



Optimization of the Cellulase Free Xylanase Production by Immobilized *Bacillus Pumilus*

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Background: The extracellular xylanase secreted by microorganisms is a hydrolytic enzyme, which arbitrarily cleaves the β -1, 4 backbone of the polysaccharide xylan; an enzyme used in the food processing, bio-pulping and bio-bleaching. The commercial production of the xylanase is limited because of a higher cost involvement, which can be overcome by the cost-effective production of the xylanase through immobilization of the microbial cell by the non-toxic substances.

Objectives: In this work, the optimization of the extra-cellular cellulase free xylanase production by the immobilized cell of the *Bacillus pumilus* IMAU80221 strain using Ca-alginate beads along with standardization of the various parameters for a higher xylanase production were studied.

Materials and Methods: Following to sterilization, the Na-alginate solution was mixed with the bacterial suspension of the *Bacillus pumilus* IMAU80221 and was added drop by drop into the 1 M calcium chloride solution for 1 h for obtaining a uniform sized polymeric bead of the Ca-alginate. For xylanase production, the Ca-alginate beads were then transferred into 100 mL Erlenmeyer flasks with 20 mL of the culture medium containing (w/v) 0.02% NaCl, 0.02% $MgSO_4$, 0.04% (KH_2PO_4) , 0.1% peptone, and 0.5% xylan and incubated at 34 °C in an incubator shaker (150 rpm) for 24 h. The resultant supernatant (crude enzyme) was used for enzyme assay.

Results: The maximum xylanase production by the free cell ($1.9 U \cdot mL^{-1} \cdot min^{-1}$) was recorded at 48 h which was 40.5% lower than the xylanase production by the immobilized cell ($2.67 U \cdot mL^{-1} \cdot min^{-1}$) at the same time. The beads containing the immobilized cells could be reused up to eight fermentation cycles for xylanase production and retained 83.5% of the productivity at the fourth cycle. The entrapped cells were stable after six months of storage at 4 °C and retained 68% of the xylanase productivity.

Conclusion: Cellulase free xylanase production from the immobilized *Bacillus pumilus* IMAU80221 was optimized. The xylanase production by the immobilized cells of *Bacillus pumilus* was higher by 40.5 and 132.6 % over the free cells respectively after 48 and 72 h of the incubation.

Keywords: *Bacillus pumilus*, Cellulase free xylanase, Calcium alginate, Immobilization

1. Background

Xylanase, a hydrolytic enzyme, arbitrarily cleaves the β -1, 4 backbone of polysaccharide xylan, is one of the most common renewable polysaccharides of the plant origin after cellulose, accounting 20 to 40 % of the plant dry biomass with a large scope of industrial

application. These enzymes are extensively used in the food processing, improvement of digestibility of the animal feed, important processes like bio-pulping, bio-bleaching in the paper and pulp industries (1), as well as bio-conversion of the lignocelluloses into feed-stocks and fuels (2). Xylanase is also used for coffee extraction,

extraction of oils and starch from plant origin, and for clarification of the fruit juice in combination with pectinases and cellulases, degumming and retting of bast fibres like sunnhemp, ramie, and jute (3, 4).

High amounts of extracellular xylanases are secreted by several microorganisms including bacteria, actinomycetes, and filamentous fungi. Secretion of xylanase often occurs in association with a low to a higher amount of cellulases production. Most of the xylanases producing strains like *Cellulomonas fimi* (5), *Clostridium stercoarium* (6), have a significant cellulase activity. Several xylanase producing strains with the negligible cellulolytic activities like *Bacillus* SSP-34 (7), *Pseudomonas* sp. WLUN024 (8), and *Bacillus subtilis* (9) have also been reported. Three pectinolytic bacterial strains without any cellulolytic activities viz. *Bacillus pumilus* IMAU80221, *Bacillus pumilus* GVC11, and *Bacillus pumilus* SYBC-W isolated from retting water has also been reported (10).

In spite of having advantages of using xylanase enzyme, its commercial exploitation was very limited because of higher cost involvement, which can be resolved by immobilization of the microbial cell. Immobilization of the microbial cells for extracellular xylanase production has several advantages compared to the free cell like a better substrate utilization, longer working life time, ease of separation to facilitate their reuse, protection against high shear damage, favorable microenvironment to the cell, reduction in the possibility of contamination, etc. Despite these advantages, gel degradation, severe mass transfer, low mechanical strength, and large pore size are the limitations associated with the immobilization of the microbial cells. The material and proper technique selection plays a significant role in the immobilization of the microbial cells for commercial production of the extra-cellular xylanase enzyme. The most commonly used and popular method for the microbial cell immobilization is by Ca-alginate beads (11) which has several advantages such as user-friendly lower cost involvement, easier availability, and non-toxic in nature.

The present work describes optimization of the extra-cellular cellulase free xylanase production by the immobilized cells of *Bacillus pumilus* IMAU80221 strain using Ca-alginate beads along with standardization of the various parameters for a higher xylanase production.

2. Objectives

In this work, optimization of the extra-cellular cellulase free xylanase production by the immobilized cells of

Bacillus pumilus IMAU80221 strain using Ca-alginate beads along with standardization of various parameters for higher xylanase production were studied. The outcome of the study will be very useful because of reusability and the long-term storage ability of the entrapped cell.

3. Materials and Methods

3.1. Microorganism and Culture Maintenance

The working strain, *Bacillus pumilus* (Accession no. KM091823), a very high xylanase producer was grown on a selective xylan-agar medium, after isolation from the retting water samples of the West Bengal (22.56670 N, 88.36670 E), India (10). The strain was identified as *Bacillus pumilus* IMAU80221 by 16SrDNA sequencing (ribotyping) of a 977 bp fragment and was deposited to the MTCC, IMTECH, Chandigarh, India for maintenance. For the preparation of the inoculum, *Bacillus pumilus* was mixed with 10 mL basal medium containing (w/v) 0.02% NaCl, 0.02% MgSO₄, 0.04% (KH₄)₂PO₄, 1% peptone, and 1% xylan incubated at 34 °C in a shaker (150 rpm) for 24 h. The microbial cell count in the suspension was 2.0 × 10⁸ cell.mL⁻¹.

3.2. Whole Cells' Immobilization

After dissolving sodium alginate (Hi media, Mumbai) in the boiling distilled water it was sterilized in an autoclave at 121 °C for 15 min. After sterilization, the alginate solution was mixed with the bacterial suspension (2.0 × 10⁸ cell.mL⁻¹) in a conical flask with constant shaking at 250 rpm for 1 h to get a homogeneous suspension, which was then added drop by drop into the cold (25 °C) sterile 1 M calcium chloride solution for 1 h using a syringe having nozzle of 1 mm diameter to obtain a uniform sized polymeric beads of the Ca-alginate. To remove excess calcium ions, these beads were then washed with distilled water. The whole process of Ca-alginate beads preparation was done aseptically in a laminar airflow chamber. In this study, we prepared calcium alginate beads with four different concentration of the sodium alginate.

3.3. Fermentation and Enzyme Production

The Ca-alginate beads were then kept in a 100 mL Erlenmeyer flasks having 20 mL of culture medium containing (w/v) 0.02% NaCl, 0.02% MgSO₄, 0.04% (KH₄)₂PO₄, 0.1% peptone, 0.5% xylan incubated at 34°C and shaking at 150 rpm in an incubator shaker for 24 h. After cultivation, beads were separated out from the medium, centrifugation of broth culture was carried out at 10,000 ×g, 4 °C for 10 minutes, and the resultant supernatant was used as the crude enzyme.

3.4. Enzyme Assay

The xylanase activity was measured by reducing sugar estimation method (12) using 3, 5 dinitro salicylic acid and taking xylose as a standard. In reducing sugar estimation method, the assay mixture (1 mL) containing an equal volume of the enzyme and 1 % (w/v) birch- wood xylan (Sigma, U.S.A) was dissolved in 0.1 (M) phosphate buffer (pH 6) and incubated at 50 °C for 10 minutes. The reaction was then stopped by adding 1 mL of 3, 5 dinitro salicylic acid reagents. A blank was run simultaneously that contained all the reagents with inactivated enzymes by heating at 100 °C for 30 minutes. The contents were placed in a boiling water bath for 10 min for the color development followed by cooling at room temperature. The absorbance was measured at 540 nm against the blank. The xylanase activity was determined by using a calibration curve of D-xylose (Himedia). One unit of enzyme activity was defined as the amount of enzyme that released 1 mili mole of reducing sugar by hydrolyzing xylan per milliliter per minute under assay conditions.

3.5. Optimization of the Different Parameters for Enzyme Production

The flasks containing culture were incubated at four different concentrations of the Ca-alginate beads (3, 5, 7, and 9 % i.e. 3, 5, 7, and 9 g of the sodium alginate was dissolved individually in 100 mL of water) at 34 °C for 24 h in order to study the effect of concentration of the Na-alginate on xylanase production. Similarly, the flasks having cultures in optimized media were incubated for different time intervals varying from 24 to 120 h and then measurement of xylanase activity was done to study the changes in the xylanase production under different cultivation times. To study the effect of agitation on enzyme production, flasks having cultures were incubated at 34 °C in a shaker incubator at the shaking rate of 50, 100, 150, and 220 rpm. After each fermentation cycle, beads were collected from the fermentation medium by sterile forceps, properly washed with the sterile water and further transferred into 100 mL shaking flasks containing 20 mL of sterile, fresh xylan medium for the next 24 h incubation in order to continue the repeated cycles of fermentation.

3.6. Storage of Entrapped Cell

After enzyme production by immobilized cell, beads were aseptically collected from the medium and washed several times with sterile distilled water for removal of impurities and then stored in sterile water at 4 °C for

future use.

All experiments were replicated thrice and the resultant mean data were presented along with standard errors (SE).

4. Results

4.1. Effect of Different Concentration of Na-alginate Beads on Xylanase Production

The maximum xylanase production (1.92 U.mL⁻¹) was recorded with beads containing with 5% alginate followed by 7% sodium alginate concentration (1.64 U.mL⁻¹) and the minimum concentration was recorded at 9% alginate concentration (1.53 U.mL⁻¹) (**Fig. 1**). The xylanase production with 5% alginate was higher by 17.1 and 25.5% over the production at 7% and 9% alginate concentration. From the study, it is clear that the lower and a higher concentration of alginate recorded comparatively lower xylanase concentration product.

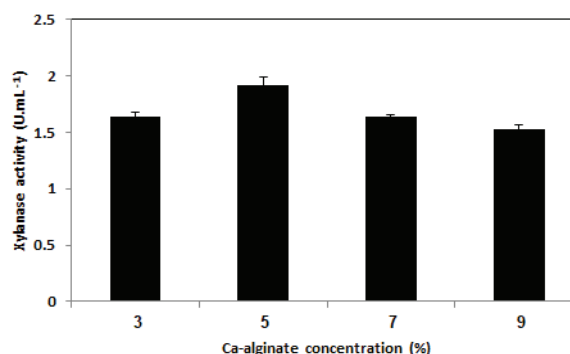


Figure 1. The effect of calcium alginate concentration on xylanase production by immobilized *Bacillus pumilus*.

4.2. Effect of Incubation Time on Xylanase Production

The xylanase production was measured under various incubation time ranging from 24 to 120 h. The maximum xylanase production (1.9 U.mL⁻¹) by free cells was recorded at 48 h, while at the same time, the xylanase production by the immobilized cell was 2.67 U.mL⁻¹, which was 40.5% higher compared to the xylanase production by the free cells (**Fig. 2**). The xylanase production by immobilized cells further increased to a maximum level (2.73 U.mL⁻¹) at 72 h and then gradually decreases with cultivation time. Although the production of xylanase by free cell was higher at 24 h compared to the immobilized cell, however after that at every point of time, the xylanase production by immobilized cells was higher by 40.5 to 132.6% over the free cells.

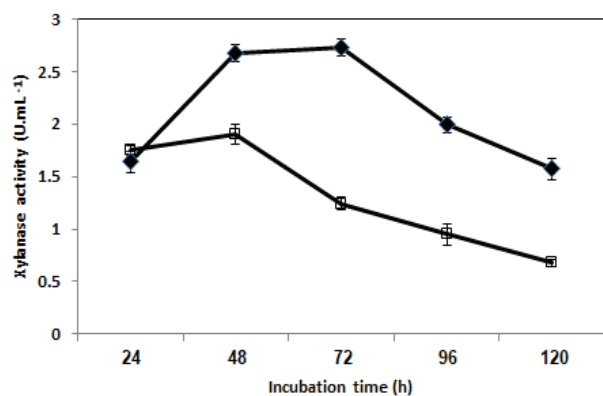


Figure 2. The effect of incubation time on xylanase production by the free and immobilized *Bacillus pumilus* cells.

4.3. Effect of Different Agitation Rate on Xylanase Production

The xylanase production capacity by the immobilized cells was studied under different agitation rate ranging from 50 to 220 rpm in the incubator shaker at 34 °C for 24 h. It was observed that the optimal agitation rate for the extracellular xylanase production by the strain under study was 100 rpm which recorded the maximum xylanase production of 2.64 U.mL⁻¹. After that, xylanase production was decreased by 28% with the increase in rpm (Fig. 3).

4.4. Reuse of the Immobilized Beads

In repeated batch fermentation, beads containing immobilized cells could be reused sequentially up to eight fermentation cycles of 24 h with an agitation rate of 150 rpm. It was noticed that the highest xylanase production of 1.64 U.mL⁻¹ could be recorded for the entrapped cell at first cycle and it retains 83.5% of the xylanase production at fourth cycle (Fig. 4). After the fourth cycle, enzyme production reduced gradually,

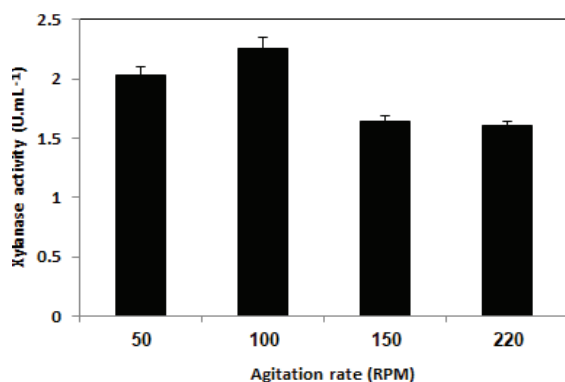


Figure 3. Effect of agitation rate on xylanase production by the immobilized *Bacillus pumilus* bacterial cells.

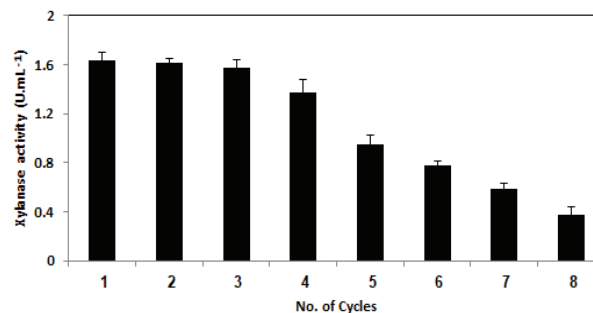


Figure 4. Study of the repeated batch fermentation of the immobilized *Bacillus pumilus* for xylanase production.

more than half of the production was lost after the fifth cycle that may be due to the abrasion of the support during reuse. Finally, xylanase production by the immobilized cell was retained 23.1% at the eighth cycle. After each cycle, the beads were in stable condition, easily separated from the medium and no leakage was observed.

4.5. The Viability of the Stored Immobilized Cell

The immobilized cells were stored in the sterile distilled water for six months and 12 days at 4 °C. After the storage time, it was noticed that, when the immobilized cells were further grown in the xylan broth medium with a temperature of 30 °C, agitation rate of 150 rpm, and incubation time of 24 h, the entrapped cells were viable and stable condition and retained 68 % enzyme production (Fig. 5).

5. Discussion

In the present study, out of the four concentrations (3, 5, 7, and 9 %) of the used alginate, the lower and the higher alginate concentration (3 and 9 %) recorded lower xylanase productivity compared to 5 and 7 %

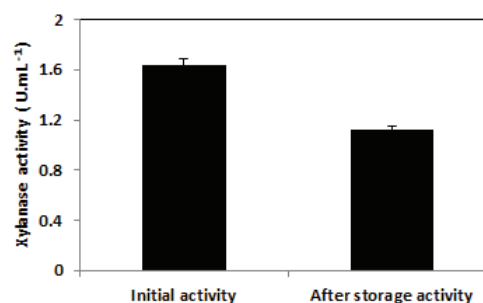


Figure 5. The viability of the stored immobilized *Bacillus pumilus* cells.

of the alginate concentration. Preparation of the beads with very low and high alginate concentration have some disadvantage because the low concentration of alginate beads allows quick leakage of the entrapped cells out of the beads (13); on the other hand, the higher alginate concentration checks cells growth and release of enzymes (14). This might have reflected in the higher xylanase production at intermediate concentrations of the alginate (5 and 7 %) compared to the lower xylanase production at lower and higher concentration (3 and 9 %) of the alginate.

The xylanase production by the immobilized cell of *Bacillus pumilus* increased with an increase in the incubation time up to 72 h and then decreased gradually due to a lower supply of nutrients to the microorganism and due to proteolysis creating unfavorable condition for physiological and enzymatic activities of the microbes (15). In case of free cells, the xylanase production was higher at 24 h compared to the immobilized cell of *B. pumilus*, but with an increase in the incubation time, the xylanase production was lowered by 40.5 to 132.6% compared to immobilized cells of *B. pumilus*. It could be because of adsorption of bacterial cells on supporting medium and thereby enhancing the residence time of bacterial cells in the medium (16).

The xylanase production by the immobilized cells of *Bacillus pumilus* was affected by agitation, which was maximum at 100 rpm, but decreased with a higher agitation up to 220 rpm. The lower xylanase production with a higher agitation rate might be due to the deleterious effect of the higher shearing force; causing the death of bacterial cells and release of proteases which can restrict xylanase production (17).

The beads containing immobilized cells of the *Bacillus pumilus* could be reused sequentially up to eight fermentation cycle- of 24 h for xylanase production, although the entrapped cells retained 83.5% of their xylanase productivity at the 4th cycle. These results are in agreement with the xylanase production by immobilized *Bacillus atrophaeus* (18).

The immobilized cells of *B. pumilus* were viable after 6 months of storage in sterile distilled water at 4°C and retained 68% xylanase productivity, respectively. This is because of the fact that mild environment of the gel network helped in maintaining a proper viability of immobilized cells in alginate during long-term storage. Similar finding were also reported earlier in which beads were active for 3 months without a decrease in their activity (13). These long-term storage properties are very important for the future applications.

The present study provides valuable information that immobilized strain of *Bacillus pumilus* in 5% calcium

alginate produced higher cellulose free extracellular xylanase compared to free cells in a very shorter duration of 48 h and continued to produce higher xylanase up to 72 h. The immobilized *Bacillus pumilus* can also be reused in eight sequential fermentation cycles for xylanase production although the entrapped cell retained 83.5% of its production at the 4th cycle. Furthermore, the entrapped cells retained 68% of xylanase production after six months of storage at 4 °C. Hence, the strain under study has a tremendous scope of the industrial application for extracellular xylanase production without any cellulase activity.

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