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Immobilization of *Saccharomyces diastaticus* on Wood Chips for Ethanol Production

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ABSTRACT. *Saccharomyces diastaticus* cells were immobilized onto beech wood chips of different particle size and three pH values. pH values in the range 5.0 – 6.0, and 1.84 – 1.92 mm particle size had a positive effect on the immobilization process. The chosen carrier – 1.84 mm-sized wood chips adsorbed 150 mg dry cell mass per g dry carrier mass. The Gibbs free energy and the activation energy for the first (monolayer) and second (multilayer) immobilization stages was 4581, 19090 and 8590 J g mol^{-1}, respectively. The kinetics of immobilized cell systems in ethanol production have been studied in a packed bed-reactor. Ethanol production and the respiration quotient (RQ) were at a maximum at a dilution rate of 0.16/h. The reactor was operated under steady-state conditions for 30 d at the dilution rate 0.16/h.

The production of ethanol by fermentation has received special attention as a consequence of the world energy crisis which enhanced the interest in renewable energy sources (Ghose and Tyagy 1979; Larsson and Mosbach 1979). As a result, there is growing interest in the utilization of starch for the production of ethanol. Many yeasts are known to grow on starch and ferment it (Lodder 1970). Recently the possible usage of some amylolytic yeasts for a one-step process of starch fermentation has been investigated (Calleja *et al.* 1982; Frelot *et al.* 1982; Laluce and Mattoon 1984). Direct alcoholic fermentation of starchy biomass has been studied using amylolytic yeast strains in batch and immobilized cell systems (Amin *et al.* 1985). *Saccharomyces diastaticus* has glucoamylase activity and has the ability to assimilate and ferment starch. The alcohol-producing capabilities of *Saccharomyces diastaticus* have not been studied extensively although there are a few reports (Duvnjak and Kosaric 1981; Laplace *et al.* 1993*a,b*) on the production of ethanol from different substrates by this yeast. One of the recent technologies used to improve the economics of ethanol fermentation is the use of immobilized biocatalysts and bioreactors. Immobilized cell reactors offer several advantages over batch type reactors, including higher production rates, continuous operation, and better conversion efficiencies. Imobilized cells show various modifications in physiology and biochemical composition when compared to suspended cells (Bärbel and Rehm 1990). The long-term effect of ethanol on the performance of an immobilized cell reactor, cell growth and death rates has been studied (Chen *et al.* 1990; Dale *et al.* 1990). Different types of immobilization techniques and carriers were used for the purpose (Masschelein *et al.* 1994; Divies *et al.* 1994). Despite many reports on ethanol-producing immobilized cell systems there has been no systematic study of process engineering parameters. We have studied the immobilization of *S. diastaticus* by adsorption on beech wood chips (Lamptey *et al.* 1981). The purpose of this paper is to report the results of a quantitative investigation of key factors (particle size, pH and critical flow velocity capable of creating hydrodynamic forces sufficient to detach the cells from the support) which influence the degree of cell loading (mg dry cell mass per g support dry mass) obtained during the immobilization step. The kinetics of cell immobilization were established at different temperatures to evaluate the time needed for accomplishing maximum cell loading. This paper reports on the fermentation of starch for ethanol production using immobilized *S. diastaticus* cells in continuous flow reactors. The aim of these investigations was to study the effect of dilution rate on the ethanol production, ethanol yield and cell metabolic activities (carbon dioxide production and oxygen consumption) during fermentation of starch into ethanol.

MATERIALS AND METHODS

Microorganism and culture conditions. Pure culture of *Saccharomyces diastaticus* from the collection of the *Faculty of Technology* in Novi Sad was used. The pure culture was maintained on a slant with the same medium composition as used by Banerjee *et al.* (1988) and stored at 4 °C. Prior to experimental use, the cells were grown aerobically for 1 d in a 250-mL Erlenmeyer flasks containing 50 mL sterile medium each (pH 4.5; 5.0; 6.0) with the following composition (g/L): soluble starch

(*Merck*) 20, diammonium sulfate 2, potassium dihydrogen phosphate 1, magnesium sulfate 0.5, yeast extract 1. The concentration of cells was increased to 15 g dry mass per L by batch centrifugation.

Equipment, immobilization and fermentation process. The packed-bed reactor used for immobilization studies and ethanol production from starch consisted of a jacketed glass column 800 mm in height and 47 mm in diameter. Sample withdrawal ports were located along the height of the column at 115 mm intervals. During immobilization a concentrated cell suspension (10–15 g/L) was circulated from the bottom of the column by a peristaltic pump and recirculated through the packed column at different flow rates of 100–250 mL/h. The whole system was maintained at the desired test temperature. The column reactor together with the support material and connecting tubes was pre-sterilized in an autoclave at 121 °C for studies of cell loading, desorption of cells immobilized on support, effect of pH and support particle size on cell loading, and the kinetics of cell immobilization. A schematic experimental setup is shown in Fig. 1.

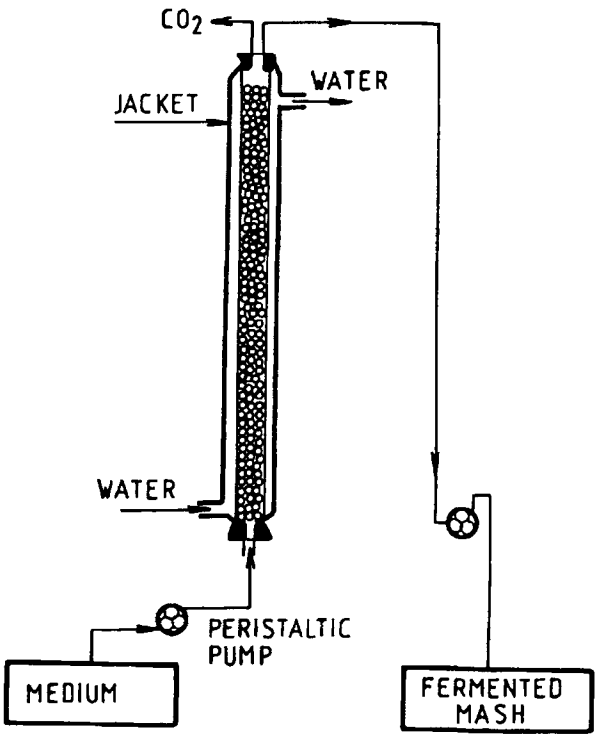

Fig. 1. Design of an experimental immobilized cell reactor.

Supports. The beech wood was used in the form of chips of various equivalent particle sizes. The chips were initially heat-treated in boiling water for 4 h followed by soaking in 10 % ethanol for 2 h and in boiling water for another 1 h to remove water and ethanol-soluble compounds from the wood. The equivalent particle size is defined as the diameter of a sphere with the same surface area as the chip. It is given by

$$d_p = w(d_c l_c + 0.5 d_c^2)^{0.5} \quad \text{(Satterfield 1970)},$$

where w is the shape factor, d_c is the diameter of the chip and l_c is the length of the chip.

Substrate preparation and fermentation. Experiments on ethanol fermentation were performed using 10 % soluble starch. The starch was solubilized by taking up 10 g starch in 100 mL boiling H_2SO_4 (pH 2.5) and then sterilized by autoclaving for 15 min at 121 °C. It was then cooled to room temperature and the pH aseptically adjusted to 6.0 by 4 mol/L NaOH. Other components of the medium were those used for cell growth. The fermentation was carried out in immobilized-cell reactor at 37 °C. The liquid medium was pumped from the bottom of the column by a peristaltic pump at different dilution rates (D) defined by the period spent in the reactor unit, and then operated at a constant dilution rate to determine the operational stability of immobilized cell systems. The product was recycled through another peristaltic pump at the top of the column.

Analytical methods. The concentration of suspended cells was measured in a spectrophotometer at 660 nm (Chen *et al.* 1990*b*). Ethanol concentration was determined by gas chromatography

(Varian-409). A column packed with Porapack Q (80 mesh) was used with a flame ionization detector. Both the injector and the detector were kept at 250 °C and the column oven operated isothermally at 150 °C. Nitrogen was used as the carrier gas at a flow rate of 30 mL/min. Total reducing sugars were determined by 3,5-dinitrosalicylic acid (DNS). The residual starch was hydrolyzed to glucose by treatment with 4 mol/L HCl at 90 °C for 2 h and the glucose was then determined by the DNS method (Miller 1959). Cell carbon dioxide production and oxygen uptake rate were measured in Warburg respirometer (Umbreit *et al.* 1972).

RESULTS AND DISCUSSION

The effects of pH and wood chip particle size on cell loading onto wood chips are shown in Fig. 2. It is evident that there is a positive effect of pH and wood chip particle size on the amount of cells adsorbed in the range 5.0–6.0 and 1.84–1.92 mm, respectively. However, as the size of the support is decreased from 3.18 to 1.84 mm, the cell loading increases significantly from about 110 mg dry mass per g support dry mass to about 150 mg/g support. This increase in cell loading is due to an increased availability of chip surface for immobilization (Moo-Young *et al.* 1980). The positive effect of pH on cell loading can be explained in the fact that the optimum initial pH of the medium for *S. diastaticus* ranged from 5.0 to 6.7 for ethanol biosynthesis (Duvnjak and Kosaric 1981). The cell metabolic activity data for *S. diastaticus* growth at different pH values confirmed the above observation (Table I). The values for specific oxygen uptake and specific carbon dioxide release are linearly dependent on the pH values as expected. The respiration quotient (RQ) decrease shows that the oxidative way of glucose degradation is intensified since the respiration quotient is the value of the ratio between energy production and efficiency of biosynthesis. This quotient appears to be a function of the citrate cycle and respiratory chain activity (Dekkers *et al.* 1981). These results indicated that wood chips were a favorable immobilization system, and they were used in all subsequent experiments.

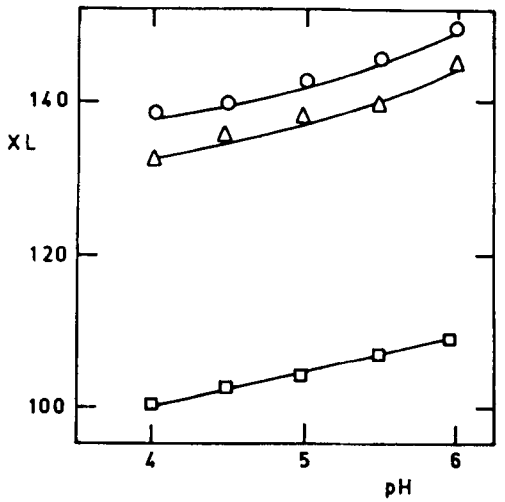

Fig. 2. Effect of pH and wood chip particle size on cell loading (XL, mg cells per g carrier). Particle size: *circles* – 1.84 mm, *triangles* – 1.92 mm, *squares* – 3.18 mm. Flow rate 100 mL/h.

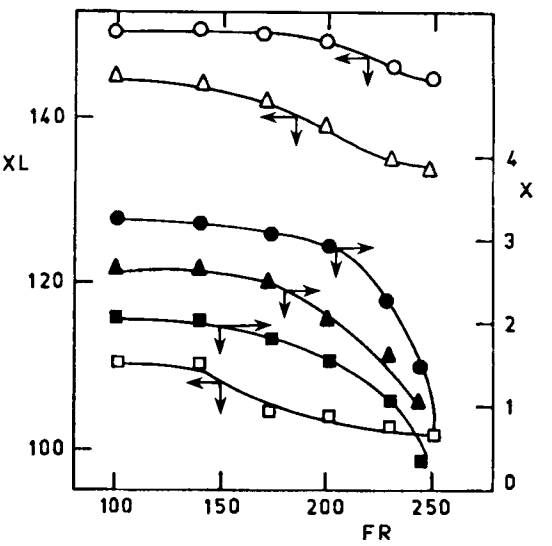

Fig. 3. Desorption of cells immobilized on different-sized wood chips at various flow rates (FR, mL/h). Cell loading (XL, mg dry cells per g dry carrier mass); Cell concentration in the exit stream (X, g/L); *circles* – 1.84 mm particles, *triangles* – 1.92 mm particles, *squares* – 3.18 mm particles; pH 6.0.

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 critical sloughing-off velocity was examined. This is defined as the minimum feed flow rate which results in cell desorption. A feed rate greater than the critical velocity will result in a significant amount of cells being washed off the carrier. Experiments conducted at different flow rates and pH 6 showed that the fast dislodgement of cells from the carrier with a larger particle size, 1.94 and 3.18 mm, occurred at much lower feed velocities, and the amount of cells dislodged was less in the case of a smaller-size carrier (1.84 mm particle size) (Fig. 3). The reduction in

cell retention was 5 and 8 % for the carrier with 1.84 mm particle size and 1.92 and 3.18 mm particles size, respectively. The critical sloughing-off velocity was found to be 20 and 45 % lower for a carrier with 1.92 and 3.18 mm particle size, respectively, as compared with a carrier with 1.84 mm particle size. This suggests that a greater hydrodynamic velocity is required to strip off the cells from the carrier with 1.84 mm size as compared with 1.92 and 3.18 mm carrier. For all wood chip particle sizes, the cell concentration in the exit stream declined with increasing flow rate. This may be due to the restricted conditions prevailing in the system and the insignificant desorption of cells from the carrier even at increased feed flow rates (Tyagi *et al.* 1992).

Table I. The metabolic activity of *S. diastaticus* during growth at different pH values[a]

pH	Specific O_2 uptake rate (Q_{O2}) mL min^{-1} mg^{-1}	Specific CO_2 production rate (Q_{CO2}) mL min^{-1} mg^{-1}	Respiration quotient Q_{CO2}/Q_{O2}
4	18.90	17.61	0.932
5	18.20	16.47	0.905
6	17.60	14.52	0.825

[a]Maximum attainable values of specific O_2 uptake rate and specific CO_2 production rate were determined after yeast harvesting.

During immobilization, the concentrated suspension of cells was recirculated through the packed column 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. The adsorption process followed first order kinetics with two distinct phases. The matrix was fully saturated with the cells in approximately 10 h at all temperatures (Fig. 4A).

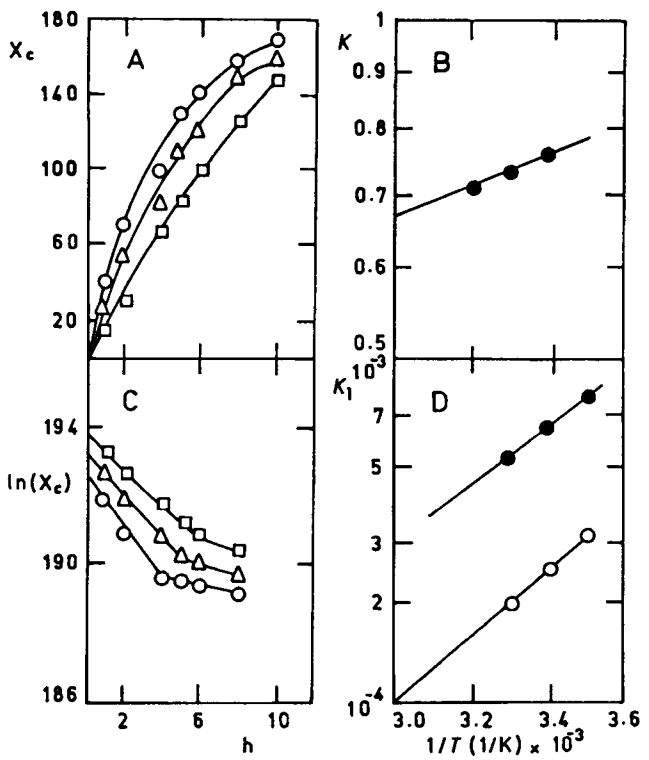

Fig. 4. A: Cell immobilization kinetics at different temperatures (X_c, mg immobilized dry cell per g dry carrier mass); B: Plot of ln$K$ *vs.* $1/T$, (1/K) $\times 10^{-3}$; C: Plot of ln(X_c) *vs.* time at different temperatures; *circles* - 10 °C, *triangles* - 20 °C, *squares* - 30 °C; D: Plot of lnK_1 *vs.* $1/T$, (1/K) $\times 10^{-3}$.

To determine the standard heat of reaction we considered the process taking place during adsorption as:

$$X_i \rightleftharpoons X_c + X_r$$

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:

$$K = ([X_c] + [X_r])/[X_i]$$

where terms in brackets indicate the concentration of the respective species at equilibrium (Tyagi *et al.* 1992). The basic relationship between the equilibrium constant (K) and Gibbs free energy (ΔH^0) can be expressed by the Vant-Hoff equation (Smith and Van-Ness 1975).

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

where R is a gas constant. A plot of ln$K$ against $1/T$ (1/K) gives the value of ΔH^0, which was calculated at 4581 J g mol^{-1} sorbent (Fig. 4B). The rate of adsorption can be related to reaction temperature by the Arrhenius equation in a logarithmic form:

$$\ln K_1 = E_{act}/RT + \ln A$$

where K_1 is the adsorption constant (1/min). A plot of lnK^{-1} against $1/T$ (K^{-1}) gives the value of activation energy E_{act} (J g mol^{-1}). The curve of ln(X_c) *vs.* time showed two distinct stages, indicating that the immobilization occurred in two phases (Fig. 4C). First, the cells are held by the free active sites of the carrier and then further attachment of the cells takes place on top of the previous cell layer (multilayer cell immobilization). The activation energies were computed to be 19.09 and 8.59 kJ g mol^{-1} for the first and the second stage, respectively (Fig. 4D).

The low values of ΔH^0 and E_{act} indicate that the nature of attachment of cells to the carrier is physical adsorption, *i.e.* adsorption equilibria are established rapidly. A positive heat of reaction for immobilization indicates the mild endothermic nature of the cell-carrier and cell-cell interaction (Tyagi *et al.* 1992).

Continuous bioconversion of starch into ethanol was studied using immobilized *S. diastaticus* on wood chips as carrier. Ethanol concentration, ethanol yield and unutilized starch as a function of dilution rate in an immobilized cell bioreactor is shown in Fig. 5. Ethanol production increased between dilution rates 0.125/h and 0.16/h but started to decrease as the dilution rate was increased above 0.16/h. Ethanol yield was at a maximum at a dilution rate of 0.16/h and decreased rapidly at dilution rates above 0.16/h. These results are in agreement with those published by Debnath *et al.* (1990) who used alginate to immobilize *S. diastaticus* cells for ethanol production from starch.

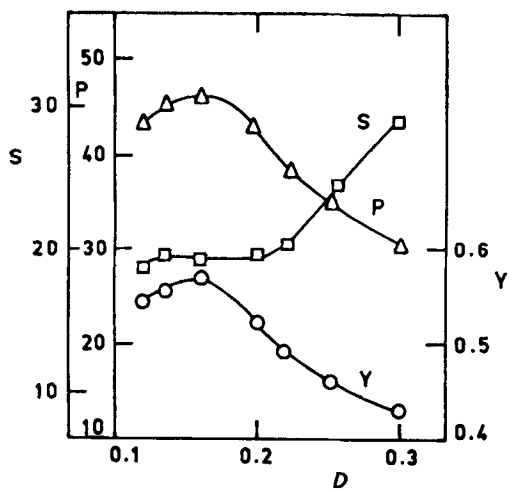

Fig. 5. Ethanol concentration (P, g/L), ethanol yield (Y, g/g utilized starch) and unutilized starch (S, g/L) as a function of dilution rate (D, 1/h) in an immobilized cell bioreactor; *triangles* – ethanol concentration, *circles* – ethanol yield, *squares* – unutilized starch. Starch content 100 g/L; pH = 6.0.

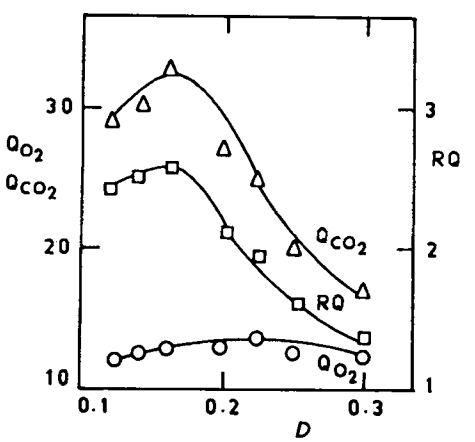

Fig. 6. Dependence of specific oxygen uptake rate (Q_{O2}), specific CO_2 production rate (Q_{CO2}, both in mL g^{-1} h^{-1}) and respiratory quotient (RQ) on the dilution rate (D, 1/h); *circles* – specific oxygen uptake rate, *triangles* – specific CO_2 production rate, *squares* – respiration quotient. Starch content 100 g/L; pH = 6.0.

The dependence of the specific oxygen uptake rate (Q_{O2}), specific CO_2 production rate (Q_{CO2}) and respiration quotient (RQ) on dilution rate D is given in Fig. 6. The values of specific carbon dioxide release were dependent on the dilution rate. The carbon dioxide production (Q_{CO2}) increased at dilution rates of 0.125–0.16/h but started to decrease as the dilution rate was increased above 0.16/h. The specific oxygen uptake rate (Q_{O2}) remained almost constant at D = 0.10–0.3/h. The respiration quotient (RQ) attained a maximum value of 2.6 at D = 0.16/h and decreased when the dilution rate exceeded 0.16/h. At all investigated dilution rates the values of the respiration quotient were RQ > 1. This showed that the fermentative way of substrate degradation is intensified since the respiration quotient is the value of the ratio between energy production and efficiency of biosynthesis (Pejin and Razmovski 1993).

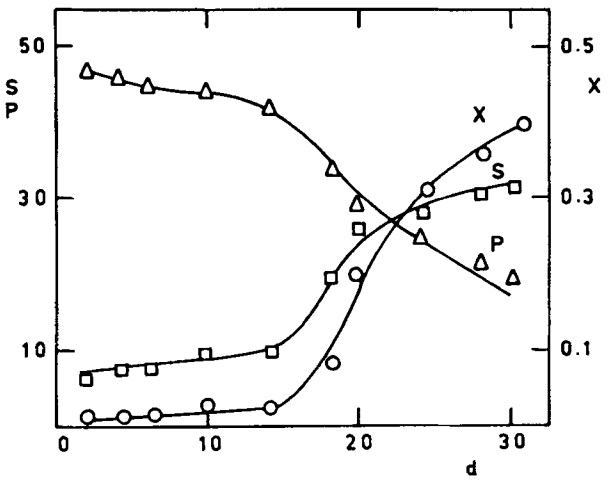

Fig. 7. Ethanol concentration (P, g/L), unutilized starch (S, g/L) and cell concentration in the exit stream (X, g/L) as a function of fermentation time (d) in an immobilized cell bioreactor; *triangles* – ethanol concentration, *circles* – cell concentration in the exit stream, *squares* – unutilized starch. Starch content 100 g/L. Dilution rate D = 0.16/h; pH = 6.0.

Fig. 7 gives the ethanol concentration, unutilized starch, and cell concentration in the exit, stream of an immobilized cell bioreactor as a function of fermentation time. For a period of 14 d the ethanol production by immobilized cells remained almost constant and then it started to decrease in parallel with an increase in unutilized starch concentration in the effluent. The low cell concentration in the exit stream up to the fourteen-day time point indicated that the immobilized system was very stable. The reactor was operated under steady state conditions for 30 d at the dilution rate D = 0.16/h.

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