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Production of xylanase by immobilized *Trichoderma reesei* SAF3 in Ca-alginate beads

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Abstract In the present study, the optimum conditions for the production of xylanase by immobilized spores of *Trichoderma reesei* SAF3 in calcium alginate beads were determined. The operational stability of the beads during xylanase production under semi-continuous fermentation was also studied. The influence of alginate concentration (1, 2, 3, and 4%) and initial cell loading (100, 200, 300, 400, and 500 beads per flask) on xylanase production was considered. The production of xylanase was found to increase significantly with increasing concentration of alginate and reached a maximum yield of 3.12 ± 0.18 U ml^{-1} at 2% (w/v). The immobilized cells produced xylanase consistently up to 10 cycles and reached a maximum level at the forth cycle (3.36 ± 0.2 U ml^{-1}).

Keywords Xylanase · Immobilization ·
Trichoderma reesei

Introduction

Enzymes are the catalytic cornerstone of metabolism and as such are the focus of worldwide intense research, not only in the biological community, but also with the process designers/engineers, chemical engineers, and researchers working in other scientific fields. In this context xylanase (β 1-4-D-xylan xylanohydrolase; EC 3.2.1.8) responsible for the breakdown of xylan, a major component of plant hemicellulose consisting of xylose, has gained importance in different industries. The enzyme is extensively used in the paper and pulp industries for biopulping and biobleaching [1]. In the paper and pulp industries, xylanase helps to remove the lignin from the cellulose network by breaking the hemicellulose chain to produce brighter papers as compared to those obtained from chemical based processing, and causes a decrease in consumption of chlorine, absorbable organic halogen (AOX), and chemical oxygen demand (COD) which improves the quality of wastewater. Therefore, the effluent of these industries does not create significant pollution to the environment. The use of xylanase in this process is not only economical but also can improve the quality of the paper. Xylanase is also used to improve the nutritive quality of poultry feed and baked products by thinning out the grains and thereby improving their digestibility [2, 10], for the extraction of coffee, plant oils, and starch [14]; in combination with pectinase and cellulase for clarification of fruit juices [4]; and degumming of plant fiber sources such as flax, hemp, jute, and ramie [13, 8]. Despite the advantages of using such enzymes, their commercial exploitation has been limited owing to its high up and downstream cost. These problems can be overcome by immobilizing the microbial cell in a suitable matrix.

The immobilization of growing microbial cells is of particular interest because of their biotransformational and biosynthetic abilities for the production of diverse valuable products such as antibiotics, organic acids, enzymes, alcohols and others. Immobilization of whole cells for the production of extracellular enzymes offers many advantages, such as the ability to separate cell mass from the bulk liquid for possible re-use, facilitating continuous operation over a prolonged period, increase in enzyme productivity, and long-term stability [15, 6]. In this regard, it is necessary to select the proper technique and supporting material for

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immobilizing the microbial cells for production of an extra-cellular enzyme. One of the most suitable and popular methods for cell immobilization is entrapment of cells in Ca-alginate, because this technique is not only simple and inexpensive but also non-toxic.

In the present study, spores of the potent xylanase producer *Trichoderma reesei* SAF3 were immobilized in Ca-alginate and various parameters were optimized for enhanced rate of enzyme production.

Materials and methods

Microorganism

A potent xylanase producing fungus, *T. reesei* SAF3 (MTCC-4876) was isolated on a selective xylan-agar medium, from soil samples of Paschim Medinipur District, West Bengal, India [9] and was preserved in xylan-containing PDA slants at 4°C for further use.

Inoculum preparation

The inoculum was prepared from the 5-day-old induced culture. A known volume (10 ml) of sterile distilled water was added to the culture, which was gently vortexed to release the spores. The quantification of the spores in the suspension was determined using a hemocytometer. In each case, a suspension containing approximately 5×10^5 spores/ml was used as inoculum.

Immobilization of whole cells

Sodium alginate (Sigma, USA) was dissolved in boiling distilled water and autoclaved at 121°C for 15 min. The sterilized alginate solution was then mixed with the spore suspension. The homogeneous suspension containing alginate and spores was dropped into the cold, sterile calcium chloride solution (1 M) using a peristaltic pump to obtain uniform sized polymeric beads (∼2 mm) of Ca-alginate. The entire process was carried out aseptically in a laminar airflow chamber. For further hardening, the newly formed beads were resuspended into fresh $CaCl_2$ solution for 24 h at 4°C. Finally, these beads were washed with distilled water to remove excess calcium ions and unentrapped spores. The beads were then transferred into 250 ml Erlenmeyer flasks containing 50 ml of culture medium containing (w/v) xylan, 1%; $CaCl_2$, 0.06%; $MgSO_4$, 0.06%; $(NH_4)_2SO_4$, 0.2%; KH_2PO_4, 0.1%; and K_2HPO_4, 0.1%. Fermentation was carried out on a rotary shaker (150 rpm) at 30°C. All experiments were performed in triplicates and the data are presented as mean ± SE.

Assay of Xylanase

Xylanase activity in the fermented media was estimated by measuring the released reducing sugar from birch wood xylan (Fluka) with 3,5-dinitrosalicylic acid (DNS) [12]. The reaction mixture constituted 0.4 ml of 0.2 M acetate buffer (pH 5.0), 0.3 ml of 1% (w/v) xylan, and 0.3 ml of enzyme solution. The enzymatic reaction was carried out at 50°C in a water bath. The enzymatic reaction was stopped by adding 1 ml of DNS (3%, w/v). The reaction mixture was then boiled for 15 min for colour development and the absorbency was measured at 540 nm against the enzyme blank. The xylanase activity was determined by using a calibration curve of D-xylose (Sigma). One unit of xylanase activity (U ml^{-1}) is defined as the amount of enzyme required to produce 1 μM of reducing sugars by hydrolyzing xylan per minute under the above assay conditions.

Repeated batch cultivation

The alginate beads were washed with sterile saline water after every experiment. Repeated batch fermentation was carried out with washed beads by decanting the fermented medium every 24 h and replacing it with a fresh xylan medium (as described above).

Results

Effect of alginate concentration

Beads prepared with different concentrations of sodium alginate (1–4%, w/v) were tested for enzyme production. Xylanase production increased with the increase of alginate concentration up to 2% (Fig. 1).
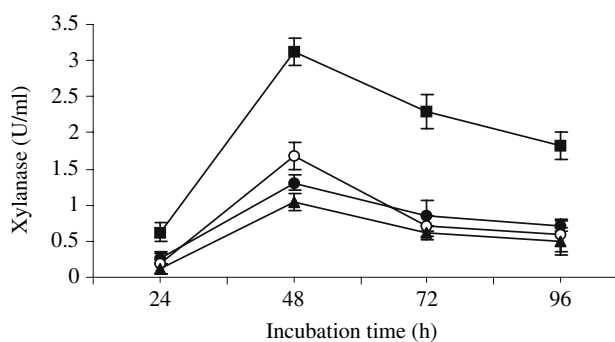

Fig. 1 Effect of different alginate concentrations (*filled circle*) 1, (*filled square*) 2, (*open circle*) 3, and (*filled triangle*) 4% on xylanase production, *T. reesei* SAF3. Fermentation was carried out at 40°C for 96 h in a 250 ml Erlenmeyer flask

Effect of initial cell loading

The effect of initial cell loading (ICL) was tested by varying the number of beads (each bead contains approximately 5×10^4 spores) from 100 to 500 per flask (250 ml Erlenmeyer flasks containing 50 ml of culture medium). Maximum amounts of enzyme were produced with the ICL of 300 beads per flask. Higher or lower numbers of the beads, i.e. the inoculum levels, resulted in reduced xylanase production (Fig. 2).

Effect of substrate concentration

Xylan acts as the sole carbon source as well as main substrate for xylanase synthesis. Enzyme production by the immobilized fungal cell was studied in media containing different concentrations of xylan (0.5–3.0%, w/v). The production of xylanase increased with increasing concentration of the xylan and reached a maximum yield of 3.64 ± 0.21 U ml^{-1} at 1% (Fig. 3) in the immobilized condition, whereas in free cell cultivation, maximum enzyme
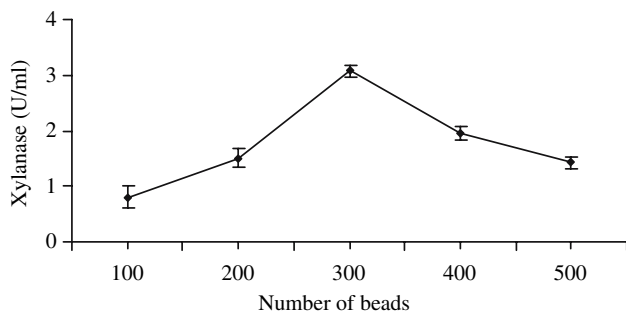
production was observed (3.42 ± 0.07 U ml^{-1}) at 1% (w/v) concentration of xylan.

Comparisons of xylanase production by free and immobilized *T. reesei* SAF3

Enzyme production in the case of free and immobilized spores was studied by varying the incubation period from 24 to 96 h. Xylanase production by free cells reached a maximum level of 3.78 ± 0.12 U ml^{-1} at 72 h, and then gradually declined. The immobilized cell produced essentially the same amount of enzyme at 48 h (Fig. 4).

Reuse of immobilized beads

The reusability of the immobilized beads for xylanase production was tested by a semi-continuous fermentation process. The highest enzyme production (3.36 ± 0.2 U ml^{-1}) was observed at the fourth repeated cycle (Fig. 5). Signifi-

Fig. 2 Effect of initial cell loading on xylanase production by immobilized *T. ressi* SAF3. Each bead contains approximately 5×10^4 fungal spores. Fermentation was carried out in shake condition for 48 h

Fig. 4 Effect of incubation time on xylanase production by free (*filled square*) and immobilized (*open circle*) *T. reesei* SAF3. Fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml of xylan containing enriched media with the same quantity of free and immobilized spores at 40°C
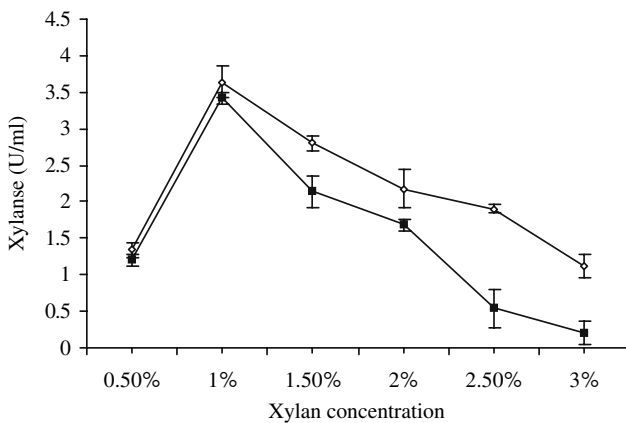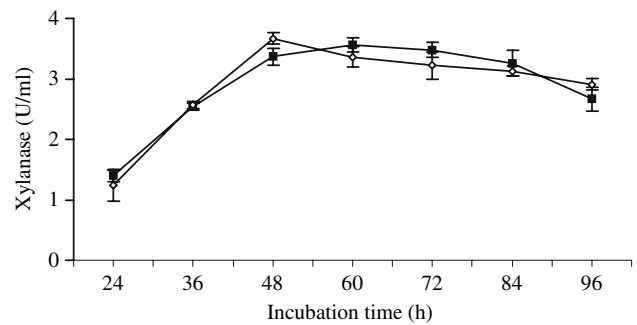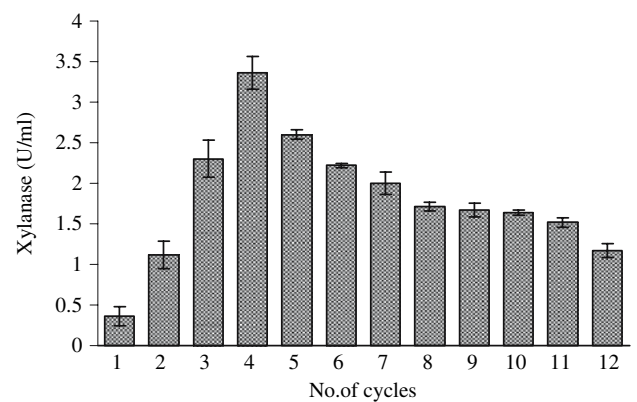
Fig. 3 Effect of xylan concentration (0.5–3.0 g%) on xylanase production by *T. reesei* SAF3 in free (*filled square*) and immobilized beads (*open circle*). Bioconversion was carried out in 250 ml Erlenmeyer flasks containing 50 ml of medium and 300 beads at 40°C for 48 h

Fig. 5 Study of repeated batch fermentation of immobilized *T. reesei* SAF3 for xylanase production. Each cycle was carried out for 48 h in a rotary shaker. After each cycle the beads were washed with sterilized saline (0.9% NaCl) and incubated with fresh xylan containing medium

cant xylanase production was observed up to eleven cycles (1.64 ± 0.05 U ml^{-1}).

Discussion

Immobilized microbial cells have attracted the attention of biotechnological industries due to high-efficiency and low-production cost. These cells are more stable, active, and reusable; and can eliminate most of the constraints faced by free cells. The demand and applications of xylanase in the paper, pulp, and food industries are increasing day by day.

In the present study, beads were prepared by varying the concentration of alginate to determine the suitable strength of beads for the entrapment of the *T. reesei* spores. Xylanase production below or above 2% (w/v) of alginate concentration was accompanied by a decrease in yield. At low-alginate concentrations, the beads were relatively soft and showed rapid leakage of cells from the beads. The strength of the beads improved at higher concentrations of alginate, which prevents the growth of the cell as well as release of enzyme from the beads. At higher alginate concentrations the rate of substrate translocation is limited due to lower porosity and thereby prevents the enzyme production, as reported by Fumi et al. [5] and Martinsen et al. [11].

The effect of ICL, in the form of the number of beads per flask, on xylanase production was studied. Xylanase production increased concomitantly with the loading up to 300 beads per flask. This result indicated that a specific ratio of cell and substrate concentration was very important for optimum xylanase production. Beshay [3] reported similar results for alkaline protease production.

Xylanase production was maximum at 1% (w/v) of xylan in the culture medium by immobilized cells (biocatalyst beads), which is similar to submerged (free fungal cell) fermentation. This indicated that a specific amount of xylan is suitable for xylanase induction from *T. reesei* SAF3. Isil and Nilufer [7] reported 1% xylan as the optimum substrate concentration for xylanase production by *T. harzianum*.

The maximum amount of xylanase was achieved at 48 h with immobilized cells in comparison to 72 h in free cell cultivation. This indicated that free cells required more time for induction of enzyme synthesis and attainment of a certain cell mass. On the other hand, active and induced cells were present in the immobilized bead from the initial period of fermentation, therefore, lower incubation periods are needed to synthesize maximum enzyme.

Xylanase production increased gradually up to the fourth repeated batch cycles. During the early repeated cycles, increased amount of xylanase production was noticed and this may be due to the proper adaptation of the fungal cells with the microenvironment and appropriate growth of cells

in the beads. Thus, Ca-alginate is a suitable and robust carrier system for immobilization of this fungal strain.

In conclusion, these studies on the immobilization of *T. reesei* SAF3 spores indicated that: (1) xylanase production was slightly higher in a shorter period of fermentation under immobilized conditions than the free cell system, (2) Ca-alginate beads containing fungal spores can be used repeatedly for xylanase production. The stability of the immobilized beads in operational state is higher because in the immobilized condition, fungal cells always remain in the optimum growth phase and they need not to adjust themselves in the production medium. Therefore, the immobilized fungal cells maintain continuous xylanase biosynthesis reducing the overall production cost of the enzyme, and (3) Ca-alginate is a promising method for immobilization of *T. reesei* SAF3. Alginate is non-toxic and therefore, can be used for large-scale xylanase production.

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