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Process Engineering Studies on Continuous Ethanol Production by Immobilized *S. cerevisiae*

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Saccharomyces cerevisiae *cells were immobilized on three supports—calcium alginate beads, raw and hexamethylene diamine treated bagasse—for their comparative assessment in terms of cell retention on to the carrier and metabolic and physiological activities of the immobilized cells. The chosen carrier-activated bagasse adsorbed 0·41 g of cells per g of carrier (on a dry weight basis). The Gibbs free energy and activation energy for immobilization for the first and second stages was computed to be 3273·45, 14621·27 and 6336·34 J g mol^{-1}, respectively. The immobilized cells were used in a packed bed reactor for the continuous bioconversion of sucrose to ethanol and the packing density of the carrier and the height to diameter ratio of the packed bed bioreactor were optimized to be 50 g l^{-1} and 2·5, respectively. The maximum productivity obtained was 31·8 g l^{-1} h^{-1} at a feed sugar concentration of 200 g l^{-1} and space velocity of 0·53 h^{-1}. The system was operationally stable in terms of productivity for 76 days. The system then could be reactivated by intermittent air sparging for further operation.*

INTRODUCTION

A whole cell immobilized system may be used to improve the performance of a classical continuous fermentation or conventional batch process by increasing the concentration of cells per unit reactor volume. Continuous operation at a dilution rate exceeding the maximum specific growth rate, change in the microbial physiology due to the physico-chemical microenvironment around the cells and reduced inhibitory effects of substrate, product or both are the characteristic features of such systems. The combined effects of these factors leads to improvements in the system's efficiency and results in increased productivity. Despite many reports on ethanol-producing immobilized cell

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systems[1–6] there has been no systematic study of process engineering parameters. The central point of the present investigation was a detailed bioreactor analysis for an immobilized packed bed yeast cell system with long term stability at a high productivity level.

MATERIALS AND METHODS

Organism and cultivation conditions

Saccharomyces cerevisiae CBS-5816 was obtained from the CBS culture collection, Baarn, The Netherlands and cultivated in a 6 litre fermenter (Bioengineering AG, Switzerland) for 10–12 h at 30 °C and pH 5·0 in a medium containing ($g\,l^{-1}$); sucrose, 20; yeast extract, 3; malt extract, 3; peptone, 5.

Fermentation medium and molasses clarification

Molasses medium used in the continuous fermentation contained: reducing sugar, 120–200 $g\,l^{-1}$; urea, 1·0 $g\,l^{-1}$; $MgSO_4.7H_2O$, 0·5 $g\,l^{-1}$; orthophosphoric acid, 0·3 $ml\,l^{-1}$; pH, 5·0. The diluted molasses (diluted with tap water to give a desired concentration of reducing sugar) was clarified by adding 6N sulphuric acid to a pH of 4·0 and heated to 60 °C. This was followed by the addition of 1% (w/w) calcium superphosphate, homogenous mixing, steaming for 2 h and subsequently cooling to ambient temperature. The supernatant was used after centrifugation at 4000 rpm (revolution per minute).

Carrier(s) preparation and immobilization procedure

The following carriers and immobilization methods were used: (1) entrapment in calcium alginate beads as described by Klein *et al.*;[7] (2) adsorption on to raw bagasse (pith portion, chipped to 1–1·5 cm size); (3) ionic adsorption on to activated bagasse (pith portion, chipped to 1–1·5 cm size, positively charged with aminohexyl group). The carrier was activated according to the method of Porath and Fornstedt.[8]

The immobilization procedure was the same for raw and activated bagasse. After harvesting the yeast was centrifuged and resuspended in a fresh sterile medium to prepare the cell suspension of the order of 50 $g\,l^{-1}$ concentration. Aliquots (100 ml) of this suspension was transferred into 500 ml capacity Erlenmeyer flasks containing 2·0 g dried and autoclaved carriers. The flasks were then put in an orbital shaker maintained at 30 °C for 80 min. The cell suspension was separated from the carrier by decantation and washed with sterile citrate buffer (0·05 mM, pH 5·0), to wash off loosely bound cells. The immobilized carrier was then packed in the column. A new column was packed each time to change the packing density while maintaining a constant column volume.

Physiological studies of the immobilized cells

Carbon dioxide evolution and oxygen uptake rates of the immobilized cells were measured in a Warburg respirometer at 10 min intervals using 1·5 ml buffered (citrate, 0·05 mM, pH 4·8) glucose (1·5%) solution.

Scanning electron microscopy

Photographs of raw and activated bagasse (with and without immobilized cells) were taken in scanning electron microscope (Cambridge Stereoscan, S4-10, UK) at different magnifications.

Analytical techniques

To arrest microbial growth, 0·1 ml of 32% formaldehyde solution was added to samples followed by centrifugation at 3000 rpm for 15 min. The supernatant was used for assay of substrate/products while the settled mass after thorough washing with 0·85% (w/v) saline solution was used for cell mass estimation.

Cell mass and viability

A linear correlation was established between cell dry weight and optical density (OD) at 520 nm (linearity range: 0·1–0·55 OD). Cell retention on to the carrier(s) was calculated by measuring the total nitrogen content in the sample by a modified Kjeldahl method[9] using ammonium sulphate as a standard to correlate cell dry weight and nitrogen content. A Walford staining technique[10] was employed for the determination of the number of viable cells using a Haemocytometer. This measurement wa· counter checked by a plate count method using growth medium being supplemented with 2% agar. The coefficient of correlation for two sets of analysis was found to be 0·96.

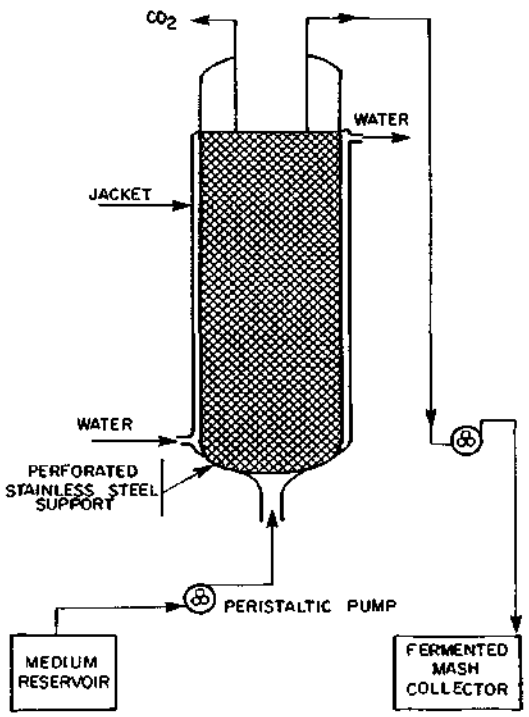

Fig. 1. Schematic diagram of immobilized cell packed bed bioreactor.

Ethanol and reducing sugars concentration

The ethanol concentration was measured in a dual column and dual detector gas liquid chromatograph (AIMIL – 5700, Nucon Engineers Pvt. Ltd). The total reducing sugars in molasses after hydrolysing with 2N HCl in a boiling water bath for 10 min and neutralizing with 1N NaOH) was measured by using dinitro-salicyclic acid (DNS) method.[11]

Experimental set-up

Glass jacketted columns (500 ml) of 6·2 cm in diameter and 16·5 cm length were used in all continuous experiments except in the studies on packing densities and optimization of height to diameter ratio. The temperature was maintained at 30 °C by circulating water through the columns' jackets. Carbon dioxide was released through a vent at the top of the column. A schematic experimental set-up is shown in Figure 1. To measure gas hold-up, the column was drained and the volume of the drained liquid measured, the gas hold-up was then calculated by subtracting the drained volume from the column void volume.

RESULTS AND DISCUSSION

A comparative study of cell entrapment was made with yeasts within calcium alginate beads and adsorbed on to raw and activated bagasse. The parameters considered in the choice of the carrier for immobilization were metabolic and physiological activities, cell desorption and maximum cell loading on to the carrier.

Metabolic activity

The specific rates of sugar uptake (ϕ_s) and ethanol formation (ϕ_p) were calculated on the basis of initial cell concentration present in the system at different dilution rates (flow rate/column volume). It was assumed that growth of the cells under anaerobic condition on to the carrier surface was insignificant and this was confirmed experimentally. The cells immobilized within the calcium alginate beads yielded lower ϕ_s (35% lower) and ϕ_p (12·9% lower) values than the corresponding values for cells immobilized on to raw and activated bagasse (Table 1). Such observations could be the result of mass transfer limitations prevailing in calcium alginate beads.

The cells supported on the activated bagasse exhibited higher values of ϕ_{CO_2} and ϕ_{O_2} than the

Table 1. Comparative study of metabolic activity of cells immobilized on to different carriers.

Carrier	*Specific O_2 uptake rate* ($l\ min^{-1}\ mg^{-1}$) *A*	*Specific CO_2 uptake rate* ($l\ min^{-1}\ mg^{-1}$) *B*	*Respiration quotient* (*B/A*)	ϕ_s^* (h^{-1})	ϕ_p^* (h^{-1})
Ca-alginate	2·58	2·33	0·864	1·197	0·74
Activated bagasse	5·47	5·12	0·936	1·841	0·85
Raw bagasse	4·89	4·61	0·942	1·844	0·85

ϕ_s. Specific sugar uptake rate; ϕ_p. specific ethanol formation rate.
*. Maximum attainable value under the experimental conditions.

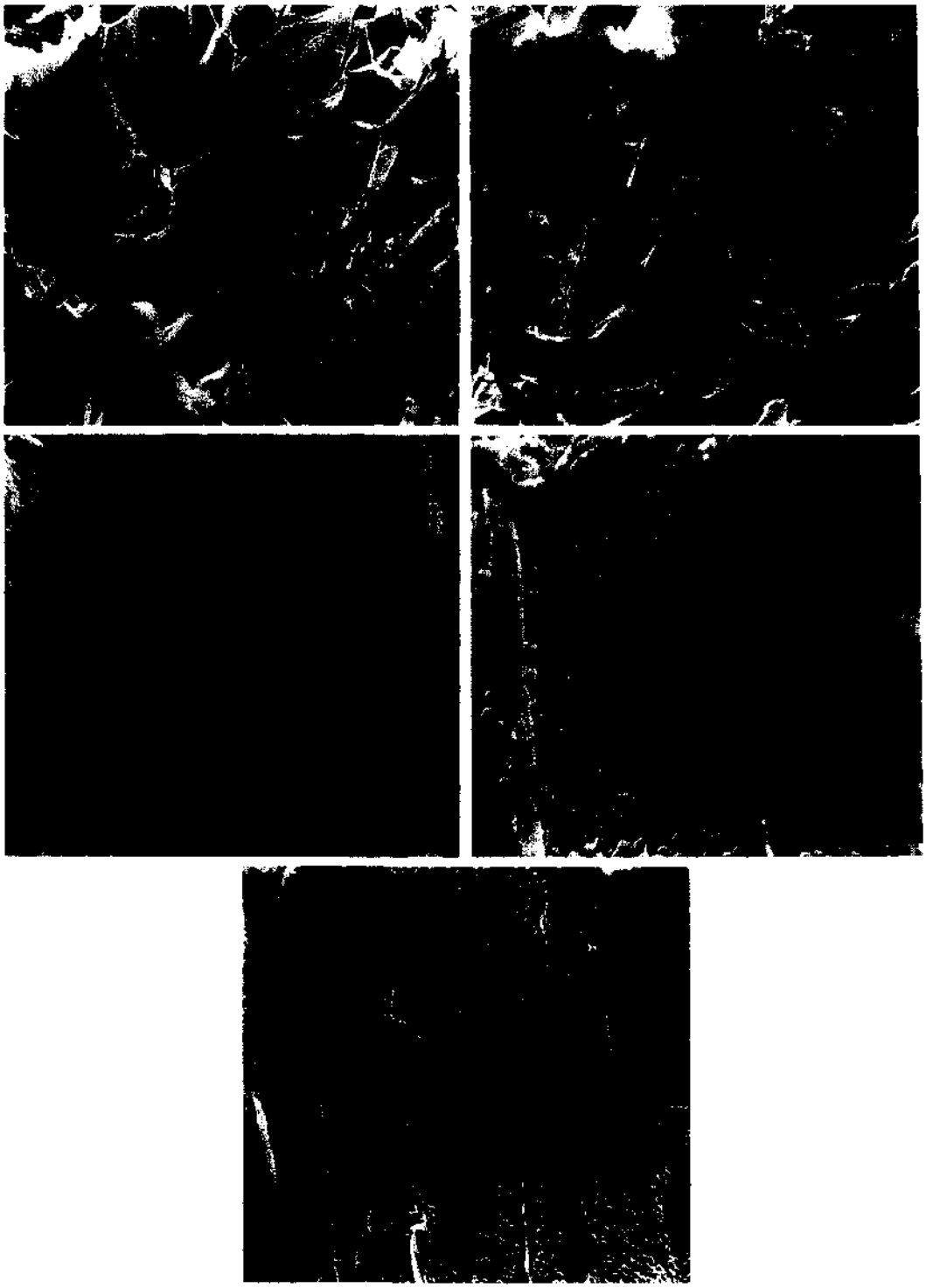

Fig. 2. (a) Electron micrograph of raw bagasse (×210). (b) Electron micrograph of activated bagasse (×210). (c) Electron micrograph of cells immobilized on to raw bagasse (×920). (d) Electron micrograph of cells immobilized on to activated bagasse (×870). (e) Electron micrograph of cells immobilized on to activated bagasse (×450).

other two carriers (Table 1). However, the ϕ_{O_2} (10·6) and ϕ_{CO_2} (9.96) values for activated and raw bagasse differed little on a percentage basis. The low values obtained in the case of calcium alginate beads confirmed the diffusional limitations of nutrients and the possible hindrance posed by the polymeric matrix either in oxygen uptake or in carbon dioxide diffusion to the surrounding medium. These results indicated that raw and activated bagasse were superior immobilization systems to calcium alginate beads and these were used in all subsequent experiments.

Microscopic examination of immobilization on to raw and activated bagasse

The structural changes brought out by the chemical treatment of raw bagasse are evident in the scanning electron micrographs (Figures 2(a) and 2(b). Raw bagasse showed distinct sacs while it seems that the structure was largely lost during chemical treatment. The immobilized raw and activated bagasse exhibited a very high population of cells (Figures 2(c) and 2(d)). A less magnified micrograph of the activated bagasse shows a very high density of immobilized cells (Figure 2(e)).

Cell desorption

While it is difficult to determine the exact binding forces (conservative, electrostatic and solvent mediated forces) for a biochemical system, the main interest was in observing the cell desorption from the immobilized carrier. The two immobilized cell preparations were examined under equivalent experimental conditions to determine the critical sloughing off velocity. This is defined as the minimum feed flow rate which results in cell desorption. A feed rate greater than this leads to a significant quantity of cells being washed from the carrier. Experiments conducted at different flow rates showed that the fast dislodgement of cells from the raw carrier occurred at much lower feed velocities and the amount of cells dislodged was less in the case of activated carrier (Figure 3). The reduction in cell retention was 13·2% and 42·1% for the activated and raw carriers, respectively. The critical sloughing-off velocity was found to be 66% lower for raw carrier as compared to activated carrier. This suggests that a greater hydrodynamic velocity is required to strip off the cells from the activated carrier due possibly to ionic bonding between cells and carrier. For both the immobilized carriers, the cell concentration in the exit stream declined with increasing flow rate. This may be due

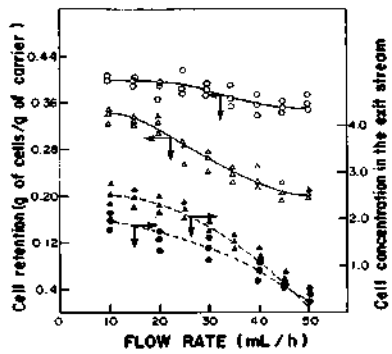

Fig. 3. Desorption of cells immobilized on to raw and activated bagasse at varying flow rates (S_F = 150 g l^{-1}; σ = 42 g l^{-1}). ○, ●: activated bagasse; △, ▲: raw bagasse.

Table 2. *S. cerevisiae* cell retention on to various carriers by adhesion to surface method.

Carrier	*Cell retention* (*g cells g carrier*$^{-1}$)	*Reference no.*
Ion exchange resin	0·133	12
Carrier A	0·380	13
PVC-flake, 1·0 cm diameter, 1·0 cm length	0·253	13
Wood chips	0·188	14
Ceramic	0·0162	14
Natural origin substrate	0·132	2
Spruce sawdust	$2·5 \times 10^{8}$*	15
Activated carbon	$1·4 \times 10^{8}$*	15
Crushed brick	$2·4 \times 10^{8}$*	15
Crushed firebrick	$10·9 \times 10^{7}$*	15
Raw bagasse	0·346†	This work
Activated bagasse	0·410†	This work

*. Expressed as number of cells per g carrier.
†. At 30 °C.

to the restricted conditions prevailing in the system and the insignificant desorption of cells from the carrier even at increased feed flow rates. The maximum cell loading on to the carrier was found to be higher in the case of activated bagasse when compared to raw bagasse and also to other immobilized preparations reported in the literature (Table 2). This evidence indicates that activated bagasse is a better support than raw bagasse and hence further studies were conducted using activated bagasse for cell immobilization.

Kinetics of cell immobilization

The kinetics of cell immobilization were established at different temperatures to evaluate the time for accomplishing the maximum cell loading. Such studies were later used to compute the Gibbs free energy and activation energy to assess the nature of cell immobilization.

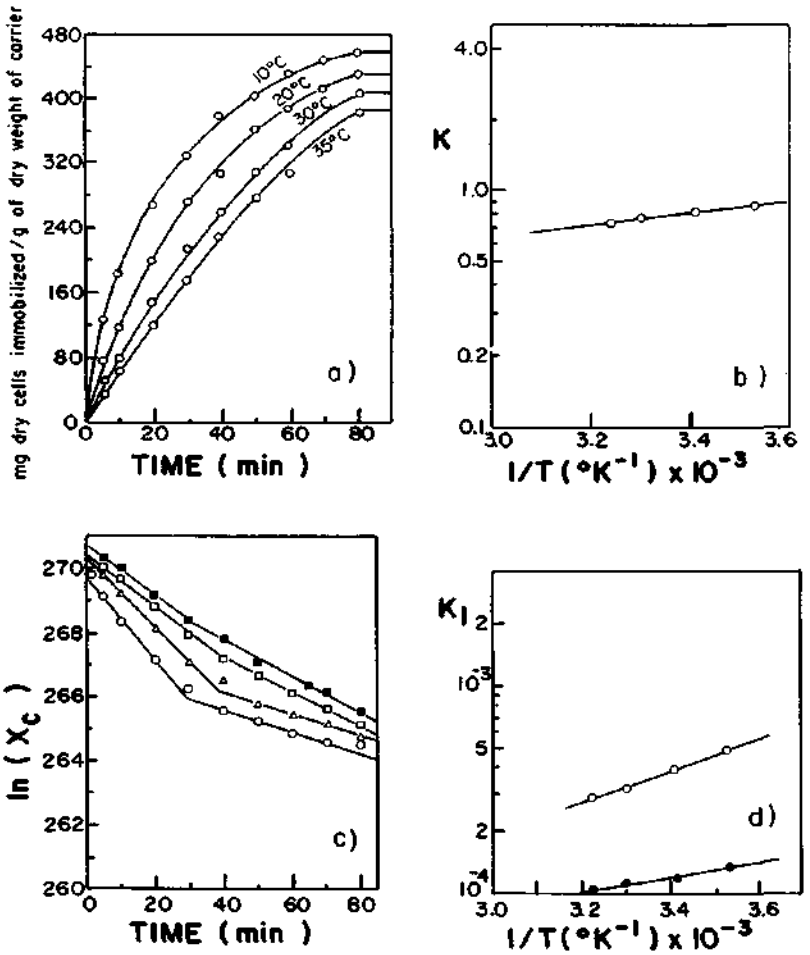

Fig. 4. (a) Cell immobilization kinetics at different temperatures. (b) Plot of $\ln K$ versus $(1/T)$ according to Eqn (3). (c) Plot of $\ln(X_c)$ versus time at different temperatures; ○: 10 °C; △: 20 °C; □: 30 °C; ■: 35 °C. (d) Plot of $\ln K_1$ versus $1/T$ on semilog scale, according to Eqn (4); ○: first stage of Figure 3(c); ●: second stage of Figure 3(c).

Immobilization was carried out for varying time intervals by agitating 1·0 g of carrier with cell suspensions at different temperatures. The adsorption process followed first-order kinetics with two distinct phases. The matrix was fully saturated with the cells in approximately 80 min at all temperatures, though 50% of the maximum loading was attained in about 30 min (Figure 4(a)).

Gibbs free energy (heat of adsorption) and activation energy computation

To determine the standard heat of reaction, considering the process taking place during adsorption as:

$$X_i \rightleftharpoons X_c + X_r \qquad (1)$$

where X_i is the initial free cell mass (g); X_c is the cell mass on the carrier (g); and X_r is the remaining free cell mass (g). The equilibrium constant K for the adsorption reaction can be expressed as:

$$K = \frac{(X_c)(X_r)}{(X_i)} \qquad (2)$$

where terms in parenthesis indicate the concentration of the respective species at equilibrium. The basic relationship between equilibrium constant (K) and Gibbs free energy (ΔH^0) can be expressed by the Vont Hoff equation:[16]

$$\Delta H^0 = -RT \ln (K) \qquad (3)$$

where R is a gas constant. A plot of $\ln K$ against $1/T$ (k^{-1}) gives the value of ΔH^0, which is calculated as 3273·45 J g mol^{-1} adsorbent (Figure 4(b)). The rate of adsorption can be related to reaction temperature by the Arrhenius equation in logarithmic form as:

$$\ln K_1 = -E_{act}/RT + \ln A \qquad (4)$$

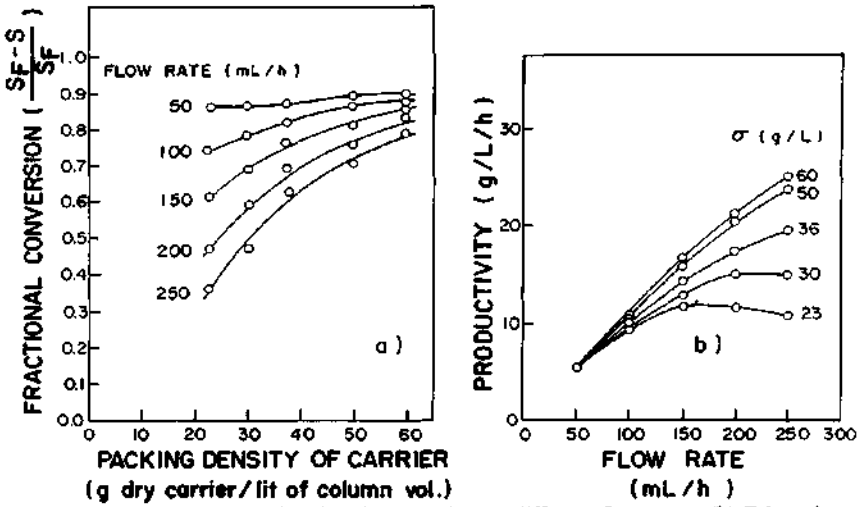

Fig. 5. (a) Effect of packing density on the fractional conversion at different flow rates. (b) Ethanol productivity as a function of packing density at various flow rates.

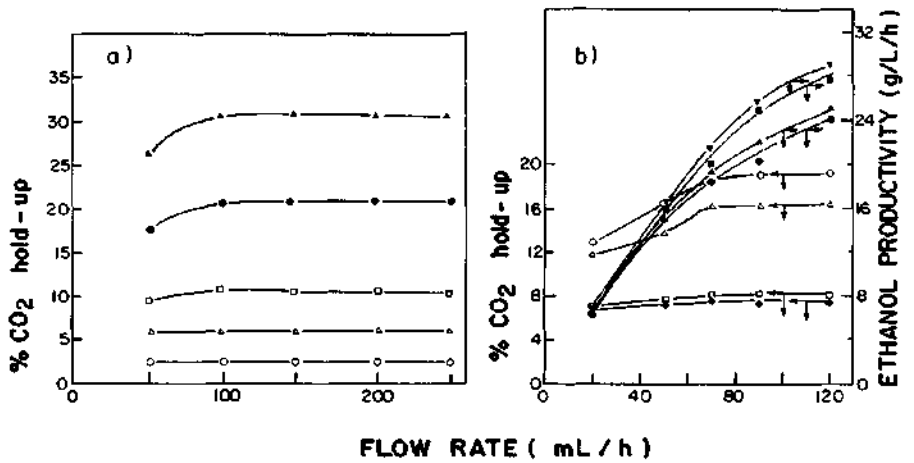

Fig. 6. (a) Gas hold-up at different packing densities at varying flow rates: ○: $\sigma = 23$ g l^{-1}; △: $\sigma = 30$ g l^{-1}; ●: $\sigma = 50$ g l^{-1}; ▲: $\sigma = 60$ g l^{-1}; □: $\sigma = 36$ g l^{-1}. (b) Effect of H/D ratio on gas hold-up and ethanol productivity at various feed flow rates; ○, ●: $H/D = 10$; △, ▲; $H/D = 5$; □, ■: $H/D = 2{\cdot}5$; ◆, ▼: $H/D =$ 1·5.

where K_1 is the adsorption constant (min^{-1}). A plot of $\ln K_1$ against $1/T$ (K^{-1}) gives the value of activation energy (E_{act}, J g mol^{-1}). The curve of $\ln(X_c)$ versus time showed two distinct stages, indicating that the immobilization occurred in two phases (Figure 4(c)). First, the cells are held by the free active sites of the carrier and then further attachment of the cells takes place on to the previous cell layer (multilayer cell immobilization). The activation energies were computed to be 14621·27 and 6336·34 J g mol^{-1} for the first and the second stages, respectively (Figure 4(d)).

The low values of ΔH^0 and E_{act} indicate that the nature of attachment of cells to the carrier is ionic and physical adsorption so that adsorption equilibria are established rapidly. A positive heat of reaction for immobilization indicates the mild endothermic nature of cell–carrier and cell–cell interactions.

Continuous bioconversion in a packed bed bioreactor

The bioreactor configuration was studied in relation to the packing density of the carrier (σ, carrier dry weight per unit reactor volume) and height to diameter ratio (H/D). Productivity was calculated on the basis of total reactor volume.

Packing density

The results obtained by conducting experiments at varying packing densities show that the fractional conversion (ε) decreased with increasing feed rate at all packing densities (Figure 5(a)). Fractional conversion was calculated by dividing the sugar converted in the bioreactor with feed sugar, i.e. $(S_F - S_i)/S_F$. It was noted that on increasing the medium flow rate from 50 to 250 ml h^{-1} the reduction in fractional conversion was 13·3% and 61·8% at $\sigma = 60$ g l^{-1} and at $\sigma = 23$ g l^{-1}, respectively, indicating a much larger loss in productivity at lower packing density. The ethanol productivity increased with flow rate at all packing densities except $\sigma \leqslant 30$ g l^{-1} (Figure 5(b)), mainly due to significant backmixing resulting in dislodgement of cells from the matrix. It was also noted that the ethanol productivities did not differ significantly at the packing densities of 50 and 60 g l^{-1}. This is attributed to increased gas hold-up at higher packing densities thus reducing the working volume. This is in agreement with the data in Figure 6(a). The gas hold-up is not influenced significantly by medium flow rate and beyond 100 ml h^{-1} it remained constant (Figure 6(a)). Since only a marginal difference in productivity resulted from increasing the packing density beyond 50 g l^{-1} it was thus considered optimal, and hence further studies were conducted at $\sigma = 50$ g l^{-1}.

Height (H) to diameter (D) ratio

Experiments were conducted using different height (H) to diameter (D) ratios of bioreactors with the same volume (200 ml). A decrease in the H/D ratio resulted in the reduction of the gas hold-up until a H/D of 2·5, beyond which there was no appreciable reduction (Figure 6(b)). The productivity data at $H/D < 2{\cdot}5$ confirmed the above observation. The reduction in gas hold-up on reducing H/D can be explained as a wider cross-sectional area facilitated better gas passage and a lower length to be traversed by gas before disengagement. Attempts have been made to reduce gas hold-up by modifying the reactor design,[17] but such designs are too cumbersome to operate at large scale. In a comparative study between horizontal (lower H/D ratio) to vertical packed bed bioreactor, Shiotani and Yamane[18] obtained 1·5 times more productivity for a horizontal bioreactor compared to a vertical one. These results were explained on the basis of reduced 'CO_2 gas phase effect'. Karanth[19] presented a model to account for the 'gas phase effect' and analysed the results of other studies.[18,20] He successfully

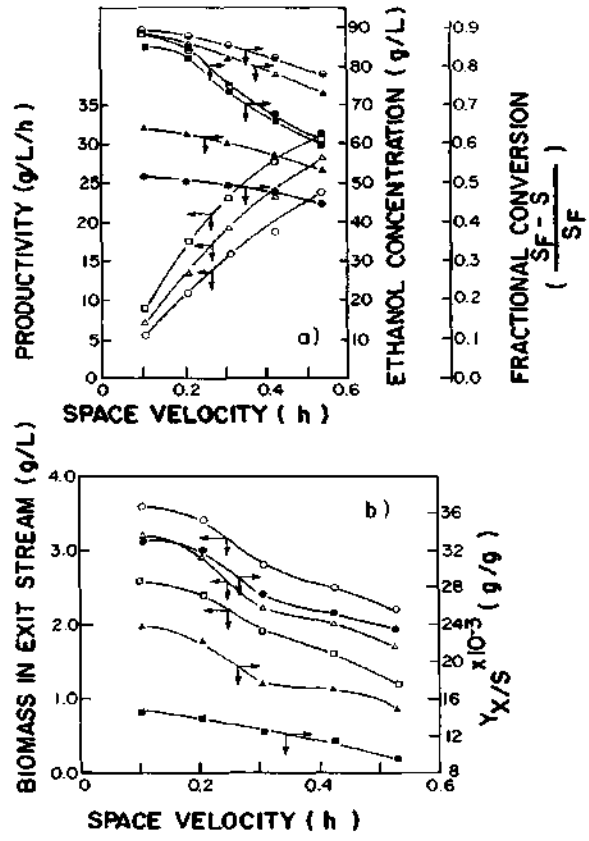

Fig. 7. (a) Immobilized cell bioreactor performance at various feed sugar concentrations and space velocity. $S_F = 120$ g l^{-1}; ○: productivity; ◒: fractional conversion; ●: ethanol concentration. $S_F = 150$ g l^{-1}; △: productivity; ▲: fractional conversion; ▲: ethanol concentration. $S_F = 200$ g l^{-1}; □: productivity; ◪: fractional conversion; ■: ethanol concentration. (b) Effect of space velocity on cell concentration and cell yield; ○, ●: $S_F = 120$ g l^{-1}; △, ▲: $S_F = 150$ g l^{-1}; □, ■: $S_F = 200$ g l^{-1}.

explained the results of both these reports and confirmed a decrease in productivity along the length of the bioreactor. This suggests that a long bioreactor should be split into several sections (reduction in H/D ratio). The present results are consistent with other reported experimental observations. Based on the above results, a H/D ratio of 2·5 was found to be the optimum and hence subsequent studies were carried out at $H/D = 2{\cdot}5$.

Reactor performance

The system was operated at various space velocities with feed sugar concentrations (S_F) 120, 150 and 200 g l^{-1}. The reactor performance is illustrated in Figures 7(a) and 7(b) in terms of ethanol and biomass concentrations in the exit stream, productivity, fractional conversion, and yield of bio-

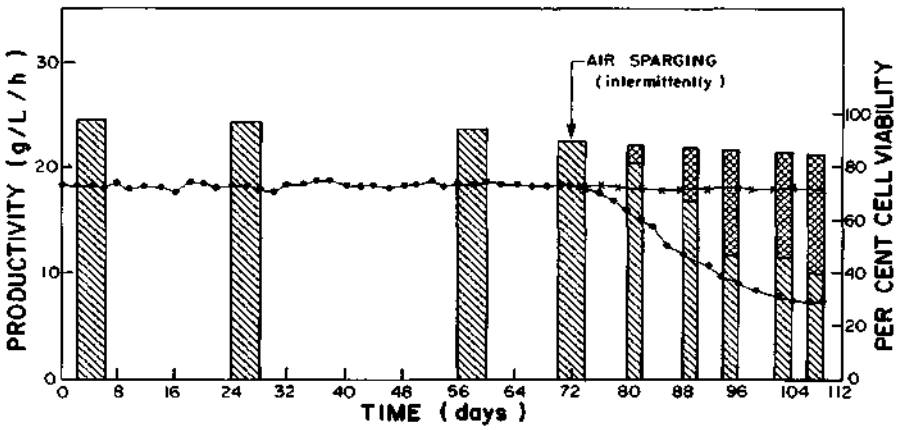

Fig. 8. Operational stability of the immobilized cell bioreactor (S_F = 200 g l^{-1}, T_i = 1·89 h); ●: productivity; ×: productivity (beyond 76 days with intermittent air sparging); ▨: per cent viability; ▩: per cent viability beyond 76 days with intermittent air sparging.

mass ($Y_{X/S}$) as a function of space velocity. At a particular dilution rate, higher productivities were obtained with increasing feed sugar concentration while fractional conversion declined. Since an appreciable part of the sugar appeared in the exit stream beyond a space velocity of 0·53 h^{-1} the system was not operated at higher values. The maximum productivity achieved was 31·8 g l^{-1} h^{-1} with S_F = 200 g l^{-1} at the space velocity of 0·53 h^{-1}. Considering the productivity data, this system performed better than those reported earlier using supports (carriers A and B),[19] natural origin[21] and bagasse.[20]. The high productivity obtained is basically a combined result of the use of different modifications such as the use of activated carrier, reduced gas hold-up and method employed for molasses clarification.

The cell yield coefficient ($Y_{X/S}$) was lower over the range of operating conditions used and with increasing feed sugar concentration and space velocity (Figure 7(b)). Such low values indicated restricted growth of the cells in the immobilized state under anaerobic condition and presumably because the cells are under stress in a packed bed system. The product yields, $Y_{P/S}$, remained almost unaffected either by feed sugar concentration or space velocity. An average value of 0·482 g g^{-1} was observed indicating nearly 95% of theoretical conversion.

System stability

The reactor was operated under steady state conditions for 76 days, being operationally stable and requiring no extra cell input (Figure 8). Beyond this period, a decline in productivity was observed due to a decrease in immobilized cell viability. The cell viability and hence productivity could be restored and maintained by sparging air intermittently. Beyond 107 days, the system's stability could not be maintained as a gradual deterioration of carrier matrix appeared which eventually resulted in the loss of cell loading on to the carrier. The system was shut down at this point. The present system is superior in terms of operational stability

Table 3. Operational stability of immobilized cell systems.

Organism	*Substrate*	*RT* (*h*)	*PD* (*g* l^{-1} h^{-1})	*P* (*g* l^{-1})	*OS* (*days*)	*Reference no.*
S. cerevisiae	Grape juice	3·125	18·0	56·25	30·0	22
Z. mobilis	Glucose	1·2	53·0	63·0	≈ 33·0	23
S. carlbergensis	Glucose	2·5	40·0	100·0	≈ 88·0	24
S. cerevisiae	Molasses	2·86	24·9	71·1	75·0	13
S. cerevisiae	Molasses	—	20·0	80–90	180·0	25
S. cerevisiae	Molasses	1·89	31·80	60·1	76 & 107*	This work

RT. Residence time; PD. Productivity of ethanol; *P*. Ethanol concentration; OS. Operational stability.
*. Operational stability with intermittent air sparging.

to all systems reported in the literature except the report of Nagashima *et al.*[25] (Table 3) who used calcium alginate beads. However, the ethanol productivity (20 g l^{-1} h^{-1}) obtained by these workers is much lower than the 31·8 g l^{-1} h^{-1} obtained in the present work. Consequently, a smaller bioreactor size is required compared to that of Nagashima *et al.*[25]

CONCLUSIONS

The following conclusions may be drawn from the foregoing study:

—Higher specific oxygen uptake rate and specific CO_2 evolution rate were obtained with cells immobilized on to raw and activated bagasse than in calcium alginate beads.

—Activated bagasse has a higher cell loading than other reported systems.

—Cell desorption occurred at much higher rates in activated bagasse (i.e. the critical sloughing-off velocity was 66% higher than raw bagasse).

—The amount of cells dislodged was low in the case of activated bagasse.

—Low values of ΔH^0 and E_{act} suggest that cell attachment on to bagasse are due to ionic bonding and physical adsorption.

—The optimum packing density to achieve maximum productivity of ethanol was found to be 50 g of carrier per litre of reactor volume.

—A ratio of $H/D = 2{\cdot}5$ was found to be optimum to achieve maximum ethanol productivity and minimum gas hold-up.

—A maximum ethanol productivity of 31·8 g l^{-1} h^{-1} at a space velocity of 0·53 h^{-1} was obtained.

—The yield of product was constant with varying space velocity and feed sugar concentration, whereas the yield of biomass decreased with space velocity.

—The immobilized cell reactor was stable in terms of ethanol productivity up to 76 days but cell viability and ethanol productivity could be maintained by sparging air intermittently up to 107 days.

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