (g (100 ml)$^{-1}$ of H_2O)

T = temperature in K

This model applies where liquid:solid ratios are 3,6:1 or greater. Due to the effect of temperature on k_3 (xylose destruction rate), hydrolysis temperatures should not exceed 130°C unless the reaction time can be limited to a few minutes by using a continuous reactor (Taylor 1987).

Based on this model, xylose yields as a function of time at different temperatures for a constant acid concentration has been plotted in Figure 2. The effect of temperature on the time required to produce a given yield is evident. Increasing the temperature from 100°C to 110°C results in a saving of over 50 percent in time. The presence of the two xylan fractions causes the rate of hydrolysis to be relatively fast initially and then slow. The implications of this are important when considering hydrolysis time and yield because to increase the yield from 165 mg xylose g^{-1} bagasse to 180 mg g^{-1} (a 9 percent increase), a 50 percent increase in reaction time is required.

The effect of acid concentration on the time required to achieve a constant yield at various temperatures is shown in Figure 3. The curves show the strong influence of acid concentration.

The effect of liquid : solid ratios on xylose yields was also investigated by Trickett (1982) using hammer milled bagasse at ratios of 5, 10, 15 and 20:1. The two lower ratios gave appreciably lower yields, possibly due to difficulties with stirring and heat transfer when liquid levels were low.

The existence of two hemicellulose fractions in bagasse was also investigated by Du Toit et al (1984) who used 5 percent HCl at 96°C or 4 percent NaOH at room temperature for the hydrolysis. The portion of easily hydrolyzable hemicellulose was slightly higher than that reported by Trickett (1982), but otherwise the results were in good agreement.

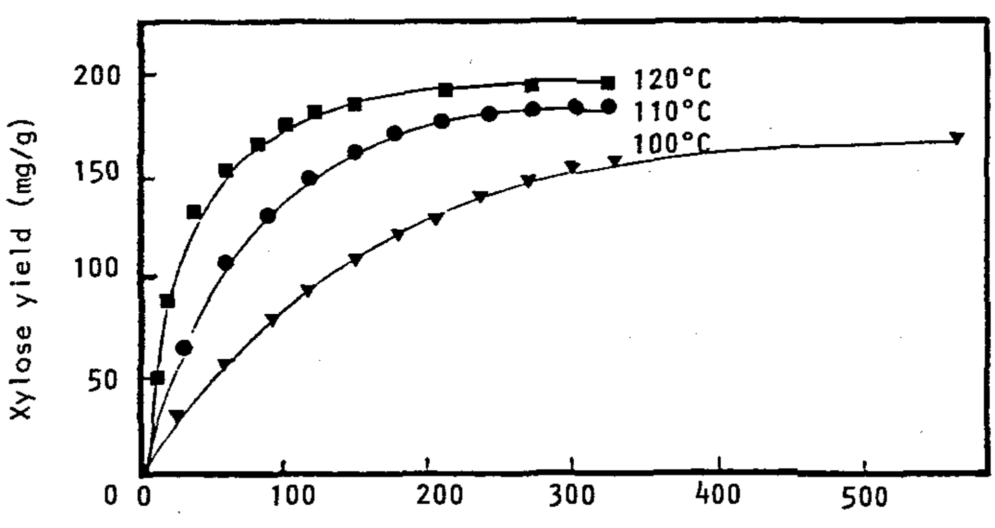

Figure 2. Yield of xylose as a function of time in 0,5 percent H_2SO_4 at different temperatures (Trickett 1982).

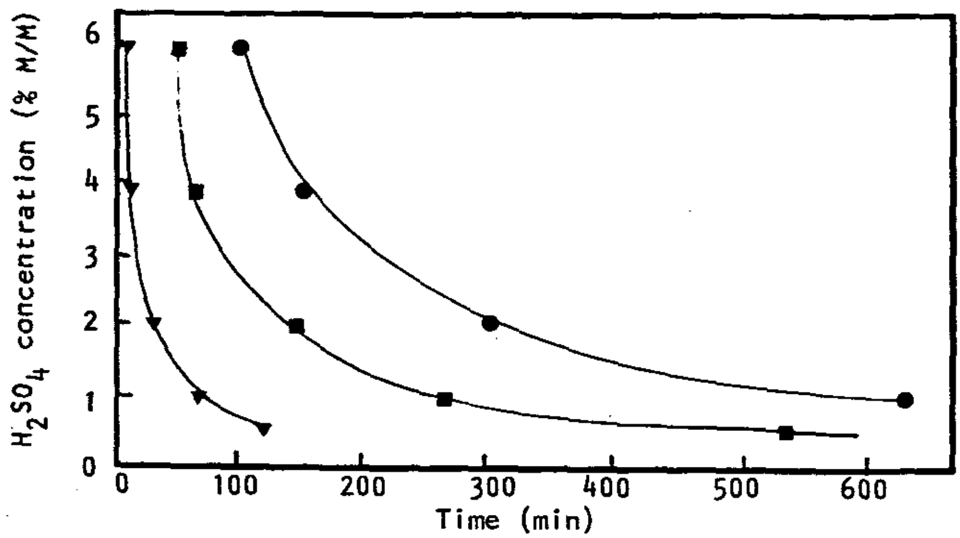

Figure 3. Acid concentrations required to produce 180 mg xylose g^{-1} bagasse at various times and temperatures. (Trickett 1982).

AUTOHYDROLYSIS

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Operation of the reactor in conjunction with washing and pressing under both batch and semicontinuous conditions allowed refinement of the model (Purchase et al 1985; Walford 1985; Project Engineering Africa 1986). The reactor was also used to make prehydrolysate for fermentation studies (Chapter 2) and to make prehydrolyzed bagasse for studies of attritor milling (Chapter 3).

Cost data based on the model showed that sulphuric acid was a major cost item when used at a concentration of 1 to 2 percent. Further, when this concentration of acid was neutralized with lime, it was evident that removal and washing of the resulting bulky calcium sulphate precipipate would be costly in terms of equipment and loss and dilution of fermentables (Walford and Proudfoot 1986). These problems could be reduced by decreasing the concentration of H_2SO_4 to 0,5 percent, but would necessitate an increase in reactor temperature to 120°C to ensure an acceptably short reaction time. The use of the high temperature conflicted with earlier ideas to limit furfural production by keeping temperatures below 100°C, but the logic of these ideas changed when it became apparent that acetic acid was the dominant toxin in the prehydrolysate (Van Zyl 1987).

Acetic acid is normally produced in prehydrolysates in the ratio of 1:5 (acetic acid:xylose) and this ratio was not increased by increasing the temperature from 95°C to 120° C (Walford and Proudfoot 1986). On a laboratory scale the acid could be removed by anion exchange resins (Watson et al 1984), but this is impractical on a commercial scale. A yeast, believed to be *Geotrichum candidum* (UOFS strain I 4), was found to remove acetate selectively from prehydrolysate under aerobic conditions (Holder 1986). Walford and Proudfoot (1986) found that when acetic acid was fed continuously to this yeast under conditions of high aeration (1 VVM air and 500 rpm stirrer speed) it was metabolized at a rate of 1,5g l^{-1} h^{-1}. An average rate of 0,6g l^{-1} h^{-1} was achieved in batch fermentations of prehydrolysate. The resulting acetate-free xylose solutions were successfully fermented to ethanol by *Pichia stipitis* even if the prehydrolysate was prepared at 120°C (Table 5).

The prolonged fermentation time for the hydrolysate prepared at 120°C showed that additional toxins were produced at the higher temperature, but these did not affect the final yield. It is probably cheaper to tolerate the prolonged fermentation than to remove the remaining toxins. No attempt has yet been made to adapt the yeast to the toxins.

Table 5. Ethanol production by *Pichia stipitis* in prehydrolysate which had been prefermented by *Geotrichum candidum* to remove acetic acid (Walford and Proudfoot 1986).

Prehydrolysate preparation temperature	Yield (p/s)	Time (h)	Efficiency (percent)
95°C	0,39	48	100
120°C	0,40	72	100

Saccharomyces cerevisiae was successfully used to remove acetate selectively from prehydrolysate. In batch fermentation it suffered a long lag period and therefore its average activity was only about one third of that of *Geotrichum candidum*, but the two yeasts gave similar results in continuous culture (Walford and Proudfoot 1986). The advantage of *Saccharomyces cerevisiae* is that it is cleared for use as a feed yeast.

The data on acetate removal and xylose fermentation confirmed that prehydrolysis could be done with 0,5 percent H_2SO_4 at 120°C and that the resulting solution could be fermented without removal of the small quantity of $CaSO_4$ precipitate which formed on neutralization of the acid (Walford and Proudfoot 1986). The data were used in drawing up a model encompassing prehydrolysis at 120°C, neutralization, acetic acid removal and xylose fermentation (Figure 4) (Project Engineering Africa 1986).

The process involving aerobic prefermentation followed by anaerobic ethanol fermentation with a different yeast is complex and costly (Project Engineering Africa 1986). An alternative is to produce single cell protein by growing *Candida utilis* aerobically on the acetic acid and the sugars in the prehydrolysate. This has been achieved (Purchase and Proudfoot 1987) and is reported more fully in Chapters 6 and 7.

SUMMARY AND CONCLUSIONS

Approximately one third of the mass of bagasse consists of hemicellulose. This can be hydrolyzed selectively with dilute acid to give a xylose rich solution with a xylose:acetic acid ratio of about 5:1. The rate and extent of the hydrolysis is affected by temperature, acid concentration and time, and these effects have been defined in a model based on laboratory studies.

The acetic acid in the hydrolysates was found to be the main toxin preventing the yeast *Pichia stipitis* from fermenting the xylose to ethanol. Prior to this discovery it was thought that furfural was the main inhibitor and that the temperature of hydrolysis had to be kept below 100°C so as to prevent excessive formation of furfural.

A 200 l batch reactor for prehydrolyzing bagasse was constructed and operated at 96°C with 1-2 percent H_2S0_4. Results obtained with this reactor and its associated bagasse dewatering mill were used to develop a computer model for a large scale reactor and to provide data for estimating the cost of the process. The high cost of H_2S0_4 and its neutralization and removal as $CaSO_4$ dictated that a lower concentration of acid be used and hence a higher temperature. Prehydrolysates prepared at 120°C with 0,5 percent H_2SO_4 for 2 h fermented slightly slower than those prepared at 96°C with 2 percent acid, but were acceptable as a source of fermentables for production of ethanol or single cell protein. When the prehydrolysates were used for ethanol production it was necessary first to remove the acetic acid and this could be done by growing a selected yeast on the prehydrolysates under aerobic conditions. This was however a costly process and less promising than the production of single cell protein by growing *Candida utilis* on the acetic acid and xylose under aerobic conditions.

Bagasse hemicellulose is a promising source of cheap fermentable sugars (Chapter 7), but further work is required to define more precisely the technology and costs of large scale prehydrolysis of the hemicellulose. The hydrolysis is a useful first stage in pretreating the bagasse for enzymic hydrolysis of the cellulose (Chapter 3).

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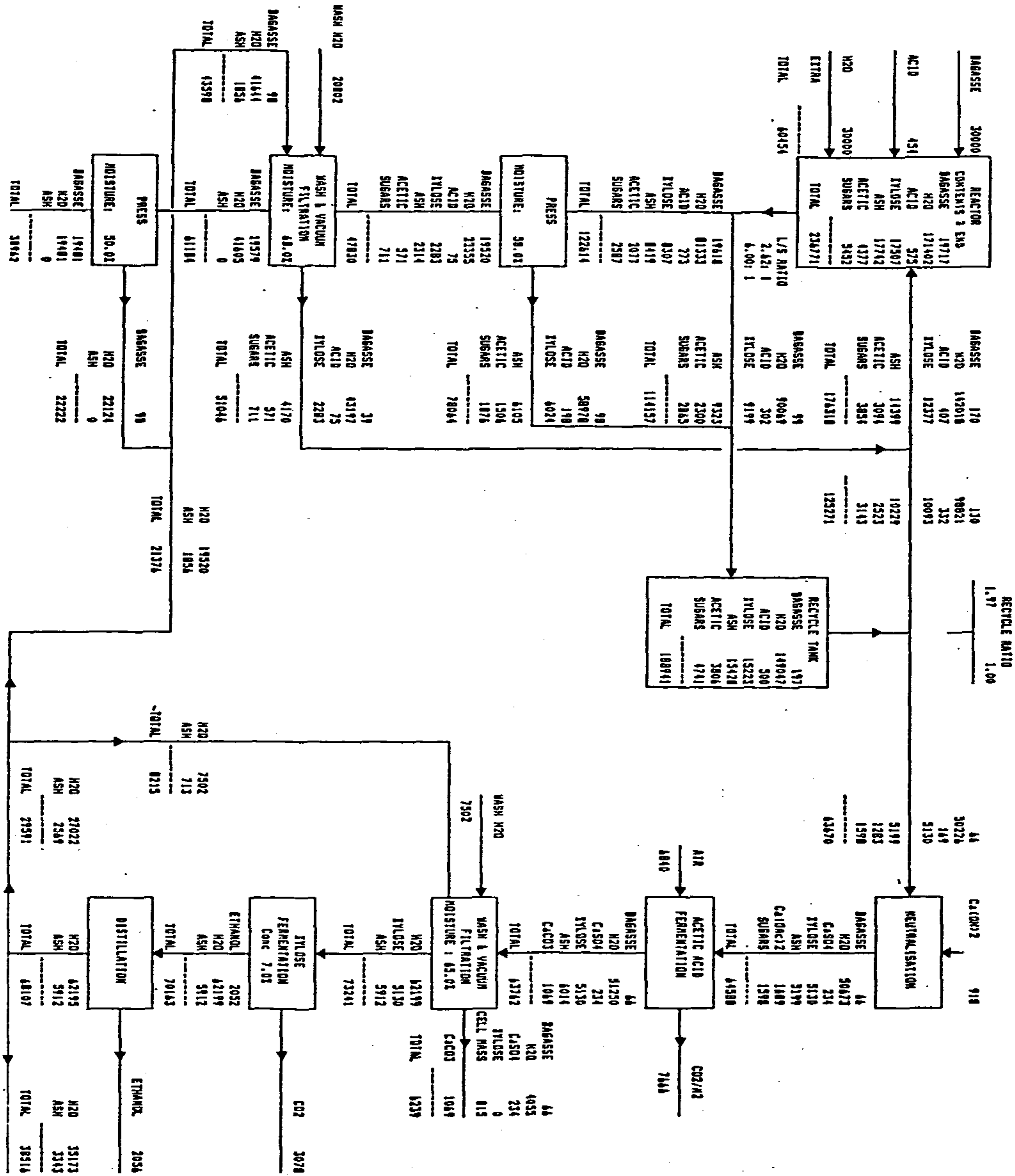

Figure 4. Computer model of a hydrolysis and fermentation process for bagasse hemicellulose.

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Italics indicate work carried out in the programme.

CHAPTER 2. FERMENTATION OF BAGASSE HEMICELLULOSE HYDROLYSATE

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ETHANOL PRODUCTION

INTRODUCTION

Hemicellulose constitutes up to 35 percent of the dry biomass of sugarcane bagasse, with D-xylose as the major sugar constituent (Dekker and Lindner 1979; Rosenberg 1980; Tsao et al 1982; Du Toit et al 1984). The extraction and hydrolysis of this xylan with dilute mineral acid can be regarded as a pretreatment to enhance subsequent enzymic cellulose saccharification (Detroy et al 1982; Lee and McCaskey 1983). The efficient fermentation of D-xylose to ethanol, therefore, is an important factor in the overall economics of ethanol production from bagasse.

Initially, aldopentoses were regarded as being non-fermentable by yeasts (Barnett 1976; Phaff et al 1978) and another avenue for achieving ethanol production from xylose was pursued, namely the enzymic isomerization of D-xylose to D-xylulose, which is fermented by *Saccharomyces cerevisiae* and especially *Schizosaccharomyces pombe* (Gong et al 1981a; Gong et al 1983; Jeffries 1981).

The ethanolic fermentation of xylose by anaerobic bacteria, including thermophilic anaerobes, has been investigated by various groups (Asther and Khan 1984; Patel 1984; Wiegel et al 1985). Xylose fermenting bacteria generally have a very low ethanol tolerance. Furthermore, bacterial xylose fermentation is characterized by extensive byproduct formation (mainly acetate) which decreases the ethanol yield and can even inhibit further fermentation.

Better xylose conversion efficiencies have been obtained with certain moulds (*Neurospora crassa* has produced 0,3 g ethanol g^{-1} xylose (Deshpande et al 1986) and the highest yields by far (0,42 g g^{-1} and higher) have been found with *Fusarium oxysporum* (Suihko and Enari 1981; Viikari et al 1984). These fermentations, however, are characteristically very slow with the rate of ethanol production of the order of 0,22 g $l^{-1}h^{-1}$ (Viikari et al 1984).

XYLOSE ISOMERIZATION

During the initial stages of this programme some attention was given to the xylose isomerization route for ethanol production via xylulose. This work was later discontinued in favour of direct xylose fermentation with yeasts.

Isomerization

Enzymic conversion of xylose to xylulose can be effected with commercial xylose isomerase (glucose isomerase) but unfortunately the reaction equilibrium is in favour of D-xylose. Displacement of the equilibrium in favour of xylulose formation is possible by increasing the temperature (Magee and Kosaric 1985; Olivier and Du Toit 1986) and/or using borate which binds to xylulose, thereby preventing reconversion to xylose (Magee and Kosaric 1985).

In this programme, xylose isomerization with a commercial immobilized enzyme (Novo Sweetzyme Q) was investigated (Olivier and Du Toit 1986). The optimal reaction conditions for both pure xylose and the hemicellulose hydrolysate were determined. Manganese and magnesium ions activated the enzyme whereas heavy metal ions had an inhibitory effect. The half-life of the xylose isomerase was 248 h at 55°C and 118 h at 70°C in the absence of stabilizing substrate. Substances in the hemicellulose hydrolysate exerted no marked inhibitory effect on the xylose isomerase. In packed column reactors, the equilibrium of the xylose to xylulose conversion was reached within 6 h at 60°C but the maximum conversion to xylulose was only 26 percent due to the unfavourable reaction equilibrium. Others obtained a slightly higher conversion (30 percent) at 70°C (Gong et al 1986) and an 80 percent conversion of D-xylose to D-xylulose was obtained by using sodium tetraborate (Hsiao et al 1982).

A purification procedure using a novel two step chromatographic separation for the production of xylulose, which is a high value fine chemical, was also developed during this programme (Olivier and Du Toit 1986). Others (Gong et al 1981a) used differential ethanol precipitation to prepare a concentrated (500 g l^{-1}) xylulose solution whereas Wijsman et al[1] (personal communication) recently patented a similar process coupled with the bacterial conversion of the residual xylose to D-xylonic acid which was then removed by ion exchange to obtain a pure xylulose preparation.

Simultaneous isomerization and fermentation

The unfavourable equilibrium for xylose isomerization can be improved by coupling the isomerization with the fermentation process. In a one step simultaneous isomerization and fermentation process, however, compromise conditions have to be used since the optimal conditions for xylose isomerization (pH 6-8, 70°C, Gong et al 1981a) and for ethanol fermentation (pH 4-6, 35°C, Chiang et al 1981) are different.

In this programme, a simultaneous isomerization and fermentation process was also studied (Du Toit 1984). In a batch process with *Saccharomyces cerevisiae* and a suspension of Sweetzyme Q, operating at pH 6-6,5 and 35°C, xylitol production occurred. With a continuous column reactor, packed with enzyme and yeast immobilized in pectin, no xylitol accumulated and up to 13,4 g l^{-1} and 6,1 g l^{-1} ethanol was produced from pure xylose and hemicellulose hydrolysate respectively. These results compare very favourably with those of Wang et al (1980) where only about 2,5 g l^{-1} ethanol was obtained in simultaneous isomerization and fermentation experiments using pure xylose. In subsequent work, Hsiao et al (1982) obtained about 20 g l^{-1} ethanol at a 90 percent conversion efficiency by using tetraborate to shift the isomerization equilibrium.

A different approach would be to use of a two step process employing two bioreactors in series (with recirculation), so that each may be operated at the optimal conditions for isomerization and fermentation respectively. Such a process calls for a much greater degree of technological sophistication. The production of proteolytic enzymes by the yeast which can inactivate the glucose isomerase has been identified as a problem in the use of a simultaneous process (Wang et al 1980).

Another drawback of the isomerization and fermentation route is the low fermentation rate of xylulose, even with the best yeast strains. According to Jeffries (1985), the fermentation rate is lower than the rate attained with the direct fermentation of xylose and the overall cost is likely to be higher than that of the direct xylose fermentation.

SELECTION OF XYLOSE-FERMENTING ORGANISMS FOR ETHANOL PRODUCTION

Direct fermentation to produce ethanol from xylose is attractive because of its simplicity and likely low cost. The fairly recent discovery that the yeast *Pachysolen tannophilus* can ferment xylose to ethanol (Schneider et al 1981) sparked worldwide interest in the direct route for xylose fermentation. An isolation and screening programme was therefore launched within this programme to identify xylose fermenting microorganisms.

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Bacteria

A total of 2 842 bacterial cultures were isolated from a wide variety of sources using both direct isolation and enrichment techniques (Wallis et al 1983). Screening of these isolates for ethanol production from xylose yielded disappointing results in that none produced more than 8,2 g l^{-1} ethanol from 5 percent xylose. Only 29 isolates produced 1 g l^{-1} ethanol or more. Mutagenesis of the ethanol producing isolates failed to improve ethanol production significantly.

Moulds

A total of 38 moulds were isolated on a selective medium containing xylose (Potgieter et al 1981). Of these, nine isolates were capable of producing ethanol from xylose. The highest ethanol concentration reached was only 1,7 g l^{-1} from 20 g l^{-1} xylose. This concentration was increased to 3,5 g l^{-1} ethanol by cultivating in a sealed Erlenmeyer flask with agitation (limited aeration) for 15 days. This rate and yield of ethanol production was still too low to warrant further investigation.

In the course of this investigation, a rapid screening method for detecting ethanol production by microorganisms on an agar plate was developed (Jacobs et al 1983). This assay was based on the enzymic determination of ethanol and produced a coloured zone around ethanol producing colonies, the diameter of which was an indication of the amount of ethanol produced.

Yeasts

The search for xylose fermenting yeasts was successful. The screening of strains in the CSIR yeast culture collection and of 674 strains isolated from natural sources turned up a number of strains capable of producing significant amounts of ethanol from xylose (Van der Walt 1983, 1984).

One of the first promising strains to be identified was *Candida shehatae* CSIR-Y492. Compared with published performance of other yeasts at that time, this yeast fermented xylose to ethanol with comparable yields but at a higher rate (Du Preez and Van der Walt 1983). A subsequent evaluation of 56 selected xylose fermenting yeast strains identified several strains which exhibited even higher ethanol yields and rates of production than this *C shehatae* strain (Table 6). Of these *Pichia stipitis* CSIR-Y633 appeared to be the most promising (Du Preez and Prior 1985).

In an independent survey of the comprehensive CBS yeast collection in Delft, *C shehatae* and *P stipitis* were also identified as the best potential candidates for the industrial fermentation of pentose sugars (Toivola et al 1984). A subsequent screening of 412 yeast isolates from various natural sources failed to turn up any strains comparable in performance to *C shehatae* and *P stipitis* (Nigam et al 1985). It seems unlikely, therefore, that further screening programmes will identify xylose fermenting yeasts which are any better than these two species.

A patent for ethanol production from pentose sugars using *C shehatae* or *P stipitis* has recently been granted in Sweden (Van Dijken and Scheffers 1984).

FERMENTATION EXPERIMENTS

During the initial phase of this programme, xylose fermentation by *Pachysolen tannophilus* was studied as it was the best xylose fermenting yeast at that time. The obligate anaerobic bacterium *Clostridium thermohydrosulfuricum* was also investigated. After the results of the screening programme became available, the emphasis shifted to fermentations with *Candida shehatae* and *Pichia stipitis*.

Table 6. The parameters for xylose fermentation by various yeast isolates in 48 h shake flask cultures with ca 50 g l^{-1} D-xylose. The values are the mean of two or more experiments (Du Preez and Prior 1985).

Yeast isolate	E (%)	Maximum ethanol (g l^{-1})	$Y_{p/s}$	Xylitol (g l^{-1})	μ max (h^{-1})	Q_p (g l^{-1} h^{-1})
C shehatae CSIR-Y492	100	17,5	0,36	3,22	0,14	0,75
P stipitis CSIR-Y633	100	21,46	0,45	0	0,14	0,92
P stipitis CSIR-Y567	100	21,28	0,42	1,48	0,16	0,85
C shehatae CSIR-57 D/1	100	20,64	0,42	0	0,13	0,80
C shehatae CSIR-117 A/1	100	20,69	0,42	0	0,13	0,82
C shehatae CSKR-Y492 P	100	17,42	0,35	2,74	0,12	0,78
C shehatae CSIR-Y492 M	100	17,56	0,34	3,56	0,12	0,78
C shehatae CSIR-Y599	100	15,44	0,33	6,62	0,17	0,75
C shehatae CSIR-Y600	82	11,60	0,28	2,70	0,14	0,26
C shehatae CSIR-Y601	100	12,43	0,24	9,39	0,15	0,33
C tenuis CSIR-Y565	83	11,11	0,26	5,09	0,20	0,37
C tenuis CSIR-Y566	95	11,06	0,24	10,94	0,17	0,40
Candida CSIR-579	72	13,66	0,36	0	0,14	0,47
Candida CSIR-41 D/1	100	17,28	0,37	5,88	0,15	0,75
Candida CSIR-III 43 A/4	88	16,35	0,41	2,28	0,16	0,57
Candida CSIR-III 43 A/5	97	16,63	0,33	2,91	0,10	0,49
Candida CSIR-56 D/1	100	18,28	0,40	3,63	0,21	0,60
Candida CSIR-58 D/2	91	15,76	0,38	1,74	0,14	0,58
Candida CSIR-62 A/2	100	20,09	0,41	0	0,13	0,76
Candida CSIR-62 A/3	100	18,84	0,41	0	0,12	0,63
Candida CSIR-64 A/1	65	11,24	0,39	0	0,14	0,45
Candida CSIR-64 A/2	92	16,04	0,34	1,98	0,12	0,44
Candida CSIR-76 D/1	100	17,99	0,36	3,43	0,12	0,70
Candida CSIR-83 D/2	75	14,41	0,44	2,11	0,14	0,38
Candida CSIR-89 D/1	87	15,89	0,36	0	0,14	0,48
Candida CSIR-91 D/1	100	19,18	0,40	2,05	0,22	0,73
Candida CSIR-94 D/1	100	17,06	0,36	3,16	0,13	0,73
Candida CSIR-96 D/2	100	18,82	0,40	1,74	0,22	0,73
Candida CSIR-96 D/4	100	14,98	0,32	5,41	0,14	0,46
Candida CSIR-100 D/2	100	17,13	0,36	4,90	0,21	0,76
Candida CSIR-103 D/3	89	13,68	0,32	2,85	0,13	0,41
Candida CSIR-104 D/1	100	18,16	0,38	4,30	0,25	0,73
Candida CSIR-105 D/4	68	12,05	0,37	2,65	0,19	0,71
Candida CSIR-113 D/1	77	12,19	0,29	5,21	0,15	0,36
Candida CSIR-114 A/1	100	17,76	0,36	5,19	0,22	0,55
Candida CSIR-114 A/3	62	10,68	0,37	2,00	0,20	0,33
Candida CSIR-114 D/2	64	11,77	0,40	0	0,19	0,55
Candida CSIR-116 A/2	100	18,16	0,40	3,66	0,12	0,71
Candida CSIR-117 D/2	100	17,67	0,38	3,44	0,11	0,65

Fermentation with Clostridium thermohydrosulfuricum

Ethanol production from xylose by this thermophilic *Clostridium* sp was investigated in a chemostat system (Potgieter et al 1981). When the xylose concentration was increased from 5 to 25 g l^{-1}, the system failed to reach a steady state. The highest ethanol concentration reached was only about 6 g l^{-1}. Furthermore, as the dilution rate was increased, the ethanol concentration decreased while the concentrations of acetate and lactate increased to about 3,5 and 6,5 g l^{-1} respectively.

This low ethanol yield due to the formation of by-products was typical of bacterial fermentations. Obviously this *Clostridium* was not a suitable organism for industrial ethanol production.

Fermentation with yeasts

General fermentation characteristics and parameters

The discovery of *Candida shehatae* CSIR-Y492 revived interest in the use of yeasts for industrial xylose fermentation (Du Preez and Van der Walt 1983). The outstanding feature of this strain was its high ethanol productivity and resultant short fermentation time. Its yield was comparable with that of *Pachysolen tannophilus* (Table 7). A significant improvement in the ethanol yield was obtained with *Pichia stipitis*, but its ethanol productivity was of the same order as that of *C shehatae*. The longer fermentation time indicated in Table 6 was primarily due to the fact that *P stipitis* produced a higher ethanol concentration than *C shehatae*, as the overall rates of ethanol produced were similar.

Although Maleszka et al (1983) claimed that by increasing the chromosome number of *P tannophilus* above the haploid level, fermentation was improved, no improvement (Du Preez and Monteiro A M T 1983) using diploid strains of C shehatae obtained by protoplast fusion (Johannsen et al 1985) was found. Subsequent work with triploid and tetraploid strains of *C shehatae* showed that the level of ploidy had a minor effect on ethanol production from xylose (Johannsen et al 1985).

The high ethanol yield (85-90 percent of the theoretical maximum) of *P stipitis* could be attributed to the fact that it produced no or very little extracellular xylitol which is an intermediate in the catabolism of xylose. In contrast, xylitol production is a typical phenomenon with xylose fermenting yeasts (Gong et al 1983), including *C shehatae* (Table 6).

In contrast with *C shehatae*, *P stipitis* had no requirement for growth factors but biotin and thiamine enhanced xylose fermentation by *P stipitis* considerably. With *C shehatae*, practically no fermentation occurred in the absence of these vitamins (Du Preez et al 1985; 1986a). Another desirable characteristic of *P stipitis* was that it was capable of fermenting a wider range of sugars than *C shehatae*, including cellobiose (Du Preez et al 1986a). Yeasts capable of producing significant quantities of ethanol from xylose as well as cellobiose are rare, the only other documented case being *Kluyveromyces cellobiovorus* (Morikawa et al 1985). *P stipitis* also had a higher fermentation rate and ethanol yield on glucose than *C shehatae*, but neither yeast was capable of fermenting L-arabinose (Du Preez et al 1986a).

The effects of pH, temperature and substrate concentration on xylose fermentation by *P stipitis* and *C shehatae* were evaluated (Du Preez et al 1986b). As these results are of cardinal importance in the industrial application of these yeasts, the data are presented in Figures 5-7. The optimum pH was in the region of pH 4-5,5. At higher pH values the fermentation time increased sharply and the ethanol yield of *P stipitis* decreased to the value of *C shehatae*. This has a bearing on the fermentation of hemicellulose hydrolysates. Without the prior removal of acetic acid, the fermentation will have to be done at a pH value closer to pH 7 to minimize the inhibitory effect of acetic acid. The optimal fermentation temperature was 30°C. The ethanol yield of *C shehatae* decreased markedly above this temperature. The maximum fermentation rate was reached at 50 g l^{-1} xylose. Xylose concentrations above 70 g l^{-1} caused a deterioration in the fermentation performance, especially in the case of *P stipitis*.

Table 7. A comparison of the parameters for xylose fermentation by *Candida shehatae* CSIR-492 and *Pichia stipitis* CSIR-Y633 with those other fungi, using synthetic media.

Microorganism	Fermentation parameters						
	μ_{max} (h^{-1})	Q_p ($g\ l^{-1}h^{-1}$)	q_p (h^{-1})	$Y_{p/s}$ ($g\ g^{-1}$)	$Y_{x/s}$ ($g\ g^{-1}$)	Maximum ethanol ($g\ l^{-1}$)	Fermentation time
C shehatae (Du Preez and Van der Walt 1983)	0,25	1,31	0,28	0,29	0,1	26,2	40 h
C shehatae (Du Preez et al 1986b)	0,17	0,96	0,48	0,37	0,09	18,5 (30,9)[a]	37 h
P stipitis (Du Preez et al 1986b)	0,15	0,86	0,3	0,43	0,12	21,5 (33,2)	48 h
P tannophilus (Slininger et al 1982)	0,18 (0,24)[a]	na	0,08 (0,12)[a]	0,3 (0,34)[a]	na	ca 15[b]	ca 130 h[b]
Kluyveromyces marxianus (Margaritis and Bajpai 1982)	0,12	0,1	na	0,28	0,16	5,6	48 h
Candida XF217 (Gong et al 1981b)	na	na	na	ca 0,42[b]	na	ca 21[b]	ca 60 h[b]
Fusarium oxysporum (Suihko and Enari 1981)	na	na	na	0,5	0,07[b]	25	6 days
Kluyveromyces cellobiovorus (Morikawa et al 1985)	na	1,6[b]	na	0,31	na	30	3 days

a maximum values obtained under another set of experimental conditions.
b value estimated from the published data.
na not available.

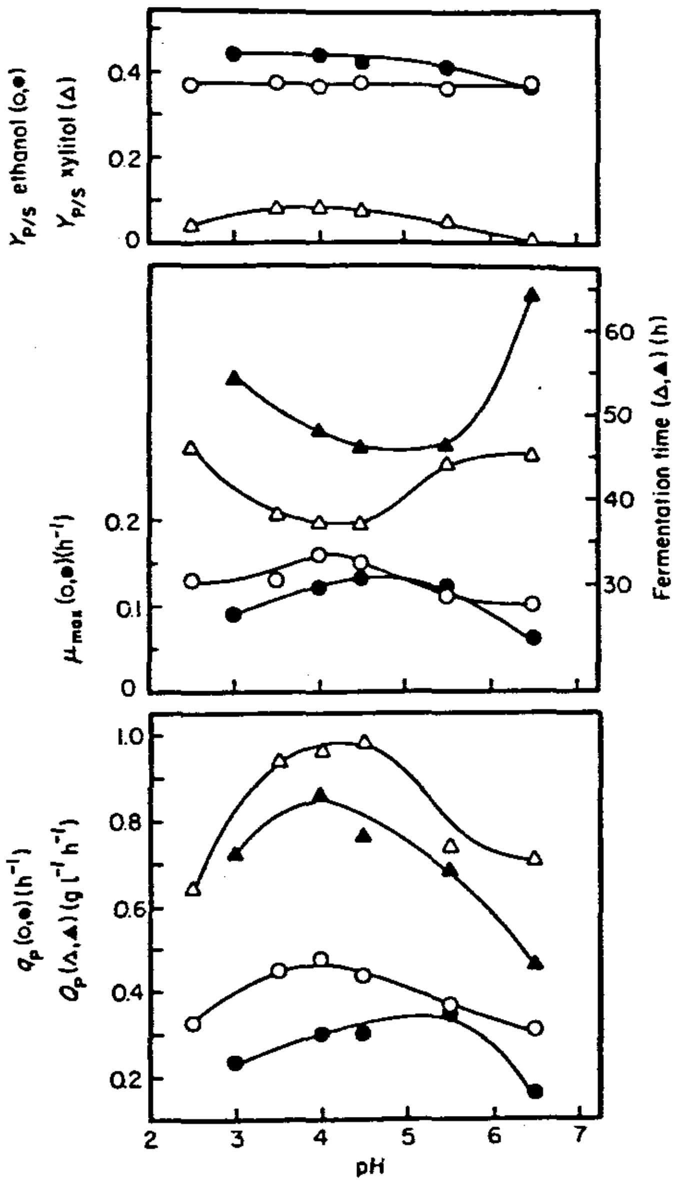

Figure 5. Effect of pH on the ethanol and xylitol yields ($Y_{p/s}$), fermentation time, maximum specific rates of growth (μ_{max}) and ethanol production (q_p) and the maximum volumetric rate of ethanol production (Q_p). Open symbols, *Candida shehatae*; solid symbols, *Pichia stipitis*. The fermentations were conducted at 30°C with an initial xylose concentration of 50 g l^{-1}. (Du Preez et al 1986b).

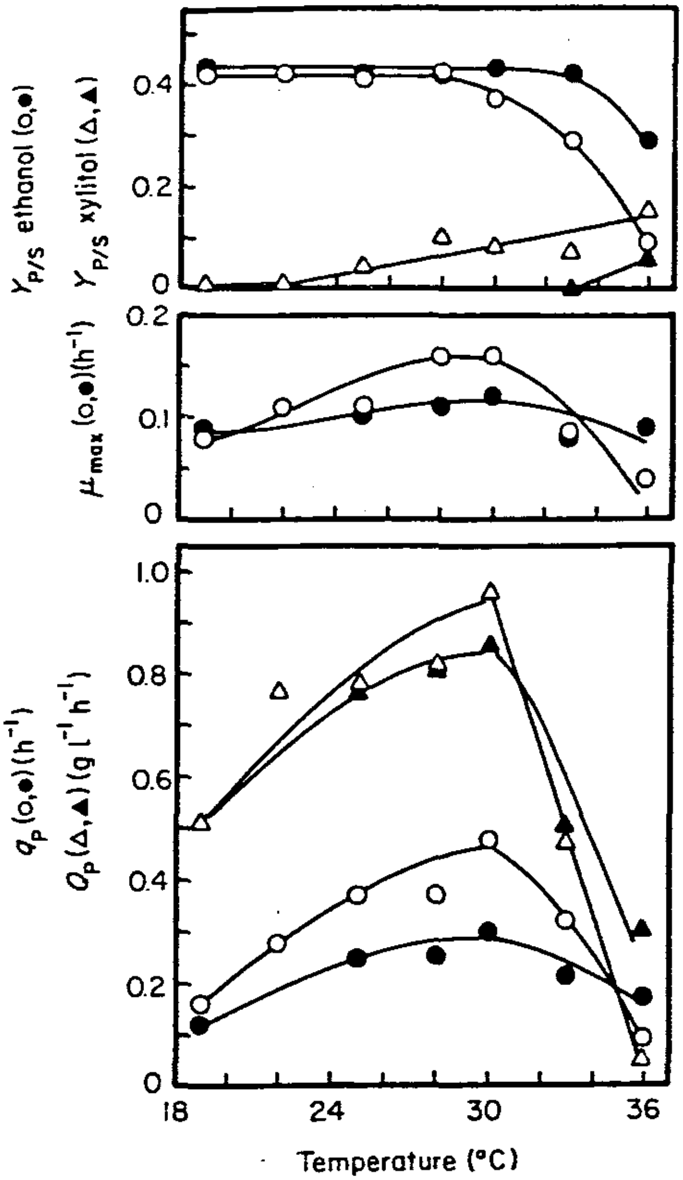

Figure 6. Effect of temperature on the ethanol and xylitol yields ($Y_{p/s}$), maximum specific rates of growth (μ_{max}) and ethanol production (q_p) and the maximum volumetric rate of ethanol roduction (Q_p). Open symbols, *Candida shehatae*; solid symbols, *Pichia stipitis*. The fermentations were conducted at pH 4 with an initial xylose concentration of 50 g l^{-1}. (Du Preez et al 1986b).

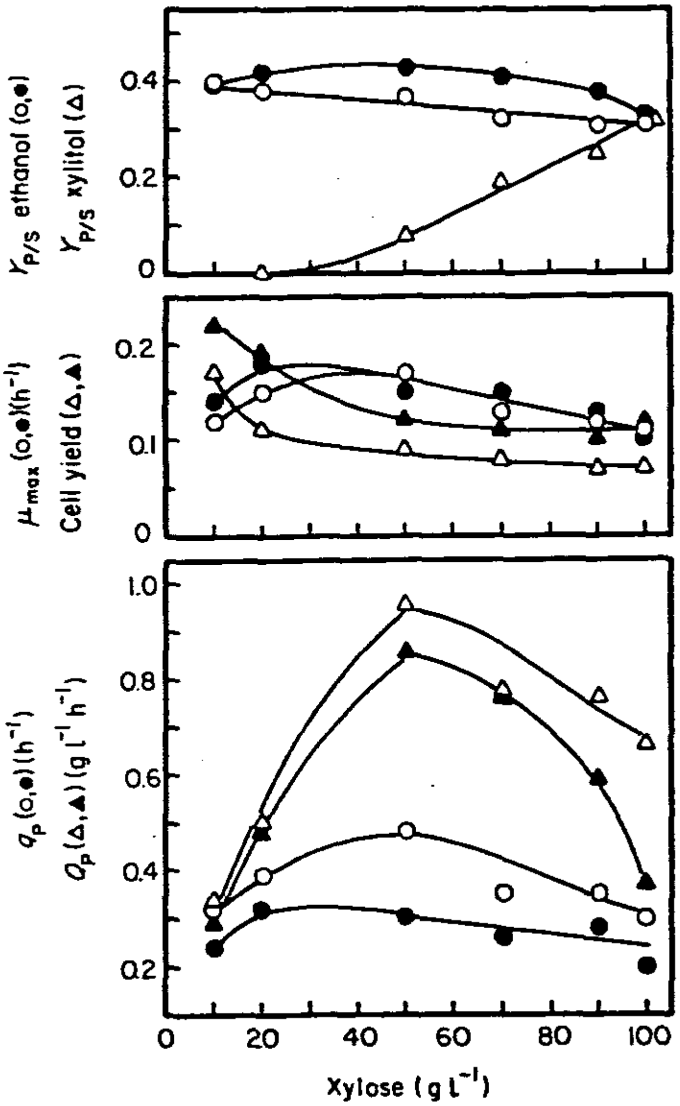

Figure 7. Effect of initial substrate concentration on the ethanol, xylitol ($Y_{p/s}$) and cell yields, maximum specific rates of growth (μ_{max}) and ethanol production (q_p) and the maximum volumetric rate of ethanol production (Q_p). Open symbols, *Candida shehatae*; solid symbols, *Pichia stipitis*. The fermentations were conducted at 30°C and pH 4. (Du Preez et al 1986b).

When evaluating the fermentation performance of different yeast strains, the substrate concentration should be taken into account. For example, Dellweg et al (1984) reported that with their *P stipitis* strain, the ethanol yield coefficient approached the theoretical maximum value of 0,51 at a low xylose concentration (5 g l^{-1}), but that it decreased to 0,24 at 50 g l^{-1} xylose.

The degree of aeration was an important factor affecting the efficiency and rate of xylose fermentation. This is clearly illustrated in Figure 8, where different stirring speeds were used to obtain different aeration rates (Du Preez et al 1984). This effect was also noted in previous reports on this project, and an inverse correlation between the degree of aeration and xylitol production has been observed (Du Preez and Van der Walt 1983; Watson et al 1984a). Although xylitol production can be minimized by control of the aeration rate, this strategy is not completely successful in maximizing the ethanol yield because the latter is also decreased by increasing the aeration level. In fact, controlling the dissolved oxygen concentration at the optimal level throughout the fermentation is one of the major practical problems in obtaining the maximum rate and yield of ethanol production, due to the very low disolved oxygen levels involved. In this respect the use of *P stipitis* or *C shehatae* has the advantage that these yeasts maintain a reasonably high rate and yield of ethanol production over a wider range of aeration levels than is the case with *P tannophilus*, facilitating somewhat easier process control (Figure 8). The fact that *P stipitis* accumulates practically no xylitol further contributes to its high ethanol yield under suboptimal aeration conditions.

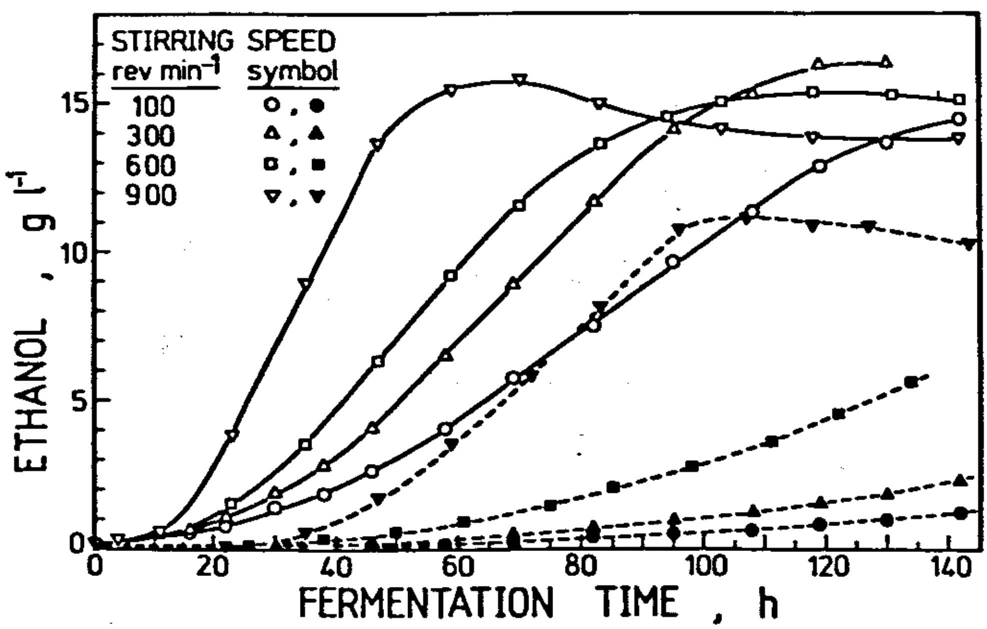

Figure 8. Ethanol production at different stirring speeds. Open symbols, *C shehatae*; solid symbols, *P tannophilus*. (Du Preez et al 1984).

Pentose metabolism

A recent publication (Bruinenberg et al 1984) elucidated the critical role of oxygen in the fermentation of xylose by yeasts. The metabolic pathway is depicted in Figure 9. According to these reseachers, yeasts such as *Candida utilis* are incapable of ethanol production from xylose because their xylose-reductase and xylitol-dehydrogenase are specific for different coenzymes (NADPH and NAD+ respectively). In the absence of oxygen, an overproduction of reducing equivalents in the form of NADH occurs (ie a shortage of NAD+) because the NADH cannot be reoxidized via the electron transport chain. In other yeasts such as *P tannophilus*, *C shehatae* and *P stipitis* the reductase (as well as the dehydrogenase) has a dual specificity for both pyridine nucleotides, so that in the absence of oxygen, the NADH can be reoxidized by being used in the initial reaction of xylose metabolism. A correlation was found between NADH linked xylose reductase activity and the xylose fermenting capacity of different yeasts. Why oxygen is required for growth remains unclear.

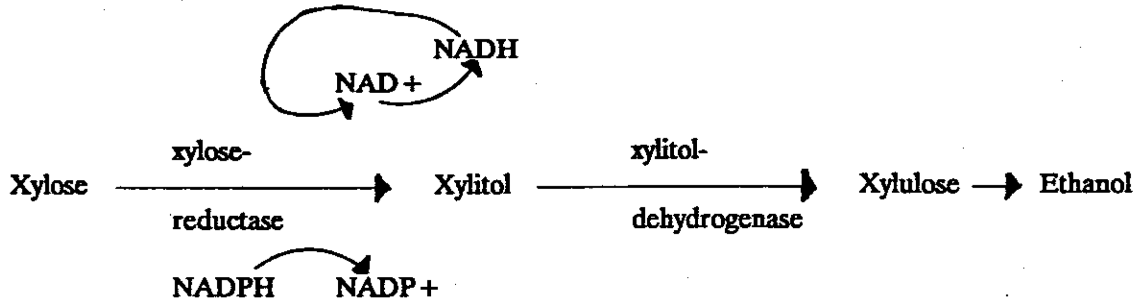

Figure 9. Metabolic pathway for the conversion of D-xylose to ethanol (adapted from Bruinenberg et al 1984).

The physiological role of oxygen in xylose fermentation was investigated as part of this programme. In agreement with other groups, we also found that *P tannophilus*, *C shehatae* and *P stipitis* required oxygen for growth but not for ethanol production, although ethanol production was greatly enhanced by a limited oxygen supply (Reid et al 1983; Du Preez et al 1984; Ligthelm 1987). On glucose, growth and fermentation occurred under anoxic conditions (Ligthelm 1987). Under anoxic conditions, the induction of the key enzymes occurred more slowly than under aerobic conditions. This, particularly, was the case with *P tannophilus* which explains in part the weaker xylose fermentation by this yeast (Ligthelm 1987). The use of acetoin as an artificial electron acceptor under anoxic conditions failed to enhance ethanol production by *C shehatae* and *P stipitis* and resulted in a slight stimulation of ethanol production by *P tannophilus*. The same effect was observed using immobilized *P tannophilus* cells in an anoxic transient continuous culture, to which of the artificial electron acceptors acetoin, acetone and acetaldehyde were added, resulting in improved ethanol production at the cost of xylitol production (Ligthelm 1987).

The mechanism of xylose transport in *P stipitis* was also investigated (Kilian and Van Uden 1987). Transport occurred via two non-repressible active symport systems with different affinities for xylose. Glucose competed with xylose for transport by the low affinity system and inhibited xylose transport by the high affinity system non-competitively. This inhibition could explain the phenomenon of sequential (diauxic) utilization of glucose and xylose observed in sugar mixtures (Du Preez et al 1986a). Du Preez et al (1986a) reported an ethanol production rate from xylose by *Pichia stipitis* that corresponded to a substrate uptake rate of 3,39 mmol $g^{-1}h^{-1}$. Application of the kinetic constants obtained in the transport studies to calculate the uptake rate under the experimental conditions of Du Preez et al (1986a) yielded a similar value (3,83 mmol $g^{-1}h^{-1}$), indicating that transport was probably the rate-limiting step in ethanol production.

Fermentation of hemicellulose hydrolysate

The dilute acid hydrolysate of bagasse consists of D-xylose (42-47 g l^{-1}), D-glucose (3-13 g l^{-1}), L-arabinose (5-12 g l^{-1}), acetic acid (10-13 g l^{-1}) and small amounts of furfural (0,6 g l^{-1}) and 5-hydroxymethylfurfural (0,04 g l^{-1}) (Bester 1987). P stipitis produced slightly more ethanol from the hemicellulose hydrolysate than C shehatae, whereas both the yield and rate of ethanol production was lower with *P tannophilus* (Cruywagen et al 1984).

Fermentations of the hydrolysate were characterized by long fermentation times and low ethanol yields. This was due to inhibitory compounds present in the hydrolysate, namely acetic acid from the hydrolysis of acetyl groups on the xylan molecule, furfural, a degradation product, and metal ions such as nickel and chromium from the milling and hydrolysis equipment (Watson et al 1984b). Various pretreatment protocols were investigated as a means of improving the fermentation by removing the inhibitory factors (Bester 1987). Some of these results are summarized in Table 8. The removal of cations improved the ethanol yield and production rate.

The greatest improvement was obtained after treatment with an anion exchange column, which removed 93 percent of the acetic acid. Phenolic compounds extracted from the bagasse and tested at a concentration equivalent to that found in the hemicellulose hydrolysate strongly inhibited xylose fermentation by *P stipitis* (Bester 1987). Walford and Proudfoot (1986) similarly found that anion exchange resins removed toxic components from the hemicellulose hydrolysate whereas a strongly acid cation exchange resin had little effect. The strongly basic anion resins used are capable of removing high molecular weight anions which may include phenol type fragments from lignin degradation. These findings implicate acetic acid and phenolic compounds as the major inhibitory substances present in the hemicellulose hydrolysate.

Table 8. Effect of various pretreatment procedures on the fermentability of hemicellulose hydrolysate by *Pichia stipitis* (Bester 1987).

Pretreatment	Fermentation parameter			
	$Y_{p/s}$	Ethanol $g\,l^{-1}$	Q_p $g\,l^{-1}h^{-1}$	Fermentation time, h
(1) $Ca(OH)_2$ pH 3 NaOH pH 6,5	0,22	10,2	0,1	117
(2) Amberlite anion exchange column $Ca(OH)_2$ pH 6,5	0,36	15,6	0,56	40
(3) Dowex-50W cation exchange column $Ca(OH)_2$ pH 6,5	0,38	14,5	0,23	72
(4) $Ca(OH)_2$ pH 10 H_2SO_4 pH 6,5	0,29	13,2	0,26	74
(5) $Ca(OH)_2$ pH 6,5	0,31	12,3	0,26	60

The inhibitory effect of acetic acid increases with a decrease in pH, due to the larger proportion of undissociated molecules at low pH values. The maximum volumetric rate of ethanol production in a synthetic medium at pH 5,1 was halved by the presence of 0,8 $g\,l^{-1}$ acetic acid, whereas the same degree of inhibition at pH 6,5 required 13,8 $g\,l^{-1}$ acetic acid. The strong inhibitory effect of the acetic acid in the hemicellulose hydrolysate (ca 10 $g\,l^{-1}$) was verified by the observation that fermentation of the hydrolysate practically ceased at pH 5 (Bester 1987). These findings also implicate inhibitory compounds other than acetic acid in the hydrolysate.

The sequential cultivation of an acetate-utilizing yeast isolate (designated I3) and *P stipitis* was investigated in an attempt to eliminate the acetic acid prior to xylose fermentation. Isolate I3 assimilated the acetic acid preferentially within 28 h, but the subsequent fermentation with *P stipitis* yielded a much lower ethanol concentration than anticipated due to the fact that the xylose was poorly utilized. No satisfactory explanation for this phenomenon was found (Bester 1987). Following a similar line of investigation, Walford and Proudfoot (1986) found that after acetic acid removal by cultivation of another yeast isolate (designated I4), fermentation of the xylose in the hydrolysate was successful. Their approach differed from that of Bester (1987) in that they separated the acetate-utilizing yeast cells from the hydrolysate prior to inoculation with *P stipitis*. This procedure has yet to be developed further, but it does appear to hold some promise as a simple and practical means for eliminating the inhibition of xylose fermentation by acetic acid.

Ethanol tolerance

It was shown that the temperature profiles of ethanol tolerance of both *C shehatae* and *P stipitis* were very similar (Du Preez et al 1987), and in close agreement with the results of Lucas and Van Uden (1985) for *C shehatae* on glucose. An increase in the ethanol concentration (added to the culture medium) severely depressed the maximum growth temperature and also increased the minimum growth temperature. The ethanol tolerance limit of about 46 g l^{-1} occurred within a narrow temperature plateau of 11 to 22°C (Figure 10). At 30°C the maxiumum ethanol concentration permitting growth was 30 to 35 g l^{-1}. These yeasts may produce higher ethanol levels than the limit for growth, as it is known that ethanol production by *Saccharomyces cerevisiae* proceeds (albeit at a slower rate) after cessation of growth. Similarly, subsequent fed-batch experiments with *C shehatae* showed that while growth at 30°C was completely inhibited by 30 g l^{-1} ethanol, ethanol production continued until 44 g l^{-1} was reached (B van Driessel, unpublished data). Others recently reported that *C shehatae* and *P stipitis* produced up to about 40 g l^{-1} ethanol (Wayman and Tsuyuki 1985; Tran and Chambers 1986).

This ethanol tolerance is much lower than that of *S cerevisiae*, and low ethanol tolerance is an unfortunate characteristic of all the xylose fermenting yeasts described thus far. Very recently, xylose fermentation by an exceptionally ethanol tolerant *Paecilomyces* strain was reported (Wu et al 1986). This mould produced up to 73 g l^{-1} ethanol from 200 g l^{-1} xylose, but the fermentation was slow as is usually the case with moulds. This *Paecilomyces* strain was also more temperature tolerant than our strains of *C shehatae* and *P stipitis*, and its ethanol yield was claimed to be little affected by a fermentation temperature of 37°C.

Mutagenesis of *C shehatae* in an attempt to isolate mutants with a higher ethanol tolerance proved unsuccessful (Reid et al 1984).

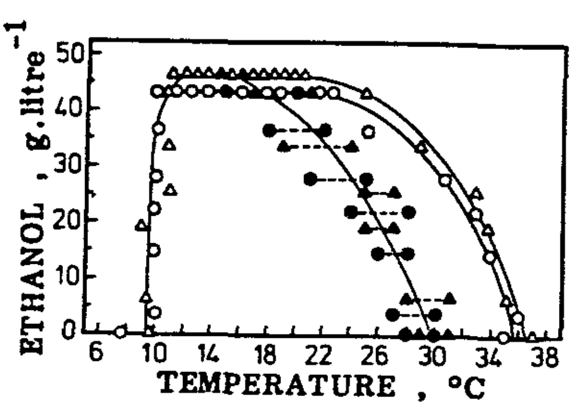

Figure 10. Temperature profiles of ethanol tolerance of *Candida shehatae* (○, ●) and *Pichia stipitis* (△, ▲). The experimental points indicate the optimum growth temperature range (solid symbols) and the highest ethanol concentration tested where growth was measurable (open symbols) (Du Preez et al 1987).

Concluding remarks

Although the rate and conversion efficiency of ethanol production from xylose has been improved significantly with the discovery of *Candida shehatae* and *Pichia stipitis*, the economic viability of the xylose fermentation is still hampered by several factors:

- the best xylose fermentation rates are much lower (at least six-fold) than with commercial hexose fermentations using *Saccharomyces cerevisiae*;
- the ethanol tolerance of the xylose fermenting yeasts is only about a third that of *S cerevisiae*. This, however, may not be such a major problem in the fermentation of hemicellulose hydrolysates because the maximum ethanol concentration possible from the dilute substrate will be well within the ethanol tolerance limit of these yeasts;
- the optimum and maximum fermentation temperatures of *C shehatae* and *P stipitis* are significantly lower than those of *S cerevisiae*;
- the xylose fermentation will have to be conducted with a low level of aeration as ethanol production is linked to respiration and growth. This renders the xylose fermentation technically somewhat more complex (and expensive) than commercial ethanol fermentations with *S cerevisiae*.

The most dramatic further improvements in xylose fermentation can be expected to come by way of strain improvement by genetic manipulation. Some research on this aspect has already been initiated by others. The xylose isomerase gene from *Escherichia coli* has been cloned into *Schizosaccharomyces pombe*, enabling this yeast to ferment xylose to ethanol directly. Although 37 g l^{-1} ethanol was produced from 100 g l^{-1} xylose, the fermentation was very slow (a 240 h fermentation time), with xylose isomerization being the probable rate limiting step. Furthermore, xylitol was a byproduct, which decreased the ethanol yield and also inhibited xylose isomerase (Chan et al 1986).

SUMMARY OF PROCESS PARAMETERS

As a result of the research undertaken in this programme the following parameters represent the best conditions for ethanol production from the bagasse hemicellulose hydrolysate. These parameters have been used for the costing of a potential commercial process and could be used as a basis for further development on a pilot plant scale.

Yeast: *Pichia stipitis* CSIR-Y633

Cultivation conditions

aeration:	oxygen-limited conditions
pH:	pH 6,5 (without prior acetic acid removal)
	pH 4,5-5,5 (with prior acetic acid removal)
temperature:	30°C (should be decreased if higher ethanol levels are reached)

Fermentation time:	40 h (pretreated with anion exchange resin)
	79 h (no resin pretreatment)

Ethanol production

yield:	0,37 g g^{-1} sugar (pretreated with anion exchange resin)
	0,38 g g^{-1} sugar (no resin pretreatment)
concentration:	15,6 g l^{-1} (pretreated with anion exchange resin)
	15 g l^{-1} (no resin pretreatment)

BUTANEDIOL PRODUCTION

A wide variety of chemicals can be prepared from 2,3-Butanediol which is a potential chemical feedstock. The derivatives include 1,3-butadiene (a major monomeric constituent of synthetic rubber), the industrial solvent methyl ethyl ketone, and styrene, an important monomer in the polymer and rubber industry (Jansen et al 1984). A further application for butanediol is as a fuel additive, as its energy content (27200 kJ kg^{-1}) is comparable with that of ethanol (29100 kJ kg^{-1}) and methanol (22100 kJ kg^{-1}) (Magee and Kosaric 1985).

We started our investigation by evaluating a number of bacterial strains for the production of 2,3-butanediol from D-xylose (Botes 1982). The best strain was an isolate identified as *Enterobacter cloacae*. As was found in the fermentation of xylose to ethanol, the degree of aeration was also a critical factor in the 2,3-butanediol fermentation. No fermentation occurred under anaerobic conditions. A high aeration rate increased the rate of butanediol production, but gave a low yield (in favour of cell growth), whereas a too low oxygen supply decreased both the rate and yield of butanediol production. Similar results were reported by Jansen et al (1984) with *Klebsiella oxytoca* (formerly *Klebsiella pneumoniae* or *Aerobacter aerogenes*). *Enterobacter cloacae* also produced high butanediol concentrations from other sugars such as D-glucose and L-arabinose (Botes and Engelbrecht 1983). The pH influenced the fermentation markedly. The maximum volumetric production rate was reached at pH 6-6,5, but the maximum yield was obtained at pH 4,5-5 (Table 9). The butanediol yield of our *E cloacae* strain surpassed the yield obtained by Jansen et al (1984) with *Klebsiella oxytoca* by far (Table 9). The yield by *K oxytoca* was increased to 0,48 g butanediol g^{-1} xylose by the addition of 5 g l^{-1} acetic acid, which also served as a substrate for butanediol production (Yu and Saddler 1982). Higher yields than in synthetic media were obtained, because of the acetic acid present in hemicellulose hydrolysates, 0,4-0,5 g g^{-1} reducing sugar (Yu et al 1984).

Table 9. Parameters for the fermentation of 40-50 g l^{-1} D-xylose to 2,3-butanediol.

Bacterium	Parameter				
	pH	$Y_{p/s}$ g g^{-1}	Butanediol g l^{-1}	Q_p g $l^{-1}h^{-1}$	Fermentation time, h
E. cloacae (Botes & Engelbrecht 1983)	5	0,46	19,9	0,55	50
E. cloacae (Botes & Engelbrecht 1983)	6	0,41	16,7	0,7	26
E. cloacae (Botes, unpublished)	7 to 5,5[a]	0,45	18,65	0,78	21
K. oxytoca (Jansen et al 1984)	5,2	0,22	11	1,0	11

[a] The pH was allowed to drop to pH 5,5. It was then controlled at this value for the remainder of the fermentation.

A neutralization procedure for the hemicellulose hydrolysate, which consisted of raising the pH to pH 3 with $Ca(OH)_2$, heating to 80°C, separating the supernatant from the $CaSO_4$ precipitate by decanting and adjusting to pH 6,8 with NaOH, proved satisfactgory (Botes and Engelbrecht 1983). After a 48 h fermentation period a butanediol concentration of 21 g l^{-1} was reached. The yield, based on the total amount of carbohydrates (31,88 g l^{-1} xylose, 4,15 g l^{-1} glucose, 4,1 g l^{-1} L-arabinose), was 0,52 g butanediol g^{-1} sugar. This value was slightly higher than the maximum theoretical yield coefficient of 0,51 and was attributed to the presence of fermentable compounds such as gluconate and trisaccharides which were not assayed by the analytical procedures employed. Little acetate utilization occurred, which suggests that it may be advantageous to use a coculture of *E cloacae* and *K oxytoca*.

The high boiling point of butanediol (180°C) renders its recovery from the fermentation broth by distillation a major expense. Jansen et al (1984) suggested that a butanediol concentration of 80-100 g l^{-1} would be required for economic product recovery so that a fed-batch fermentation with a feed of concentrated sugar solution would have to be used. The utilization of bagasse hemicellulose hydrolysate for 2,3-butanediol production would, therefore, require an intermediate concentration stage. This would increase the cost of the process.

Because butanediol was not considered a product with market potential in South Africa (Kamper et al 1983), its production from hemicellulose hydrolysate was not investigated further.

NOMENCLATURE

E — efficiency of substrate utilization; g xylose utilized g^{-1} initial xylose x 100 percent

q_p — maximum specific rate of ethanol production; g ethanol g^{-1} biomass h^{-1}

Q_p — maximum volumetric rate of ethanol production, calculated from the slope of the curve of ethanol versus time; g ethanol $l^{-1}h^{-1}$

$Y_{p/s}$ — product (ethanol or xylitol) yield coefficient; g product g^{-1} xylose utilized

$Y_{x/s}$ — cell yield coefficient; g biomass g^{-1} xylose utilized

μ_{max} — maximum specific growth rate; h^{-1}

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Italics indicate work carried out in the programme.

CHAPTER 3. PRETREATMENTS

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INTRODUCTION

Cellulose in plant material is partially protected against enzymic hydrolysis. This protection is the result of a combination of factors which include the following:

- the crystalline nature of cellulose, with molecules being tightly packed into hydrophobic microfibrils;
- the matrix of lignin and hemicellulose which surrounds the cellulose and restricts access by large enzyme molecules;
- the low surface area of the lignocellulose (Dale 1985).

For efficient hydrolysis of the cellulose it is necessary to reduce the influence of these protective factors. This requires pretreatment of the lignocellulose prior to hydrolysis.

The methods used for pretreatment have been the subject of a number of reviews including those by Millet et al (1975), Dunlap et al (1976), Horton et al (1980), Trickett and Neytzell-de Wilde (1982) and Dale (1985). These reviews generally classify pretreatments as either physical or chemical, or a combination of both. The physical methods include gamma radiation, ball-milling, hammer-milling, disc refining and compression milling. The chemical treatments include hemicellulose removal (prehydrolysis) with acid, alkali or steam; lignin degradation with hydrogen peroxide/ferrous ions or ozone; delignification with sodium hydroxide, ammonia or alcohols; cellulose solubilization with Cadoxen and "decrystallisation" with ethylene diamine. Combined pretreatments include steam prehydrolysis followed by steam explosion to disrupt the cells physically and disc refining in the presence of alkali. For bagasse, a combined treatment involving acid hydrolysis of hemicellulose followed by attritor milling of the residue has proved promising (Purchase 1981, 1983; Trickett and Neytzell-de Wilde 1982).

The most recent and most comprehensive overview of pretreatments (Dale 1985) suggests that most pretreatments are still at the stage of laboratory techniques. Their commercial potential usually cannot be assessed because their energy and chemical requirements have not been adequately determined. Furthermore, the methods used for assessing the effectiveness of pretreatments have not been uniform and therefore the various pretreatments cannot be compared meaningfully. The best pretreatment for a particular substrate may not be the best for a different substrate, thus further complicating pretreatment comparisons.

An objective of the CSIR programme was to develop processes for the enzymic hydrolysis of bagasse. This necessitated the development of pretreatment processes specifically for bagasse. To assess the more promising pretreatments fully, it was necessary to test hydrolysis of the pretreated material under anticipated industrial conditions. It therefore became important to interlink pretreatment and hydrolysis studies and to optimize conditions for the hydrolysis of the variously pretreated bagasse materials.

PRETREATMENTS TESTED LOCALLY ON BAGASSE

ACID PREHYDROLYSIS

Acid prehydrolysis alone causes only a slight increase in the susceptibility of the residual bagasse to enzymic hydrolysis. Cellulose conversions increased from about 6 percent for whole (untreated) bagasse to 12 percent (Purchase 1981) or 25 percent (Purchase et al 1982) or 34 percent (Trickett and Neytzell-de Wilde 1982) for prehydrolyzed bagasse, the discrepancies in responses being due to differences in hydrolysis conditions, particularly enzyme loading.

DELIGNIFICATION

Extensive tests of delignification of bagasse and subsequent enzymic hydrolysis were conducted by the Chemical Engineering Group at the University of Natal. They were summarized by Trickett and Neytzell-de Wilde (1982) who confirmed that an inverse linear relationship existed between lignin content and extent of hydrolysis. Most of these delignification trials were done on prehydrolysed bagasse and four different pulping systems were tried.

Soda pulping was tried with NaOH concentrations ranging from 0,16 to 0,32 g NaOH g^{-1} bagasse and temperatures from 100 to 170°C. The treated residues had lignin contents between 2,6 and 12,9 percent, but the cellulose loss due to pulping was unacceptably high at 15 to 35 percent (Trickett and Neytzell-de Wilde 1982).

Ammonia pulping was investigated in the hope that it would cause less cellulose loss. Prehydrolyzed bagasse was treated at 120 and 170°C with ammonia concentrations between 2,38 and 2,96 g NH_4OH g^{-1} bagasse. Cellulose losses were low but the lignin contents of the final residue were too high at 20-22 percent and the enzymic hydrolysis was only slightly improved.

Acid sulphite pulping of prehydrolysed bagasse for 5-8 hours at 132°C with various amounts of sulphite solution caused negligible cellulose loss, but gave residues with a minimum of 10 percent lignin. More severe conditions would be necessary for good pretreatment and the process would be expensive, particularly at the relatively small scale envisaged (Neytzell-de Wilde and Lussi 1981).

"Organosolve" pulping with butanol/water and ethanol/water mixtures at 178°C for 2-4 h removed up to 75 percent of the lignin without removing cellulose. Decreasing the temperature to 150°C and the time to 1 h made the process almost ineffectual as a pretreatment. Its cost, even with the milder conditions, would be prohibitive (Trickett and Neytzell-de Wilde 1982).

Ozone treatment of prehydrolysed bagasse was tested by bubbling ozone through an aqueous suspension of bagasse for six hours (Neytzell-de Wilde and Lussi 1980). This had no appreciable effect on subsequent enzymic hydrolysis, even when the ozone was applied in the presence of ultraviolet light. More detailed work on ozone pretreatment is reported by Neely (1984) who concluded that an ozone consumption of 4-6 percent of the dry mass of lignocellulose was necessary for effectiveness and that the effectiveness rises sharply when the critical minimum amount of ozone is applied. Neely also found that excessively wet material cannot be effectively ozonised, thus possibly explaining the failure of Neytzell-de Wilde and Lussi's (1980) treatment. The cost of ozone for effective pretreatment was estimated as R42 t^{-1} lignocellulose (Neely 1984) which is too expensive if ethanol is the endproduct.

In general, delignification involves high chemical costs which are acceptable if the endproduct is of high value, such as paper, but are uneconomical for the production of fermentable substrates.

COMMINUTION

Ball-milling

Grinding of lignocellulose to fine particles is an effective pretreatment, but is generally expensive in terms of energy requirements. Datta (1981) assessed the energy requirements of various pretreatment processes. For ball-milling of municipal solid waste, the energy requirement depended on final particle size according to the relationship shown in Table 10.

Table 10. Energy requirements for grinding municipal solid waste to various particle sizes (Datta 1981).

Particle size (μm)	Energy required ($kWh\ t^{-1}$)
420	100
178	330
149	400
74	1 670
53	2 860

A particle size of less than 100 μm is generally necessary and Datta (1981) therefore concluded that the energy requirement was often greater than the energy content of the material being ball-milled.

Compression milling

As an alternative to ball-milling, Tassinari et al (1980) tried compression milling between differential speed rolls. They found this pretreatment to be effective for a wide variety of materials. The enzymic conversion of bagasse was increased from 5 percent to 37 percent by four minutes of milling. Further optimization (Tassinari et al 1982) enabled newspaper to be milled with a specific energy input of 0,46 $kWh\ kg^{-1}$.

Local trials of compression milling of bagasse proved unsuccessful for whole bagasse, but partially successful for prehydrolysed bagasse. Comparative trials, however, showed that attritor milling was more effective than compression milling, the cellulose conversions being 60 percent and 47 percent respectively (Neytzell-de Wilde and Lussi 1981).

Attritor milling

When prehydrolyzed bagasse was ball-milled it disintegrated much more readily than did whole bagasse (Purchase 1980). This observation suggested that the energy requirement for milling of prehydrolyzed bagasse might be much lower than that estimated for most lignocelluloses and might be low enough to make the process acceptable as a pretreatment. Subsequent trials involved a version of ball-milling in which the balls are stirred with an agitator while the drum remains stationary. Such mills are more energy-efficient than ball-mills and they enable greater energy input per unit volume. They are called stirred bead mills or attritor mills.

Using a batch attritor mill with 10 mm diameter ceramic balls and an agitator speed of 200 rpm it was found that 10 minutes of milling of prehydrolyzed bagasse was an adequate pretreatment to enable 60 percent of the cellulose to be hydrolysed within 24 h by only 10 filter paper units (IU) of enzyme g^{-1} cellulose. The energy requirement was less than 0,45 $kWh\ kg^{-1}$ bagasse (dry basis) (Purchase 1981) and so the process seemed more promising than compression milling in terms of effectiveness and energy requirement. The pretreated material compared favourably with commercially pulped bagasse and wood and was only slightly less reactive than soda-pulped bagasse involving 0,32 g NaOH g^{-1} bagasse at 170°C for 0,25 h (Trickett and Neytzell-de Wilde 1982).

In further optimizing the attritor milling process it was found that:

- when steel balls were used in place of ceramic balls the balls rusted rapidly and released a material which could cause severe inhibition of saccharification of the milled bagasse;

- dry attritor milling of prehydrolyzed bagasse was impossible. All milling was done with a bagasse slurry and if the slurry level exceeded the ball level then the efficiency declined because the balls tended to circulate around the mill en masse (Figure 11);

- when sulphuric acid was used for hemicellulose hydrolysis, the subsequent cellulose conversion yields in standard tests never exceeded 70 percent even when all cells were disrupted by thorough milling. Conversion yields of 70-80 percent were achieved when the acid prehydrolysis at 100°C was replaced by steam autohydrolysis at 182°C. A minimum of 0,25 h steaming was required to ensure trouble-free milling (Purchase 1981, 1983)

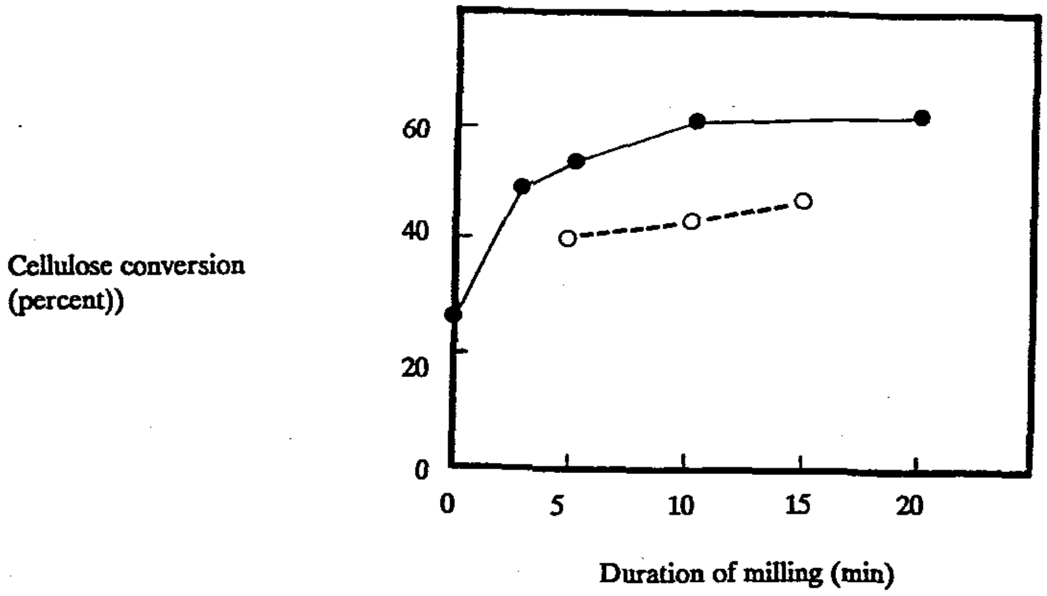

Figure 11. Cellulose conversions as affected by duration of milling and by mill flooding (–●– = 2,5 l slurry; --o-- = 3,0 l slurry). (Purchase 1983).

Performance of a continuous attritor mill

The promising results obtained with the batch attritor mill suggested that adequate milling would be achieved by a single pass through a larger, continuous machine. To assess this possibility, a mill with a 7,5 kW motor, 26,5 l grinding chamber and a screw feeder was constructed. This mill was filled with 6 mm diameter steatite balls and generally operated at 200 rpm. An indication of the performance of the machine is given in Table 11.

Table 11. Comparison of continuous mill performance when operated with different slurry concentrations (Purchase 1985).

Slurry concentrations in Mill (percent)	Feed rate (dry kg h^{-1})	Mill power at 200 rpm (kW)	Specific energy input (kWh kg^{-1})
15	25*	4,8-5,2	0,20
8-9	42*	4,4	0,10
8-9	32	3,4	0,11
8-9	18	2,8	0,15
6	42	2,7	0,06

* Maximum possible feed rate

The machine could handle a slurry concentration of 15 percent, but, at this concentration, the maximum throughput was 25 kg h^{-1} (dry basis) and the specific energy input was relatively high. With lower slurry concentrations a specific energy input as low as 0,06 kWh kg^{-1} could be achieved. The resulting material was slightly fibrous but in standard hydrolysis tests 65 percent of its cellulose was converted to glucose in 24 h. This conversion was only 2 percent less than that for material which had received 0,15 kWh kg^{-1}. For cost calculations, a figure of 0,1 kWh kg^{-1} with a throughput of 42 kg h^{-1} seems *reasonable (Purchase 1985).*

The mean mass loss of the beads, based on the mass of new beads is shown in Table 12.

Table 12. Mass losses of steatite beads in an attritor mill after various energy inputs (Purchase 1985).

kWh Readings		(Mass Loss)	
Initial	Final	(percent kWh^{-1})	(g kWh^{-1})
15,9	56,3	0,026	9,8
56,3	64,0	0,019	7,2

The declining rate of wear is probably due to the existence of a ridge around the beads. This has sharp edges which are particularly prone to wear in the initial stages.

The beads are slightly oblong and over the first 80 kWh of energy input the dimensions changed as shown in Table 13.

Table 13. Wear of beads expressed as change in size.

	Mean "diameters" (mm) Long	Short
New beads	6,57	6,23
Old beads (80 kWh)	6,51	6,22
Difference (percent)	0,86	0,27

If the beads are assumed to be spheres then the calculated volumetric losses are 0,03 and 0,01 percent kWh^{-1}, based respectively on the long and short diameters. For cost calculations, a wear loss of 0,02 percent kWh^{-1} seems reasonable (the percentage being based on new beads, not worn beads). With a bead load of 37,8 kg this translates to 7,6 g of wear for each of the first 80 kWh applied (Purchase 1985).

Design concepts for scale-up

When scaling-up an attritor mill, the diameter of the grinding chamber is limited because, with large diameters, the speed of the ends of the stirrer arms becomes excessive (together with the torque requirement). The high speed causes the beads to circulate en masse around the vessel and this diminishes the grinding effect. For large-scale milling, a new concept is therefore necessary.

The new concept (Purchase 1985) involves multiple stirrers in a tank of beads and it thus avoids the cost of building and maintaining individual vessels for each strirrer. This is a major cost saving because the vessels have to be stainless steel to resist acid corrosion. The use of multiple small stirrers diven by a common shaft enables the use of relatively few motors thus introducing another cost saving and possibly an energy saving.

The envisaged feeder system (Figure 12) is much simpler than a screw feeder. Its proposed design is based on the following practical observations:

- the screw feeder discharged compacted lumps of bagasse into the mill and energy was wasted in dispersing these lumps. The lumps sometimes jammed the mill;

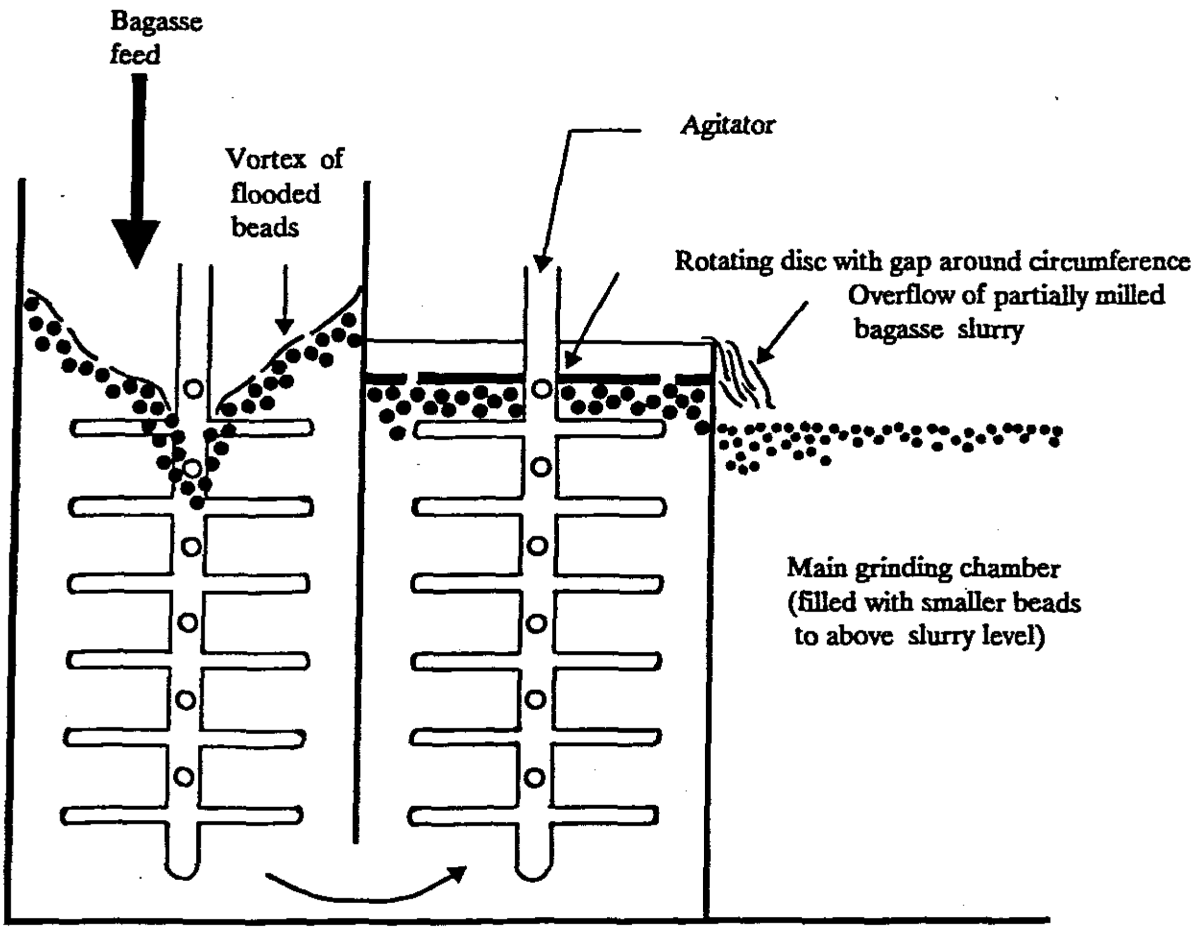

Figure 12. Details of the inlet design concept for a large attritor mill.

- the screw feeder was the most problematic component of the mill and is likely to involve high maintenance costs;

- experience with the batch mill showed that top feeding was easy if the water level in the mill was above the bead level. Under these conditions a vortex forms and pulls the bagasse down among the beads. If there is no water to help buoy up the top beads then the bagasse remains on top of the beads. However, if the top beads are buoyed up then the milling efficiency is adversely affected. The proposed design enables a high water level to be maintained only in the first section of the tank and thus it does not compromise milling efficiency in the main tank.

The proposed design would facilitate simple transfer of the slurry through zones of diminishing bead sizes. This would increase milling efficiency by matching particle size to optimum bead size. The separation of zones with different bead sizes would be achieved by means of a series of screens with diminishing aperture sizes. Through wear, the beads would become smaller and would thus move through the zones.

If all the bagasse from a medium sized sugar mill was prehydrolyzed and then attritor milled, the required milling capacity would be 20 t h^{-1} (dry mass). Judging from the existing experimental mill the required mill volume for 20 t h^{-1} is 12,6 m^3 and the estimated number of stirrers is 136 (assuming a tank depth of 1 m).

Some costs of attritor milling prehydrolyzed bagasse have been estimated approximately as follows (Purchase 1985):

Item	Cost (R t^{-1})
Capital	3,50
Bead wear	4,90
Electricity (@4c kWh^{-1})	4,00
	12,40

The cost of bead wear is based on the price of a small consignment of beads and on the high initial rate of wear. It could probably be reduced substantially by buying beads in bulk or by developing a cheaper grinding medium.

Perspectives on attritor milling as a pretreatment

There is very little information available on attritor milling of fibrous materials. The information generated on the subject in this programme is novel and sufficient to enable a preliminary commercial assessment of the process. It indicates that attritor milling has promise as a pretreatment for bagasse, but only if the bagasse is first prehydrolyzed. The energy requirement of about 100 kWh t^{-1} prehydrolyzed bagasse is probably the lowest ever reported for grinding lignocellulose to a point where very few intact cells remain. The process cannot, however, be regarded as a general pretreatment for all prehydrolyzed lignocelluloses because the responses of different plant species to milling are very different (Millet et al 1975).

Pretreatments are assessed in terms of the digestibility of the treated material but this depends on the conditions during digestion, especially the enzyme concentration. The digestibility of a 5 percent slurry of prehydrolyzed, attritor milled bagasse at 50°C and pH 4,8 with different enzyme:substrate ratios is shown in Figure 13 (Purchase 1983). This indicates that, if enough enzyme is used, almost 100 percent cellulose conversion can be achieved within 24 h but when realistic quantities of enzyme are used then the conversion is about 60 percent in 24 h. The use of non-enzymic additives (polyethylene glycol or polyvinylpyrrolidone) increases this to about 75 percent, but, if the slurry concentration is increased from 5 percent to 14 percent, then the 24 h cellulose conversion declines from 77 percent to 58 percent due to endproduct inhibition (Purchase et al 1985).

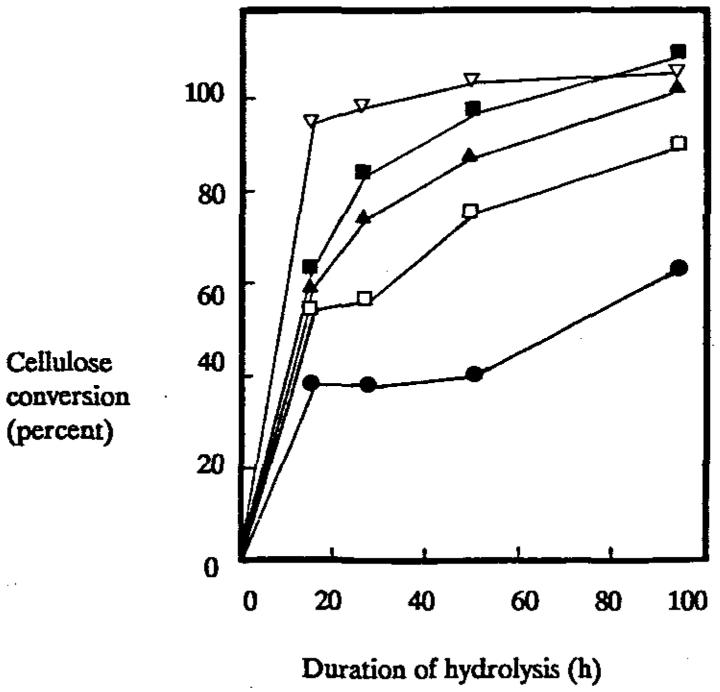

Figure 13. Hydrolysis dynamics for a 5 percent slurry of attritor milled bagasse with different enzyme (IU): cellulose ratios (▽= 142:1; ■= 57:1; ▲= 28:1; □= 14:1; ●= 7:1). (Purchase 1983).

A disadvantage of milling as a pretreatment is that lignin remains in the bagasse and passes through subsequent processes as insoluble solids. Despite this the bagasse has been successfully hydrolyzed and fermented (Waugh 1986). In the absence of the lignin it is probable that there would be no need for additives such as polyethylene glycol, which are thought to prevent irreversible adsorption of enzyme onto the lignin. On the other hand, the finely ground lignin rich residue which remains after hydrolysis can be recovered and probably has value as a raw material for adhesives (van der Klashorst 1986) or as a boiler fuel. When dried, it had an energy value of 19270 kJ kg^{-1} and was calculated to contain about 40 percent of the fuel value of the original bagasse from which it was derived (Walford et al 1984).

A disadvantage of wet milling is that the milled product has a high bulk density and cannot be easily pumped or mixed at *concentrations above about 9 percent. The maximum solids content which the mill could reasonably handle was 15 percent.* This limits the final ethanol concentration to about 2,5 percent. Against this disadvantage there is the advantage of being able to use the last stages of the mill for blending enzyme into the bagasse and of generating sufficient heat to warm the thick paste to the required 50°C and thus avoiding difficult heat transfer problems (Purchase et al 1985).

Steam explosion

Explosive decompression of steam pressurized lignocellulose has proved effective as a pretreatment (Saddler et al 1982, Puri and Maners 1983, Dekker and Wallis 1983, and Grous et al 1985). In the manufacture of furfural, bagasse is steamed at 183°C and then explosively decompressed. The steam effectively prehydrolyses the bagasse and converts the resulting xylose into furfural. The explosive decompression shatters the prehydrolyzed bagasse to a powdery residue. This furfural factory residue is normally used as a boiler fuel. In South Africa there is enough of it to provide the substrate for about 40 x 10^6 l ethanol annually and it is all produced at a single factory (SmithChem, Sezela). The existence of this quantity of steam exploded bagasse warranted investigations of the material to establish whether it had been effectively pretreated.

A series of investigations (Purchase 1980, Perrow et al 1983, Waugh and Proudfoot 1985, Waugh 1986) showed that the material had been very effectively pretreated and was more susceptible to enzymic hydrolysis than was attritor milled bagasse. By using only 5 international units (IU) of enzyme g^{-1} solids in a simultaneous saccharification and fermentation it was possible to convert more than 90 percent of the cellulose to ethanol within 64 h giving a high enzyme efficiency of 107 mg glucose IU^{-1} enzyme. Furthermore, the maximum solids concentration which could be handled was higher than for attritor milled bagasse (20 percent as against 15 percent) thus enabling a final ethanol concentration of 5 percent to be achieved. Points of detail which were learnt from work with furfural factory residue included:

- freshly exploded material is better that old or dried material but the material could be stored in a refrigerator for a few weeks without loss of activity;

- attritor milling improved the reactivity of old or dried material but had negligible beneficial effect on fresh material;

- if the material is not washed with water prior to hydrolysis, inhibitors interfere with the fermentation;

- in simultaneous saccharification/fermentation a ß-glucosidase (IU):cellulase (IU) ratio of 1:1 was necessary for maximum rate of hydrolysis;

- sparging of the slurry with air during hydrolysis had a beneficial effect whereas nitrogen gas had a deleterious effect (Waugh 1986).

The furfural factory residue is undoubtedly a better substrate for enzymic hydrolysis than is acid-prehydrolyzed, attritor milled bagasse. The limitation to the use of furfural factory residue is that the quantity available is sufficient for only one medium sized distillery.

It is unlikely that steam explosion could stand alone as a replacement for attritor milling because the costs of equipment and steam are high and can be justified only when a valuable endproduct such as furfural is made. The most advanced equipment for steam explosion of bagasse is probably that produced by Stake Technology Ltd in Canada. The standard "Stake II" system is continuous and processes 3 t h^{-1} bagasse (dry basis). It has a total connected electrical power of 150 kW and consumes approximately 4,8 t steam t^{-1} bagasse. The capital cost is R3 400 000 (J D Taylor [2]). This equipment would be too expensive and energy demanding to replace attritor milling.

A steam explosion system operating at 200°C and using carbon dioxide or nitrogen gas to produce additional pressure was investigated by Puri and Mamers (1983). Even with this relatively energy efficient process, the steam requirement was calculated to be at least 0,9 t^{-1} bagasse treated. This is equivalent to at least nine times the energy required for attritor milling.

SUMMARY AND CONCLUSIONS

Enzymic hydrolysis of lignocellulose can be facilitated if the lignocellulose is first pretreated to make the cellulose more accessible to the enzymes. Various pretreatment processes are effective, but the best one for a particular substrate may be inferior for a different substrate, thus making it important to test and develop pretreatments specifically for the substrate involved.

For many proposed pretreatment processes the costs of commercial application cannot be estimated because neither have the energy and chemical requirements been determined adequately, nor has a realistic standard test been used to measure the effectiveness of the pretreatment.

In this programme, various pretreatment processes were tested on bagasse and the costs of the most promising ones were

[2] Personal communication - letter dated 19 February 1987.

estimated. The processes tested included chemical treatments, physical disruption and a combination of chemical and physical treatments. Most of the chemical treatments involved acid prehydrolysis to remove hemicellulose and then chemical delignification using either sodium hydroxide or ammonia or acid sulphite or organic solvents or ozone. Physical disruption tests involved two-roll milling, ball milling, attritor milling and steam explosion.

Steam exploded bagasse from a furfural factory was found to be the form of bagasse most susceptible to enzymic hydrolysis, but the amount of this material available is enough for only one medium sized factory (40 x 10^6 l ethanol per year).

The steam explosion process was thought to be too expensive for use solely as a pretreatment without an associated income from sale of furfural. The most promising alternative was a combined pretreatment involving acid prehydrolysis followed by attritor milling. The prehydrolysis generated a xylose rich liquid stream which could be fermented to ethanol or converted to single cell protein. The prehydrolysed solid residue was unusual in that it required surprisingly little energy (0,1 kWh kg^{-1} (dry basis)) for reduction to subcellular particles by attritor milling. The milled residue was readily digested by enzyme and compared favourably with delignified bagasse. A process development unit, including a 7,5 kW continuous attritor mill, was used to determine the technical requirements of the pretreatment process and to develop a concept and preliminary cost estimate for an industrial scale process. The successful development of this process could be important for any future biological exploitation of bagasse.

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Italics indicate work carried out in the programme.

CHAPTER 4. CELLULASE : PRODUCTION AND CHARACTERIZATION

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INTRODUCTION

At the outset of the programme four basic processes for the conversion of bagasse cellulose to fermentable sugar were tabled. These were dilute acid hydrolysis, concentrated acid hydrolysis, enzymic hydrolysis and vacuum pyrolysis. The enzymic route, operated under mild conditions and with good specificity of hydrolysis, seemed to offer, in the long term, the best potential solution and was adopted.

Cellulase production technology was at that time not well developed although several laboratory and pilot scale studies had been reported. In the Natick process, using a mutant strain of the fungus *Trichoderma reesei*, a yield of cellulase of 4,6 International Units (IU) ml^{-1} using the standard cellulase assay method of Mandels et al (1976) had been achieved in a 250 litre pilot scale fermenter at a productivity of 35 IU $l^{-1}h^{-1}$ (Allen et al 1979). Despite these promising results, enzyme costs were a major obstacle to the economic exploitation of an enzyme based process.

An urgent need was seen as part of the proposed programme to reduce enzyme costs and the following three approaches were chosen:

- to select from nature and possibly mutate more promising cellulase producing microorganisms than were available at that time;
- to optimize enzyme production technology using both the available strains from the Natick programme of the United States Army as well as promising microorganisms from the local selection programme;
- to achieve the most economical use of enzyme in the process.

In fulfilling the latter objective, a detailed characterization of the enzyme was considered an important aspect and is included, together with the studies on microbial selection and fermentation optimization and scale-up, in this chapter.

MICROBIAL SELECTION

The CSIR Microbiology Research Group isolated some 500 strains of cellulolytic Streptomycetaceae from soil and compost using elective media with bagasse as sole source of carbon and screened them for cellulase activity. Certain of the isolates had enzyme activities slightly in excess of 0,2 IU ml^{-1}. Optimum conditions for extracellular cellulase production and activity, thermal stability at 50°C and absorption onto bagasse were reported for three of the isolates having the highest cellulase activity (Van Zyl 1985). Studies aimed at generating hypercellulase producing mutants from these isolates using the mutagen N-methyl-N-nitrosoguanidine (NTG) were unsuccessful and no isolates were considered promising enough for fermentation optimization studies.

A similar study of 50 cellulolytic fungi did produce a strain of *Aspergillus terreus* having a slightly higher cellulase activity than the reference cellulase producer *Trichoderma reesei* QM9414 used in the Natick pilot scale trials. When grown in bagasse medium, 0,28 IU ml^{-1} were obtained. This was increased to 0,35 IU ml^{-1} after mutagenesis with NTG. Since the activity was still well below the approximately 1 IU ml^{-1} *routinely obtained at that time by T reesei* QM9414 grown in cellulose medium, the *A terreus* mutant was not considered for further optimization studies. Workers in other centres had also attempted to improve upon the Natick strains by screening highly cellulolytic fungi. Saddler (1982) for example had screened over 100 of the 2 000 strains of cellulolytic fungi present in the Forintek culture collection in Ottawa and only one, *Trichoderma strain* E58 was comparable to *Trichoderma reesei* QM9414 in its cellulase activity.

Meanwhile, researchers at the University of Natal had also met with only very limited success in the screening of 50 isolates of cellulolytic fungi. Consequently, they shifted their attention to the selection of anaerobic and thermotolerant bacteria (Wallis et al 1982). Over 275 isolates were tested for cellulolytic activity. It was concluded, however, that none of the isolates was able to produce extracellular cellulase at levels or rates high enough to be considered a likely source for industrial cellulase production and the project was terminated.

ENZYME PRODUCTION TECHNOLOGY

Despite attempts during the early part of this programme to isolate and select hypercellulase producing microorganisms from nature, mutants derived from a parent strain of *Trichoderma reesei* (isolated from a deteriorated cartridge belt in New Guinea during World War II), still remain the first choice for enzyme production technology (Reese and Mandels 1984). Several of these strains, viz QM9414, MCG77 and RUT-C30 were used during the course of this study.

Preliminary experiments on the optimization of cellulase production using QM9414 were conducted in the Department of Microbiology, University of the Orange Free State and the Department of Biochemistry, University of Fort Hare. By the end of 1980, the University of the Orange Free State workers had achieved an extracellular cellulase yield of 2,0 IU ml^{-1} after approximately 140 h in a 2 litre batch fermentation using 0,75 percent cellulose powder and automatic control of pH to a minimum value of 3,0 with NH_4OH (S G Killian 1980, personal communication). The Fort Hare group also demonstrated the advantage of pH control and achieved 1,3 IU ml^{-1} after 140 h using 1,5 percent cellulose at a minimum pH of 3,5. They attempted to increase cellulase yield further by raising the cellulose concentration to 6 percent, but without success (Brand 1981).

A third group at the CSIR were also active in this field and had by this time achieved a cellulase yield and productivity of 3,6 IU ml^{-1} and 29 IU $l^{-1}h^{-1}$ in laboratory scale fermentations. At a pilot scale (150 l), however, only 1,4 IU ml^{-1} of cellulase were obtained, probably as a result of poor inoculum development (Watson et al 1980).

All subsequent work within the programme was undertaken solely at the CSIR. During 1981, as a result of numerous further laboratory scale and several pilot scale fermentations using carefully optimized inoculum development, a cellulase yield and productivity of 7,5 IU ml^{-1} and 54 IU $l^{-1}h^{-1}$ were achieved in a pilot scale trial with 5 percent cellulose (Watson and Anziska 1982). A method was also perfected for the concentration of enzyme broth by ultrafiltration using membranes of 10 000 nominal molecular mass limit, followed by freeze drying, to provide a stable enzyme preparation for use by other participating groups in the programme.

Further enhancements in enzyme production were obtained when an improved mutant *T reesei* RUT-C30 (Montenecourt and Eveleigh 1979) became available to us. This strain has the advantage of reduced sensitivity of cellulase production to glucose catabolite repression. According to Tangnu et al (1981) it can be cultured under optimum conditions for growth and ß-glucosidase production ($pH \geq 5{,}0$) without forfeiting cellulase production. They achieved 14,4 IU ml^{-1} after 8 days fermentation at pH 5,0.

Contrary to the work of Tangnu et al (1981), rapid growth of RUT-C30 could not be sustained at a pH of 5,0 beyond an initial phase during which the complex nitrogen source was being utilized. After approximately 30 h, respiration more or less ceased and no enzyme production was detected (Watson and Nelligan 1982). Good growth, enzyme yield and productivity were obtained at pH 4,0, however, a condition also conducive to the production of ß-glucosidase, a rate limiting enzyme in the hydrolysis of cellulosic materials by many T reesei enzyme preparations including those previously prepared in this programme. In a 150 litre pilot scale fermentation trial using 5 percent cellulose, the cellulase yield and productivity were increased to 12 IU ml^{-1} and 75 IU $l^{-1}h^{-1}$ with a ß-glucosidase yield of 5,5 IU ml^{-1} (Watson and Nelligan 1983a) measured according to Sternberg et al (1977).

Fermentation media still included expensive complex nitrogen sources, viz yeast extract (0,4 percent) and peptone (0,1 percent) and attention was directed to replacing these with cheaper substrates such as corn steep liquor as part of the optimization programme. Although results were promising, it became clear that these supplements played a less important role in the physiology of *T reesei* than previously thought. It seemed that they were only providing an easily assimilable carbon source which did not repress cellulase induction. The use of lactose to fulfil this role was then investigated, lactose being a readily available and cheap byproduct of the cheese manufacturing industry. The results were moderately successful although the onset of enzyme production was delayed somewhat (Watson and Nelligan 1983b). Later, through careful optimization of the fermentor operating conditions it was found possible to dispense with both supplementary complex nitrogen sources and lactose without significantly decreasing enzyme yields and productivities (Watson and Nelligan 1983c).

Optimization studies had been carried out exclusively using a batch culture technique in which cellulose was added only at the start of each fermentation. Cellulose was progressively consumed until near exhaustion at maximum enzyme yield after a period of approximately one week. Continuous culture techniques had not proved particularly successful for cellulase production and although significant improvements to enzyme productivity had been reported, these were invariably achieved at the expense of low enzyme yields (Ghose and Sahai 1979; Ryu et al 1979).

Experience gained during this optimization programme suggested that batch fermentations were limited to cellulose concentrations of not much more than 5 percent, especially when scale-up to large production fermenters is envisaged. Problems of slow initial cellulase production, inadequate mixing and oxygen transfer and a large concentration of fungal mycelia, built up at the expense of enzyme production, occur. For these reasons, lactose was again examined, this time as an alternative soluble energy source and cellulase inducer in place of cellulose. Lactose was not as effective an inducer as cellulose and since it was in any case more rapidly assimilated than glucose, its use led to oxygen limited conditions early in the fermentation unless low concentrations were employed. Given these restraints it became apparent that significant progress in batch culture was no longer possible in the absence of future microbial strain improvement (Watson et al 1984a).

Attention was therefore directed towards a fed-batch system in which cellulose was added intermittently during the course of a fermentation. In this way problems associated with high initial concentrations of cellulose were avoided whilst still gaining advantages from high total effective concentrations. Considerable success had already been achieved by Hendy et al (1982) using this technique. An enzyme yield of 30 IU ml^{-1} and productivity of 106 IU $l^{-1}h^{-1}$ were obtained by them using an effective cellulose concentration of 150 g l^{-1}.

Fed-batch fermentations were conducted at 0,25, 0,5 and 1,0 g cellulose $l^{-1}h^{-1}$ after initial batch growth on 20 g l^{-1} cellulose for 42 h. In each case a plateau in biomass concentration resulted which was subsequently maintained throughout the remaining period of feeding (Figure 14). During the growth phase of the culture, biomass concentrations built up until the level reached was such that the requirement of that population density for energy and carbon source for cell maintenance and enzyme production was met precisely by the cellulose feed rate. At this point no further increase in biomass occurred although the level was maintained, corresponding to an average specific maintenance coefficient of 0,029 g cellulose g^{-1} biomass h^{-1} (Table 13). Specific enzyme production rates were also essentially the same during these quasi-steady state conditions (9,6-11,8 IU g^{-1} biomass h^{-1}) and since the protein content of the biomass remained constant at approximately 52 percent, ammonia uptake by the cells could be equated with extracellular protein production.

The maximum cellulose feed rate which can usefully be employed in a fed-batch culture is limited by the ability of available fermentation equipment. In the experiments described here, oxygen supplementation of the air supply became necessary at a mean feed rate of 1,0 g cellulose $l^{-1}h^{-1}$, the situation being particularly critical immediately after each addition. For production scale operation, a continuous feed of cellulose would be feasible and would probably overcome these problems experienced in oxygen supply. A maximum enzyme yield of 57 IU ml^{-1} at an overall productivity of 201 IU $l^{-1}h^{-1}$ was achieved in these experiments (Table 14) using a feed rate of 1,0 g $l^{-1}h^{-1}$. During the course of the fermentation, 70 g l^{-1} soluble protein was produced in the culture broth. The enzyme yield achieved remains the highest published value for cellulase production (Watson et al 1984b).

Table 13. Effect of cellulose feed rate on biomass and cellulase production during quasi-steady state conditions in fed-batch culture of *Trichoderma reesei* RUT-C30.

	Cellulose feed rate ($g\ l^{-1}h^{-1}$)		
	0,25	0,50	1,00
Residual cellulose concentration ($g\ l^{-1}$) (mean; prior to each fresh addition)	2,3	3,3	8,8
Biomass yield (mean) ($g\ l^{-1}$)	10,0	15,1	35,9
Cellulase productivity ($IU\ l^{-1}h^{-1}$)	96,0	159,0	427,0
Specific maintenance coefficient (g cellulose g^{-1} biomass h^{-1})	0,025	0,033	0,028
Specific enzyme production rate ($IU\ g^{-1}$ biomass h^{-1})	9,6	10,5	11,9

Table 14. Overall cellulase yields and productivities by *Trichoderma reesei* RUT-C30 in fed-batch culture.

	Cellulose feed rate ($g\ l^{-1}h^{-1}$)		
	0,25	0,50	1,00
Duration of cellulose feed (h)	42-242	42-242	42-274
Total effective cellulose concentration ($g\ l^{-1}$)	70,0	120,0	252,0
Maximum cellulase yield ($IU\ ml^{-1}$)	15,5	26,8	57,0
Overall cellulase productivity at maximum yield ($IU\ l^{-1}h^{-1}$)	60,0	104,0	201,0
Efficiency of enzyme production ($IU\ g^{-1}$ cellulose)	221,0	223,0	226,0

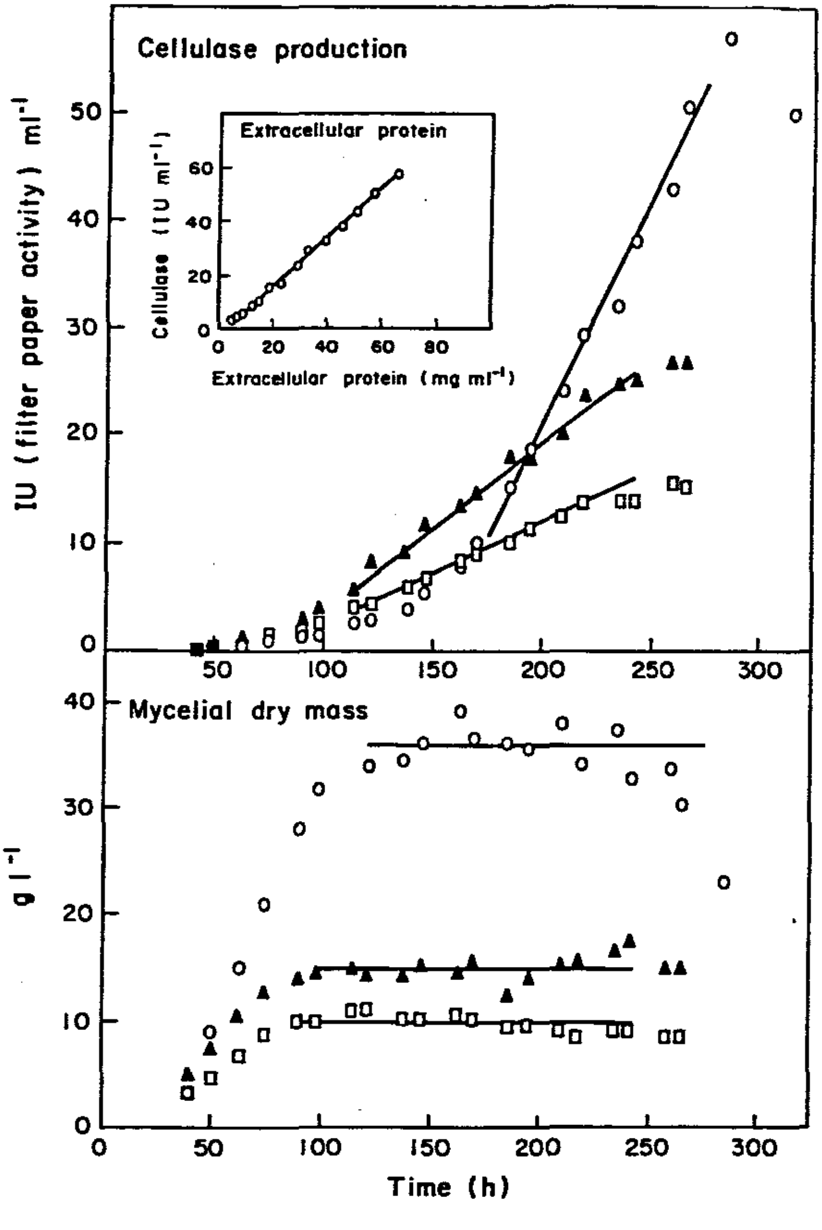

Figure 14. Fed-batch Fermentation Profile of *T Reesei* RUT-C30 Cellulose feed rates (from 42h):
□ 0,25 g $l^{-1}h^{-1}$;
▲ 0,50 g $l^{-1}h^{-1}$;
o 1,0 g $l^{-1}h^{-1}$.

The optimum temperature of fermentation for cellulase production by *T reesei* is considerably lower than the optimum growth temperature of 35°C. Generally, a temperature of not more than 27°C is recommended, and 25°C was used in the experiments reported above. On an industrial scale, it would be desirable for cellulase production to be satisfactorily maintained at a temperature closer to the optimum growth temperature. This aspect is especially important in climates of high ambient temperature, such as Natal, where difficulties could be encountered in providing adequate fermenter cooling at an economic level. Recent results suggest that acceptable enzyme yields and productivities are obtainable at up to 30°C although ß-glucosidase production is considerably lower at 30 °C than at 25°C (Watson et al 1985).

The cellulose source of choice throughout this study has been an industrial hardwood pulp[3] (sulphite process), bleached, flash dried and ball milled. Attempts were made, however, to replace it with cheaper alternatives such as bagasse pulp or cotton linters. In both cases good rapid growth of *Trichoderma reesei* occurred but rather weak enzyme production.

ENZYME CHARACTERIZATION

Limited studies on the characterization of cellulases from *Trichoderma reesei* QM9414 were carried out in the Department of Biochemistry of the University of Fort Hare. Brand (1981) investigated the effect of fermentation conditions on the glycoprotein nature of the cellulase enzyme complex. He found that enzymes produced later in the growth phase contained a greater proportion of carbohydrate than the enzymes produced earlier.

Work on the characterization of the cellulase of *Trichoderma reesei* RUT-C30 within this programme was conducted exclusively in the Department of Biochemistry of the University of the Orange Free State and is described here.

The cellulase complex of *T reesei* RUT-C30 was fractionated into three main components with regard to cellulolytic activities. An endo- and exoglucanase as well as ß-glucosidase enzymes were isolated, purified and characterized. Properties of the enzymes were:

- relative molecular mass: exoglucanase 53700, endoglucanase 56250 and ß-glucosidase 17400;
- diffusion coefficient: exoglucanase 6,27x10^{-7} cm^{2}s^{-1} and endoglucanase 6,58x10^{-7} cm^{2}s^{-1};
- Stokes radii: exoglucanase 34,5x1O^{-8} cm and endoglucanase 36,1x10^{-8} cm.

The exoglucanase contained 5 percent carbohydrate and the endoglucanase 2 percent (m m^{-1}). The enzymes were characterized with regard to catalytic properties and displayed an optimum pH of 4,5 to 5,0. Cellulosic residues of bagasse were hydrolyzed at a lower rate than pure cellulose probably due to inhibition by phenolic substances and resultant lignins present in the preparation (Du Toit, Olivier, Van Wyk et al 1985).

Xylanases and xylosidases were also present in the cellulase complex. When the cellulase complex was used for the hydrolysis of bagasse residue subsequent to pentose extraction, xylose was initially released from the hemicelluloses still present in the cellulosic material at a faster rate than glucose. When different hemicellulose substrates were used for growth of the *T reesei*, the resulting extracellular enzyme complex was enriched with xylanase and xylosidase activities and the use of this complex to hydrolyze a bagasse "cellulose" preparation resulted in a relatively higher rate of hydrolysis than when the enzyme complex had been produced by growth on pure cellulose. The overall yield of cellulase enzyme complex was, however, lower when hemicellulose was used for enzyme production (Du Toit, Olivier, Van Rensburg and Kriel 1985).

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[3] A generous gift of SAICCOR, Umkomaas.

CONCLUDING REMARKS

The ultimate goal of continuing cellulase research is the large scale enzymic saccharification of lignocellulosic material in the production of fuel ethanol, with possible spin-offs in single cell protein production and other fermentation products as alternative uses of the intermediate glucose.

At present, although encouraging progress in cellulase production technology has taken place, problem areas in economical enzyme usage still exist, namely the recalcitrance of lignocellulosic material to enzyme attack and the low specific activity of cellulase for its substrate.

Despite the low specific activity, commercial cellulase preparations have found a small, but expanding, industrial market in the pharmaceutical industry as a digestive aid and in animal feed supplementation. Also particularly promising is the use of cellulase in brewing to improve filterability and increase ethanol yield and in the textile and fruit processing industries (Lessing and Watson 1985).

The technology is now available locally, as a result of the Biological Utilization of Bagasse Subprogramme, to exploit these markets through the production of cellulase at the highest yield and productivity attainable internationally.

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Italics indicate work carried out in the programme.

CHAPTER 5. CELLULOSE HYDROLYSIS AND FERMENTATION

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INTRODUCTION

Several processes for the acid hydrolysis of cellulose to produce glucose exist and are reviewed by Grethlein and Converse (1983). They include both dilute and concentrated acid processes, but, apart from operation during world wars, the processes have generally failed in the west because of the high cost of equipment and acid. Enzymic hydrolysis has the advantage over acid hydrolysis of mild reaction conditions and high reaction specificity (Mukataka et al 1983). It suffers though from high enzyme cost per unit of glucose produced and low volumetric productivity.

Although some attention has been given to the direct production of ethanol from cellulose using a single microorganism capable of hydrolysis and fermentation (Cooney et al 1978; Zertuche and Zall 1982; Wu et al 1986), the more common approach to enzymic cellulose hydrolysis for ethanol production has been via the two stage conversion, first its hydrolysis to glucose and then fermentation of the glucose to ethanol. It has become clear from this work that improvement of the hydrolysis stage is critical to commercial success.

It is theoretically possible to produce 0,56 g ethanol g^{-1} cellulose. This implies a potential production of 280 l ethanol t^{-1} bagasse. Unacceptably high enzyme concentrations and long reaction times have been needed to produce this theoretical yield. Authors of early publications on process development studies accepted yields of about 0,2 g ethanol g^{-1} cellulose (Spano et al 1980; Wilke et al 1981), only 35 percent of the theoretical maximum.

FACTORS AFFECTING ENZYMIC HYDROLYSIS

The complexity of the cellulase-cellulose system is evident from published work, and numerous parameters which affect the rate and extent of hydrolysis have been identified (Spano et al 1980). These include the following:

- the degree of crystallinity and lignin content of the substrate as determined by its nature and extent of pretreatment;
- the composition and source of the enzyme;
- the temperature and pH of the reaction;
- the initial substrate concentration;
- the enzyme:substrate ratio;
- the degree of agitation;
- the nature and concentration of non-enzymic additives.

The ethanolic fermentation of glucose can also affect the rate and extent of hydrolysis of cellulose because hydrolysis is more successful under conditions where the glucose endproduct is removed by fermentation as soon as it is formed (Takagi et al 1977; Blanco et al 1982; Ghose et al 1984; Ooshima et al 1985).

TEMPERATURE AND pH

The temperature and pH optima for the action of *Trichoderma* cellulase have long been established as 50°C and pH 4,8 respectively (Mandels and Weber 1969). The temperature optimum, however, is time dependent (Nystrom and Andren 1976), shifting from 50°C to 40°C as the reaction time is increased above 48 hours (Purchase et al 1982). This is probably due to thermal inactivation of the enzyme and is significant when considering enzyme recycle. According to Bisset and Sternberg (1978) ß-glucosidase is the most thermostable component of the enzyme complex, having a half life of around 20 hours at 55°C. The most temperature sensitive component is exoglucanase.

Peitersen et al (1977) found that enzyme adsorption was strongly temperature dependent, decreasing with increasing temperature between 20°C and 50°C. Lee et al (1982), however, noticed no significant difference between the adsorption at 50°C and that at 4°C.

AGITATION

Agitation is necessary to ensure good contact between the enzyme and its substrate and to remove hydrolysis products from the reaction sites. However, it has been found that shear forces, particularly those acting at the air-liquid interface, have a detrimental effect on enzyme stability (Kim et al 1982; Mukataka et al 1983).

Mukataka et al (1983) found that mild agitation, even in the presence of an air-liquid interface, was beneficial to the hydrolysis up to an agitator speed of around 200 rpm. Numerous authors (eg Sakata et al 1985) confirmed that agitation was beneficial. This led to investigations of surfactant additives as a means of stabilizing the enzyme during agitation by displacing it from the air-liquid interface (Kim et al 1982).

Turbine impellers were ineffective for the initial mixing of the enzyme into the high viscosity pastes encountered at the start of hydrolysis of milled bagasse (Purchase et al 1985a). A food mixer was used for the preliminary incorporation of enzyme, followed by intermittent agitation (Blackbeard et al 1982) or by no agitation for eight hours, by which time the material was liquid enough for agitation with turbine impellers (Purchase et al 1985a).

SUBSTRATE CONCENTRATION

The hydrolysis of concentrated slurries is economically desirable because it enables smaller reactors to be used and higher endproduct concentrations to be achieved. Milled bagasse could be dewatered by vacuum filtration to a solids content of between 18 percent and 20 percent (Walford et al 1984) and steam exploded bagasse to 30 percent (Perrow et al 1983). These dewatered materials presented severe mixing problems and the concentrations were generally limited to 15 percent for milled bagasse and 20 percent for steam exploded bagasse (Waugh 1986). This limited final ethanol concentrations to 4 percent and 5,3 percent respectively. Dekker and Wallis (1983) used steam exploded bagasse up to concentrations of 40 percent but admitted that on an industrial scale mixing problems would limit concentrations to between 10 and 15 percent.

With milled bagasse, the yield of glucose from the hydrolysis of the cellulose declined with increasing concentration from about 75 percent for a 5 percent slurry to 60 percent for a 14,6 percent slurry (Purchase et al 1985). Similar results were obtained with steam exploded bagasse (Waugh and Proudfoot 1985). As expected the higher substrate concentrations gave higher concentrations of glucose (Figure 15), but this was accompanied by a decrease in reaction rate (or a drop in conversion at a given time) (Figure 16) and a decrease in enzyme efficiency (Figure 17). This decline must, in part, have been due to the increased severity of endproduct inhibition with increased substrate concentration (Spano et al 1980, Dekker and Wallis 1983), but even initial (0-4 hour) rates of reaction were found to be slower at higher substrate concentrations (Figure 18), indicating a drop in enzyme adsorption efficiency with increasing substrate concentration (Lee et al 1982).

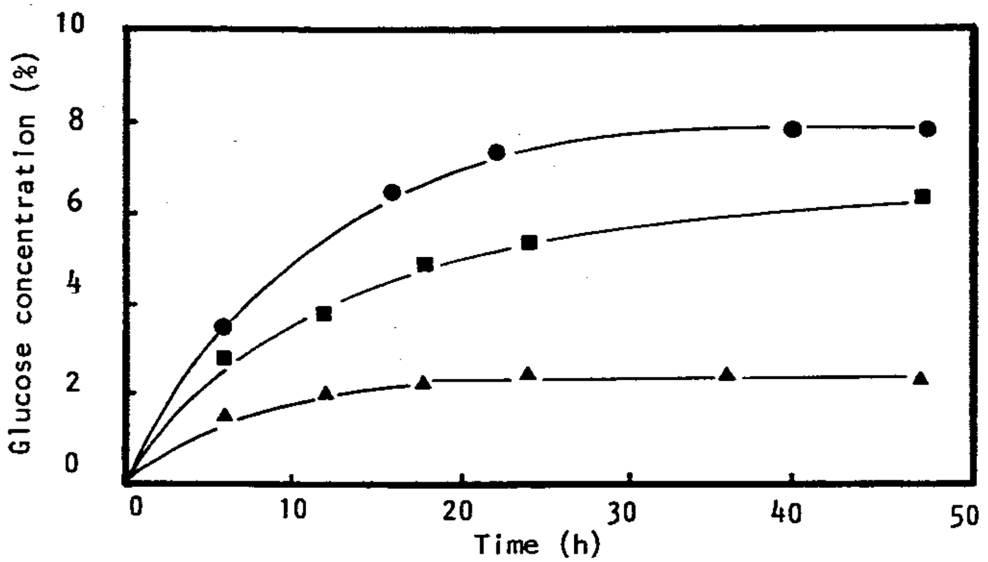

Figure 15. Hydrolysis dynamics of 5 percent (▲), 15 percent (■) and 20 percent (●) steam exploded bagasse with 5 IU cellulase g^{-1} solids.

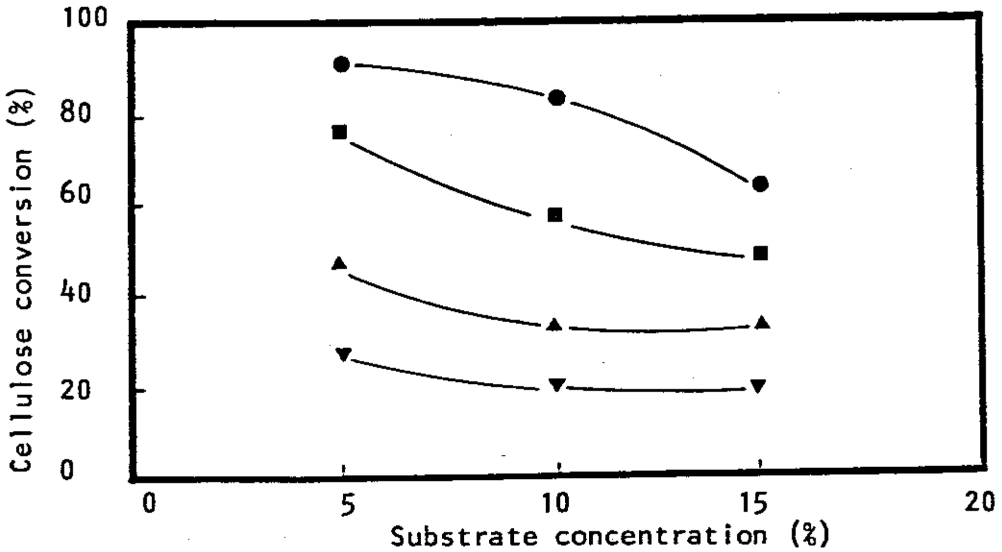

Figure 16. Cellulose conversions at different substrate concentrations and different enzyme loadings (▼: 1,0 IU g^{-1}; ▲: 2,5; ■: 5,0; ●: 10,0).

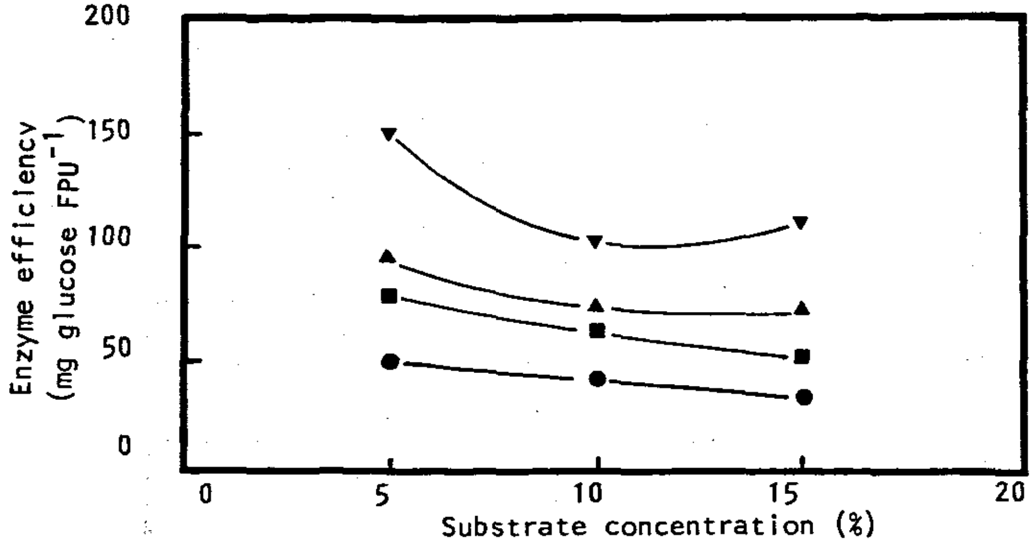

Figure 17. Enzyme efficiencies at different substrate concentrations and enzyme loadings (▼: 1,0 IU g^{-1}; ▲: 2,5; ■: 5,0; ●: 10,0).

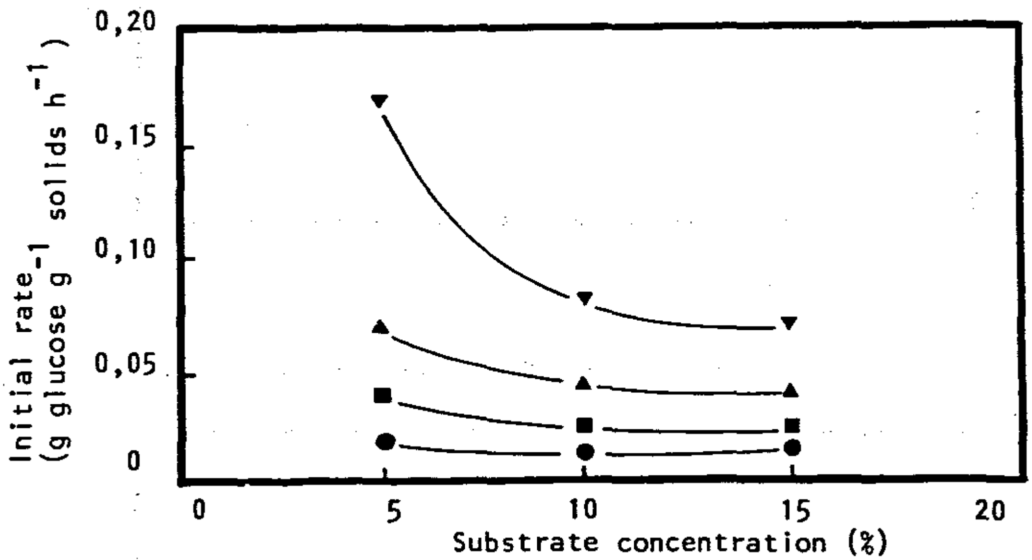

Figure 18. Initial hydrolysis rates at different substrate concentrations and enzyme loadings (▼: 10,0 IU g^{-1}; ▲: 5,0; ■: 2,5; ●: 1,0).

It is significant that the negative trends shown in Figures 16-18 were less severe at the lower enzyme loadings and at the higher solids concentrations. No differences in conversion or enzyme efficiency were noticed in hydrolyses starting with 15 or 20 percent solids (Waugh and Proudfoot 1985).

The highest glucose concentration produced in batch hydrolysis was 8 percent from 20 percent steam exploded bagasse in 36 hours with an enzyme:substrate ratio of 5 IU g^{-1} solids. This represents an average volumetric productivity of 2,2 g glucose l^{-1} h^{-1} (or 11 mg glucose g^{-1} solids h^{-1}) and an enzyme efficiency of 80 mg glucose IU^{-1} (Waugh and Proudfoot 1985). By contrast milled bagasse gave only 5,2 percent glucose in 48 hours, a productivity of 1,1 g glucose l^{-1} h^{-1} (7 mg g^{-1} solids h^{-1}) and an enzyme efficiency of 60 mg glucose IU^{-1} (Waugh 1986). The difference was partly due to the lower initial solids concentration of the milled bagasse (15 percent as opposed to 20 percent for steam exploded bagasse), but also to the poorer reactivity of the milled bagasse.

ENZYME:SUBSTRATE RATIO

The hydrolysis dynamics of steam exploded and milled bagasse were determined at cellulase loadings of 1, 2.5, 5 and 10 IU g^{-1} solids and initial solids concentrations of 5, 10 and 15 percent (Waugh and Proudfoot 1982; 1985). The conversion at any given time increased with increasing enzyme:substrate ratio but in a manner which indicated a significant decline in enzyme efficiency with increasing enzyme loading (Figures 19 and 20). Using average reaction rates, a linear relationship was found to exist between percent conversion of cellulose to glucose (Figure 21) and log enzyme:substrate ratio, following the empirical relationship determined by Reese and Mandels (1971) and mentioned also by Dekker and Wallis (1983). The initial rate of reaction, however, followed Michaelis-Menten type kinetics within the range of enzyme:substrate ratios tested (Figure 22) since the initial hydrolysis rate was proportional to the enzyme concentration.

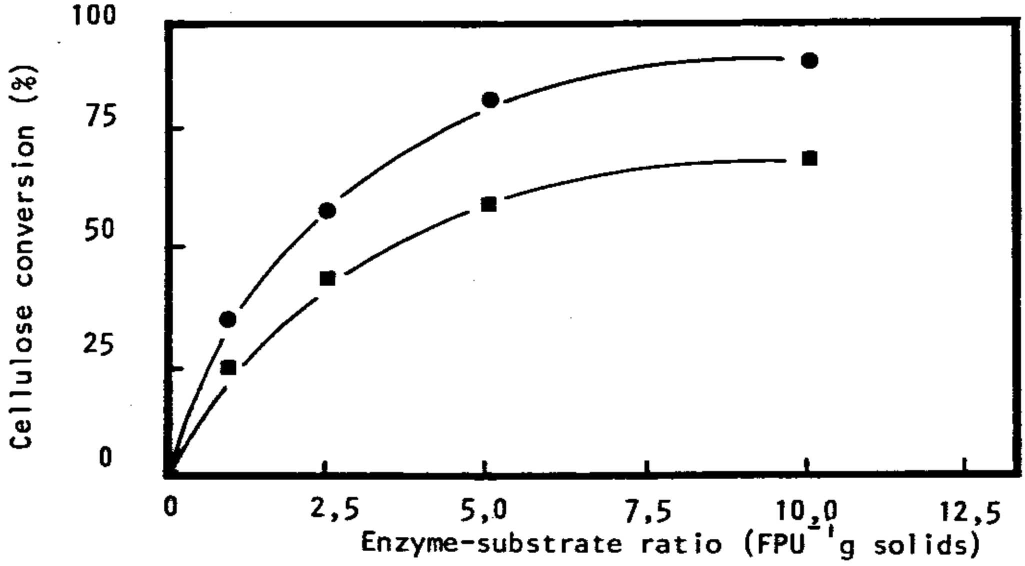

Figure 19. Cellulose conversions at different enzyme loadings and substrate concentrations (●: 5% solids; ■ : 15%).

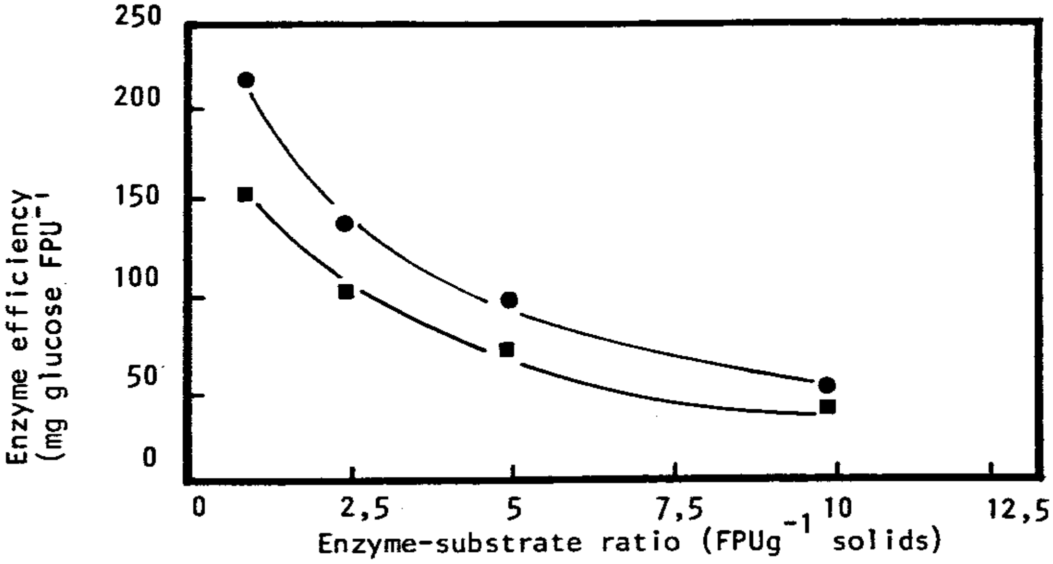

Figure 20. Enzyme efficiencies at different enzyme loadings and substrate concentrations (●: 5% steam exploded bagasse; ■ : 15%).

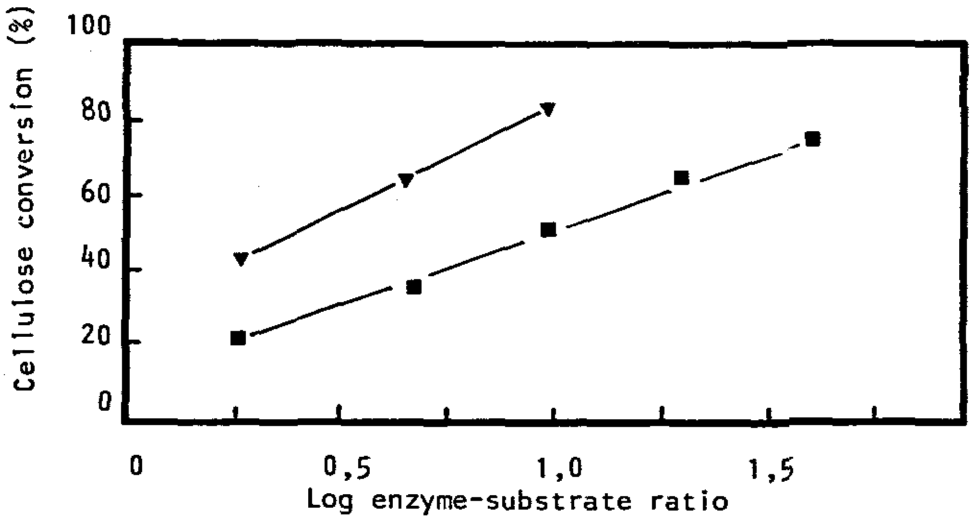

Figure 21. The relationship between cellulose conversion and log enzyme- substrate ratio for 5 percent milled bagasse (■) and 5 percent steam exploded bagasse (▼).

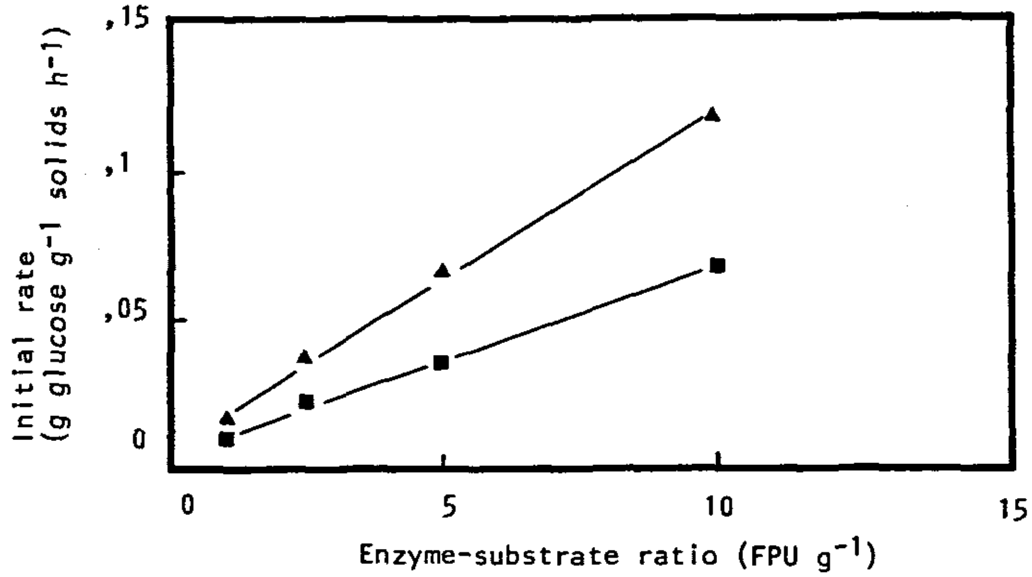

Figure 22. The effect of enzyme:substrate ratio on initial hydrolysis rate for 5 percent (▲) and 15 percent (■) steam exploded bagasse.

Use of a very broad range of enzyme:substrate ratios are recorded in the literature, from 2.5 IU g^{-1} solids (Blanco et al 1982) to 212 IU g^{-1} cellulose (Vallander and Eriksson 1985). The optimum enzyme loading has not been clearly defined. Addition of enough enzyme to saturate the cellulose substrate should result in the maximum hydrolysis rate, but, due to the high cost of enzyme, economic factors may dictate the use of subsaturated enzyme loadings.

Watson and Carstens (1985) showed that the binding of cellulase on Avicel cellulose followed a Langmuir type adsorption isotherm with a saturation value of 36 mg protein g^{-1} cellulose (40 IU enzyme g^{-1} cellulose) with almost 100 percent adsorption efficiency below saturation. In work on a variety of pure cellulose substrates (Lee et al 1982) the measured saturation values were between 10 and 25 IU g^{-1} cellulose. The linear relationship found between initial hydrolysis rate and enzyme loading (Figure 22) shows that the cellulose in steam exploded bagasse is not saturated at enzyme:substrate ratios below 10 IU g^{-1} (Waugh 1986).

For bagasse, the most efficient enzyme usage, in terms of glucose produced per unit of enzyme added to the system, occurred at the lowest enzyme:substrate ratios tested. Enzyme efficiencies in excess of 100 mg glucose IU^{-1} were achieved using steam exploded bagasse at enzyme:substrate ratios less than 2,5 IU g^{-1}. Cellulose conversions under these conditions, however, were less than 50 percent in 48 hours on 15 percent solids material. A good compromise between enzyme efficiency and extent of cellulose hydrolysis was achieved at an enzyme:substrate ratio of 5 IU g^{-1} solids (Waugh 1986).

ß-GLUCOSIDASE SUPPLEMENTATION

In the enzymic hydrolysis of cellulose, glucose causes feedback inhibition of the ß-glucosidase and this in turn leads to a buildup of cellobiose which strongly inhibits the action of the exo- and endo-glucanases. This slows down further hydrolysis. The inhibition can be alleviated to a great extent by supplementing the cellulase with extra ß-glucosidase (Sternberg et al 1977; Mandels 1981). Several commercial preparations of ß-glucosidase are available.

The amount of ß-glucosidase needed to eliminate inhibition by cellobiose varies with substrate and with a change in enzyme:substrate ratio (Mandels 1981). Steam exploded bagasse required more ß-glucosidase than milled bagasse (Perrow et al 1983). This could be related to the observation that approximately 25 percent more ß-glucosidase was adsorbed onto steam exploded wheat straw than onto other substrates (Desphande and Eriksson 1984). This binding may reduce the effectiveness of the ß-glucosidase.

The effect of ß-glucosidase supplementation on the batch hydrolysis of steam exploded bagasse is shown in Figures 23 and 24 (Waugh 1986). The amount required for maximum hydrolysis rate was found to be between 1 and 1.5 IU ß-glucosidase IU^{-1} cellulase, with the higher ratio being necessary to prevent completely the accumulation of cellobiose. Mandels et al (1980) gave the optimum ratio as 1.5, Dekker and Wallis (1983) as 1.25 and Grous et al (1986) as 0,94. Depending on the cost of ß-glucosidase, the optimum cost effective amount may be less than that required for maximum rate of hydrolysis.

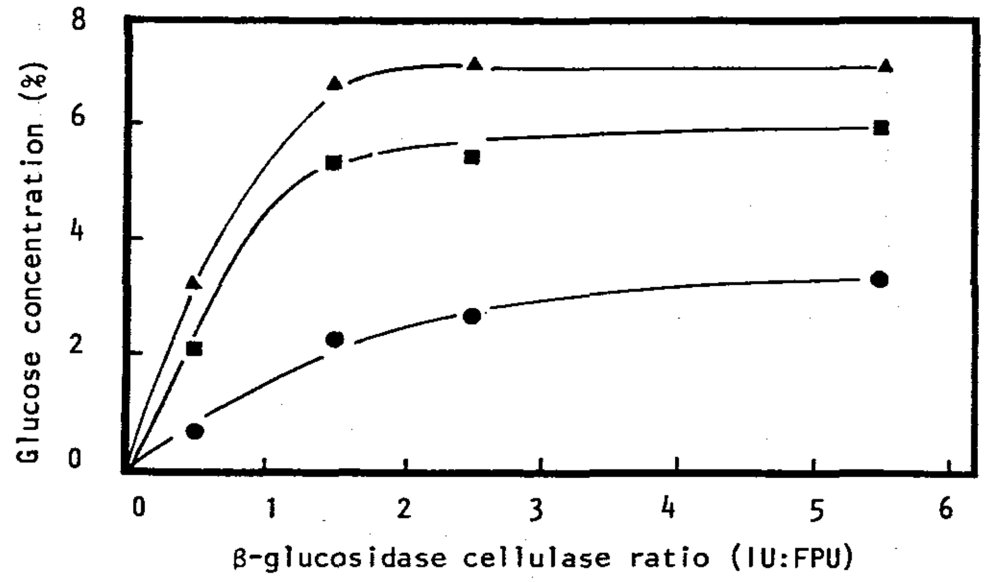

Figure 23. Glucose production from 15 percent steam exploded bagasse with 5 IU g^{-1} solids and various ß-glucosidase:cellulase ratios (▲: after 48h; ■ : 24h; ●: 6h).

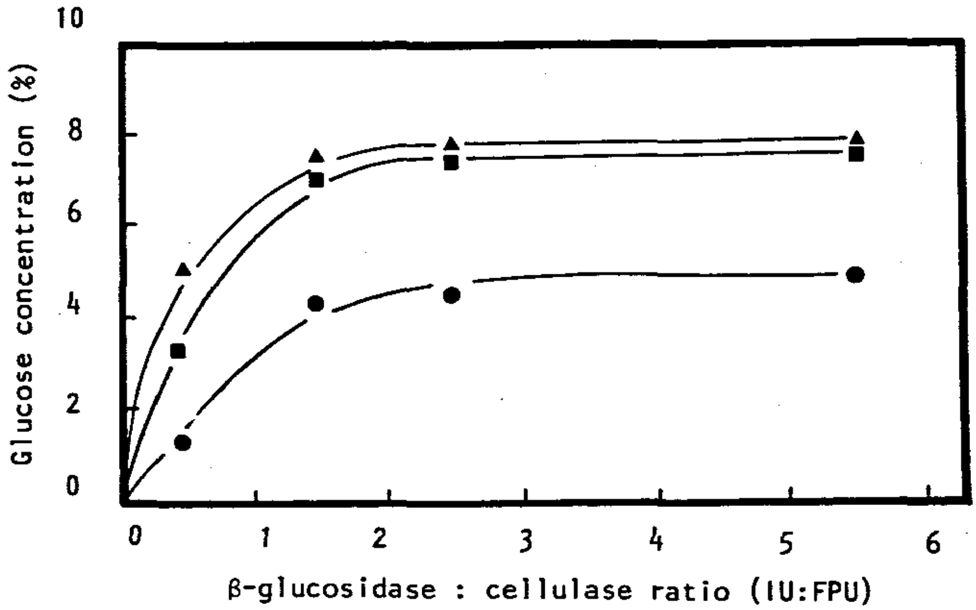

Figure 24. Glucose production from 15 percent steam exploded bagasse with 10 IU g^{-1} solids and various ß-glucosidase:cellulase ratios (▲: after 48h; ■: 24h; ●: 6h).

NON-ENZYMIC ADDITIVES

A range of additives has been tested in an effort to improve hydrolysis rate, increase enzyme stability and aid enzyme recovery (Kim et al 1982; Reese and Mandels 1980). These additives can be divided into four classes:

- proteins, added to increase the bulk protein concentration in order to reduce the extent of non-specific enzyme binding and reduce the concentration of enzyme at the air-liquid interface;
- surfactants, added to reduce the detrimental effects of shear at the air-liquid interface;
- lignin complexing agents, added to reduce non-specific enzyme binding;
- biocides and antibiotics, added to prevent glucose loss by microbial contamination.

The effects of the additions of urea, peptone, tryptone, triton X-100, polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) were investigated (Purchase et al 1982; Walford et al 1984). The greatest improvements in hydrolysis were provided by PVP and high molecular weight PEG (PEG 20 000). Triton X-100 also improved hydrolysis. Of the proteins, peptone proved most effective but at higher concentrations than the other compounds mentioned.

The most effective additive was PEG (Walford et al 1984); probably because it has surfactant properties and the ability to complex with phenols, thereby reducing the irreversible binding between lignin and enzyme. As shown in Figure 25, its effect increased with increasing concentration up to 1 percent on solids when tested on 5 percent milled bagasse, improving conversion by between 5 and 20 percent in 48 hours, depending on the enzyme loading employed (Purchase et al 1985b). This represents a return of between 3,5 and 12 mg glucose mg^{-1} PEG. When tested with a higher concentration (15 percent) of milled bagasse solids, the benefit was only 0,6 - 3 mg glucose mg^{-1} PEG (Purchase et al 1985b) whereas with 15 percent steam exploded bagasse it had a marked beneficial effect (Figure 26) (Waugh and Proudfoot 1985). It was also beneficial to the simultaneous hydrolysis and fermentation of steam exploded bagasse, causing a 25 percent increase in ethanol production when added at 1 percent on initial solids (Waugh and Proudfoot 1985).

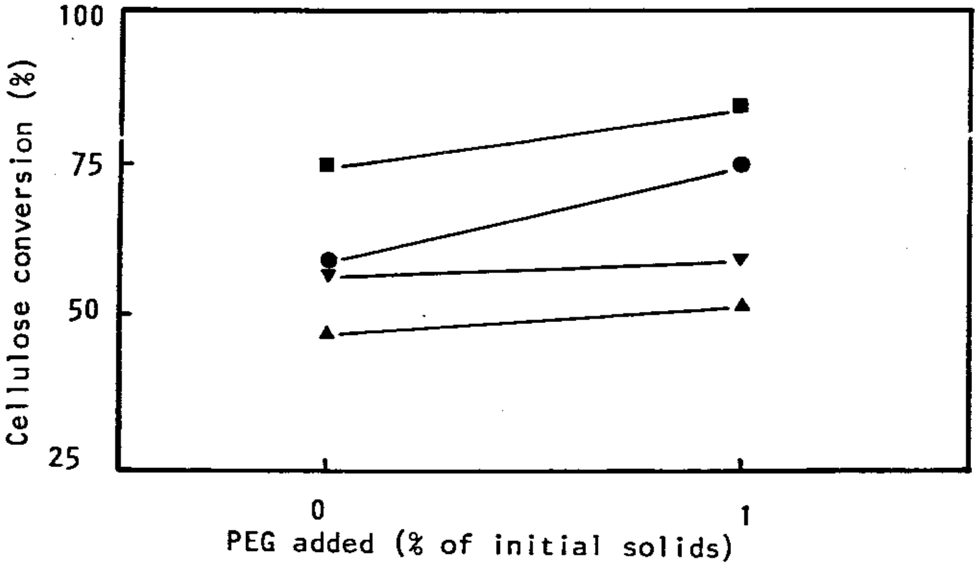

Figure 25. The effect of PEG on 5 percent milled bagasse at 5 IU g^{-1}(●) and 10 IU g^{-1} (■) and on 15 percent milled bagasse at 5 IU g^{-1} (▲) and 10 IU g^{-1} (▼).

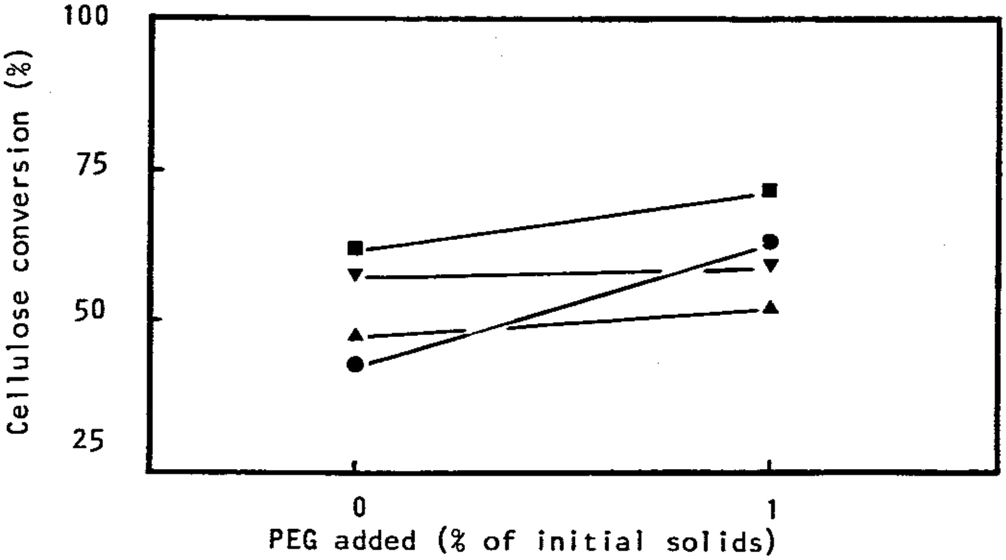

Figure 26. The effect of PEG on 15 percent milled bagasse at 5 IU g^{-1}(▲) and 10 IU g^{-1} (▼) and on 15 percent steam exploded bagasse at 5 IU g^{-1} (●) and 10 IU g^{-1} (■).

ETHANOL ACCUMULATION

The development of a simultaneous hydrolysis and fermentation system has enabled glucose accumulation to be prevented. Ethanol, however, has also been identified as an inhibitor of the cellulase enzymes, although to a much lesser extent than the sugars (Takagi et al 1977; Ghosh et al 1982). Ethanol is thought to decrease the extent of exoglucanase adsorption (Ooshima et al 1985).

The hydrolysis rate was found to decrease linearly with increasing concentrations of glucose and ethanol (Waugh and Purchase 1987). The levels of glucose and ethanol causing a 50 percent reduction in rate were found to be 5,4 percent and 14,4 percent respectively, confirming and quantifying the comparatively small effect of ethanol.

ENZYME RECOVERY AND RECYCLE

The major barrier to the commercial viability of enzymic cellulose hydrolysis is the high cost of the cellulase enzyme (Nystrom and Andren 1976). Wilke et al (1981) and Desphande and Eriksson (1984) attributed between 50 and 60 percent of plant operating costs to enzyme production. Desphande and Eriksson (1984) claimed the need for a 90 to 95 percent recovery and reuse of enzyme to make enzymic hydrolysis competitive with acid hydrolysis.

It is theoretically possible to make a substantial reduction in enzyme consumption by recycling the enzyme. Studies on enzyme recycle, however, have proved disappointing, indicating at best the reuse of between 50 and 60 percent of the initial enzyme charge (Wilke et al 1981 and Desphande and Eriksson 1984). The longterm feasibility of recycling cellulase remains unknown.

The amount of enzyme which can be recycled is dependent on cellulase adsorption-desorption patterns, enzyme stability and the extent of non-productive irreversible complexing. Two different enzyme adsorption patterns have been noted. Castanon and Wilke (1980) observed a continuous depletion of free enzyme from solution during hydrolysis. Most other workers, however, have noted a strong initial adsorption followed by release into solution during reaction (Purchase et al 1985; Vallander and Eriksson 1985; Stutzenberger and Lintz 1986). Lee et al (1982) attributed the difference to the availability of adsorption sites and suggested that a well pretreated material should follow the latter pattern.

Purchase et al (1985) studied enzyme distribution in a 5 percent slurry of milled bagasse after 4, 8 and 24 hours of hydrolysis. The slurry was centrifuged to give a high solids component (pellet) and a supernatant component. Each was tested for enzyme activity by adding fresh milled bagasse and monitoring glucose production. After 24 hours, the total enzyme activity had declined to 62 percent of its value at 4 hours and the activity associated with the supernatant had increased in absolute terms. This clearly indicated desorption of enzyme from the pellet. The drop in total activity was probably due to temperature and sheer inactivation. It is also likely that there is a disruption in reaction synergism due to changes in exo- and endo-glucanase ratios with time (Mandels et al 1981).

Nystrom and Andren (1976) found it virtually impossible to remove freshly bound enzyme from cellulose. Raising the pH to neutrality assisted the removal (Sinitsyn et al 1983). Desphande and Eriksson (1984) were able to remove between 30 and 40 percent of bound enzyme by elution with a phosphate buffer.

The difficulty in achieving economical enzyme recovery caused Waugh and Proudfoot (1985) to investigate the alternative of using low enzyme loadings without provision for recycle. This approach proved promising and an enzyme loading of 5 IU g^{-1} bagasse was adopted as a reasonable compromise between enzyme efficiency and reaction rate.

FERMENTATION OF HYDROLYSATES

YEAST SELECTION

Eighteen yeasts were screened for their ability to ferment the glucose in a typical bagasse hydrolysate containing 5 percent glucose. With simultaneous hydrolysis and fermentation in mind, 37°C was chosen as the screening temperature. The strain *Saccharomyces cerevisiae* CSIR Y718 performed best with an average productivity of 3 g ethanol l^{-1} h^{-1} and a conversion efficiency of 0,42 ethanol g^{-1} glucose.

FERMENTABILITY OF HYDROLYSATES

Milled bagasse

The fermentation of unsupplemented, milled bagasse hydrolysates was characterised by a low conversion efficiency of the glucose to ethanol. Nutrient supplementation in the form of yeast extract (1 percent) improved ethanol production in an eight hour fermentation by 115 percent and the ethanol yield coefficient increased from 0,42 to 0,47 g ethanol g^{-1} glucose. Addition of calcium and magnesium made no improvements to the fermentation (Blackbeard et al 1982).

Steam exploded bagasse

The fermentation of untreated steam exploded bagasse hydrolysates was less successful than that of milled bagasse hydrolysates (Waugh and Proudfoot 1985). The steaming treatment is known to produce potential inhibitors such as furfural, hydroxymethylfurfural, acetic acid and soluble lignin derivatives (Chung and Lee 1985). Acetic acid was suspected to be the major inhibitor, but added on its own to thoroughly washed steam exploded bagasse at concentrations between 0,1 and 0,5 percent, it caused only mild inhibition (Waugh and Proudfoot 1985). Acetic acid added to the hydrolysate of unwashed steam exploded bagasse caused severe inhibition which indicated a combined effect of acetic acid and other inhibitors present.

The inhibitors in the steam exploded bagasse could be removed simply by washing with a minimum amount of water (2 l kg^{-1} dry solids). Further improvements in the fermentation were achieved by nutrient supplementation although the nutrients alone were not able to counter the effect of the inhibitors (Waugh and Proudfoot 1985).

SIMULTANEOUS HYDROLYSIS AND FERMENTATION

The removal of glucose by its simultaneous fermentation to ethanol during hydrolysis has been demonstrated to be a simple yet effective solution to the problem of glucose inhibition (Takagi et al 1977). The two reactions can run simultaneously in a single vessel using *Trichoderma* cellulase and an ethanol producing microorganism. The process has been demonstrated to be feasible for a variety of *Saccharomyces* spp (Takagi et al 1977; Blotkamp et al 1978), *Zymomonas spp* (Viikari et al 1980) and *Kluyveromyces fragilis* (Mansoor 1982).

Because of the poor thermotolerance of these microorganisms, the optimum temperature for simultaneous reaction is between 35 and 40°C, well below the optimum for hydrolysis (Takagi et al 1977). A comparison between simultaneous reaction at 35°C and simple hydrolysis at 50°C showed the simultaneous reaction to be the faster particularly when a high initial substrate concentration (20 percent) was employed (Waugh 1986). At this concentration, the inhibition was such that only 8 percent glucose was produced (67 percent conversion) in simple hydrolysis whereas the equivalent of 11 percent glucose (5 percent ethanol) was produced when simultanous fermentation was involved. This represents a 92 percent conversion of the initial cellulose. Another advantage of the simultaneous system is that it obviates the need for a further fermentation step.

The reaction rate in simultaneous hydrolysis and fermentation is controlled by the rate of hydrolysis, making the overall reaction rate independent of inoculum size (Takagi et al 1977).

Ghosh et al (1982) showed that the requirement for ß-glucosidase during simultaneous hydrolysis and fermentation reaction is reduced in comparison with simple hydrolysis. This was not the case, however, with steam exploded bagasse (Waugh 1986).

Since ethanol itself inhibits hydrolysis (Takagi et al 1977; Ghosh et al 1982; Waugh 1986), Ghose et al (1984) have proposed that the simultaneous reaction should be run in fed-batch operation with periodic ethanol removal under vacuum. They showed that ethanol could be produced at a rate of 4 g l^{-1} h^{-1}. This compared with a productivity of 1,25 g l^{-1} h^{-1} under batch operation without ethanol removal.

An alternative approach to simultaneous hydrolysis and fermentation in one vessel is the coupling of the two reactions in separate vessels so that each can operate at its optimum temperature, with continuous circulation of hydrolysate between them (Blanco et al 1982). This is technically a more complex process, however, as well as being more energy intensive and requires continuous heating and cooling of the circulating hydrolysate and continuous solid-liquid separation. In comparison with the single vessel system, a higher residual glucose concentration must be maintained in the hydrolysis vessel in order to provide an adequate feed to the coupled fermenter. This means that glucose inhibition cannot be eliminated from such a system.

SUMMARY AND CONCLUSIONS

The effects of various parameters on the enzymic hydrolysis of bagasse have been investigated so that the optimum conditions for hydrolysis can be defined. Steam exploded bagasse from a furfural factory proved more suitable than attritor milled bagasse as a substrate for hydrolysis. The former could be used at an initial solids concentration of 20 percent whereas the latter was unmanageable above a concentration of 15 percent. The steam exploded bagasse was also more reactive, giving 10-20 percent higher absolute cellulose conversion yields than the milled bagasse under comparable conditions.

The conditions for the most economic hydrolysis of the steam exploded bagasse were:

- prewashing of the bagasse with water, adjusting its pH to 4,8 and then dewatering to a solids concentration of 20 percent;
- adding polyethylene glycol at a concentration of 0,5 percent on solids.
- adding cellulase enzyme at a concentration equivalent to 5 IU g^{-1} solids and adding ß-glucosidase to give a cellulase IU:ß-glucosidase IU ratio of 1:1;
- inoculating with Saccharomyces cerevisiae Y718 and blending all the components with a dough mixer before incubating at 35°C;
- initiating mild agitation when the mixture is liquid enough to stir, after about eight hours.

Under these conditions it was possible to produce 5 percent ethanol in 48 hours; this represents a cellulose conversion of 92 percent and an enzyme efficiency of 107 mg glucose IU^{-1}.

An assessment of the profitability of this process (Chapter 7) showed that the process would be reasonably profitable if the ethanol could be sold for 60c l^{-1}.

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Italics indicate work carried out in the programme.

CHAPTER 6. SINGLE CELL PROTEIN PRODUCTION FROM BAGASSE HYDROLYSATES

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INTRODUCTION

The term "single cell protein" refers to cells of microorganisms such as yeasts, moulds, bacteria, actinomycetes and algae cultivated on a large scale for use as protein sources in animal feeds or in human food products (Goldberg 1985). Yeasts have been associated with human nutrition (bread and alcoholic beverages) for many centuries and of all the microbial groups yeasts are the most acceptable from a psychological as well as a nutritional and safety viewpoint. *Candida utilis* is a well known pentose utilizing yeast and thousands of tons of this yeast were produced in Germany during both world wars for use in human foods as a meat substitute and extender (Goldberg 1985). Single cell protein production from various substrates has been investigated extensively and several recent reviews on the subject have been published (Rolz 1982; Litchfield 1983; Rosales 1984; Tusé 1984; Goldberg 1985).

A comparative techno-economic study carried out within the programme (Kamper et al 1983) identified single cell protein, like ethanol, as a product with a market potential in South Africa . During the last three years of the programme, research which concentrated on the use of *C utilis* was undertaken at the University of the Orange Free State and at the Sugar Milling Research Institute.

UTILIZATION OF HEMICELLULOSE HYDROLYSATE

Because the hemicellulose hydrolysate contained up to 13 g l^{-1} acetic acid, it was preferable that the yeast used for single cell protein production utilized acetic acid as well as xylose. A screening programme (Holder 1987) identified seven yeast strains capable of growing on both substrates and three which utililized acetate, but not xylose. Four isolates gave yield coefficients of 0,4 to 0,48 on xylose in a synthetic medium. The biomass yield of *Candida utilis* strains on xylose was 0,3 to 0,36 g cells g^{-1} xylose, and 0,4 to 0,46 on acetic acid. The higher yield on acetic acid was surprising. Rychtera et al (1977) reported a corresponding cell yield coefficient of 0,36 on acetate, whereas the yield of *C tropicalis* on xylose was low (0,38).

The cultivation of *C utilis* in the hemicellulose hydrolysate, with added nutrients and neutralized as described in Chapter 2, resulted in a final cell concentration of 13 g l^{-1} (dry mass) after 50 - 60 h (Holder 1987). The xylose (40 g l^{-1}), glucose (2,5 g l^{-1}) and acetic acid (13 g l^{-1}) components of the hydrolysate were utilized simultaneously. About half of the initial 5 g l^{-1} L-arabinose was also utilized. The cell yield coefficient based on the total assimilated mass of the latter four substrates was only 0,22 with a cell concentration of 13 g l^{-1}. A higher cell concentration of 19,5 g l^{-1} was obtained by the cultivation of an isolate identified as *Geotrichum candidum*. The cultivation time increased to 100 h, however. The use of a coculture for single cell protein production offered no advantage over a mono culture of *C utilis*.

The growth kinetics of *C utilis* in a synthetic medium simulating the hemicellulose hydrolysate were not significantly different, indicating that inhibitory factors other than acetic acid were absent in the hydrolysate (Holder 1987).

The low *C utilis* yields obtained on xylose and especially on the hemicellulose hydrolysate were disappointing. Chahal (1984) reported cell yields considerably higher than the theoretical maximum for the cultivation of the moulds *Trichoderma reesei* and *Chaetomium cellulolyticum* and the edible mushroom *Pleurotus sajor-caju* on wheat straw hemicellulose hydrolysate. These high yields were attributed to the utilization of reducing compounds other than sugars for the synthesis of mycelial biomass. The microbial biomass concentration only reached 5-7 g l^{-1} (dry mass), however, due to the low sugar concentrations (about 10 g l^{-1} reducing sugars) in the hydrolysate with a rather long cultivation time of 23-96 h, depending on the fungal species used.

The inhibitory effect of monocarboxylic acids on yeast growth is well documented. This toxic effect is pH dependent (Shennan and Levi 1974; Cama and Edwards 1970), because a low pH favours permeation of the free acid, which is more lipid soluble than the ionized form, into the cell and thereby enhances its toxicity (Pirt 1975). The action of carboxylic acids may be to uncouple the metabolism in a similar manner as unsaturated acids (Slater 1963), and Sestakova (1979) noted that a high concentration of acetic acid (3-6 g l^{-1}) caused a decrease in the cell yield of *C utilis*.

In subsequent experiments the yield (per g substrate utilized, sugars plus acetic acid) and final cell concentration of *C utilis* were increased to 0,38 and 29,7 g l^{-1} (dry mass), respectively, by using a fed-batch cultivation procedure to minimize acetic acid toxicity as well as by using a hemicellulose hydrolysate prepared at 120°C in 0,5 percent (m v^{-1}) H_2SO_4 containing 62 g l^{-1} xylose, 2 g l^{-1} glucose, 4 g l^{-1} L-arabinose and 10 g l^{-1} acetic acid (Purchase and Proudfoot 1987). This markedly higher yield was achieved only in hydrolysates which had not been recycled through a second load of bagasse (to obtain a higher xylose and inadvertently also a higher acetic acid concentration) and which had not been autoclaved after pH adjustment. The use of recycled hydrolysate prolonged the initial lag period of the cultivation and also resulted in cell yields as low as 0,24. It was noted that autoclaving neutralized hydrolysate caused xylose degradation and also a decrease in the cell yield per unit of substrate utilized, which suggested that toxic compounds were formed during heat treatment (Purchase and Proudfoot 1987).

ATP determinations proved unreliable as a rapid method of monitoring biomass production in the turbid hydrolysate due to fluctuations in the intracellular ATP content (Holder 1987).

UTILIZATION OF CELLULOSE HYDROLYSATE

With a cellulose hydrolysate containing 40 g l^{-1} glucose, a cell concentration of 15 g l^{-1} was obtained with *C utilis* after a 12 h cultivation period resulting in a yield coefficient of 0,38. The latter value was considerably lower than the theoretical maximum yield of 0,51 g cells g^{-1} glucose. Again no inhibitory factors in the hydrolysate were evident.

UTILIZATION OF COMBINED CELLULOSE AND HEMICELLULOSE HYDROLYSATES

The cultivation of the yeasts in an equal volume mixture of the C5 and C6 hydrolysates resulted in respective cell yield coefficients (g dry biomass g^{-1} substrate utilized, sugars plus acetic acid) of 0,36 (16,5 g l^{-1} dry cell mass) and 0,4 (19,5 g l^{-1} dry cell mass) with *C utilis* and *G candidum* after 25 h. A contributory factor to the shorter cultivation time was undoubtedly the lower xylose concentration in the hydrolysate mixture. The crude protein content of these two yeasts was similar (46 percent and 48 percent respectively) so that *G candidum* produced 9 g l^{-1} crude protein as opposed to 7,9 g l^{-1} obtained with *C utilis* (Holder 1987).

G candidum therefore compared favourably with *C utilis* as far as yield and rate of protein production from a C5/C6 hydrolysate mixture were concerned. With a pure C5 hydrolysate the higher cell yield was offset by the long cultivation time. Harvesting of *G candidum* may prove easier and thus cheaper than with *C utilis*: Its large arthrospores sedimented quite rapidly, whereas its mycelium tended to remain in the top layer of the culture where it could probably be skimmed off. One drawback at this stage is that *G candidum* is not a recognized food or feed yeast.

PROCESS PARAMETERS

The parameters given in Table 15 represent the best conditions for biomass production from the bagasse hydrolysates as deduced from these preliminary results. It is not yet clear whether single cell protein production from the hemicellulose hydrolysate alone or from the combined C5 and C6 hydrolysates has the best potential for commercial realization.

Table 15. Process parameters for biomass production from bagasse hydrolysates.

Parameter	Hydrolysate			
	C5	C6	C5 + C6	
Yeast	*Candida utilis*[a]	*Candida utilis*[b]	*Candida utilis*[b]	*Geotrichum candidum*[b]
Cultivation conditions				
procedure	fed-batch	batch	batch	batch
aeration	aerobic	aerobic	aerobic	aerobic
pH	7,5	6,0	6,0	6,0
temperature	30°C	30°C	30°C	30°C
Cultivation time	48 h	12 h	25 h	25 h
Biomass production				
yield, g g^{-1} substrate	0,38	0,38	0,36	0,4
cell concentration g l^{-1}	29,7	15,0	16,5	19,5
crude protein content (Nx6,25) (percentage)	60	48	46	48

[a] Purchase and Proudfoot 1987
[b] Holder 1987

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Italics indicate work carried out in the programme.

CHAPTER 7. PROCESS SUMMARIES AND COST ESTIMATES

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INTRODUCTION

This summary is written for the commercial reader rather than the technical reader. It provides an overview of the state of the art, including abridged technical details and an indication of the profit potential of the most promising processes.

BAGASSE PRODUCTION AND SURPLUS

Bagasse is the fibrous residue which remains after sugarcane has been crushed. In South Africa approximately 3×10^6 t (dry mass) are produced annually by a total of 16 factories located mainly along the Natal coast. The major use of the bagasse is as a boiler fuel for the sugar factories.

It is not easy to specify how much surplus bagasse could be generated because this depends on the type of equipment installed at the factories and on whether sugar refineries are attached to the factories. Surplus bagasse can be generated, but at the expense of installing energy efficient equipment such as high pressure boilers. The newest South African factory (Felixton) was designed for high thermal economy because an associated paper factory created a demand for bagasse. Ultimately this factory could operate with a bagasse surplus of 45 percent, but this will be achieved only with the installation of expensive vapour recompression equipment.

If there was an economic incentive it would be possible to increase surplus bagasse by delivering the cane without prior removal of tops and trash. This would increase the quantity of bagasse by at least 15 percent.

At present the value of bagasse is equivalent to its coal replacement cost. One tonne of coal replaces approximately 3,8 t of wet bagasse (50 percent moisture) and so the coal replacement value of bagasse is approximately R25 t^{-1} (dry) in the Durban area.

EXISTING USES IN BYPRODUCTS

Approximately 9 percent of bagasse is currently used for purposes other than boiler fuel. About 75 percent of this (ie 190 000t) is used for paper making. The remainder is used for producing furfural, animal feed and chipboard, and a small but unknown quantity is used for generating electricity for irrigation.

PHYSICAL AND CHEMICAL PROPERTIES

The cellular composition of bagasse is shown in Table 16.

Unbaled bagasse is expensive to transport because of its low bulk density. On a dry mass basis the bulk density varies between 80 and 180 kg m^{-3} depending on compression. When piled freely in a railcar it has a dry bulk density of 97 kg m^{-3} which can be increased to 148 kg m^{-3} by careful packing and compression. The bulk density of baled bagasse is about 300 kg m^{-3}.

Table 16. The cellular components of bagasse.

Cellular component	Mass percent	Length (mm)	Length/ width
True fibre (rind and vascular tissue)	55	1,5	70
Vessel segments	20	1,0	9
Pith (parenchyma cells)	20	0,3	5

The chemical compositions of the pith and fibre are almost identical.

The major chemical components of dry bagasse are shown in Table 17.

Table 17. The major chemical components of bagasse.

Component	(percent)
Cellulose	38
Hemicellulose	33
Lignin	22
Ash	3
Fresh bagasse contains 46-52 moisture	

The hemicellulose component can be selectively hydrolyzed with dilute acid to yield a xylose rich process stream containing some acetic acid, glucose and arabinose.

The cellulose can be hydrolyzed enzymatically to yield a glucose rich stream, but acceptable yields are achieved only if the bagasse is pretreated in some way to make the cellulose more accessible to the enzyme.

The lignin is chemically more reactive than that from wood species and could therefore be particularly well suited for use as raw material in adhesives manufacture.

RECENT RESEARCH ON ALTERNATIVE USES FOR BAGASSE

The major thrust of recent research on bagasse in South Africa has been aimed at hydrolysing the bagasse to produce fermentable sugars. This has involved the development of procedures for the following:

- selective acid hydrolysis of the hemicellulose component;
- detoxification of the resulting xylose rich solution and its bioconversion to chemicals or single cell protein;

- production of cellulase enzymes for hydrolysis of the cellulose rich residue which remains after acid hydrolysis;
- pretreatment, enzymic hydrolysis and bioconversion of the cellulose rich residue which remains after acid hydrolysis.

These topics are the subjects of previous chapters.

PROCESS ROUTES

Some of the endproducts which have been considered include ethanol, single cell protein, animal feed and lignin. The various process routes involved are summarized in Figure 27 and are described in the following sections.

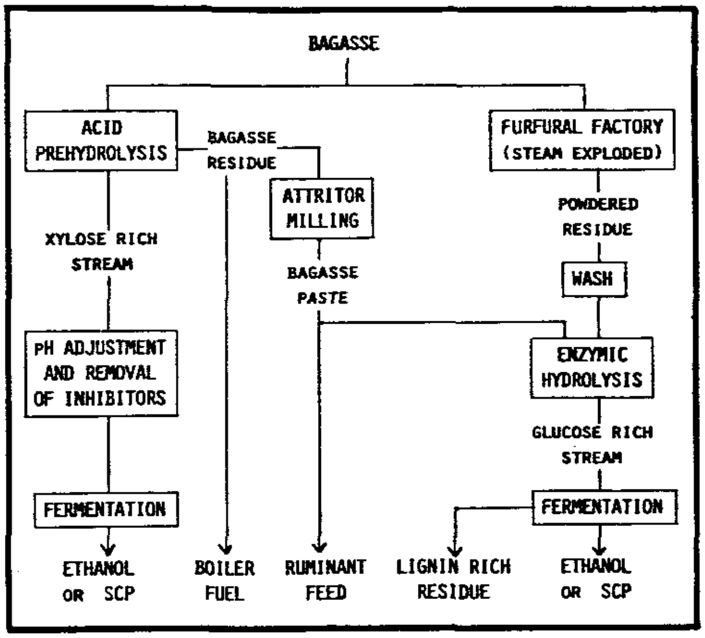

Figure 27. Processes for converting bagasse to various products.

Acid hydrolysis of hemicellulose (prehydrolysis)

The hemicellulose is more susceptible to hydrolysis than is cellulose. It can be hydrolyzed by using various combinations of acid concentration, temperature and time, but a practical combination is 0,5 percent H_2SO_4 (M V^{-1}) at 120°C for 3,5 hours. Higher acid concentrations reduce the required heating time, but create problems of high acid costs and high volumes of $CaSO_4$ in the neutralized solutions. Temperatures below 100°C are impractical because of the long heating times required with 0,5 percent acid. Above 120°C the rate of furfural formation increases rapidly and this can cause inhibition of the yeasts used in subsequent bioconversions. The 3,5 hour heating period is necessary to achieve a xylose yield equivalent to 18 percent of the original dry mass of bagasse. Longer times give only very small increases in yield but appreciable increases in furfural accumulation.

The solid residue remaining after acid hydrolysis retains the appearance of bagasse and can be dewatered and burnt. Its sulphur content is less than that of most coals. Its mass is approximately 66 percent of that of the original bagasse and so it could provide sufficient fuel for a very fuel efficient raw sugar factory.

Bioconversion of the hemicellulose hydrolysate

The xylose in the hydrolysate can be fermented to ethanol by yeasts such as *Pichia stipitis*, but the fermentation is slow and the yeasts are incapable of tolerating final ethanol concentrations above about 30 g l^{-1} (Chapter 2). Furthermore, the acetic acid in the hydrolysate has to be removed prior to fermentation otherwise it is inhibitory.

A simpler process for bioconversion of the hydrolysate involves growing yeast on it under aerobic conditions to produce maximum cell yield for use as single cell protein. Under the aerobic conditions, the acetic acid is not inhibitory, but is used by the yeast as a substrate for growth. Harvesting of the final product is less expensive than recovery of ethanol from dilute (3 percent) solution and the technology and yeast strains are well known. *Candida utilis* grows efficiently on xylose and acetic acid and it is acceptable as a feed yeast. A demand for single cell protein is anticipated in South Africa, particularly for the poultry industry, and so there is a likely market for the product. If all of the bagasse from a medium sized (250 t cane h^{-1}) sugar factory was prehydrolyzed and the hydrolysate used for single cell protein production, the amount produced annually would be about 20 000 t. This could be increased by using molasses as the substrate during the 3-4 month period when cane is not harvested. Mass balances are shown in Figure 28 and cost estimates are presented later in this chapter.

Attritor milling and steam explosion

Attritor milling and steam explosion are pretreatments which make the cellulose in bagasse more susceptible to enzymic hydrolysis. Normally a physical pretreatment such as attritor milling consumes too much energy to be cost effective and this is true when applied to whole bagasse. Prehydrolyzed bagasse differs, however, in that it is brittle and readily disrupted by attritor milling with an energy input of only about 0,1 kWh kg^{-1} (dry basis) of milled material (Chapter 3). A machine for continuous milling of bagasse was designed and tested. It is essentially a stirred ball mill filled with 6 mm diameter ceramic balls. Prehydrolyzed bagasse and water are fed to the base of the mill and they overflow from the top as a fine slurry which can be mixed with enzyme for hydrolysis.

If the slurry is air dried it regains its resistance to hydrolysis, but if it is rapidly drum dried it forms flakes which are readily hydrolyzed and could probably be extensively digested by ruminant animals (which themselves produce cellulase enzymes).

An alternative to prehydrolysis combined with attritor milling is autohydrolysis followed by steam explosion. This is part of the process of making furfural from bagasse. The bagasse is heated with steam to 180°C thus causing hydrolysis of the hemicellulose and conversion of the resulting xylose to furfural. The prehydrolyzed bagasse is then explosively decompressed and thereby converted to fine particles which are even more susceptible to enzymic hydrolysis than is the attritor milled material. This furfural factory residue is presently produced at Sezela (Natal South Coast) in sufficient quantity to make $40\text{-}50 \times 10^6$ l ethanol annually.

Enzymic hydrolysis

The enzyme complex for hydrolysing cellulose is obtained from the fungus *Trichoderma reesei* and has been produced very successfully in a pilot plant at the CSIR in Pretoria (Chapter 4). As it is expensive, research has been concentrated on improving its production and increasing its efficiency. Its efficiency during hydrolysis can be improved by for instance the following:

- ensuring that the substrate has been well pretreated;
- ensuring that the complex of enzymes is well balanced and contains enough ß-glucosidase to prevent feedback inhibition by accumulated cellobiose;
- running the hydrolysis simultaneously with fermentation so that the hydrolysis endproduct does not accumulate;
- adding non-enzymic chemicals, such as polyethylene glycol (PEG), which seem to prevent irreversible binding between enzyme and lignin.

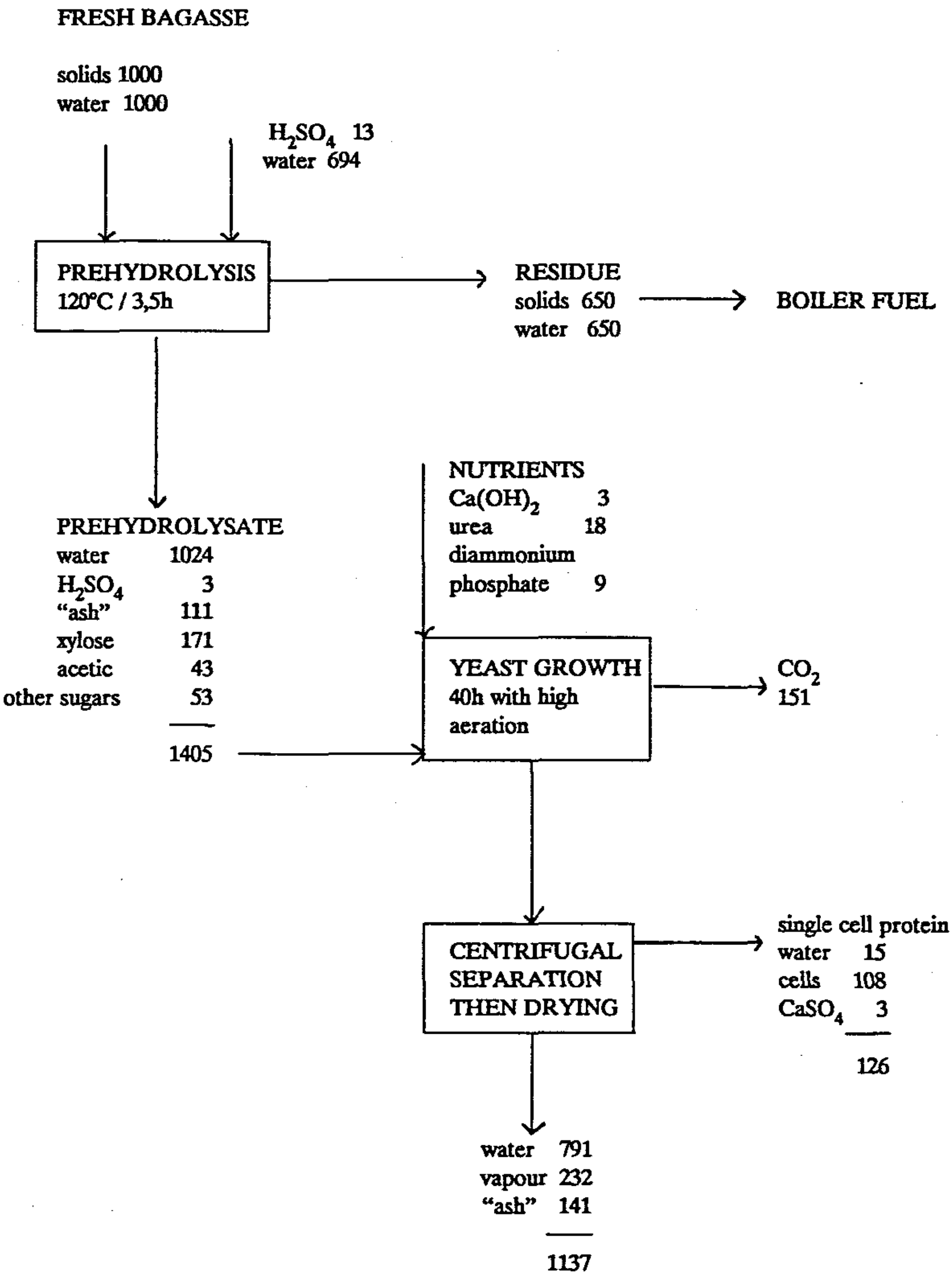

Figure 28. Simplified mass flow diagram for single cell protein production from bagasse hemicellulose hydrolysate.

The optimum temperature for the enzyme reaction is 50°C and the optimum pH is 4,8. The concentration of the enzyme complex is generally expressed in terms of international units (IU). One IU of enzyme is the quantity of enzyme which produces one µmole glucose per minute when hydrolysing filter paper under specified conditions. Under realistic practical conditions for hydrolysis of steam exploded bagasse (furfural factory residue), the maximum efficiency achieved has been 119 mg glucose produced per IU of enzyme used. Efficiencies with attritor milled bagasse were about 60 mg glucose IU^{-1} (Chapter 5).

Fermentation of hydrolyzed furfural factory residue

Furfural factory residue contains an inhibitor which diminishes the rate of fermentation of the glucose produced by hydrolysis. This inhibitory effect can be eliminated by washing the furfural factory residue with water before hydrolysis. After washing, the furfural factory residue can be hydrolyzed and fermented simultaneously so that 90 percent of the cellulose is converted to alcohol which accumulates to a maximum concentration of 5 percent (M/V). Mass balances for the process are shown in Figure 29.

COST ESTIMATES

The technical requirements of the various processes have been determined in a small process development unit and used by a consulting engineer to estimate the viability of ventures based on these processes. Some details of the cost estimates are presented in Tables 18 to 23.

Judging from the estimated rates of return on investment, the following processes appear to be most promising:

- ethanol production from steam exploded bagasse;
- production of single cell protein by growth of yeast on the xylose rich stream generated by dilute acid hydrolysis of the hemicellulose component of bagasse.

The economics of both of these processes are improved if molasses is used as the substrate during the 3-4 months each season when no cane is being crushed. Storage of bagasse for use during the off-crop is not economically attractive.

The amount of furfural factory residue available annually is sufficient for the production of $40\text{-}50\times10^6$ l ethanol, ie only one medium sized distillery. A material similar to furfural factory residue can be produced by bead milling bagasse after removing the hemicellulose component by acid hydrolysis. The cost of milling the prehydrolyzed bagasse is likely to be at least R15 t^{-1} and the cost of hydrolysing it is higher than for furfural factory residue because it requires more enzyme per unit of glucose produced. The process based on bead milled bagasse has therefore not been costed in detail, but appropriate technical information is available.

ETHANOL PRODUCTION FROM FURFURAL FACTORY RESIDUE

Assuming a sale price for ethanol of 70c l^{-1} (delivered to Durban), an after tax internal rate of return of 15 percent was estimated for a factory running during the cane harvesting season (Project Engineering Africa 1986). The internal rate of return increased to 24 percent if molasses was assumed to be fermented during the off-crop. Decreasing the sale price to 52c l^{-1} decreased the internal rate of return from 24 percent to 8,8 percent. Changing the inflation rate from 0 percent (base case) to 10 percent increased the maximum internal rate of return to 34 percent.

The capital cost of the plant was estimated to be R45 576 000 (Table 18), with 67 percent of this involving local expenditure, the major import item being distillation equipment which could be made locally if necessary.

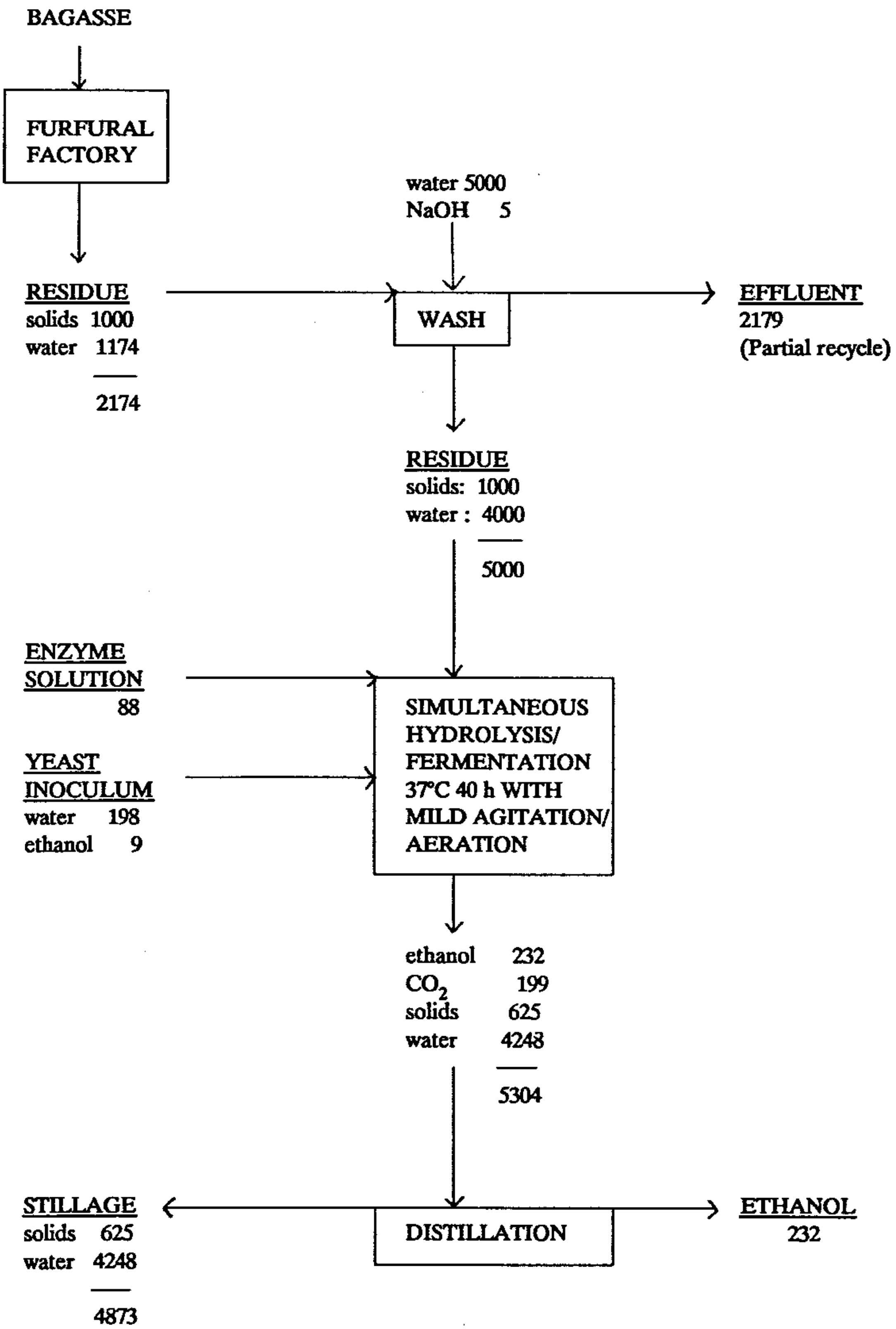

Figure 29. Simplified mass flow diagram for ethanol production from furfural factory residue.

The variable operating costs were R375 kl^{-1} of ethanol, 44 percent of this being for enzyme and 27 percent for polyethylene glycol (PEG). The cost of the substrate (furfural factory residue) at its coal replacement value (R25 dry t^{-1}) is R35 kl^{-1} ethanol assuming that the undigested lignin rich residue from the fermenters can be dewatered and returned to the sugar factory for credit against the original bagasse value. There is a chance that this residual material will have a high value as a raw material for adhesives manufacture.

Table 18. Capital costs of a factory for production of 40 x 10^3 t ethanol from 175 x 10^3 t furfural factory residue. (Project Engineering Africa 1986).

Item	Cost (R)	Percentage of total
Site development	1 083 500	2,4
Civils and buildings	1 122 920	2,5
Hydrolysis and fermentation	6 188 840	13,6
Distillation	11 219 200	24,6
Tankage	391 400	0,9
Utilities	6 186 300	13,6
Piping and valves	793 000	1,7
Electrics	1 086 750	2,4
Instrumentation	272 400	0,6
Installation	3 208 300	7,0
Subtotal	31 552 610	69,3
Process licence and engineering	539 773	1,2
Engineering	1 972 480	4,3
Site supervision	821 215	1,8
Spares	465 078	1,0
G S T	3 531 910	7,7
Contingency	4 228 870	9,3
Escalation allowance	2 463 630	5,4
Total	45 575 566	100

Enzyme production costs

The cost of enzyme is important. It was estimated at R165 kl^{-1} ethanol when used at a concentration of 5 IU g^{-1} furfural factory residue. This estimate is based on ethanol yields obtained in simultaneous saccharification and fermentation in a 20 l fermenter (Waugh and Proudfoot 1985) and on an enzyme costing based on results with a 150 l fermenter (Watson 1983).

For an enzyme factory producing $4{,}2 \times 10^{11}$ IU enzyme annually, the enzyme cost was estimated at R11,70 per million IU. The total capital cost, excluding land, was estimated at R16 700 000 (1982 prices).

After making the enzyme cost estimate, Watson et al (1984) reported considerable technical improvement in the process. The original estimate of enzyme cost did not include the cost of interest on capital and this cost would largely offset the savings due to process improvements.

The high cost of the enzyme, together with the recent progress in production technology, make it imperative that the enzyme cost be carefully reassessed by anyone contemplating exploitation of the technology.

SINGLE CELL PROTEIN PRODUCTION FROM HEMICELLULOSE HYDROLYSATE

A costing of single cell protein production from hemicellulose hydrolysate showed a potential ten year internal rate of return of 17 percent if the factory operated purely on bagasse hydrolysate (Project Engineering Africa 1987). The internal rate of return increased to 22 percent when molasses was assumed to be fermented during the sugarcane off-crop. The viability of the project is, of course, dependent on there being a market for the single cell protein at the anticipated price of R1 000 t^{-1} pure product (R730 t^{-1} after correction for moisture and ash). This price is not currently realisable, but the world price for protein is increasing faster than most other prices. A sale price sensitivity analysis showed that a 25 percent decline in the assumed price would cause the internal rates of return to decline to 9 percent and 12 percent respectively whereas a 10 percent increase in price resulted in internal rates of return of 22 percent and 29 percent.

The project is very sensitive to inflation. The base case outlined above assumes no inflation. Inflation of 10 percent increases internal rates of return by almost 10 percent.

The major capital cost items are the hydrolysis reactor and bagasse dewatering equipment (Table 19). These are particularly expensive because they must withstand exposure to hot 0,5 percent sulphuric acid. If their costs could be reduced by 10 percent through research on alternative materials of construction then the impact on internal rates of return would be 2-3 percent. The capital cost for a plant producing 25 000 t single cell protein annually was estimated as R33 000 000, including R3 400 000 for effluent evaporation.

The costing assumes a cell yield of 38 percent on fermentables and this has been achieved in laboratory studies (Chapter 2). The concentrations of fermentables and single cell protein assumed in the costing exercise are, however, about twice what now seems reasonable from fermentation studies and so the estimated capital cost of fermenters (8 percent of total) is presumably erroneously low.

Pilot plant studies will be necessary to confirm the cost estimates, but the existing estimates and the potential for improvement are encouraging for future production of protein.

THE COST OF FERMENTABLES

It is appropriate to estimate the cost of producing fermentable sugars from bagasse because this information may be useful for assessing costs of bagasse based endproducts other than ethanol and single cell protein. A precise cost estimate is impossible because the fermentable streams are impure and may require different amounts of purification for different end usages, eg acetic acid in prehydrolysate may act as a feedstock in one fermentation, but as an inhibitor in another. Futhermore, the cost of capital is a major component and it varies with time and between different companies.

Fermentables in hemicellulose hydrolysate

It is estimated that a factory producing 44 200 t fermentables annually from bagasse hemicellulose by acid hydrolysis would cost R12 729 000 (Table 20). The costs of fermentables for various costs of capital are organized in Table 21.

Table 19. Budget estimate of capital requirements for a factory producing single cell protein from the hemicellulose hydrolysate from all the bagasse from a 250 t cane h^{-1} sugar factory. (Project Engineering Africa 1987).

Item	Cost (R)	Percentage of total
Site development	836 850	2,5
Civils and building	997 350	3,0
Hydrolysis and dewatering	5 791 600	17,3
Neutralize and fermentation	2 739 650	8,2
Effluent evaporation	3 407 800	10,2
Single cell protein drier	1 831 000	5,5
Tankage	122 800	0,4
Utilities	1 294 300	3,9
Piping and valves	1 070 000	3,2
Electrics	713 750	2,1
Instrumentation	344 900	1,0
Installation	2 212 100	6,6
Subtotal	21 362 100	64,0
Process knowhow, licence engineering	2 420 740	7,2
Site supervision	654 930	2,0
Spares	242 490	0,7
G S T	2 388 500	7,2
Contingency	3 980 790	11,9
Subtotal	31 049 550	93,0
Escalation allowance	2 347 610	7,0
Total	33 397 160	100

This compares favourably with molasses, which at 42 percent fermentables and R80 t^{-1} shows a fermentables cost of R190 t^{-1}, but it is emphasized that acetic acid has been regarded as a fermentable in the bagasse product. Molasses has advantages of being more concentrated and containing more nutrients.

Any surplus bagasse which could be used without requiring coal replacement would reduce the cost of fermentables (or increase the revenue to the sugar factory) by about R36 for each ton of fermentables produced, assuming that the residue remaining after hydrolysis is credited as boiler fuel.

Effluent treatment costs have not been included because there would be no major effluent production from the hydrolysis stage. Effluent treatment in subsequent stages might, however, be expensive because of the relatively low concentration of fermentables.

Table 20. Deviation of estimated costs of producing fermentables from bagasse by acid hydrolysis of hemicellulose (Project Engineering Africa 1987). Assumed yields (as percent of dry bagasse) were 17,1 for xylose, 5,3 for other sugars and 4,3 for acetic acid (total 26,7 percent). Annual production of fermentables 44 200 t, assuming 230 working days.

Capital costs of hydrolysis and recovery equipment for producing hemicellulose hydrolysate from 30t h^{-1} bagasse (dry basis):

Item	Cost (R)	Percentage of total	Portion •
Site development	418 000	3,3	1/2
Civils and buildings	498 000	3,9	1/2
Hydrolysis and dewatering	5 292 000	41,0	1/1
Utilities (boilers etc)	858 000	6,7	2/3
Piping and valves	357 000	2,8	1/3
Electrics	238 000	1,9	1/3
Instrumentation	115 000	0,9	1/3
Installation	737 000	5,8	1/3
Engineering	807 000	6,3	1/3
Site supervision	218 000	1,7	1/3
G S T	1 082 000	8,5	
Contingency	1 327 000	10,0	1/3
Escalation allowance	782 000	6,0	1/3
Total	12 729 000		

• The estimates used are based on the indicated portion of cost estimated for a facility for hydrolysis, fermentation and distillation, ie an ethanol factory.

Variable operating costs

Item	Cost ($R\ t^{-1}$ fermentables)
Bagasse	36
Acid	15
Lime	4
Electricity	6
Steam	4
Total	65

Fixed costs (assuming 50 percent of estimated costs for single cell protein production).

Item	Cost ($R\ t^{-1}$ fermentables)
Total salaries, wages and salary burden	8
Maintenance materials	3
Total	11

Fermentables produced by enzymic hydrolysis of cellulose

When using cellulase enzymes to hydrolyse bagasse to glucose, the hydrolysis is very inefficient if the glucose is not simultaneously removed. It is therefore prohibitively expensive to produce glucose as the endproduct unless it is acceptable at a concentration of less than 2 percent. The cheapest glucose will be produced under conditions of simultaneous hydrolysis and fermentation and will be produced most cheaply from furfural factory residue which requires no expenditure on pretreatment. An approximate indication of the cost of glucose feedstock for ethanol production from furfural factory residue is shown in Table 22. The cost calculations are shown in Table 23.

Table 21. Estimated costs of fermentables, including acetic acid, produced by acid hydrolysis of bagasse hemicellulose.

Annual cost of capital (percentage)	25	20	15
Annual cost of capital (R t^{-1} fermentables	72	58	43
Variable costs (R t^{-1} fermentables)	65	65	65
Fixed costs (R t^{-1} fermentables)	11	11	11
Total (R t^{-1})	148	134	119

Table 22. Estimated costs of fermentables produced by enzymic hydrolysis of furfural factory residue.

Annual cost capital (percent of capital)	25	20	15
Annual cost of capital (R t^{-1} fermentables)	28	22	15
Variable costs (R t^{-1} fermentables)	163	163	163
Fixed costs (R t^{-1} fermentables)	4	4	4
Total (R t^{-1} fermentables)	195	189	182

Fermentables produced by enzymic hydrolysis of cellulose

When using cellulase enzymes to hydrolyse bagasse to glucose, the hydrolysis is very inefficient if the glucose is not simultaneously removed. It is therefore prohibitively expensive to produce glucose as the endproduct unless it is acceptable at a concentration of less than 2 percent. The cheapest glucose will be produced under conditions of simultaneous hydrolysis and fermentation and will be produced most cheaply from furfural factory residue which requires no expenditure on pretreatment. An approximate indication of the cost of glucose feedstock for ethanol production from furfural factory residue is shown in Table 22. The cost calculations are shown in Table 23.

Table 23. Deviation of estimated costs of producing fermentables from bagasse by enzymic hydrolysis of furfural factory residue (Project Engineering Africa 1986). Fixed costs (at 33 percent of costs for ethanol production R4 t^{-1} glucose).

<u>Capital costs</u> of a factory for production of 90 x 10^3 t glucose from 175 x 10^3 t furfural factory residue.

Item	Cost (R)	Portion *
Site development	361 000	1/3
Civils and buildings	374 000	1/3
Hydrolysis and dewatering	3 094 000	1/2
Utilities (boilers etc)	1 546 000	1/4
Piping and valves	198 000	1/4
Electrics	271 000	1/4
Instrumentation	68 000	1/4
Installation	802 000	1/4
Engineering	493 000	1/4
Site supervision	205 000	1/4
G S T	889 000	
Contingency	1 057 000	
Escalation allowance	616 000	
Total	9 974 000	

* The estimates used are based on the indicated portion of the cost estimated for a facility for hydrolysis, fermentation and distillation, ie a complete ethanol factory.

<u>Variable operating costs</u>

Item	Cost (R t^{-1} glucose)
Furfural factory residue (at coal replacement value)	18
NaOH	1
Enzyme	87
Polyethylene glycol	54
Electricity	3
Total	163

The price is similar to that of fermentables in molasses. The major cost components are enzyme and polyethylene glycol, the latter being a cost effective additive for increasing enzyme efficiency. These figures emphasize the relevance of enzyme efficiency. Further progress in reducing the cost of enzyme is now particularly relevant because the price of glucose from furfural factory residue is close to that of fermentables from molasses and the latter have a ready market.

If the starting material is prehydrolyzed bagasse instead of furfural factory residue then a milling cost of approximately R15 t^{-1} of bagasse would be necessary for pretreatment. This is equivalent to about R40 t^{-1} of glucose. Hydrolysis of the milled material gives a cellulose conversion of about 75 percent whereas 90 percent is achieved with furfural factory residue. This suggests that the enzyme cost would increase by R17 t^{-1} glucose. The PEG cost would increase by R11 t^{-1} glucose and there would be a slight increase in capital cost to accommodate the increased bagasse input per unit of glucose produced. Thus the glucose cost would be approximately R262 t^{-1}.

SUMMARY AND CONCLUSIONS

Processes for producing various endproducts from bagasse have been developed and tested in a small process development unit. A xylose rich stream can be produced by acid hydrolysis of the hemicellulose component and fermentables in this stream are estimated to cost less than those in molasses. The main fermentable sugar is xylose and there is a xylose:acetic acid ratio of 5:1. The acetic acid is toxic in anaerobic fermentations and although it can be removed by an aerobic prefermentation, it prevents economic ethanol production. The production of single cell protein on the hydrolysate by growing *Candida utilis* under aerobic conditions is promising.

By comparison with other sources of protein, single cell protein is estimated to be worth about R600 t^{-1} and, at this price, an internal rate of return of 16 percent is estimated for a single cell protein factory using bagasse during the harvesting season and molasses during the off-crop. A 25 percent increase in the value of single cell protein would increase the internal rate of return to approximately 23 percent. The internal rates of return have been calculated assuming zero inflation and increase by almost 10 percent if inflation is assumed to be 10 percent.

A readily fermentable glucose rich stream was produced by enzymic hydrolysis of steam exploded bagasse residue from a furfural factory. Ethanol produced on this stream was estimated to generate internal rates of return of 24 percent and 9 percent for assumed sales prices of 70c l^{-1} and 53c l^{-1} respectively. These internal rates of return increase under conditions of inflation. There is currently enough furfural factory residue to support a medium sized alcohol factory (450×10^6 l annually). A product similar to furfural factory residue can be produced by attritor milling of prehydrolyzed bagasse, but this product is more expensive and more difficult to hydrolyse than is the furfural factory residue. Nevertheless, the potential for large scale production of fermentable sugars from bagasse has been demonstrated and high cost areas have been identified for future research.

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