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Ethanol production from sulphuric acid wood hydrolysate of *Pinus radiata* using free and immobilized cells of *Pichia stipitis*

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SUMMARY

Ethanol was produced by a strain of *Pichia stipitis* adapted to an inhibitory acid wood hydrolysate of *Pinus radiata*. The best ethanol productivity for batch cultures was 0.21 g/l h at 0.7% ethanol. Varying culture conditions increased ethanol concentration to 0.76%, however the productivity decreased to 0.18 g/l h. A decrease in ethanol concentration in the culture fluid was noted late in the batch which suggested ethanol catabolism. Values of kinetic parameters (K_m, K_s, μ_{max}, and V_{max}) were evaluated for this system. The use of calcium alginate immobilized cells in a continuous-flow stirred tank reactor lead to enhanced fermentative performance, namely a maximum productivity of 0.27 g/l h and 1.13% ethanol yield. The immobilized cells in continuous flow reactors represent an attractive option for fermenting sugars released by sulphuric acid hydrolysis of *P. radiata* wood.

INTRODUCTION

During the recent years there has been an ongoing interest concerning the production of ethanol from plant materials, particularly from the abundant supply of wood residues.

Hydrolysis of wood can be carried out either by dilute acids or enzymes [7]. Acid hydrolysis of pine wood is currently the preferred route to hydrolysis because enzymatic hydrolysis is uneconomic [7]. However, the acid hydrolysate contains a number of fermentation inhibitory compounds, especially sugar degradation products [1,12,13]. Although sodium sulphite treatment has been used to minimise these inhibitors [6], the hydrolysate so treated may remain inhibitory. The aim of the present work was to adapt a culture of *Pichia stipitis* to inhibitory sulphuric acid wood hydrolysate and to compare growth and ethanol fermentation kinetics in batch and immobilized systems.

MATERIALS AND METHODS

Wood hydrolysate and feedstock medium

Sulphuric acid wood hydrolysate of *Pinus radiata* of following composition was obtained from the Forest Research Institute, Rotorua (New Zealand): glucose, 20 g/l; xylose, 0.9 g/l; mannose, 1.3 g/l; and galactose, 0.3 g/l. The pH of this hydrolysate was adjusted to 5.0 with calcium hydroxide slurry (500 g/l) after which it was treated with sodium sulphite (0.5 g/l) and supplemented with yeast extract (5 g/l, Oxoid, U.K.).

This medium was autoclaved at 121 °C for 15 min. On cooling, the clear supernatant was aseptically decanted from the thick precipitate and used throughout all studies. It contained reducing sugars at 22.5 g/l and is referred to as W.H. medium.

Yeast culture

The adapted yeast strain used for these studies was derived from *Pichia stipitis* NRRL 1724. The adaptation was carried out using the following steps. All cultures were incubated at 30 °C.

Adaptation to xylose. Initially the culture was grown on YM agar (dextrose, 10 g/l; yeast extract, 3 g/l; malt extract, 3 g/l; peptone, 5 g/l; and agar 15 g/l) slopes. Following this, the culture was transferred to agar slopes containing YM medium and D-xylose, 50 g/l. Three successive transfers were made to adapt the culture to xylose. After the third transfer cell growth was quicker and the incubation period was reduced from several days to two days. Further transfers were made to agar slopes containing xylose (as the sole carbon source), 100 g/l; proteose peptone (Difco Laboratories, Detroit, MI, U.S.A.), 10 g/l; yeast extract (Oxoid, U.K.), 5 g/l and agar (Oxoid), 15 g/l in distilled water. Three successive transfers were made

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on slopes of the above composition. The final slope was used as the inoculum for further adaption studies.

Adaptation to sulphuric acid wood hydrolysate. Following growth on the above slopes the culture was grown in liquid medium in 250-ml shake flasks containing 100 ml of 25% (diluted four times with distilled water) wood hydrolysate; xylose, 50 g/l; proteose peptone, 10 g/l; and yeast extract, 5 g/l in distilled water. The pH of the medium was adjusted to 5.0 by adding Ca $(OH)_2$ solution aseptically. Cells from one of the latter xylose-adapted culture slopes were transferred to a flask containing the above medium and agitated at 100 rpm. Once cell growth was observed 20 ml of this culture was inoculated into another flask containing 100 ml of 50% wood hydrolysate and the above medium ingredients. Cell growth was developed and then 20 ml of this cell culture was inoculated into a liquid medium containing 100 ml of 75% wood hydrolysate. The next transfer was made in W.H. medium and growth allowed to develop.

Maintenance of the culture. Cells from the above flask (W.H. medium) were inoculated onto agar slopes of W.H. medium supplemented with peptone, 3 g/l; malt extract, 3 g/l, and agar 20 g/l. These were incubated at 30 °C until full cell growth developed and then stored at 4 °C until required.

Inocula preparation

Inocula were prepared by inoculating a loopful of yeast cells from slopes into W.H. medium and agitating for 24 to 30 h. Experimental studies (cell growth and ethanol production) used W.H. medium (100 ml in 250-ml conical flasks) with a 10% (v/v) of inoculum. These studies were conducted into two parts. Firstly, where the culture was agitated throughout the growth and ethanol production phase (aerobic culture) and secondly, where the culture was agitated only during growth and initial ethanol production phase. In the latter case agitation was terminated after growth and initial phase of ethanol production and then fermentation proceeded anaerobically. 5-ml samples were withdrawn for estimation of biomass, ethanol and residual sugars.

Immobilization

Cell mass grown aerobically as described above was harvested by centrifugation at 5000 rpm for 8 min, followed by washing with sterile physiological saline. Washed cells were immobilized in calcium alginate gel as described elsewhere [10]. 80 ml of suspended cells containing 41 g cell mass/l were mixed thoroughly with 50 ml of 2.7% sodium alginate slurry which was extruded through a 22-gauge syringe needle into 0.2 M $CaCl_2$ solution. The cells in the alginate beads (2.5 mm diameter) were stored at 4 °C for 12 h before their activation in W.H. medium. Beads equivalent to 3.28 g (dry weight) cells were used in a 1-l glass Continuous Stirred Tank Reactor (CSTR). The liquid volume in the reactor was kept constant at 400 ml.

Analyses

Ethanol was determined by gas chromatography (Model GC-8A, Shimadzu Corporation, Kyoto, Japan) using a flame ionization detector and a column of Porapak Q. Total reducing sugars were measured by a modified Somogyi-Nelson colorimetric method as described below.

Low-alkalinity copper reagent. Potassium sodium tartarate (12 g) and anhydrous sodium carbonate (24 g) were dissolved in about 250 ml of distilled water. A solution of 4 g of cupric sulphate pentahydrate made in distilled water was added to the above solution with stirring followed by addition of 16 g of sodium hydrogen carbonate. 500 ml of anhydrous sodium sulphate (180 g in distilled water) was made and boiled to expel air followed by adding and mixing with potassium sodium tartarate and sodium carbonate solution. The total volume of this solution was made-up to one l. After 3–7 days of storage the clear supernatant solution was used for analysis.

Arseno-molybdate reagent. 21 ml of 96% sulphuric acid and 3 g of di-sodium hydrogen arsenate heptahydrate dissolved in 25 ml distilled water was added to 450 ml of aqueous ammonium molybdate (25 g in distilled water). This solution was incubated at 37 °C for 24 h followed by storage in a glass stoppered brown bottle.

Samples containing sugars were diluted to contain 50–250 μg total sugars per ml. 2 ml of the low alkalinity copper reagent were added to 2 ml of diluted sample. The above mixture was heated in a boiling water bath for 10 min followed by cooling under running water. To this 1 ml of arseno-molybdate reagent was added and the sample was mixed thoroughly. After mixing the samples were diluted with 20 ml distilled water and mixed. Test tubes containing above samples were allowed to stand for 5 to 10 min. The absorbances of the samples were read at 500 nm against a reagent blank (where sugar containing sample was replaced by 2 ml distilled water) using a Shimadzu Spectrophotometer UV-120-02 (Shimadzu Corporation, Kyoto, Japan). The sugar concentration of the samples was read from a D-xylose standard curve (xylose concentration vs. optical absorbance) prepared on the same day. All the samples were analysed in duplicate.

Cell mass was estimated as dry weight at 650 nm from a standard curve showing cell dry weight vs. optical density.

Kinetic studies employed diluted W.H. medium at total reducing sugar concentrations of 4.5, 9, 13.5, 18.5, and 22.5 g/l and yeast extract at 5 g/l. The kinetic parameters measured were cell yield coefficient (g cell/g total

reducing sugar utilized), ethanol yield coefficient (g ethanol/g total reducing sugar metabolized), ethanol productivity (g/l h), specific ethanol productivity (h^{-1}), μ_{max} (h^{-1}), K_s (g/l), K_m (g/l) and V_{max} (h^{-1}). Ethanol productivity (g/l h) in the batch culture experiments was expressed as maximum ethanol concentration (g/l) divided by elapsed time (h), while for continuous culture experiments it was expressed as ethanol concentration in the overflow (g/l) multiplied by dilution rate (D). The dilution rate is defined as feed flow rate divided by the total reactor volume. Specific ethanol productivity (g/l h/g cell/l) was expressed as ethanol productivity per unit cell concentration at the time of measurement. The extent of culture incapacitation brought about by inhibitors in the wood hydrolysate was expressed as the ratio μ/μ_{max} and V/V_{max}. The calculation of μ_{max} and K_s used Monod equation ($1/\mu = 1/\mu_{max} \cdot 1/S + 1/\mu_{max}$) while V_{max} and K_m were evaluated from a Lineweaver-Burk plot ($1/V = 1/V_{max} \cdot 1/S + 1/V_{max}$) [9].

RESULTS AND DISCUSSION

A maximum ethanol concentration of 7 g/l was achieved after 33 h of aerobic fermentation with a cell concentration of 1.8 g/l (Table 1). Prolonging fermentation from 33 h to 55 h increased the cell mass to 3.1 g/l and reduced ethanol to 2.6 g/l indicating that ethanol utilization occurred in the later stages of fermentation. The utilization of ethanol during the aerobic phase by *Pichia stipitis* has been observed by other authors [2,4,11]. To prevent this, the yeast was grown in the W.H. medium aerobically for 33 h (to allow full population development) and for the next 22 h fermentation proceeded without agitation (static). This resulted in the production of 1.8 g/l cells at 33 h which diminished to 0.64 g/l at 55 h. Ethanol concentration reached a maximum at 7.6 g/l at 41.5 h and decreased to 5.5 g/l at 55 h. The ethanol productivity and specific productivity at 41.5 h were 0.18 g/l h and 0.1 h^{-1}, respectively. The cell yield and ethanol yield were of the order of 0.09 and 0.34, respectively. Results from other authors are given in Table 1.

From kinetic analysis a μ_{max} of 0.16 h^{-1} was obtained (Table 1). Other reported values of μ_{max} are 0.22 h^{-1} [3] and 0.12 h^{-1} [4] for *Pichia stipitis*. These authors used different media containing xylose as carbon source and this may reflect different μ_{max} values. Our μ_{max} value obtained from sulphuric acid wood hydrolysate fermentation can be compared with the other two values. It appears that the medium had no inhibitory effect on cell growth.

To study this inhibitory effect, further W.H. medium was diluted to reduce inhibitor concentrations. It should be noted that the yeast extract concentration was maintained at 5 g/l. Obviously, lower values of μ and V would be expected if inhibitory components inhibited cell growth and ethanol production and this would decrease

TABLE 1

Values of kinetic and other fermentation parameters for *Pichia stipitis* for the production of ethanol from wood hydrolysate and xylose

Fermentation parameters							Reference
P (g/l)	R (g/l h)	R_s (h^{-1})	$Y_{p/s}$ (g/g)	$Y_{x/s}$ (g/g)	X (g/l)	μ_{max} (h^{-1})	
Batch culture/fed batch culture							
7.0	0.21	0.12	0.35	0.09	1.8	–	This work*[1]
7.6	0.18	0.10	0.34	0.09	1.8	0.16	This work*[2]
52	0.30	0.20	0.40	–	–	–	13
–	–	0.03	0.40	–	–	0.22	3
47	0.24	0.02	0.34	–	15.8	0.12	4
Immobilized cell reactor							
11.3	0.27	0.08	0.50		3.2		This work*[1]
22	0.30	–	–		–		5
10.3	2.60	0.05	0.47		49		8
Kinetic constants* V_{max}, 0.25 h^{-1}; K_m, 9.8 g/l; K_s, 1.0 g/l.							

* Sulphuric acid wood hydrolysate of *Pinus radiata*.

1. Aerobic.

2. Aerobic phase followed by anaerobic phase.

μ/μ_{max} and V/V_{max} ratios. To test this possibility values of μ and V were evaluated using W.H. medium diluted 1.25 to five times with distilled water. These data are plotted as μ/μ_{max} and V/V_{max} against total reducing sugar in the medium (Fig. 1). No decreasing relationship was observed between μ/μ_{max} and V/V_{max} with increasing wood hydrolysate sugar concentrations indicating that there was no inhibition from wood hydrolysate components within the experimental range of wood hydrolysate sugar concentrations. At higher concentrations of wood hydrolysates, however a precipitate formed during yeast growth which interfered with cell measurements and produced deviations in the μ/μ_{max} ratios.

Further, immobilized cell studies conducted in a CSTR showed an ethanol concentration, yield and sugar utilization of 11.3 g/l, 0.5 and 100%, respectively at a dilution rate of 0.024 h^{-1} (Fig. 2, Table 1). At this dilution rate a specific ethanol productivity and an ethanol productivity of 0.08 h^{-1} and 0.27 g/l h were obtained, respectively. Ethanol productivity values from other authors are compared in Table 1. Linko et al [5] reported a productivity of 0.30 g/l h in an immobilized cell reactor of *Pichia stipitis* fed on xylose. Parekh et al. [8] used a rotating immobilized cell reactor of *Pichia stipitis* to produce ethanol from wood hydrolysate and reported a productivity of 2.6 g/l h. Some of the discrepancies observed in the results of xylose/wood hydrolysate fermentation by *Pichia stipitis* and *Candida shehatae* have been discussed recently by du Preez et al. [4].

The absence of detectable reducing sugars in the overflow indicated that the adapted strain used all major sugar components from wood hydrolysate in free cell and immobilized cell reactors. The immobilized cell CSTR produced higher ethanol concentrations (Table 1) and it

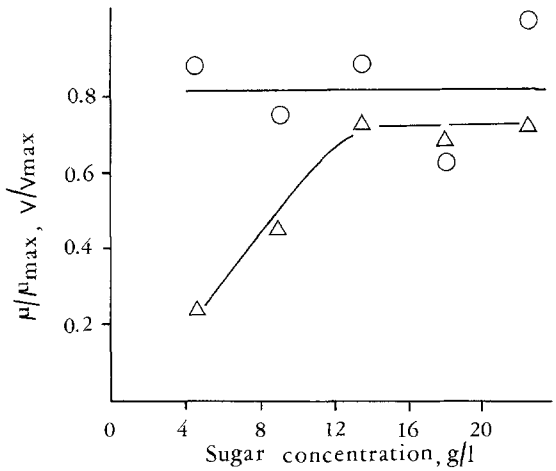

Fig. 1. Ratios of μ/μ_{max} and V/V_{max} at various sugar concentrations in sulphuric acid wood hydrolysate. μ/μ_{max}, ○; V/V_{max}, △.

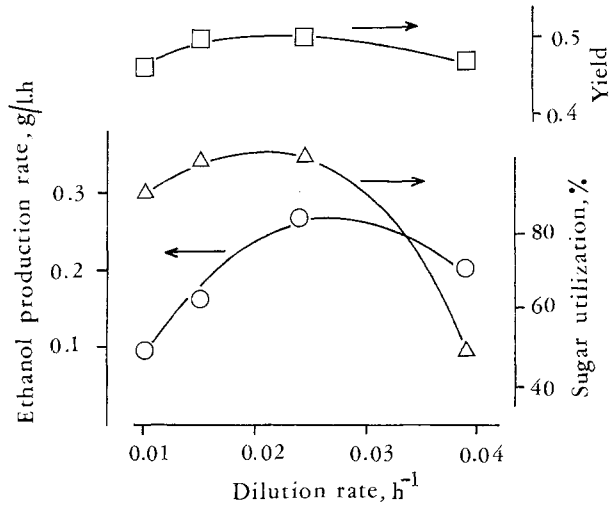

Fig. 2. Production of ethanol in an immobilized cell CSTR of *Pichia stipitis* from sulphuric acid wood hydrolysate of *Pinus radiata*. Ethanol productivity, ○; Sugar utilization, △; Ehanol yield, □.

seemed possible that ethanol was not being consumed even though full sugar utilization had occurred. This was in contrast to free cell batch cultures where ethanol utilization occurred during the final stages of fermentation. The kinetic data indicated that immobilized cell CSTR of *Pichia stipitis* is an attractive option for the production of ethanol from sulphuric acid wood hydrolysate of *Pinus radiata*. This information is seen to be of use in the assessment of *P. stipitis* in the larger scale utilization of locally produced sulphuric acid hydrolysate.

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NOMENCLATURE

K_m = Kinetic constant for ethanol production [g/l]
K_s = Kinetic constant for cell growth [g/l]
S = Substrate concentration [g/l]
V = Specific rate of ethanol production [h^{-1}]
V_{max} = Maximum specific rate of ethanol production [h^{-1}]
X = Cell concentration [g/l]
$Y_{p/s}$ = Ethanol yield [g/g]
$Y_{x/s}$ = Cell yield [g/g]
P = Ethanol concentration [g/l]
R = Ethanol productivity [g/l h]
R_s = Specific ethanol productivity [h^{-1}]

μ = Specific growth rate constant [h^{-1}]
μ_{max} = Maximum specific growth rate constant [h^{-1}]

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