IMMOBILIZATION OF *KLUYVEROMYCES MARXIANUS* CELLS WITH INULINASE IN AGAR

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(Received March 22, 1985)

The inulinase-active whole cells of yeast *Kluyveromyces marxianus* were immobilized by entrapment into agar gel with 76% retention of the enzymatic activity. The enzymatic properties of the immobilized cells were investigated and compared with those of free cells. The immobilization procedure did not alter the optimum pH of the enzymatic preparation. The optimum pH of both free and immobilized cells was 6. The optimum temperature for inulin hydrolysis with the immobilized cells was 5°C higher than that with the free cells. Activation energies for the reaction with the free and immobilized cells were calculated to be 25.62 kJ/mol and 13.27 kJ/mol, respectively. K_m values were 8.3 mM inulin for the free cells and 10.5 mM for the immobilized cells. Free and immobilized cells showed fairly stable activities between pH 4 to 7 but free cell inulinase was more labile at other pH values compared to the immobilized counterpart. Thermal stability of the enzyme was improved by immobilization. There was no loss of activity of the immobilized cells on storage at 4°C for 30 days.

Fructose has recently received much attention due to renewed interest in natural sweeteners. In addition, fructose has some advantages to sucrose in sweetness, solubility, viscosity and dental health characteristics (1). Syrups containing fructose are currently manufactured from hydrolyzed corn starch which is subsequently enzymatically isomerized to give a blend of fructose and glucose (2, 3). Until 1976, such syrups were composed of approximately 50% glucose, 42% fructose and 8% other saccharides. More recently, however, fractionation of this product has led to the marketing of syrups containing up to 90% fructose.

Polyfructans, also appear to be an attractive source of fructose (4–6). These

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are found in tubers and rhizomes of the compositae family and are composed of multiple fructose units and a terminal glucose unit linked via an α -1-2 bond. Polyfructans of this configuration occur in chicory, dahlia, and Jerusalem artichoke tubers but they vary in their degree of polymerization. Jerusalem artichoke tubers contain a homologous series of polyfructans of DP₂ to approximately DP₃₅ (5). Production of fructose from Jerusalem artichoke extract is possible using chemical hydrolysis which requires considerable energy expenditure and causes modification in the syrup such as darkening in colour (7-10).

The utilization of enzymatic hydrolysis by inulinases is an attractive alternative. The presence of inulinases have been reported in plants, yeast, bacteria and fungi (11-13). Only those inulinases which free fructose by exowise steps are useable for making fructose. Such inulinases occur in yeasts and their use in the production of fructose have been suggested by many researchers (14-16). The production of fructose from Jerusalem artichoke tubers on a continuous or semi-continuous basis would require the use of immobilized inulinase. A thorough literature search has revealed that there are very few reports on immobilization of inulinase (17-19). BAJPAI and MARGARITIS (20, 21) have recently reported the immobilization of *Kluyveromyces marxianus* cells containing active inulinase enzyme in calcium alginate gels and their use in batch and packed bed bioreactors.

In this paper we report a method for immobilization of *Kluyveromyces marxianus* cells having inulinase activity in agar gel. The enzymatic characteristics of the immobilized and free cells are presented. The results of batch and packed bed column performance to produce high fructose syrup from artichoke tubers by using immobilized cells are presented in a later paper (22).

MATERIALS AND METHODS

Microorganism and cultural conditions. Dead cells of *Kluyveromyces marxianus* were used as a source of enzyme inulinase. The culture was kindly provided by Dr. A. Lachanche of the Plant Science Department, University of Western Ontario. The culture was grown with shaking in a medium containing 5% Jerusalem artichoke tuber extract, 0.3% yeast extract, 0.1% (NH₄)₂HPO₄, 0.05% MgSO₄·7H₂O and 0.015% FeSO₄ at 35°C for 24 hr. The total sugar concentration in Jerusalem artichoke tuber extract was about 50 g·l⁻¹. The cells were collected by centrifugation at 3,440 × g for 10 min and killed with 95.0% ethanol.

Immobilization method. 20 ml of 2.5% agar dissolved in distilled water and 5 ml of cell suspension (0.1 g dry weight) were mixed thoroughly at 40°C. The mixture was cooled to 5°C to form a gel. The gel formed was soaked in a toluene-tetrachloroethylene (1 : 1) mixture at 4°C for 24 hr to increase the gel strength. The resulting stiff gel was cut into small cubes (2.5-3.0 mm) and washed thoroughly with toluene and tetrachloroethylene.

Inulinase activity measurement. The reaction mixture for assaying the inu-

linase activity of free cells contained 2.5 ml of 2% inulin solution, 2.5 ml of 0.1 M sodium acetate buffer (pH 6.0) and 0.01 g dry weight of the cells. After incubating at 30°C for 15 min, the reaction was stopped by heating the reaction mixture in a boiling water bath for 10 min. Reducing sugars were determined by the dinitro-salicylic acid method (23). The inulinase activity of the free cells usually varied between 30–40 units/g dry weight of the cells. For determining the inulinase activity of immobilized cells, the reaction mixture contained 2.5 ml of 2% inulin solution, 2.5 ml of 0.1 M sodium acetate buffer (pH 6.0) and 2.5 ml of immobilized cells containing 0.01 g dry weight of the cells. The reaction mixture was incubated at 30°C for 15 min with shaking. After incubation was completed the reaction was stopped as described above.

Effect of agar concentration on enzyme activity. The effect of 5 different concentrations of agar ranging from 1 to 5% on the activity of the immobilized cells was studied. The cell concentration was kept constant.

pH activity profile. The pH activity profile of the free and immobilized cells was determined. The enzyme reaction was carried out in 0.1 M sodium acetate buffer in the pH range from 2 to 8 at 30°C for 10 min.

Temperature activity profile. The enzyme reaction was carried out at various temperatures ranging from 20 to 65°C with both free and immobilized cells in 0.1 M sodium acetate buffer (pH 6.0) for 10 min with 2% inulin as the substrate. Arrhenius plots were used to determine activation energies.

K_m values of inulinase of free and immobilized cells. The K_m values of the enzymes of both the free and immobilized cells were determined by conducting the enzyme reaction at different substrate concentrations at 30°C for 10 min. The concentration of the enzymes was kept uniform.

pH stability. The pH stability of the free and immobilized cells were determined by incubating them in 25 ml of 0.1 M sodium acetate buffer of different pH values at 30°C for 24 hr. The remaining activities were determined according to the standard assay procedure.

Thermal stability. Both free and immobilized cells were incubated at different temperatures varying from 30 to 65°C for varying lengths of time up to a maximum of 140 min. The residual activities were evaluated according to the standard assay procedure.

Storage stability. The storage stability of agar-entrapped cells was determined over a period of time at 4°C and at room temperature, 30±2°C.

RESULTS AND DISCUSSION

Agar is a polysaccharide consisting of β -1, 4-D galactopyranoside and α -1, 3, 3, 6-anhydro- α -galactopyranoside. It becomes gel under mild conditions and represents a suitable matrix for microbial immobilization.

Figure 1 shows the effect of agar concentration on the inulinase activity of

Fig. 1. Effect of agar concentration on inulinase activity of immobilized cells.

○ : activity yield (%) = $\frac{\text{activity of the immobilized cells}}{\text{activity of the free cells}} \times 100$,

△ : cell leakage (g dry weight/l)

the immobilized cells. The activity of agar entrapped cells decreased with the increase in agar concentration. However, with an increase in agar concentration, the immobilized matrix became more stable in regard to retaining the cells. From an increase of 1% up to 5% (w/v) agar concentration, there was about a 42% decrease in activity. This decreased activity is due to the fact that with an increase in agar concentration, there is more resistance to diffusion of the substrate into and the product out of the matrix. The increased density of the carrier probably makes the substrate inaccessible to the enzyme's active center. From these results, an agar concentration of about 2.5% can be taken as optimal because it represents a compromise between two factors, namely inulin diffusion to the matrix and cell leakage. So in further experiments 2.5% agar was used as the matrix for the entrapment of the cells. The activity retention by 2.5% agar entrapped cells was found to be about 76% of the intact cells.

The effect of two different sizes of agar cubes (2–3 mm and 4–5 mm) on the inulinase activity of the immobilized cells was studied. It was observed that the enzyme activity of the smaller agar cubes was higher. Small cubes provide larger solid-liquid interface areas per unit volume for mass transfer of substrate and product. In addition, small cubes minimize the mass transfer limitation problem of substrate and product within the cubes. Due to the minimal mass transfer problem the fraction of active immobilized cells is maximized, resulting in higher activity. So for further studies, experiments were performed with agar cubes of 2–3 mm size.

The pH activity profile of free and immobilized cells was studied. Immobilization did not alter the optimum pH for enzyme activity. The optimum pH of both free and immobilized cells was 6. Similar results have been reported by KIM and BYUN (19) who immobilized purified inulinase on aminoethyl cellulose.

The effect of temperature on the rate of hydrolysis of inulin by free and im-

mobilized cells was investigated. With the immobilized cells, the highest activity for inulin hydrolysis was obtained at 55°C. With the free cells, maximum activity occurred at 50°C. Such increases in optimum temperature have been observed in many examples of biocatalyst immobilization in gels. The slope of the Arrhenius plot for immobilized cells yielded activation energy of approximately 13.27 kJ/mol which was lower than the value of 25.62 kJ/mol for the free cells. This apparent decrease in activation energy indicates diffusional limitations in the gel matrix with diffusion possibly being rate limiting (24). Higher temperature caused partial inactivation of the immobilized-cell enzyme as has been observed for the free counterpart.

Figure 2 shows the effect of inulin concentration (2–32 mM) on the activity of

Fig. 2. Effect of substrate concentration on inulinase activity of free and immobilized cells.

○ : free cells, △ : immobilized cells.

Fig. 3. pH stability of inulinase of free and immobilized cells.

○ : free cells, △ : immobilized cells.

Fig. 4. Thermal stability of inulinase of free cells with preincubation at different temperatures.

□: 30-50°C, ▽: 55°C, ○: 60°C, ●: 65°C.

Fig. 5. Thermal stability of inulinase of immobilized cells with preincubation at different temperatures.

○: 30-55°C, ▽: 60°C, ●: 65°C.

the free and immobilized cells. K_m values were calculated from the Lineweaver-Burk plots. The K_m value for the free cells was found to be 8.3 mM. For the immobilized cells, it was slightly greater, i.e. 10.52 mM. These results also indicate diffusion limitation of the substrate and product in the gel matrix of the agar.

pH stability of the free and immobilized cells is presented in Fig. 3. The inulinase activity of both free and immobilized cells was quite stable between pH 4 to 7 but, compared to the immobilized-cell inulinase, free-cell inulinase was more labile at pH values lower than 4 and higher than 7.

Figures 4 and 5 show the thermal stability of free and immobilized cells respectively. Figure 4 shows that after a 140 min exposure at 30, 40 and 50°C, the inulinase activity of the free cells was quite stable but at temperatures above 50°C, the activity decreased rapidly with exposure time. On the other hand, the activity

of immobilized cells after treatment for 140 min remained fairly stable up to 55°C and decreased beyond that. At 60°C about 75% of the activity was lost in 90 min and at 65°C about 90% of the activity was lost in 30 min. Thus, immobilization improves the thermal stability of the enzyme.

The storage stability of immobilized cells was studied. Agar cubes were stored at 4°C and at room temperature $30 \pm 2^\circ\text{C}$ for 30 days and the enzyme activity of the immobilized cells was determined. There was no loss of activity over a period of 30 days at 4°C, but at room temperature the activity lost about 25% of the original during the same period. It seems that these immobilized cells can be stored for a long time at 4°C without significant loss of activity.

This work is being supported by strategic grant No. G0470 of the National Sciences and Engineering Research Council of Canada.

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