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Selective oxidation of glucose for facilitated trehalose purification

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ABSTRACT

To facilitate the purification of trehalose, an enzymatic process for the selective conversion of glucose was investigated. An epoxy-activated acrylic matrix was used for the immobilization of glucose oxidase. Due to the relatively hydrophobic nature of carrier surface, significant enhancement in enzyme load was observed in the presence of 1000 mM phosphate without observable enzyme denaturation. Upon immobilization glucose oxidase, with optimal activity at 40 °C and pH 7.0, was shown to have higher residual activity at elevated pHs and temperatures. In repeated-batch operations, the immobilized glucose oxidase, with a catalytic activity of 214.06 ± 5.34 U/g gel at an enzyme load of 11.31 ± 0.19 mg/g gel, exhibited sound operation stability for up to 12 cycles, beyond that the activity declined steadily, due to the sequential inactivation of catalase and glucose oxidase by the accumulated hydrogen peroxide. It was shown that the inactivation of enzymes can be alleviated by the addition of hydrogen peroxide scavengers. It was also shown that the gluconic acid thus formed can be readily adsorbed with an ion exchanger leaving trehalose in the solution. The results obtained in this study demonstrate that the proposed process could facilitate the purification of trehalose enzymatically converted from maltose.

1. Introduction

Trehalose, D-glucopyranosyl D-glucopyranoside, a non-reducing disaccharide commonly found in diverse organisms [1], is widely used in pharmaceutical, cosmetic, and food industries [2–4]. In light of this, processes involving microbial fermentation and chemical synthesis for the production of trehalose have been studied [5,6]. However, they are generally not cost-effective and thus are not feasible for large-scale production.

Alternatively, various enzymatic processes for trehalose synthesis have been reported [7,8]. For example, the transglucosylation of maltose to trehalose with trehalose synthase, an enzyme capable of converting α-1,4 linkage into α-1,1 linkage, has been proposed [8]. A process involving the use of immobilized *Picrophilus torridus* trehalose synthase for the production of trehalose has thus been reported [9]. Compared to other enzymatic processes [10–12], the employment of trehalose synthase as the catalysts for trehalose production has the advantage of ease of operation because only one enzyme is involved. To this end, various recombinant trehalose synthase from different species exhibiting distinctive activities have been reported [13–23].

Employment of enzymatic processes for the synthesis of trehalose

, however, always leads to the formation of glucose, a by-product of the transglucosylation reaction [9], and/or the presence of residual reactants, maltose. For example, in our previous study we have shown that the product stream containing 23.6 mol% of residual maltose and 18.2 mol% of glucose. Even though the conversion of maltose can be enhanced by manipulating operation parameters such as reaction temperature [23], the presence of glucose and the maltose in the product stream is inevitable. It is thus necessary to develop cost-effective processes for the purification of trehalose. Unfortunately, this cannot be readily accomplished with conventional separation operations, because of the similarity in chemical structures and thus physicochemical properties among trehalose, maltose and glucose.

In light of this, a separation strategy involving the selective conversion of maltose and glucose to molecules that exhibit distinctive physicochemical properties has been proposed [24,25]. For example, in our earlier study we have proposed that by sequentially converting maltose to glucose and gluconic acid, it may be possible to separate trehalose from gluconic acid by adsorption and precipitation [26]. To demonstrate the feasibility of this approach, we have recently shown that under the catalysis of glucoamylase

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maltose can be selectively and quantitatively hydrolyzed to glucose without measurable degradation of trehalose [26].

Glucose oxidase has found applications in clinical analysis for the development of biosensors [27] and in food industry for the removal of glucose and oxygen [28–30] and for the production of gluconic acid and hydrogen peroxide [31,32]. Since gluconic acid and its derivatives, such as calcium gluconate, are widely used in food and pharmaceutical industries [33,34], we believe the conversion of glucose into gluconic acid under the catalysis of glucose oxidase can not only facilitate the downstream processing of trehalose, but also improve the process economics of the system.

In this study, the oxidation of glucose with immobilized β-D-glucose: oxygen 1-oxidoreductase (EC 1.1.3.4, glucose oxidase), is reported. The removal of gluconic acid thus formed from trehalose in the product stream by adsorption is also demonstrated.

2. Materials and methods

2.1. Immobilization of glucose oxidase

One hundred milligrams of glucose oxidase powder (from *Aspergillus niger*, 1.5 U/mg, Amino Enzyme, Inc., Japan) was added into 2.0 ml of phosphate buffer (pH 7.0) and the suspension was mixed in a rotary agitator for 10 min. After centrifugation at 8000 × *g* for 5 min, the supernatant containing dissolved glucose oxidase was collected. Protein concentration of the supernatant was determined by Bradford method as described in Section 2.4 [35].

Covalent immobilization of glucose oxidase was performed by mixing of 200 mg (wet weight) Immobead 150 (Sigma–Aldrich, USA) with 2.0 ml of 1000 mM phosphate buffer (pH 7.0), containing 1.94 mg/ml glucose oxidase (from *A. niger*, 1.5 U/mg, Amino Enzyme, Inc., Japan). The immobilization of glucose oxidase was allowed to proceed for 48 h at 25 °C, after which, the immobilized glucose oxidase was collected by filtration and then washed thoroughly twice with 1.0 ml of 50 mM phosphate buffer (pH 7.0). The filtrate was collected for protein assay as described in Section 2.4.

The effect of phosphate concentration on the covalent immobilization of glucose oxidase on Immobead 150 was investigated by mixing 200 mg of Immobead 150 with 2.0 ml of pH 7.0 phosphate buffer, at concentrations ranging from 200 mM to 1600 mM, containing 1.94 mg/ml glucose oxidase [36]. The effect of protein concentration on the efficiency of glucose oxidase immobilization was also examined by adding 200 mg Immobead 150 to 2.0 ml of 1000 mM phosphate buffer (pH 7.0) containing glucose oxidase at concentrations ranging from 0.39 mg/ml to 3.87 mg/ml.

2.2. Characterization of immobilized glucose oxidase

For the determination of the optimal reaction pH of the immobilized glucose oxidase, 200 mg immobilized enzyme was added to a cylindrical reactor (ID = 5.0 mm, *H* = 7.0 mm), containing 50 ml of 50 mM phosphate buffer containing 100 mM glucose at pHs ranging from 6.0 to 9.0, equipped with a pH meter (DG111-SC, Mettler Toledo) and a pH-stat system (DL50, Mettler Toledo) to maintain the pH of the reaction mixture at the desired value. Humidified oxygen at a constant flow rate of 100 ml/min, 2.0 vvm, was introduced to the reactor. The reaction was allowed to proceed at 40 °C for 20 min. To assess the optimal reaction temperature, the activity of the immobilized glucose oxidase was determined by adding 200 mg of the immobilized enzyme into the aforementioned reactor containing 50 ml phosphate buffer (pH 7.0) with 100 mM glucose. The reactions were performed at temperatures ranging from 20 °C to 60 °C. The thermostability of the immobilized enzyme was evaluated by measuring the activity of the immobilized glucose oxidase after being incubated at the prescribed temperatures, ranging from 20 °C to 60 °C, for 1 h. All the reactions were terminated by heating in boiling water for 10 min.

2.3. Oxidation of glucose

The product solution containing 64 mM trehalose and 72 mM glucose, obtained from the sequential conversion of maltose into trehalose with the immobilized recombinant *P. torridus* trehalose synthase and the residual maltose into glucose with the immobilized *Rhizopus oryzae* glucoamylase as reported in our previous studies [9,26], was used as the substrate solution for glucose oxidation with the immobilized glucose oxidase. Glucose oxidation was carried out in the aforementioned reactor at 40 °C. The reusability of the immobilized glucose oxidase during batch operations was investigated. Each cycle of reaction was allowed to proceed under the conditions based on the foregoing results for 90 min no residual glucose remained in the reaction product. After each cycle, the immobilized enzyme was recovered by filtration and then washed thoroughly with 50 mM phosphate buffer (pH 7.0) for the subsequent operations.

2.4. Analysis

The concentrations of trehalose, glucose and gluconic acid were measured by HPLC. Samples (20 μl each) were injected into an HPLC system (Jasco, Japan) equipped with a NH_2 column (5 μm, 250 mm × 4.6 mm, Phenomenex, USA) maintained at 40 °C and eluted isocratically with a mobile phase consisting of acetonitrile (J.T. Baker, USA) and Milli-Q water with 200 mM formic acid (Sigma, USA) at a volume ratio of 80:20 at a flow rate of 1.5 ml/min. The eluent was monitored with a refractive index (RI) detector [23]. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of glucose per minute under the assay conditions. Catalase activity was determined by measurement of the decline in absorbance of H_2O_2 at 240 nm [37]. Protein concentrations of all samples were analyzed by Bradford method with protein dye (Bio-Rad, USA) at 595 nm with BSA as the standard [35].

3. Results and discussion

3.1. Immobilization of glucose oxidase

Matrices containing epoxy groups have been used as carriers for the covalent immobilization of enzyme via the linkages between the oxirane groups on the surface of matrices and the nucleophile groups, amino, hydroxyl or thiol groups, of protein molecules [38]. In this study, Immobead 150, an epoxy-activated acrylic matrix [39], was used as the carrier for glucose oxidase immobilization. It has been reported that physical adsorption of proteins via hydrophobic interactions onto Eupergit® C, an epoxy support similar to Immobead 150, is required for the formation of covalent linkages [36]. To elucidate if that is also the case for Immobead 150, the effect of phosphate concentration on glucose oxidase immobilization was thus investigated. As shown in Fig. 1, the amount of enzyme immobilized on the carrier increased monotonically with phosphate concentration. At a phosphate concentration of 1600 mM, the enzyme load, 14.48 ± 0.23 mg/g gel, was four times higher than that at a phosphate concentration of 200 mM, implying that high ionic strength is conducive to covalent immobilization by facilitating the adsorption of protein molecules onto the carrier, as suggested in the literature [36]. Although further increase in phosphate concentration might lead to an even higher enzyme load, this was not attempted to avoid possible salt-induced enzyme denaturation.

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