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ORIGINAL PAPER

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Production of palatinose using *Serratia plymuthica* cells immobilized in chitosan

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Abstract In recent decades, the production of palatinose has aroused great interest since this structural isomer of sucrose has interesting potential. We describe a simple and effective method of immobilizing *Serratia plymuthica* cells in chitosan. The sucrose isomerase activity of immobilized preparations was enhanced many times by activation with fresh nutrient medium and subsequent drying. The preparations obtained were physically very stable with high enzyme activity and excellent operational stability. The effect of temperature, pH and substrate concentration on enzyme activity of the immobilized cells was investigated. Using immobilized cells, a complete conversion of sucrose (40% solution) into palatinose was achieved in 4 h in a ''batch''-type enzyme reactor. The use of free or immobilized cells had no effect on the composition of the solution, in particular the sugar content. The palatinose content was 80% and that of trehalulose 7%.

Keywords *Serratia plymuthica* · Palatinose · Immobilized cells · Chitosan

Introduction

The food industry is constantly seeking novel ingredients to improve existing products or to allow the introduction of new products. Palatinose (isomaltulose, 6-*O*-α-D-glucopyranosyl-D-fructose) is a naturally occurring compound that is valuable as a sweetener and parenteral nutrient. This sugar has a sweet taste and very similar physical and organoleptic properties to sucrose, but it is noncariogenic [9, 11] and shows a much slower rate of release of monosaccharides into the bloodstream [7, 12]. Therefore insulin release is correspondingly reduced compared with other sugars, creating the possibility of application in diabetic and sports foods and drinks. Side-effects of palatinose have not been reported. Palatinose is currently considered to be a promising sugar substitute.

Chemical synthesis of isomaltulose is very difficult, but a small number of bacterial strains can convert sucrose to isomaltulose. *Protaminobacter rubrum* [15], *Serratia plymuthica* [6] and *Erwinia rhapontici* [3] produce mainly isomaltulose (75–80%) and a small quantity of trehalulose. The microbial formation of palatinose has attracted commercial interest. The earliest process (1976; South German Sugar, Nürnberg, Germany) employed a continuous process, but the patent literature has recently described the use of immobilized cells. In 1985, the Mitsui Seito company (Tokyo, Japan) industrialized a bioreactor, using *P. rubrum* containing α-glucosyl transferase, for industrial production of palatinose [10]. The pioneering work on the use of immobilized cells in the production of isomaltulose employed strains of *E. rhapontici* [2, 4, 5]. Of the many different immobilization methods tested, by far the most successful was entrapment of intact cells in beads of calcium alginate. The beads were used most effectively in packed bed reactors. The high operational stability of the immobilized *Erwinia* cells is related to the use of structurally intact immobilized cells rather than isolated enzyme, disrupted cells, or nonimmobilized cells, and to the use of high concentrations of sucrose as substrate and the achievement of a high degree of conversion of the concentrated sucrose into product.

Recently, many authors have investigated the mechanism of sucrose conversion by the sucrose isomerase of *S. plymuthica* [8, 13, 14]. Due to the very high initial activity [5] we consider *S. plymuthica* ATCC 15928 to be very attractive for palatinose production from sucrose. There are no publications on using immobilized *S. plymuthica* cells for this purpose. In order to contribute to

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the creation and understanding of an effective process for conversion of sucrose into palatinose, we focused on immobilization of *S. plymuthica* cells and their use.

Materials and methods

Growth conditions

Stock cultures of *S. plymuthica* ATCC 15928 were maintained on nutrient agar slopes at 0–4°C. The growth medium contains (%): sucrose, 5.0; beef extract, 0.3; Bacto peptone, 1.0; yeast extract, 0.5; NaCl, 0.3; Na_2HPO_4, 0.2. A standard inoculum was prepared by growing the organism in 5 ml medium in a test tube and, when growth reached mid-log phase ($OD_{600} = 0.5–1.0$), inoculum was transferred into a 500 ml baffled Erlenmeyer flask containing 100 ml medium. The cultures were shaken at 28°C at pH 6.8. Cells were removed from the medium by centrifugation at 7,000 *g* for 20 min.

Preparation of chitosan solutions

A 2% (w/v) viscose (viscosity ~50–150 cP) chitosan-acetate solution was obtained by mixing 2 g chitosan (Wako Pure Chemical, Tokyo, Japan) with 75 ml H_2O and 13 ml 5% acetic acid while stirring and heating (80°C). To prepare a sterile solution, chitosan was suspended in water and autoclaved.

Immobilization methods

Method A

Method A (adapted from [14]): 3.5 g wet cells was suspended in 6.5 ml 0.9% NaCl solution (pH~5, adjusted with acetic acid) and mixed with 20 ml chitosan acetate solution. This suspension was added dropwise to 500 ml of a gently stirred 2% polyphosphate solution (pH 8.2). After 3–4 h the beads were collected and stored overnight in 0.9% NaCl solution at 4°C.

Method B

Method B (adapted from [14]): the same as method A but the suspension was added dropwise to 1.5% polyphosphate solution (pH 5.7). After 40 min the polyphosphate solution was decanted and the chitosan beads were washed with 0.1 M phosphate buffer (pH 7.5) and stored for 3 h in Na-tripolyphosphate solution (pH 8.2). The beads were washed again with phosphate buffer, collected and stored overnight in 0.9% NaCl solution at 4°C.

Method C

Method C: 3.5 g wet cells (this quantity can be varied) is suspended in 6.5 ml 0.9% NaCl solution (pH~5, adjusted with acetic acid) and mixed with 20 ml chitosan acetate solution. An appropriate volume of 25% (or 12.5%) glutaraldehyde solution was added and the suspension was immediately mixed. After gelatinizing the preparation, it was fragmented into small particles (1 mm), washed with 0.1 M phosphate buffer (pH 6.5) and stored overnight in 0.9% NaCl solution at 4°C.

Sucrose isomerase activity of immobilized preparations

Enzyme activity of the preparations was measured by incubating 1 g immobilized cells with 10 ml 40% sucrose solution in 50 mM Na-phosphate buffer (pH 6.0) at 37°C with gentle agitation in a 50 ml baffled Erlenmeyer flask. Reducing sugars (like palatinose) were determined by the Somogyi-Nelson method (at 660 nm) and high performance liquid chromatography (HPLC). Sucrose conversion into palatinose is the main enzyme activity of the cells; thus, the international unit of activity (U) is defined as the amount of enzyme that can release 1 μmol isomaltulose per minute at the initial stage under the standard assay conditions and is calculated as U/g preparation.

Analysis of sugars

Qualitative and quantitative determinations of sugars were conducted by thin layer chromatography (TLC) and HPLC. TLC analysis was performed on silica gel plates developed with isopropanol-ethyl acetate-water (7:1:2, v/v) at room temperature. The plates were then stained with carbazole-sulfuric acid reagent according to the method of Adachi [1]. HPLC analysis was performed on an ULTRON PS-80C column, Pump 880-PUi (Jusco, Chiba, Japan), Intelligent RI Detector 830RI (Jusco) and Column oven CTO-6A. The mobile phase was water and the flow rate was 0.3 ml/min at 80°C.

Effects of pH, temperature and substrate concentration on sucrose isomerase activity of immobilized preparations

The effect of pH and temperature on sucrose isomerase activity was investigated by measuring enzyme activity of preparations at pH values varying from 5.4 to 7.2 (at 37°C) with 50 mM Na-phosphate buffer and temperatures from 30°C to 60°C (pH 6.0). The effect of substrate concentration was investigated by measuring the activity under standard assay conditions for different concentrations of sucrose (146–1,460 mM) and the results were treated according to the method of Lineweaver-Burk in order to calculate the K_m value.

Conversion of sucrose into palatinose by immobilized cells

The process was conducted in a 200 ml enzyme batch type reactor with equipment for temperature and stirring control. After adjustment of an appropriate temperature in sucrose solution, different quantities of immobilized preparations were added and the process carried out until no further conversion occurred.

Results

We examined and compared three methods for immobilizing *S. plymuthica* cells. Methods A and B are described in the literature, while method C is original and was developed by us.

Comparing the activity of the immobilized preparations (Fig. 1) shows that those produced by methods A and B have approximately equal activity (13–15 U/g), while the preparations produced by method C are more than twice as active (38 U/g). The gel obtained after immobilization is relatively fragile and unstable, which would negatively affect the operational stability of the immobilized cells. With the aim of achieving preparations that are more stable and have a higher specific activity, they were dried at room temperature for 24 h. As a result, the specific activity of cells immobilized using methods A and B reached 38–40 U/g and that of cells immobilized by method C, 138 U/g. The dried

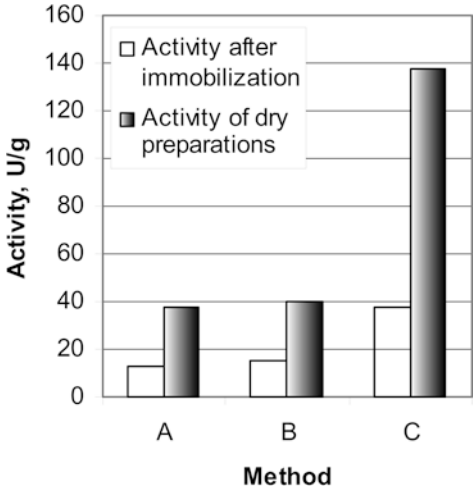

Fig. 1 Activity of *Serratia plymuthica* cells immobilized using different methods

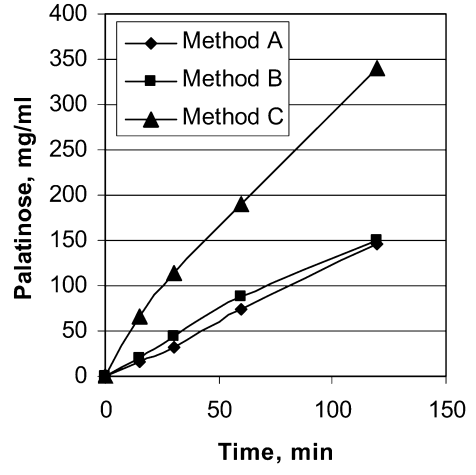

Fig. 2 Production of palatinose using immobilized *S. plymuthica* cells obtained by different methods of immobilization

immobilized preparations thus produced were used for conversion of saccharose (40% solution) into palatinose in a "batch"-type enzyme reactor (Fig. 2). The initial velocity of the enzyme conversion was several times higher when the process was carried out with cells immobilized in chitosan using method C; 120 min after the start of the process, the amount of liberated palatinose in the solution was almost 350 mg/ml (87% conversion) compared to only 150 mg/ml palatinose (37% conversion) when using cells immobilized using methods A and B. These results clearly demonstrate the advantages of method C, which is why all subsequent studies were conducted with cells immobilized in chitosan using this method.

Two consecutive processes for obtaining stable immobilized preparations with high specific activity were studied. In the first, after the cells were immobilized, the gel particles were placed in fresh culture medium for a period of time at 30°C with gentle shaking. As a result, within 5–6 h we observed growth of the cells within the gel and an increase in cell concentration within the gel. This led to an increase in specific activity of 65–70%, with a maximum level achieved 14–15 h after the start of the process (Fig. 3). After that the activity decreased and the gel particles were broken up by division of the cells, which continued to develop outside the gel. Microscopic observations showed that this treatment sharply increased the titer of cells nearest to the surface layer of the gel and that 12 h after the start of the process, cells appeared on the surface of the gel globules.

In the second process, following activation of the preparations (which resulted in an increase in the titer of the cells), the gel particles were dried at room temperature. The changes in the weight of the immobilized preparations and in their activity are shown in Fig. 4. During drying, the mass of immobilized preparations decreased in proportion to the increase in activity. Maximum sucrose isomerase activity was obtained after 36 h, when three times the initial activity was observed. When drying continued longer than 36 h, a decrease in enzyme activity of about 5% occurred until 48 h due to the formation of hardened gel particles. This hampered diffusion of the substrate to the cells and reduced the rate of conversion of palatinose. The reductions in mass

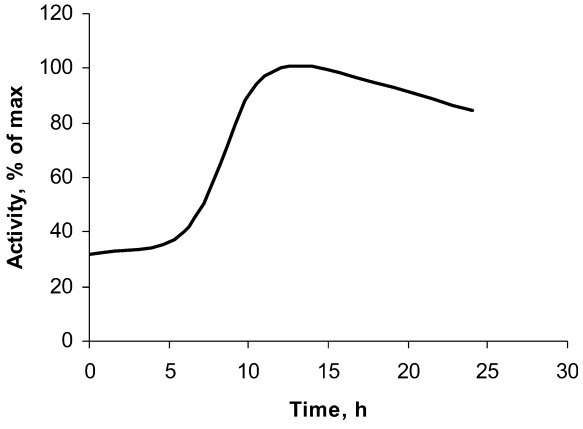

Fig. 3 Time-course of re-activation of immobilized preparations in fresh nutrient medium (growth of immobilized cells inside the gel)

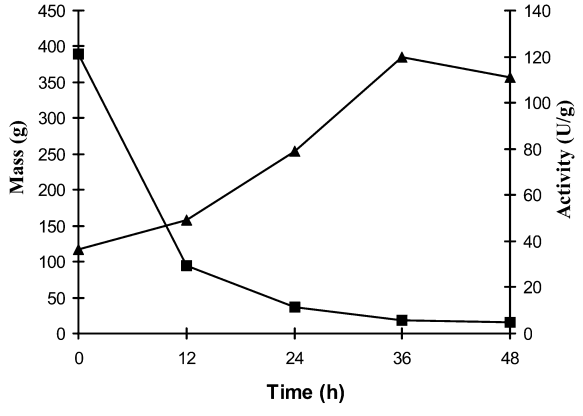

Fig. 4 Time-course of variation of the mass (■) of immobilized preparations and their activity (▲) during drying

were around 95% and the resulting preparations had a stable gel structure, very good hydrodynamic properties and high enzyme activity. The disadvantages of this procedure had to do with partial inactivation of the cells immobilized in the gel during drying and the presence of diffusional restrictions in the gel structure. The absolute value of the incremental enzyme activity of the immobilized preparations during drying depends on cell loading in the preparations. The relative value of the incremental enzyme activity was reduced when immobilizing a larger amount of biomass in the same amount of gel. It is possible to achieve a 3-fold increase in enzyme activity if preparations with a low cell loading are subjected to prolonged drying (over 48 h). It is evident that immobilizing a larger number of cells per unit gel results in a preparation with a higher activity. The relationship between the former and the latter is demonstrated in Fig. 5. The activity of the preparations rises sharply when increasing the cell loading (biomass content) above 25%. This renders the gel particles unstable, fragile, brittle and unsuitable for re-use. The optimal cell loading level was 20%. After subsequent re-activation in fresh nutrient medium and drying, we obtained preparations of *S. plymuthica* cells immobilized in chitosan with an activity of 120 U/g, corresponding to 2.6 g product per gram of immobilized cells per hour.

The optimum pH for isomaltulose production activity was 6.2–6.3 (Fig. 6), which corresponded to the optimum pH for free cells [13], while immobilized cells display maximum activity within a wider pH range (6.0–6.5). The optimal temperature for sucrose isomerase activity was 44–45°C (Fig. 7). Raising the temperature above 45°C reduced activity and at 55°C it stood at 85% of the maximum. The apparent K_m and V_{max} values of free cells for sucrose conversion to isomaltulose are 66.6 mM and 625.2 μmol min^{-1} g^{-1} respectively. K_m and V_{max} values of *S. plymuthica* cells immobilized in chitosan are 83.3 mM and 131.37 μmol min^{-1} g^{-1}, respectively.

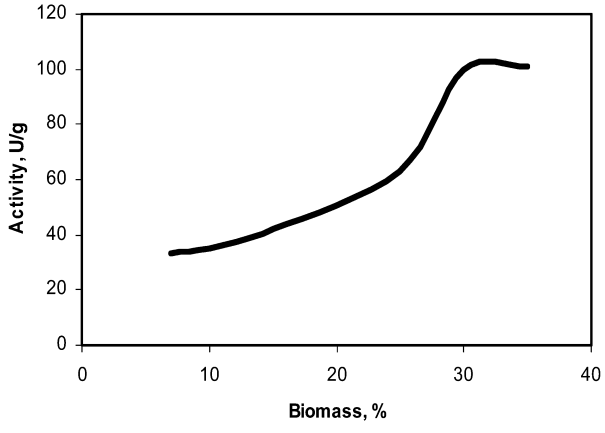

Fig. 5 Dependence of the activity of immobilized preparations on quantity of immobilized biomass per unit of gel

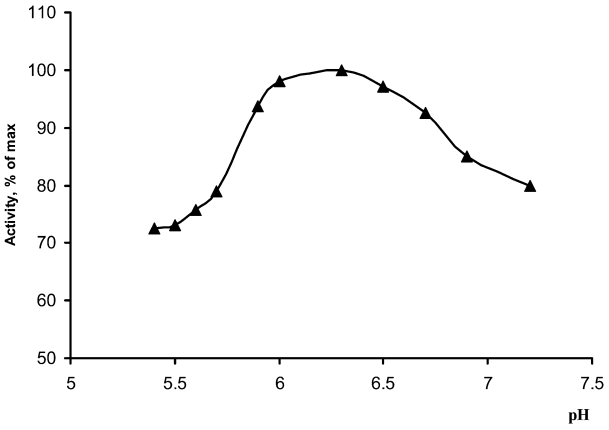

Fig. 6 Effect of pH on sucrose isomerase activity of *S. plymuthica* cells immobilized in chitosan

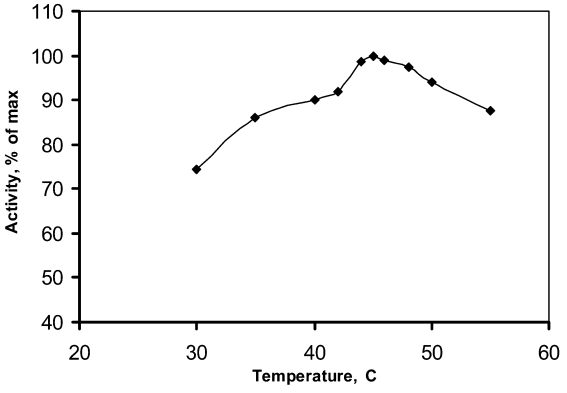

Fig. 7 Effect of temperature on sucrose isomerase activity of *S. plymuthica* cells immobilized in chitosan

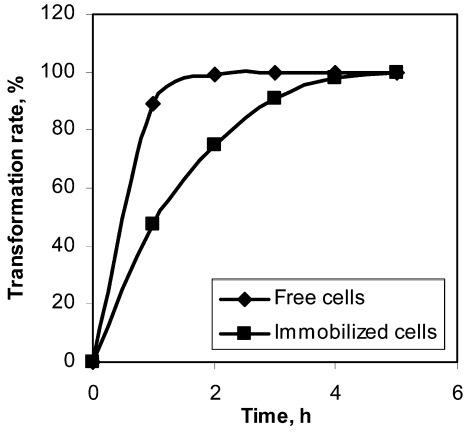

Fig. 8 Transformation of sucrose into palatinose by free and immobilized *S. plymuthica* cells in a "batch"-type enzyme reactor

We compared transformation of saccharose into palatinose and other sugars using equal quantities of free cells and cells immobilized in chitosan (Fig. 8). The initial speed of conversion with free cells is considerably higher than that of immobilized cells, thus achieving 90% transformation only 60 min from the beginning of

Table 1 Contents (%) of the sugar solution obtained after 4 h of conversion

Sample	Glucose	Fructose	Trehalulose	iso-Maltose	Palatinose	Sucrose	Other
Free cells	2.90	5.84	7.04	1.30	79.95	0.18	2.79
Immobilized cells	2.89	5.67	6.95	1.20	79.84	0.88	2.57

the process; 2 h later there was a 100% conversion rate. Using immobilized cells, complete (100%) conversion was achieved only after 4 h. The results were achieved using a 40% solution of saccharose at 37°C (the same temperature as with all analyses of enzyme activity) and correlation of gel to substrate solution 1:13 (mass/volume).

The content of the sugar solution after 4 h of conversion is shown in Table 1. The use of free or immobilized cells made no difference to the composition of the solution, in particular its sugar content. The palatinose content was 79–80%, that of trehalulose 7%, and of the other analyzed sugars according to our table.

Discussion

In establishing an effective process for obtaining palatinose with immobilized cells the activity and stability (both physical and with respect to enzyme activity) are of major importance. The studies of Cheetham are fundamental in this field [2, 3, 4, 5]. Cells of *E. rhapontici* immobilized in alginate have a high operational stability; this microorganism was chosen because of this fact. Cheetham reported that *S. plymuthica* cells possess high initial activity but that the immobilized preparation is unstable. Obtaining palatinose with immobilized cells of this microorganism is not mentioned in the literature and it is definitely a method of interest, not only because of its high enzyme activity, but also for the relatively small quantity of side products produced during the transformation of sucrose into palatinose.

Our aim was to obtain active, stable preparations of immobilized cells of *S. plymuthica*. All methods of immobilization mentioned by Cheetham were tested. The preparations were active, but unstable with the exception of those immobilized in alginate cells. However, the mechanical stability of this preparation was unsatisfactory. Given the advantages of chitosan as a carrier for immobilization, we directed our attention to this material. Cells of *S. plymuthica* were immobilized in chitosan using two methods (A and B) proposed by Vorlop and Klein [14] and a method (C) developed here. The activity of cells immobilized using method C is almost 2-fold and 3.5-fold higher without and with drying of the preparations, respectively, compared to the activity of preparations obtained using methods A and B. Accordingly, during the transformation of sucrose into palatinose, the initial rate and the degree of transformation are twice as high. These results show the advantages of method C over the others and all subsequent research was carried out with preparations obtained using this method.

By creating conditions that are appropriate for reproduction of cells in the gel, and through a suitable rate of the subsequent drying step, a significant increase in their activity resulted. Two effects were obtained: an increase of cell loading, and preparations with good mechanical stability and good compression strength, which can be used in both batch and continual processes. The drying should be done at a rate that does not allow a decrease in activity at the expense of diffusion limitations in gel particles that are too hard. Preparations with high cell loading can be obtained if a bigger quantity of biomass is used initially in the same amount of gel. If the content of immobilized cells in the gel is greater than 20–25%, the activity of the preparations increases (Fig. 5) but their physicochemical characteristics rapidly decline.

The difference between the apparent K_m values of free cells and immobilized preparations is not dramatic, which shows that immobilization does not lead to a rapid decrease in α-glucosyltransferase activity of the cells. The 9 vector value of K_m of free cells compared to that of immobilized preparations is due to diffusion limitations resulting from double immobilization of the intracellular enzyme (α-glucosyltransferase). First the enzyme is ''immobilized'' in the cells—localized in the periplasmic space—and then it is immobilized in the gel particles.

The most important advantage of the method of immobilization developed here is the achievement of the same degree of transformation and almost the same content of the outgoing solution in the process of transformation of sucrose into palatinose with immobilized and free cells. Logically, immobilized cells need more time (about 4 h) to reach 99% transformation. Also, the product composition of outgoing solution (Table 1) is almost the same (~80% palatinose) compared to cases using purified enzyme [8, 13].

An important aspect of this work is the choice of organism. *S. plymuthica* can be used in immobilized form for palatinose production from sucrose. Using the method described yielded very stable and active immobilized preparations. They can be applied in either batch or continuous flow processes for transformation of sucrose into palatinose.

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