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Alcohol Production from Cassava Starch by Co-immobilized *Zymomonas mobilis* and Immobilized Glucoamylase

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ABSTRAK

Pensakaridaan dan penapaian serentak kanji ubi kayu terdekstrin kepada glukosa dan alkohol, masing-masing, dilakukan oleh Zymomonas mobilis *dan glukoamilase tersekat-gerak yang tersekat-gerak bersama. Sel-sel dan glukoamilase tersekat-gerak yang terperangkap di dalam kalsium alginat (nisbah 4 : 1) memberi produktiviti alkohol sebanyak 0.30 g/g berat basah/jam untuk proses penapaian sekumpul. Bagi penapaian selanjar, 54.3 g/1 alkohol dihasilkan pada kadar pencairan 0.3/h. 60% daripada aktiviti awal terhapus dalam masa 3 hari dan selepas itu, sistem tersebut memasuki fasa penghasilan alkohol yang menurun dengan perlahan.*

ABSTRACT

Simultaneous saccharification and fermentation of dextrinized cassava starch to glucose and alcohol, respectively, were carried out by co-immobilized Zymomonas mobilis *and immobilized glucoamylase (IG). Calcium alginate-entrapped cells and IG (4 : 1 ratio) gave an alcohol productivity of 0.30 g/gww cells/h in a batch fermentation process. For continuous fermentation, 54.3 g/l alcohol was produced at a dilution rate of 0.3/h. 60% of the initial activity was lost within 3 days and, thereafter, the system entered a slowly decreasing phase of alcohol production.*

INTRODUCTION

Co-immobilizing microbial cells and an enzyme together in the same support in order to carry out a certain biochemical reaction has some merits. Besides supplementing the array of intracellular enzymes of the cells, the product from the enzyme reaction can be channelled directly to the cells for further conversion or modification. This is especially advantageous if further conversion or modification of the product requires a sequential reaction. It may be possible to reduce production costs with such an arrangement since both reactions are carried out in a single reactor.

Few reports have been published on co-immobilized enzymes and cells. One of the earliest reports on such a system concerned the co-immobilization of glucoamylase and *Saccharomyces cerevisiae* (Hough and Lyons, 1972). $TiCl_4$ was used to immobilize the enzyme on the surface of the yeast cells. Hirano *et al.* (1979) and Karube *et al.* (1977) studied systems where aminoacylase and glucose oxidase, respectively, were immobilized on the natural mycelial pellets of *Aspergillus ochraceus*. Glutaraldehyde was used as the coupling agent and albumin was found to stabilize the pellets by preventing the leakage of the enzymes. Ghazali and Cheetham (1983) pursued the approach of co-immobiliza-

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tion by using a system where an enzyme that had already been immobilized was further immobilized with a microorganism in a common support. They demonstrated that continuous bioconversion of solubilized corn starch into alcohol was possible when using a co-immobilized system comprising immobilized glucoamylase (IG) and *Saccharomyces uvarum*. Both the enzyme and the yeast were immobilized in calcium alginate pellets. However, the stability of the system in continuous fermentation was low owing to cell inactivation in the presence of high alcohol concentrations.

Zymomonas mobilis has been shown to be a better alcohol producer than yeast (Rogers *et al.*, 1980) and has also been reported to be able to tolerate high concentrations of alcohol (Lavers *et al.*, 1980). The bacterium, however, can only ferment glucose, fructose and sucrose into alcohol and CO_2, but it cannot utilize other carbon sources such as maltose and starch (Swings and De Ley, 1979). The inability to utilize starch, which is a cheap substrate compared with sugars, can be overcome by saccharifying the starch first.

This paper reports on the use of a co-immobilized system, comprising *Z. mobilis* ATCC 2304 and immobilized glucoamylase, to carry out the simultaneous saccharification and fermentation of dextrinized cassava (*Manihot esculenta*, L) starch into alcohol.

MATERIALS AND METHODS

Maintenance and Preparation of Z. mobilis

Z. mobilis ATCC 2304 was maintained in a growth medium containing glucose (100 g/l), yeast extract (10 g/l), ammonium sulphate (1 g/l), potassium dihydrogen phosphate (1 g/l) and magnesium sulphate heptahydrate (0.5 g/l) (Rogers *et al.*, 1979). The culture was grown at 30°C for 24 h and stored at 4°C between successive transfers of 3 to 4 days.

For immobilization, a total of 2 l of the above medium in shake-flasks was inoculated with *Z. mobilis* and incubated with shaking (70 rpm) for 24 h at 30°C. The bacterial cells were then harvested by centrifugation at 13000 g for 15 minutes at 4°C. The weight of the cells used for immobilization was expressed in gram wet weight (gww) cells.

Preparation of Starch Substrate

The cassava starch was dextrinized according to the method described by Subhi and Ghazali (1986). The dextrinized starch used for the fermentation process has a pH of 5.5 and a glucose content of 3 to 5%. Cassava starch was bought locally.

Immobilization of Glucoamylase

The method used for the immobilization of glucoamylase was that described by Daniels and Farmer (1981) and Subhi and Ghazali (1986).

The Extent of Saccharification of Substrate by Alginate-entrapped IG

A 5% (w/v) suspension of IG (support particle size of $355 - 600$ μm) in 5% sodium alginate (BDH Chemicals Co. Ltd, Poole, England) solution was prepared by mixing in a 10 ml hypodermic syringe. The slurry formed was extruded from a height of 20 cm into a beaker containing a continuously stirred 0.1M $CaCl_2$ solution. The calcium alginate pellets formed had a diameter of $0.4 - 0.5$ cm and were left in the liquor for 1 h. The pellets were removed and rinsed with distilled water by decantation. They were then added to 100 ml of substrate and incubated with shaking (100 rpm) at 55°C. Samples were withdrawn at regular intervals and analysed for glucose (Barton, 1966). A similar set-up was used for the control using non-entrapped IG.

Co-immobilization of Z. mobilis *and IG*

A 5% cell load was prepared by mixing 0.5 g of freshly harvested *Z. mobilis* and IG in 5% sodium alginate solution in a 10 ml syringe. The weight (gww) of IG was varied to give the desired cell-to-enzyme ratio. The slurry formed was then extruded into a continuously stirred 0.1M $CaCl_2$ solution containing 15% glucose to obtain beads

of calcium alginate pellets. The pellets were left in the liquor for 1 hr and were then rinsed by decantation with distilled water before use.

Continuous Fermentation of Substrate

Pellets containing a 4 : 1 ratio of *Z. mobilis*-to-IG were packed into a jacketed glass column, 37 cm × 1 cm internal diameter. 4 g (ww) of cells were used for the column. The temperature of the column and the flow rate of the substrate were maintained at 30°C and 0.3/h, respectively. The substrate solution was fed upward into the column so as to facilitate the removal of CO_2 formed during fermentation. 10 ml of eluate was collected at the time of analysis and assayed for glucose and alcohol (Amerine and Ough, 1974).

RESULTS AND DISCUSSION

The effect of enzyme carrier particle size on the activity of the IG was studied in order to obtain the best enzyme preparation for co-immobilization with *Z. mobilis*. As shown in *Figure 1*, decreasing the carrier particle size increased the saccharification activity of IG. The increase in activity could be attributed to greater amounts of glucoamylase being bound to the carrier: the smaller the particle size, the larger the specific surface area. However, decreasing the particle size further did not give an enzyme preparation of greater activity (Ghazali, 1981). IG bound to charcoal of less than 300 μm in diameter was found to have very low activity.

In subsequent studies, glucoamylase immobilized on charcoal of particle size between 355 and 600 μm was used. Furthermore, it was found that it was easier to extrude the alginate-*Z. mobilis*-IG mixture from the syringe into the $CaCl_2$ bath to form even sized, spherical pellets that contained the IG evenly dispersed throughout the gel matrix, when using particles in this size range.

Immobilizing the IG in calcium alginate pellets caused a decrease in the amount of glucose produced, but the rate of glucose production after an initial lag period was similar to

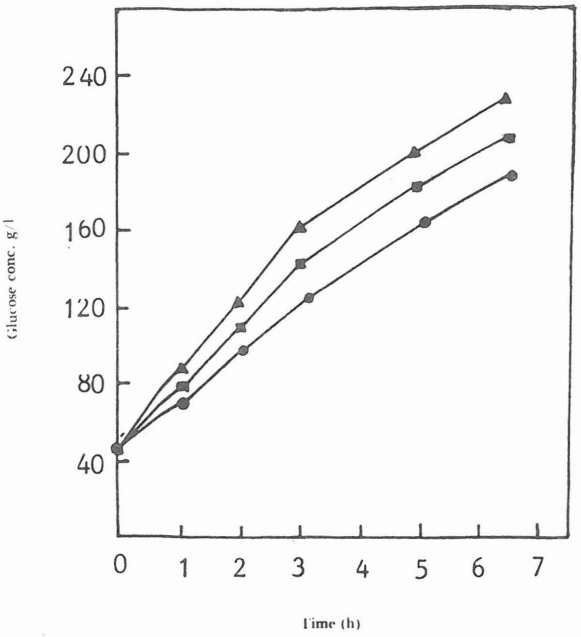

Fig. 1: The effect of support particle size on the efficiency of immobilization of glucoamylase. 2 g of IG were used to saccharify 100 ml substrate.
▲ *355 – 600 μm* ■ *> 600 – 710 m*
● *> 710 – 1000 μm*

that with free IG *(Figure 2)*. The lag period observed can be ascribed to establishing a diffusion gradient and to the diffusion limitations of the higher molecular weight components of the dextrinized cassava starch. Kierstan (1982) reported that diffusion characteristics of lower molecular weight substances were unaffected by alginate and ion concentrations; however, diffusion of higher molecular weight components was affected by alginate concentration. That the rate of glucose production was similar in both cases indicated that immobilizing IG in alginate gel did not affect its activity significantly. Therefore, in a co-immobilized system, the glucose that was produced by the gel-entrapped IG within the gel would be in contact with the bacteria at all times and could be utilized immediately for fermentation.

The effect of varying the amount of IG (gww) in calcium alginate pellets containing a fixed amount of *Z. mobilis* (gww) on glucose and alcohol production is shown in *Figure 3*. The

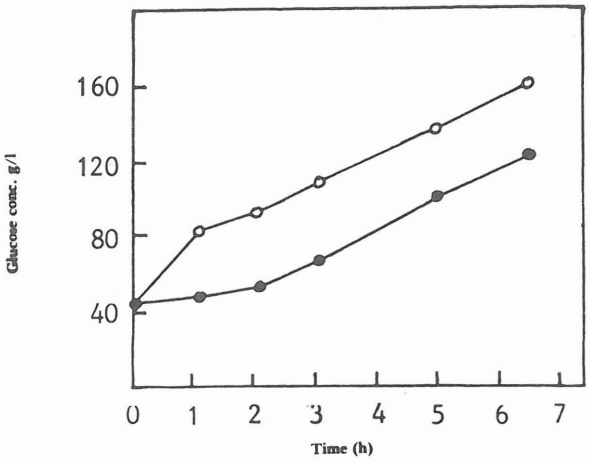

Fig. 2: The effect of gel entrapment on IG. 1 g of IG was used for the saccharification process.
○ free IG ● alginate entrapped IG

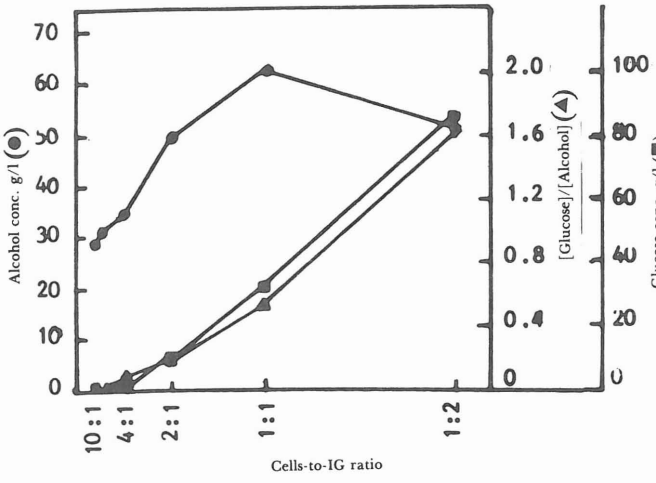

Fig. 3: The effect of Z. mobilis-*to-IG ratio on saccharification and fermentation. The processes were carried out for 24 hrs.*

study was carried out by batch fermentation, whereby pellets of known cells-to-IG ratio were added to 100 ml of substrate and incubated with shaking (100 rpm) at 30°C for 24 h. The results obtained indicated that the ratio of cells-to-IG of 10 : 1 appeared to be best in terms of glucose utilization (based on the amount present after fermentation) and a ratio of 1 : 1 for alcohol production. However, at the ratio at which glucose was best utilized, the concentration of alcohol produced was low (28.6 g/l) and, at the ratio of 1 : 1, glucose was produced in excess (33.5 g/l) where it remained unutilized. The optimal cells-to-IG ratio was taken to be 4 : 1, where the availability of glucose was neither in excess nor deficient, based on the glucose to alcohol ratio *(Figure 3)*. At this ratio, the concentration of alcohol produced was 36 g/l, which corresponds to a productivity of 0.3 g/gww cells/h.

Continuous fermentation by co-immobilized *Z. mobilis* and IG (4 : 1 cells-to-IG ratio) was carried out to study the stability of the system with respect to time *(Figure 4)*. During the first three days of operation, the concentration of alcohol produced dropped by about 60% of the initial value of 54.3 g/l and thereafter the system entered a gradually decreasing phase of alcohol production. A corresponding initial increase was observed for glucose but eventually the amount produced gradually decreased with time. The decrease in the production of both alcohol and glucose can be attributed to the decline in the activities of the bacteria and the IG in the alginate pellets.

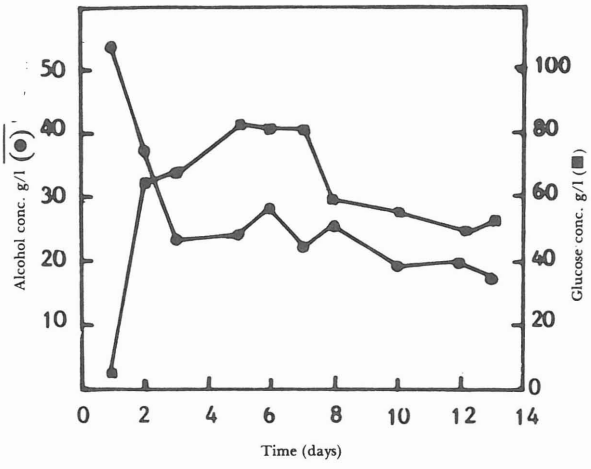

Fig. 4: Time course on the stability of co-immobilized Z. mobilis *and IG at a cell-to-enzyme ratio of 4 : 1.*

A similar trend for alcohol production was observed by Ghazali and Cheetham (1982) when using co-immobilized *S. uvarum* and IG. With the yeast system, 50% of the initial alcohol producing activity was lost within 8 days of operation. The major limiting factor in the overall stability of the system was the yeast cells, since *in situ* regeneration of the cells with growth medium could restore the activity of the system.

The low operational stability of the *Z. mobilis*-IG system may be overcome by intermittent supply of growth medium or by continuous supply of diluted growth medium so that a 'growing immobilized cells' system is obtained. Alternatively, strains of *Z. mobilis* with greater tolerance to higher concentrations of glucose when maintained in a non-growing state can be sought.

Visual observations showed that CO_2 evolution during the first day of fermentation was very vigorous indicating that the immobilized bacteria were very active. Although most of the gas evolved escaped from the column through the eluate outlet at the top end of the column reactor, some CO_2 was retained as bubbles in the column. The escaping gas caused the pellets to be more closely packed at the top of the column. New pockets of CO_2 were formed as fermentation continued and these replaced the CO_2 bubbles that eventually escaped from the column. The installation of a multiple-disk shaft (McGhee *et al.*, 1982) in the column may help to eliminate gas hold-up and uneven distribution of the alginate pellets.

CONCLUSION

Cassava starch, an important agricultural by-product in this region, can be further modified to produce value-added products such as glucose syrup, high fructose-glucose syrups and alcohol. The work described in this paper shows an example of a system that can be used to produce alcohol. *Zymomonas mobilis,* a bacterium capable of producing and tolerating high oncentrations of alcohol but incapable of utilising starch as a substrate, can be made to produce alcohol from starch by supplementing its cellular enzyme system externally with glucoamylase, a starch hydrolysing enzyme. However, it is important to evaluate the potential of this work for larger scale operations since the demand for alcohol, especially as fuel, is dependent on current market price for petroleum.

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