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Immobilization of yeast inulinase on chitosan beads for the hydrolysis of inulin in a batch system

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ABSTRACT

An extracellular inulinase was partially purified by ethanol precipitation and gel exclusion chromatography from a cell free extract of *Kluyveromyces marxianus*. Partially purified inulinase exhibited 420 IU/mg specific activity and it was immobilized on chitosan beads. Activity yield of immobilized inulinase was optimized with glutaraldehyde concentration (1–5%), glutaraldehyde treatment time (30–240 min), enzyme coupling-time (2–16 h) and enzyme loading (5–30 IU) as functions. Under the optimized conditions maximum yield 65.5% of immobilized inulinase was obtained. Maximum hydrolysis of inulin 84.5% and 78.2% was observed at 125 rpm after 4 h by immobilized and free enzyme, respectively. A retention-time of 4 h and 5 h was found optimal for the hydrolysis of inulin under agitation (125 rpm) by free and immobilized enzyme, respectively. The recycling of the developed immobilized biocatalyst was carried out after 5 h of inulin hydrolysis in a batch system. The developed immobilized biocatalyst was successfully used for the hydrolysis of inulin for 14 batches. This is the first report on the immobilization of yeast inulinase on chitosan beads for the hydrolysis of inulin in a batch system.

1. Introduction

Inulin-acting enzymes belong to glycoside hydrolase (GH) family 32 and 91. On the basis of their hydrolytic activity on inulin, these enzymes are categorized as inulin transferases (GH-91) and inulin hydrolases (GH-32). The inulin transferases (EC 4.2.2.17) and (EC 4.2.2.18) produces difructose anhydrides (DFA-I) and (DFA-III), respectively from inulin by exo-acting intermolecular transfructosylation process [1]. Since the transfer is intermolecular and the reaction is elimination, these enzymes are called as lyases instead of transferases and hence termed as inulin lyases. Inulin hydrolases have strong exo- and endo-inulolytic activity to produce high fructose syrup (Exoinulinase, EC 3.2.1.80) and fructooligosaccharides (Endoinulinase, EC 3.2.1.7). Both fructose and fructooligosaccharides have wide applications in food and pharmaceutical industries [2–4]. Inulin is widely used as a substrate for the enzymatic production of high fructose syrup [2]. It occurs as a reserve carbohydrate in roots/tubers of many plants like Asparagus, Jerusalem artichoke and Dahlia *etc*. It is mainly made up of fructose molecules joined by β-2,1 glycosidic linkages and is terminated at the reducing end by a glucose moiety attached to the polyfructan chain through a sucrose-type (α-1,2 glycosidic) linkage. Inulin is a second most abundant plant polymer after starch and can be used as a substrate for the production of fructose syrup.

Fructose is a low caloric natural sugar which is almost 2-times sweeter than sucrose and has much functional and technical superiorities over sucrose, and also it is organoleptically desirable. Thus, fructose is considered as a safe and ideal substitute of sucrose. Fructose can be produced by chemical and enzymatic processes from starch and inulin. Chemical process has many drawbacks such as production of off-flavored colored product and formation of other by-products like difructose anhydride [5]. So enzymatic method of production of fructose is preferred over chemical synthesis. The conventional enzymatic method of fructose production is a low yielding, multi-enzymatic process using starch as substrate. The production yield by this process is approximately 45% [6]. While, the single-step enzymatic production of fructose from inulin yield upto 95%. This process is economically more viable for commercialization.

Enzymes can be immobilized onto or into a solid matrix to increase their thermostability, operational stability and ease of downstream processing. The additional advantages of immobilization include the repeated and continuous use of biocatalyst [7]. Enzyme interactions with material surfaces are of interest for industrial food and pharmaceutical transformations, biosensors, artificial cells, cell free reactions, drug and nutrition delivery tech-

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nologies and imaging [8]. A number of matrices/supports have been used for immobilization of microbial inulinases and the immobilized inulinase have been successfully used for the production of high fructose syrup from inulin in batch [9–11] and continuous systems [12–15]. In present investigation, chitosan was selected as matrix for immobilization of inulinase. Chitosan [poly-β(1 → 4)-2-amino-2-deoxy-D-glucose] is the product of deacetylation of chitin derived from the exoskeleton of crustacean and shows enhanced solubility in dilute acids as compared with the parent chitin. Chitosan has attracted great attention, and it has been reported as a promising polymer in medical and food industry. Recently, chitosan beads were used as an enzyme carrier for the white wine protein stabilization in a continuous system [16]. Chitosan could serve as carrier for both enzymes and whole cells. It was selected as a support for the immobilization of inulinase, because it is inexpensive, biodegradable, non-toxic, physiologically inert, biocompatible support and most importantly possess appropriate functional groups which covalently attach with the proteins [17,18]. In the present study, partially purified exoinulinase from *K. marxianus* YS-1 was immobilized on the chitosan beads and the developed immobilized biocatalyst was evaluated for the production of fructose from pure inulin in a batch system.

2. Materials and methods

2.1. Yeast culture and inulinase production

Kluyveromyces marxianus YS-1 an isolate of our laboratory was maintained on malt yeast agar slants as described previously [19]. Shake-flask fermentations were carried out in an inulin-based medium for the production of inulinase as described earlier [20].

2.2. Partial purification of inulinase

The partial purification of inulinase from *K. marxianus* YS-1 was carried out using a two-step purification strategy as discussed earlier [9]. Briefly, crude enzyme was precipitated with chilled 85% (v/v) ethanol. The precipitates obtained were dissolved in sodium acetate buffer (0.1 M, pH 5.5) and then loaded onto Sephadex G-100 column for gel exclusion chromatography. The fractions with high inulinase activity were pooled and used as partially purified enzyme for immobilization experiments. The partially purified enzyme contained 420 IU/mg specific activity.

2.3. Preparation of chitosan beads

Chitosan beads were prepared according to the method of Zhang et al. [21] after slight modifications. Chitosan solution was prepared by dissolving 1% (w/v) chitosan (from shrimp shells, Molecular weight 190–375 kDa, Deacetylation ≥75%, Sigma Aldrich, USA) in acetic acid (1%, v/v). Beads were prepared by dropping the bubble free chitosan solution in gently agitated aqueous solution of tripolyphosphate (5%, w/v) by a glass syringe fitted with a 22-gauge needle. The beads were separated by decantation after 1 h curing-time, washed thoroughly with distilled water and the beads are shown in Fig. 1.

2.4. Immobilization of partially purified inulinase on chitosan beads

Freshly prepared chitosan beads were used for the immobilization of partially purified inulinase. Immobilization procedure was standardized in order to get the maximum inulinase activity yield. Activity yield was calculated as follows:

$$\text{Activity yield}\,(\%) = \frac{\text{Immobilized enzyme activity}}{(\text{Totalenzyme activity added} - \text{Enzyme activity in supernatant})} \times 100$$

Beads were first activated using glutaraldehyde with gentle stirring at room temperature followed by incubation with enzyme at 4 °C. Schematic representation of inulinase immobilization on chitosan beads is shown in Fig. 2.

2.4.1. Activation of chitosan beads

The surface activation of the chitosan beads were carried out using glutaraldehyde to activate the amino groups on chitosan. The effect of different concentrations of glutaraldehyde (1–5%, v/v) on activation of support and consequently on activity yield of inulinase was studied. After activation of beads, glutaraldehyde was decanted and beads were washed with deionized water. Immobilization can be carried out either by entrapment into chitosan beads or by covalent binding to transport chitosan films or by using glutaraldehyde by formation of the Schiff's base [22,23].

2.4.2. Effect of activation time

To investigate the influence of activation time on enzyme activity yield, chitosan beads were kept in an aqueous solution of glutaraldehyde (2%, v/v) for 30–240 min.

2.4.3. Effect of coupling-time

Chitosan beads were incubated with enzyme (20 IU) in sodium acetate buffer (0.1 M, pH 5.5) for various time periods (2–16 h), to study the optimal coupling-time.

2.4.4. Effect of enzyme loading

Enzyme units (5–30 IU/2 g beads) were loaded to the support to optimize the enzyme loading.

2.5. Hydrolysis of inulin by free and immobilized inulinase in a batch system

Operational parameters like agitation and hydrolytic time were investigated for the hydrolysis of inulin in a batch system using free and immobilized enzyme. Operational stability of the developed immobilized biocatalyst has also been studied by recycling the developed immobilized biocatalyst in a batch system.

2.5.1. Effect of agitation on hydrolysis of inulin

To investigate the influence of agitation on hydrolysis, 50 mL of inulin solution (5%, w/v) was incubated individually with free and immobilized inulinase (25 IU) at 50 °C, under agitation (75–150 rpm) for 4 h.

2.5.2. Effect of time-course on hydrolysis of inulin

The effect of time-course (1–7 h) on the hydrolysis of inulin (5%, w/v) was studied using free and immobilized inulinase (25 IU) at 50 °C.

2.5.3. Recycling of immobilized biocatalyst in a batch system

Immobilized biocatalyst (25 IU) was added to 50 mL of inulin (5%, w/v) and incubated at 50 °C, under shaking (125 rpm) for 5 h. After the completion of each batch, immobilized biocatalyst was recovered, washed thoroughly with sodium acetate buffer (0.1 M, pH 5.5) and recycled for a new bioconversion run.