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Continuous hydrolysis of pullulan using covalently immobilized pullulanase in a packed bed reactor

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ABSTRACT

Pullulanase covalently immobilized on Duolite XAD761 has been used for developing a continuous flow reactor for the hydrolysis of pullulan. Packed bed reactor containing 34.10 U of immobilized biocatalyst with feeding of pullulan solution (0.44%, w/v) at a flow rate of 5 mL h^{-1} supported maximum pullulan hydrolysis at 60 °C. The reactor was run continuously for 32 days and immobilized biocatalyst lost half of its original activity after 31 days of continuous operation at 60 °C. The volumetric productivity and yield of reducing sugars were 3.38 ± 0.02 g L^{-1} h^{-1} and 4.40 ± 0.01 mg mL^{-1}, respectively during the beginning of the hydrolysis. The developed immobilized biocatalyst has shown good operational and mechanical stability and can be successfully used for the hydrolysis of pullulan in a continuous system. Literature survey reveals no report on continuous hydrolysis of pullulan using whole cells or immobilized enzyme.
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1. Introduction

Pullulan is a linear α-D-glucan built of maltotriose subunits i.e. α-(1→4)Glu*p*-α-(1→4)Glu*p*-α-(1→6)Glu*p*-, connected by (1→6)-α-D-glucosidic linkages and synthesized by the yeast-like fungus *Aureobasidium pullulans* (Leathers, 2003). However, other structures particularly the tetramer or maltotetraose, α-(1→4)Glu*p*-α-(1→4)-Glu*p*-α-(1→4)Glu*p*-α-(1→6)Glu*p*-, may be present in the pullulan polymeric chain (Wallenfels, Keilich, Bechtler, & Freudenberger, 1965). Pullulan is generally viewed as a succession of α-(1→6)-linked (1→4)-α-D-triglucosides i.e. maltotriose (G3). The regular alteration of α-(1→4) and α-(1→6) bonds results in two distinctive properties of structural flexibility and enhanced solubility. Pullulan and its derivatives have numerous potential for food and pharmaceutical industrial applications (Shingel, 2004; Singh, Saini, & Kennedy, 2008).

Pullulanase (EC 3.2.1.41, pullulan-6-glucanohydrolase) is a debranching enzyme which hydrolyses the α-1,6-glucosidic linkages in pullulan and other amylaceous polysaccharides belonging to a family of 13 glycosyl hydrolases, also termed as the α-amylase family (Matzke, Herrmann, Schneider, & Bakker, 2000). Pullulanases are widely distributed among animals, plants, fungi and bacteria (Domań-Pytka & Bardowski, 2004). Pullulan can undergo enzymatic hydrolysis by both (1–6)-α-D- and (1–4)-α-D-pullulanases. The (1–6)-α-D-pullulanases cleave the (1–6)-α-D-glucopyranosidic linkages. Complete hydrolysis of pullulan using (1–6)-α-D-pullulanase yields maltotriose as major product along with traces of maltotetraose. Whereas, (1–4)-α-D-pullulanases act on (1–4)-α-D-glucosidic linkages at their reducing ends adjacent to (1–6)-α-D linkages and its complete hydrolysis gives isopanose as the main product. Products of enzymatic pullulan degradation are used in food industry (Domań-Pytka & Bardowski, 2004). Maltotriose rich syrups are being produced by cation exchange resin chromatography of maltose syrups. Maltotriose syrup can also be produced by enzymatic hydrolysis of the polysaccharide 'pullulan' using the debranching enzyme, pullulanase (Singh, Saini, & Kennedy, 2010a). Maltotriose syrup possess excellent properties as low freezing point depression, mild sweetness, keeps in moisture, prevention of retrogradation of starch in foodstuffs, less color formation compared with maltose syrups, glucose syrups or sucrose, good heat stability, low solution viscosity, high fermentability and favoring glassy states. These properties are very useful in food and pharmaceutical industries (Zoebelein & Böllert, 2001). High maltotriose syrup may be applied in the food industry for the manufacturing of desserts, baking and brewing, as well as in the pharmaceutical industry for replacing glucose in intravenous feeding.

The specificity of enzymes and their catalytic abilities make them suitable for many industrial applications. This approach is particularly more effective if an immobilized biocatalyst is used,

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since it allows either reuse of biocatalyst or a continuous mode of operation and also prevents contamination of the processed product. The economic considerations dictate the use of cheap and simple, yet effective immobilization method with carefully characterized system when scale-up is foreseen. Nevertheless, despite of its appreciable stability, pullulanase from *Bacillus acidopullulyticus* was easily inactivated in free form at higher temperatures (Singh, Saini, & Kennedy, 2010a). Therefore, the improvement in the stability of pullulanase as well as possibility of its reuse in a batch system or continuous mode of operation, are the targets of considerable importance.

In our previous studies, covalent immobilization with a simple low cost method for the hydrolysis of pullulan in a batch system has been discussed (Singh, Saini, & Kennedy, 2010b). The improved thermal stability of the immobilized biocatalyst in a batch system prompted us to investigate its application in a continuous system. Here, we report the continuous hydrolysis of pullulanase covalently immobilized in a packed bed reactor. This is the first report on continuous hydrolysis of pullulan.

2. Materials and methods

2.1. Immobilized biocatalyst

Pure pullulanase (Source: *B. acidopullulyticus*) procured from Sigma, USA was covalently immobilized on Duolite XAD 761 (SD Fine-Chem. Ltd., India) as described earlier (Singh, Saini, & Kennedy, 2010b). Briefly, the resin was first modified with glutaraldehyde (1.25%, v/v) for 2 h at room temperature to generate an activated support containing carbonyl groups and then incubated with pullulanase for 24 h. Covalent immobilization of pullulanase was based on the formation of Schiff's base between the aldehyde group of glutaraldehyde derivatized resin and amino group of the enzyme. By this technique, the immobilized biocatalyst contained 3.10 U g^{-1} of wet resin with 65 ± 1.65% recovery yield.

2.2. Substrate

Pullulan was produced from *A. pullulans* FB-1 in shake-flask fermentations as reported earlier (Singh, Singh, & Saini, 2008) and purified by the method as described by current authors (Singh, Saini, & Kennedy, 2009). Purified pullulan was used for hydrolysis in continuous system. The number-average molecular weight (Mn) of the pullulan was 185 kDa.

2.3. Reactor system

To develop a continuous system for the hydrolysis of pullulan, a jacketed column (1 cm × 20 cm, GE Healthcare Biosciences Ltd., USA) was used for the packed bed reactor. The column was packed with 11 g (wet weight) of resin containing immobilized pullulanase (34.10 U). The bed height of the bioreactor was 18 cm. Total volume and the void volume of the reactor were 15.7 mL and 6.5 mL, respectively. Void volume was calculated as under:

$$V = V_t - V_s$$

where V = void volume; V_s = volume of support; V_t = total reactor volume

The temperature of the packed column was maintained at 60 °C by circulating water through the outer jacket. The upward flow of the pullulan solution was maintained for hydrolysis.

2.4. Continuous hydrolysis of pullulan in a packed bed reactor

Pullulan (0.44%, w/v) in sodium phosphate buffer (0.1 M, pH 5.5) was fed continuously into the column using a peristaltic pump. This concentration of pullulan was selected on the basis of kinetic characterization of immobilized pullulanase for the hydrolysis of pullulan in a batch system (Singh, Saini, & Kennedy, 2010b) to maintain the operational stability for a longer period. The upward flow of the pullulan solution was maintained. The effect of flow rate on the performance of the continuous system was investigated. Hydrolysis of pullulan in the packed bed reactor (PBR) was operated at varied flow rates (1.0–10 mL h^{-1}). The column was equilibrated for 2–10 h for each change of flow rate according to the residence time at the respective flow rate to achieve a steady state concentration before the collection of sample for analysis. Residence time (τ) was calculated as under:

$$\text{Residence time (h)} = \frac{V}{F}$$

where, F = flow rate (L h^{-1}) and V = reactor volume (L).

The samples were analyzed for reducing sugars. Volumetric productivity (g L^{-1} h^{-1}) was calculated as under:

$$\text{Volumetric productivity}\,(g\,L^{-1}\,h^{-1}) = C \times \frac{F}{V}$$

where, C = reducing sugars produced (g L^{-1}), F = flow rate (L h^{-1}) and V = reactor volume (L).

2.5. Operational stability of immobilized biocatalyst

The continuous hydrolysis of pullulan was carried out successfully in a packed bed reactor by immobilized pullulanase (34.10 U). The operational stability of the system was investigated by continuously running the system under standardized conditions, until enzyme activity of the immobilized biocatalyst was reduced to half. The system was operated continuously for 32 days by feeding pullulan (0.44%, w/v) at a flow rate of 5 mL h^{-1}. The samples were collected at 6 h intervals and analyzed for the products. Hydrolysis (%) and volumetric productivity (g L^{-1} h^{-1}) were also calculated at each interval.

2.6. Pullulanase activity

Pullulanase activity was determined as described previously (Singh, Saini, & Kennedy, 2010b). Briefly, reaction mixture (3 mL) consisting 0.5 mL of pullulan (1%, w/v), 0.5 mL of appropriately diluted enzyme and 2.0 mL of 0.1 M phosphate buffer (pH 5.0) was taken in a test tube and incubated at 50 °C in a water bath for 20 min. After incubation, the test tube was kept at 100 °C for 10 min to inactivate the enzyme. Reaction mixture was assayed for reducing sugars by the DNSA method (Miller, 1959). One unit of enzyme is defined as amount of enzyme that produces 1 μmole of reducing sugars (as maltotriose equivalents) per minute under standard assay conditions. The enzyme units are expressed per gram of the matrix.

2.7. Statistical analysis

Experiments were carried out in triplicates and the mean values were calculated. One-way analysis of variance (ANOVA) and pair wise multiple comparison procedures (Tukey's test) were carried out using the statistical software SigmaStat, version 2.0 (Jandel Corp., San Rafael, CA, USA). Values are expressed as the mean ± s.e.m. The level of significance was set at $P < 0.001$.

3. Results and discussion

3.1. Biocatalyst immobilization

Due to the relatively high cost of enzymes used in food processing, it is often advantageous to have a system in which the enzyme can be recovered or utilized repeatedly. A common method of achieving this aim is to immobilize the enzyme on a solid support. Among the many advantages of using immobilized enzymes, such as continuous processing and enzyme reuse, a property of particular significance to the food industry is avoiding the presence of extraneous compounds in the final product. With this aim, the feasibility of immobilization of pure pullulanase on Duolite XAD761 was carried out. Duolite XAD761 consists of a synthetic hydrophobic matrix made of phenol–formaldehyde copolymer. Pullulanase can be adsorbed directly on it but this form of immobilization is reversible and therefore not preferred. In the present study, pullulanase was covalently immobilized on Duolite XAD761. The developed enzyme system contained 3.1 U g^{-1} of wet resin. In a separate study, the developed immobilized biocatalyst has been very well investigated for kinetic and thermodynamic characteristics (Singh, Saini, & Kennedy, 2010b). Moreover, the system has been reported very effective in terms of enzyme stability for batch hydrolysis of pullulan. *B. acidopullulyticus* pullulanase has been reported to be immobilized by adsorption on porous glass (PG-Pase), covalent binding on chitosan beads treated with glutaraldehyde (GA-CB-Pase) and ionic binding on Amberlite IRC-50 (IRC-Pase) with high pullulanase activities (Kusano, Shiraishi, Takahashi, Fujimoto, & Sakano, 1989).

3.2. Immobilized enzyme reactor

PBR is the most frequently used bioreactor with minimum enzyme linkage and carrier attrition problems. These types of reactors are continuing to dominate the large scale industrial applications of immobilized enzymes. The good stability of the developed immobilized biocatalyst for the hydrolysis of pullulan in a batch system (Singh, Saini, & Kennedy, 2010b) has prompted us to investigate its applications in a continuous system. PBR was loaded with 11 g of wet resin having 34.10 U of pullulanase. The operation of the reactor was facilitated by the upward flow of substrate so that the self compression of the immobilized enzyme biocatalyst causing clogging problems can be minimized. The most important operating parameters affecting the performance of an immobilized enzyme reactor are temperature, substrate concentration, flow rate of substrate feeding and operational stability. Amongst these, column temperature is very important parameter to maintain the initial enzyme activity for a longer operational time. The optimal temperature of 60 °C and substrate concentration (0.44%, w/v) was selected for continuous system based on our earlier findings of batch hydrolysis of pullulan (Singh, Saini, & Kennedy, 2010b). PBRs have been reported very efficient and operationally stable immobilized enzyme reactors for continuous systems (Gill, Manhas, & Singh, 2006; Singh, Dhaliwal, & Puri, 2008; Wenling, Huiying, & Shiyuan, 1999).

3.3. Continuous hydrolysis of pullulan in packed bed reactor as a function of flow rate of substrate

PBR was operated at various flow rates (1.0–10 mL h^{-1}) to optimize the hydrolysis of pullulan. The observations were made up to the steady state at all investigated flow rates (Fig. 1). A decrease in pullulan hydrolysis was observed with the increase in flow rate of pullulan solution. Maximum pullulan hydrolysis of 99.80 ± 0.15% was obtained at flow rate 1–5 mL h^{-1} (τ = 6.50–1.30 h). Further increase in flow rate resulted in decrease of pullulan hydrolysis. Decrease in hydrolysis rate with increasing flow rate of the substrate may be attributed to the decrease in contact time of substrate and immobilized biocatalyst. Our results corroborate the earlier findings of continuous hydrolysis in packed bed reactors where inulinases have been used as biocatalyst (Kim & Rhee, 1989; Nakamura, Ogata, Shitara, Nakamura, & Ohta, 1995). A specific residence time of the substrate in PBR is required to obtain maximum hydrolysis which directly depends upon the volume of the reactor used. The flow rate of 5 mL h^{-1} (τ = 1.3 h) was considered optimal for the hydrolysis of pullulan in PBR and selected for further experiment.

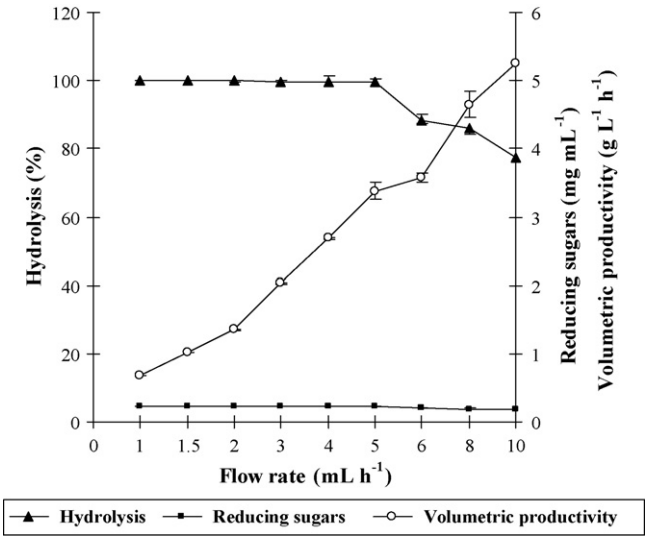

Fig. 1. Pullulan hydrolysis at 60 °C as a function of flow rate in a packed bed reactor containing immobilized biocatalyst (34.10 U).

3.4. Operational stability of immobilized biocatalyst in packed bed reactor

The success of any developed bioreactor is dependent largely upon the maintenance of the biocatalytic activity over the operational time. The operational stability of the developed reactor was studied for a prolonged period under the above optimized conditions using purified pullulan. A continuous hydrolysis of 0.44% pullulan at a flow rate of 5 mL h^{-1} (τ = 1.3 h) was monitored at a column temperature of 60 °C by measuring the reducing sugars and volumetric productivity in the hydrolyzate (Table 1). Hydrolysis of pullulan to reducing sugars was continued for 32 days (Fig. 2). Hydrolysis recorded after 15 days was 80%. The extent of pullulan hydrolysis gradually decreased to almost 50% after 31 days of continuous operation. Immobilized biocatalyst lost half of its original activity after 31 days of continuous operation at 60 °C and the sys-

Table 1
Reducing sugars released and volumetric productivity from pullulan (0.44%, w/v) during continuous operation of packed bed reactor at 60 °C and a flow rate of 5 mL h^{-1}.

Day	Reducing sugars (mg mL^{-1})	Volumetric productivity (g L^{-1} h^{-1})
1	4.40 ± 0.01	3.38 ± 0.02
5	4.29 ± 0.04	3.29 ± 0.03
10	3.99 ± 0.06	3.06 ± 0.05
15	3.51 ± 0.05	2.69 ± 0.04
20	3.06 ± 0.06	2.44 ± 0.09
25	2.46 ± 0.08	1.89 ± 0.06
30	2.23 ± 0.05	1.72 ± 0.04
31	2.18 ± 0.04	1.68 ± 0.03
32	2.13 ± 0.05	1.64 ± 0.04

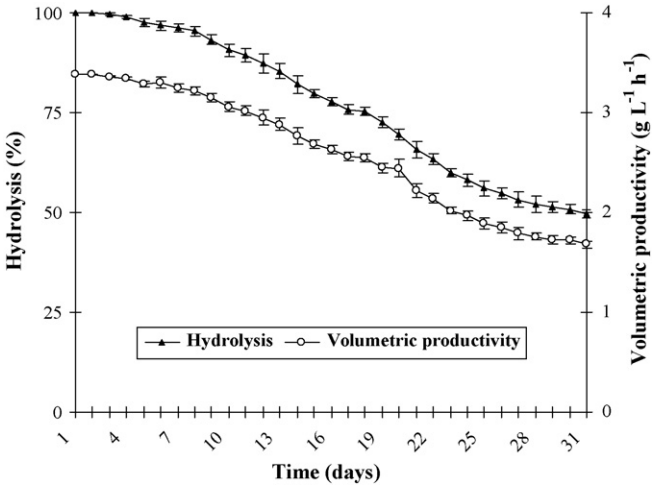

Fig. 2. Operational stability of immobilized biocatalyst in a packed bed reactor at 60 °C with feeding of pullulan solution (0.44%, w/v) at a flow rate of 5 mL h^{-1}.

tem was terminated after 32 days. Activity loss can be resulted from enzyme denaturation, pore blockage and physical loss of enzyme from the carrier due to erosion or from the fracture of the bonds between enzyme and carrier. Factors other than thermal deactivation may also be responsible for the low operational stability especially in packed bed reactors (Singh, Dhaliwal, & Puri, 2008). There was no microbial contamination observed in the column over the operational time. The literature survey reveals no report on continuous system developed for the hydrolysis of pullulan using whole cells or enzyme immobilization.

4. Conclusions

Immobilized pullulanase was successfully used in a continuous flow reactor for pullulan hydrolysis. In an earlier study, the repeated use of the immobilized biocatalyst in successive batches supported the feasibility of using the system in continuous mode. Simple enzyme packing of the column, no distinct mass transfer limitations, high capacity and long-lasting activity of the enzyme made the PBR set-up very suitable for continuous hydrolysis of pullulan. The half-life of the developed system was 31 days at 60 °C, which is very encouraging. The remarkable operational and mechanical stability of the immobilized biocatalyst suggests that it is a promising candidate for the development of a highly effective set-up on higher scale for pullulan hydrolysis.

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