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The use of monochloroacetic acid for improved ethanol production by immobilized *Saccharomyces cerevisiae*

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***Saccharomyces cerevisiae* adsorbed on acid-treated glass beads produced 5.4 g l^{-1} ethanol at 96 h in batch process. Precoating the acid-treated glass beads with gelatin (25 g l^{-1}) before immobilization of the cells increased ethanol production to 26.3 g l^{-1} at 72 h. Cell leakage into the medium was decreased when the immobilized cells were cross-linked with increasing concentrations of glutaraldehyde (0–100 g l^{-1}). Although monochloroacetic acid inhibited ethanol production and cell multiplication at concentrations greater than 0.1 g l^{-1}, at a concentration of 0.01 g l^{-1} it not only increased the ethanol production to 52.1 g l^{-1} but also shortened the ethanol production time to 48 h. In a semi-continuous batch process with the feed containing 0.01 g l^{-1} monochloroacetic acid, immobilized cells showed no significant change in ethanol-producing ability for 40 days when incubated with nutrient medium intermittently.**

Key words: Glutaraldehyde, immobilization, monochloroacetic acid, *Saccharomyces cerevisiae*.

Glass supports in different forms (Navarro & Durand 1977; Rouxhet *et al.* 1984; van Haecht *et al.* 1984; van Haecht *et al.* 1985; Parascandola *et al.* 1987) have been reported as suitable for the immobilization of yeast cells. To improve the immobilization of cells by adhesion, either the support was modified or the property of the cell was altered. Glass was modified by coating with $Al(OH)_3$ or Fe_2O_3 (van Haecht *et al.* 1985) or gelatin and treating with formaldehyde or glutaraldehyde. The adhesion property of cells was altered by starving them (van Haecht *et al.* 1984). Adhesion of the yeast cells to porous glass altered their metabolic behaviour and improved ethanol production (Navarro & Durand 1977).

Improved ethanol production by yeast was achieved either by using mutants having high pyruvate decarboxylase and alcohol dehydrogenase activities (Sharma & Tauro 1986) or by an extraction system to remove the product (Lee & Wang 1982) thereby avoiding product inhibition. Metabolic inhibitors such as dinitrophenol (Amin *et al.* 1984), azide (Hahn-Hägerdal & Mattiasson 1982; Hahn-Hägerdal *et al.* 1986) and arsenate (Amin *et al.* 1984) were used to reduce biomass and increase product formation.

In this paper, cross-linking of *Saccharomyces cerevisiae* immobilized on to glass beads using glutaraldehyde, and the effect of monochloroacetic acid on ethanol production are reported.

Materials and Methods

Organism

A locally isolated strain of *Saccharomyces cerevisiae* was used.

Materials

Glass beads, undrilled (0.5 mm diameter, BDH Chemical Company, UK) were used. All the chemicals used in these studies were of analytical grade.

Cultivation of Saccharomyces cerevisiae

Cells were grown before immobilization in a 5-l fermentor containing nutrient medium having the following composition in g l^{-1} tap water: sucrose, 100; yeast extract, 4.0; $(NH_4)_2SO_4$, 1.0. Growth was at 35 °C and pH 4.5. Cells were harvested in the late exponential phase of growth (18 h) by centrifugation (10 min at 10,000 rev/min at 10 °C) and washing three times by suspend-

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ing in 300 ml normal saline followed by centrifugation. The cells were then suspended in distilled water for 24 h

Analytical Methods

Total cell number in the medium was determined using a haemocytometer. The samples were filtered through a 0.45 μm pore size membrane (Millipore). Ethanol was estimated by GC (column contained 10% Carbowax^R 20M on 80/100 Chromosorb^R (w/w, stationary phase, Superleco park, Belletonue, USA; Larsson *et al.* 1988) and residual sugar in the filtrate by the dinitrosalicylic acid method (Miller 1959) after acid hydrolysis (Arasaratnam 1989).

Coating of Glass Beads with Gelatin and Immobilization of Yeast Cells

Sterile glass beads were washed with sterile distilled water and 5 M HNO_3 and kept in 5 M HNO_3 at 90 °C for 18 h. Treated beads were finally washed with double distilled water and kept in an oven at 105 °C. A portion of glass beads (25 g) was mixed with 25 g gelatin l^{-1} (250 ml) while the other portion (25 g) was treated with double distilled water (250 ml) and incubated at 30 °C overnight.

Treated glass beads (25 g) were mixed with yeast cells suspended in distilled water (100 ml, 26×10^6 cells ml^{-1}) for 24 h at 30 °C and then washed with distilled water. The amount of cells immobilized by adhesion to the beads was calculated from the difference between the amount of cells present in the suspension before and after immobilization. The number of cells present in the washing was also determined and considered for the above calculations.

Nutrient medium (250 ml) was inoculated with yeast cells immobilized to glass beads precoated with gelatin (25 g) and to glass beads uncoated with gelatin (25 g) and incubated at 35 °C and pH 4.5. Ethanol produced and cells leaked into the media were monitored.

Cross-linking of Immobilized Cells with Glutaraldehyde

The yeast cells immobilized to glass beads (25 g) were cross-linked with glutaraldehyde (Husain & Saleemudin 1986) of different concentrations (0.0, 25.0, 50.0, 75.0 and 100.0 g l^{-1}) at pH 7.0 and 4 °C for 18 h. The beads were washed with phosphate buffer saline (0.01 M phosphate buffer, pH 7.0–9.0 g l^{-1} NaCl). The nutrient medium (250 ml) was inoculated with cross-linked immobilized yeast cells (25 g) and immobilized yeast cells (25 g) as described above.

Effect of Monochloroacetic Acid on Ethanol Production

Nutrient medium was inoculated with immobilized yeast cells cross-linked with glutaraldehyde and incubated for 48 h at 35 °C. The immobilized cells were transferred to nutrient medium containing different concentrations of monochloroacetic acid (0.0, 0.01, 0.1, 1.0 and 10.0 g l^{-1}).

Semi-continuous Process for Ethanol Production

Nutrient medium was inoculated with yeast cells immobilized and cross-linked at optimized conditions and incubated for 48 h at 35 °C. The immobilized cells were transferred to nutrient medium containing an optimized amount of monochloroacetic acid. The fermentation was continued by replacing the spent medium with fresh medium at regular intervals. Whenever there was a decrease in ethanol production, the immobilized cells were treated with nutrient medium.

Results and Discussion

Effect of Gelatin on Immobilization

The number of yeast cells immobilized to acid-washed glass beads by physical forces was 20.8×10^7 cells/25 g beads and the total number of cells used for immobilization was 26×10^8 in 100 ml distilled water. Thus 8% of the total added cells were immobilized to the glass beads and this is equal to 8.32×10^6 cells/g beads. Van Haecht *et al.* (1984) reported that 8.96% of the total added cells were immobilized to glass plates. Thus our results are in good agreement with that reported previously. They concluded that the amount of cells brought into contact with the support was immaterial due to cell–cell and cell–support repulsion (van Haecht *et al.* 1984). The repulsion was due to the net negative charges on the cell and the support (Rouxhet *et al.* 1984). Such repulsion was reduced by modifying the cell membrane by starving the cells for 24 h (van Haecht *et al.* 1984). Starvation of the cells led to modifications of cell wall and release of ionic substances, which in turn decreased the electrostatic repulsion between the cells and support (van Haecht *et al.* 1984). We also suspended the harvested yeast cells in distilled water for 24 h to take advantages of such changes.

To increase the cell adhesion from 8.0%, acid-treated glass beads were precoated with gelatin. Total number of cells immobilized to 25 g of glass beads was 10.4×10^8. This is equivalent to 40% of the total cells added.

When the alcohol produced by the two immobilized cell preparations was compared, yeast immobilized to gelatin-precoated glass beads produced 4.9 times more alcohol than by the yeast immobilized to uncoated glass beads (Figure 1). This is in exact proportion with the number of cells immobilized to gelatin-precoated glass beads and uncoated glass beads. Highest ethanol production time for yeast cells immobilized to gelatin-precoated and uncoated glass beads was 72 h and 96 h respectively. Cell leakage into the medium is also related to immobilization efficiency (Figure 1). The glass beads used were undrilled and hence the cells only adhered to the surface of the beads unlike in porous glass beads (Navarro & Durand 1977).

Effect of Glutaraldehyde

Because cell leakage was very high with cells immobilized on gelatin-precoated glass beads, it was decided to attempt to fix the immobilized cells and the daughter cells more firmly onto the glass beads by cross-linking the immobilized cells with glutaraldehyde. Yeast cells immobilized to gelatin-precoated glass beads were cross-linked with different concentrations of glutaraldehyde (0.0–100.0 g l^{-1}). The nutrient medium was inoculated

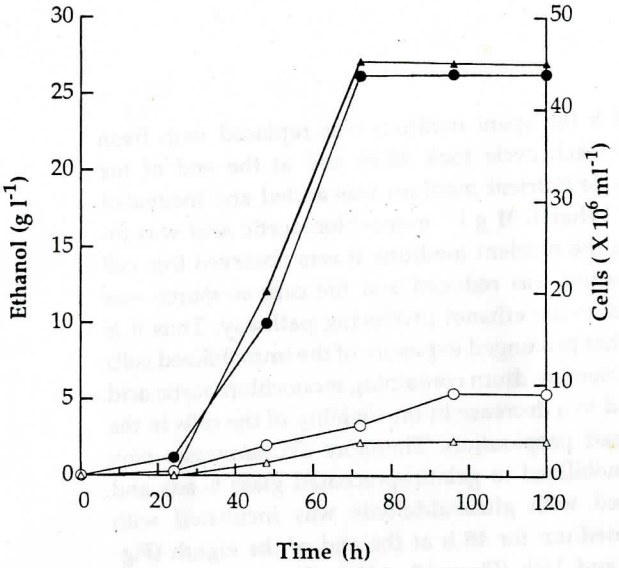

Figure 1. Fermentation of sucrose to ethanol by locally isolated *Saccharomyces cerevisiae* immobilized to (•) gelatin-precoated (test) and (○) uncoated (control) glass beads at pH 4.5 and 35 °C. *S. cerevisiae* cells leaked into the medium from cells immobilized to (▲) gelatin-precoated and (△) uncoated glass beads.

with the cross-linked immobilized cell preparations. The number of cells leaked into the medium decreased from 45.2×10^6 to 8.3×10^6 cell ml^{-1} (at 72 h) when the glutaraldehyde concentration used for cross-linking of immobilized cells was increased from 0.0 to 100.0 g l^{-1} (Figure 2). On the other hand when the glutaraldehyde concentration used for cross-linking was increased from 0.0 to 50.0 g l^{-1}, ethanol production also increased from 26.3 to 39.1 g l^{-1} (at 72 h, Figure 2) but further increase in glutaraldehyde concentration (up to 100 g l^{-1}) for cross-linking led to decreased ethanol production to 14.8 g l^{-1}. Increased cross-linking of the cells by glutaraldehyde may have either affected the cellular metabolism or retained the daughter cells and led to mass transfer problems which could have decreased ethanol production. Previous reports (Navarro & Durand 1977; Parascandola *et al.* 1984) showed that treatment of the supports with glutaraldehyde prior to immobilization of yeast had inhibited the cellular activity. Use of other cross-linking agents such as polyacrylamide also inhibited cellular metabolism (Siess & Divies 1981; Pines & Feeman 1982). In our studies, even though glutaraldehyde (50.0–100.0 g l^{-1}) had decreased ethanol production, cellular metabolism was not completely affected.

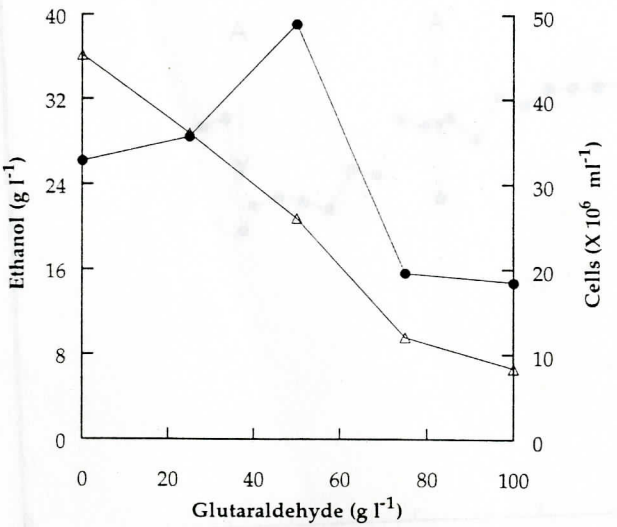

Figure 2. Fermentation of sucrose to ethanol by *Saccharomyces cerevisiae* immobilized to gelatin-precoated glass beads and cross-linked with different concentrations of glutaraldehyde. Incubation was for 72 h at pH 4.5 and 35 °C. (•) Ethanol and (△) *Saccharomyces cerevisiae* cells leaked into the medium.

These results show that glutaraldehyde cross-linking of *Saccharomyces cerevisiae* immobilized to gelatin-precoated glass beads is better than using the immobilized cells without cross-linking. However, the amount of glutaraldehyde used for cross-linking should not exceed 50 g l^{-1}.

Effect of Monochloroacetic Acid

When *Saccharomyces cerevisiae* cells immobilized to gelatin-precoated glass beads were cross-linked with 50 g glutaraldehyde l^{-1} and inoculated into nutrient medium, ethanol production was 39.1 g l^{-1} giving a yield of 72.7% (at 72 h). At 72 h the carbon source was completely consumed. To minimize cell multiplication and to channel the sucrose utilization for fermentation, monochloroacetic acid of different concentrations was added to the nutrient medium. To increase the initial cell growth of *Saccharomyces cerevisiae* immobilized to glass beads and cross-linked with glutaraldehyde, the immobilized cross-linked cells were incubated at 35 °C in nutrient medium for 48 h before the start of the experiment.

With an increase in monochloroacetic acid concentration from 0.0 to 0.01 g l^{-1}, ethanol production increased from 25.4 to 52.1 g l^{-1} at 48 h (Table 1). Further increase in monochloroacetic acid concentration above 0.01 g l^{-1} that is from 0.1 to 1.0 g l^{-1}, inhibited ethanol production and at 10.0 g l^{-1}, ethanol production was completely inhibited. The addition of 0.01 g l^{-1} monochloroacetic acid to the medium improved ethanol yield from 36.1 to 96.8% at 48 h. Cell leakage into the medium also decreased with an increase in monochloroacetic acid concentration. Decreased cell leakage and increased ethanol production can be attributed to increased channeling of the carbon source for fermentation rather than for cell mass production. Previous reports suggest that fluroacetic acid inhibited cisaconitase activity and

Table 1. Effect of different concentrations of monochloroacetic acid on ethanol production by and leaking of *Saccharomyces cerevisiae* immobilized on glass beads precoated with gelatin and cross-linked with glutaraldehyde (50 g l^{-1}). Incubated at pH 4.5 and 35 °C for 48 h.

Monochloroacetic acid (g l^{-1})	Ethanol (g l^{-1})	10^{-6} × Cell number (ml^{-1})
0	25.4	26.00
0.01	52.1	14.10
0.10	10.4	10.30
1.00	6.9	00.04
10.0	0.0	00.00

increased citric acid productivity in *Aspergillus niger* (0.01 M) (Manonmani & Srikantiah 1987) and *Candida* Sp. Y_2 (0.5 mM) (Tani *et al.* 1990). Monochloroacetic acid might have acted, in a similar way to fluroacetic acid.

When nutrient medium was inoculated with *Saccharomyces cerevisiae*, sucrose in the medium could have been metabolized via the Embden–Meyerhof Pathway (Reed & Peppler 1973) to pyruvate and utilized for the production of ethanol and cell mass and completely oxidized via the tricarboxylic acid cycle. However, when 0.01 g l^{-1} monochloroacetic acid was incorporated into the nutrient medium, complete oxidation of pyruvate through the tricarboxylic acid cycle might have been inhibited. Monochloroacetic acid is a structural analogue of acetyl CoA (Mayes 1993) and might condense with oxaloacetate to form chlorocitrate. The chlorocitrate would then inhibit cisaconitase. This block in the tricarboxylic acid cycle could have led to the accumulation of pyruvate and its channeling for ethanol production. Increase in monochloroacetic acid concentrations above 0.01 g l^{-1} seems to affect the metabolism of *Saccharomyces cerevisiae* adversely.

Previous studies showed that azide (Hahn-Hägerdal & Mattiasson 1982; Hahn-Hägerdal *et al.* 1986), dinitrophenol (Amin *et al.* 1984) and arsenate (Amin *et al.* 1984) either inhibited or uncoupled the respiratory chain and helped in the channeling of the carbon source to ethanol production. In our studies the metabolic block seems to be effected in the tricarboxylic acid cycle and not in the respiratory chain.

To study the value of adding monochloroacetic acid to improve ethanol production, 0.01 g l^{-1} was added to the nutrient medium and used in a semi-continuous process.

Semi-continuous Process for Ethanol Production

Saccharomyces cerevisiae immobilized on to gelatin-precoated glass beads and cross-linked with glutaraldehyde was incubated with nutrient medium for 48 h. The immobilized cell preparation was inoculated into nutrient medium containing 0.01 g monochloroacetic acid l^{-1}. Every 48 h the spent medium was replaced with fresh medium (each cycle took 48 h) and at the end of the eighth cycle nutrient medium was added and incubated for 48 h. When 0.01 g l^{-1} monochloroacetic acid was included in the nutrient medium, it was observed that cell multiplication was reduced and the carbon source was channeled to the ethanol producing pathway. Thus it is obvious that prolonged exposure of the immobilized cells to the nutrient medium containing monochloroacetic acid might lead to a decrease in the viability of the cells in the immobilized preparation. Therefore *Saccharomyces cerevisiae* immobilized to gelatin-precoated glass beads and cross-linked with glutaraldehyde was incubated with nutrient medium for 48 h at the end of the eighth (Figure 3, A) and 16th (Figure 3, A′) cycles to increase the viable cell number. Such intermittent addition of nutrient medium had helped to store the ability of the immobilized preparation to ferment sucrose to ethanol, as observed in the initial cycles of the process. This semi-continuous process was continued for 40 days and discontinued although the fermenting ability of the cells was still observed. Yeast cells immobilized by adhesion on tuff granules (for 15 days; Parascandola *et al.* 1987), yeast entrapped on pebbles by carrageenan (for 40 days; Amin *et al.* 1984) and *Saccharomyces cerevisiae* entrapped in alginate (for 40 days; Arasaratnam 1994) showed similar operational stability. Our results indicate that the inter-

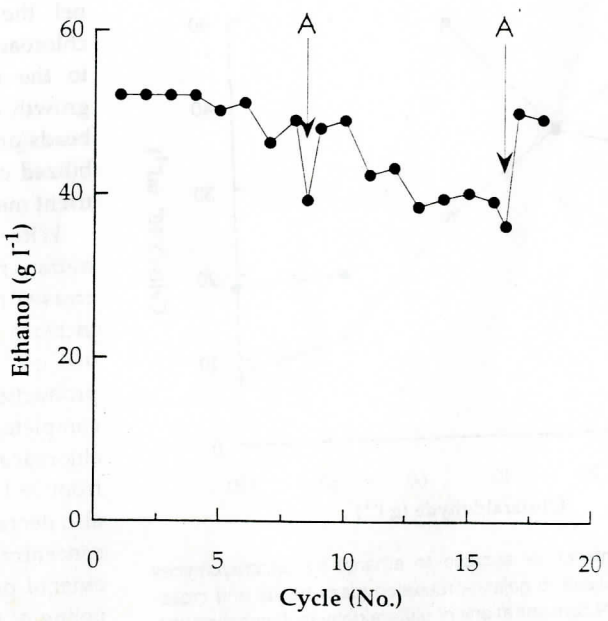

Figure 3. Semi-continuous process for ethanol production by *Saccharomyces cerevisiae* immobilized on glass beads precoated with gelatin and cross-linked with glutaraldehyde at 35 °C and pH 4.5. The nutrient medium containing 0.01 g monochloroacetic acid l^{-1} was used, and to activate the cells nutrient medium was passed intermittently (A and A′).

mittent addition of nutrient medium to the immobilized cells could sustain high ethanol yield for at least 40 days.

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