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# Agar–agar entrapment increases the stability of endo-β-1,4-xylanase for repeated biodegradation of xylan

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ABSTRACT

Microbial xylanases, specially endo-β-1,4-xylanase catalyzes the hydrolysis of xylan, is considered one of the most significant hydrolases. It has numerous applications but most extensively is utilized in paper and pulp industry as a bio-bleaching agent. Immobilization technique is comprehensively studied with the expectation of modifying and improving enzyme stability and characteristics for commercial purposes. Currently, matrix entrapment technique is applied to immobilize endo-β-1,4-xylanase within agar–agar gel beads produced by *Geobacillus stearothermophilus* KIBGE-IB29. Maximal enzyme immobilization yield was achieved at 2.5% of agar–agar concentration. Optimized conditions demonstrated an increase in the optimal reaction time from 05 min to 30 min and incubation temperature from 50 °C to 60 °C with reference to free enzyme whereas; no effect was observed for optimum pH. Entrapment technique uniquely changed the kinetic parameters of immobilized endo-β-1,4-xylanase ($K_m$: 0.5074 mg min$^{-1}$ to 0.5230 mg min$^{-1}$ and $V_{max}$: 4773 U min$^{-1}$ to 968 U min$^{-1}$) as compared to free enzyme. However, immobilized enzyme displayed broad thermal stability and retained 79.0% of its initial activity at 80 °C up to 30 min whereas; free enzyme completely lost its activity at this temperature. With respect to economic feasibility, the immobilized enzyme showed impressive recycling efficiency up to six reaction cycles.



## 1. Introduction

Xylan is amongst abundantly available hetero-polysaccharides and is composed of β-1,4 linked D-xylopyranosyl residues with diverse side chains. A synergistic action of endo-β-1,4-xylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase and acetyl xylan esterase is usually required to completely hydrolyze the complex xylan structure [1,2]. Endo-β-1,4-xylanase randomly cleaves β-1,4 linkages in xylan and as a result xylose units are released. This hydrolase has several commercial applications in various industrial processes including ethanol production, clarification of fruit juices, animal feed processing, textile, baking and paper industry [3]. The catalytic efficiency of any enzyme is a very important aspect for its commercialization. Most of the times, free enzymes are unable to accomplish the requirement of industries due to either lower operational stability, difficult enzyme recovery procedure or no recycling efficiency [4]. For the continuous economic operation, enzyme immobilization is an encouraging way not only to improve the catalytic properties of an enzyme onto/within different synthetic and non-synthetic matrices but also to ensure the reusability of costly enzymes by multiple times [5]. Therefore, immobilization of different enzyme using numerous techniques is now considered an important aspect of biotechnological processes. These immobilization techniques involve covalent binding, physical adsorption, crosslinking-adsorption and entrapment methods. Covalent binding and physical adsorption mostly decreases enzyme activity by affecting the substrate binding sites of an enzyme and act as a strong barrier for substrate diffusion [6,7]. Entrapment technique physically restricts the enzyme within a structured polymer matrix and causes negligible effect on enzyme catalytic properties. However, crosslinking-adsorption using glutaraldehyde as a crosslinking agent also proved to be an effective technique for immobilizing commercial xylanase and cellulase [8]. Different matrices such as calcium alginate beads, agar–agar and polyacrylamide gel have been used for the entrapment of different enzymes and cells [9–11] and amongst them agar–agar is a biocompatible, non-toxic and strong solidifying agent to immobilize various enzymes [12,13].

In the current investigation, endo-β-1,4-xylanase produced by *Geobacillus stearothermophilus* KIBGE-IB29 is immobilized within agar–agar matrix using entrapment technique. The characteristic catalytic properties of immobilized and free (native) enzymes were

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studied including kinetic activity (optimum pH, optimum temperature, $V_{max}$ and $K_m$), thermal stability and reusability in terms of recycling efficiency for the development of continuous use in industrial bioprocesses. Xylanase bound polymer was also examined using scanning electron microscopy (SEM).

## 2. Material and methods

### 2.1. Endo-β-1,4-xylanase production

*Geobacillus stearothermophilus* KIBGE-IB29 [GenBank Accession: KF241865.1] used in the current study was previously isolated from a blast furnace site of steel mill industry [14]. Endo-β-1,4-xylanase was produced using optimized fermentation medium ($g\,L^{-1}$): Nutrient broth, 13.0; xylan, 5.0; $K_2HPO_4$, 2.5; $KH_2PO_4$, 0.5; $CaCl_2$, 0.1 and $(NH_4)_2SO_4$, 0.5 with pH 6.0 at 60 °C for 24 h of incubation time [14]. Cells were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C and the supernatant was precipitated using 40.0% ammonium sulfate saturation. The precipitates were dissolved in phosphate buffer (25.0 mM, pH 7.0) and was desalted using dialysis tubing (12,000 kDa cut-off, Servapor®) in same buffer. These dialyzed precipitates were further used for the entrapment procedure within agar–agar support.

### 2.2. Immobilization of endo-β-1,4-xylanase within agar–agar matrix

The entrapment of endo-β-1,4-xylanase was initiated by mixing an equal amount of dialyzed enzyme with agar–agar solutions in 1:1 ratio. Initially, 2.5% agar–agar solution was dissolved by vigorous shaking at 100 °C. The solution was cooled down in between 40 °C and 45 °C and afterwards the enzyme was mixed thoroughly before the gelling temperature. This mixture was immediately poured into a clean petri plate and let stand for solidification at room temperature. The enzyme containing agar–agar gel was cut into small beads (5.0 mm by metallic cork borer) and was washed with phosphate buffer (25.0 mM, pH 7.0) to remove unentrapped or loosely entrapped enzyme. Finally, immobilized enzyme beads were weighed (0.5 g) and used for further analysis. An agar–agar bead without immobilized enzyme was used as a control.

### 2.3. Enzyme assay

The catalytic activity of free and immobilized endo-β-1,4-xylanase was measured by estimating the reducing sugar using 3′5′-dinitrosalicylic acid (DNS) method [15] in the presence of xylan (substrate) and xylose was used as a standard to plot the standard curve. Free enzyme (100 μl) was mixed with 2.0% xylan (1.0 ml) which was solubilized in phosphate buffer (25.0 mM, pH 7.0) and incubated at 50 °C for 5.0 min. Afterwards DNS solution (1.0 ml) was incorporated and kept in boiling water bath for 5.0 min. The reaction mixture was allowed to cool at room temperature and 9.0 ml deionized water was mixed. However, slightly modified protocol was followed for immobilized enzyme. Initially, 0.5 g of immobilized beads (1336 U $mg^{-1}$) were mixed with 2.0% xylan (1.0 ml) and incubated at 50 °C for 30 min. From this reaction mixture 1.0 ml was transferred to another tube containing DNS solution (1.0 ml). Rest of the procedure was followed exactly the same as for free enzyme assay. Optical density (OD) was measured at 546 nm against reagent blank. One unit of endo-β-1,4-xylanase is defined as "*the amount of enzyme required to produce 1.0 μmol of xylose per minute under standard assay conditions*".

### 2.4. Effect of agar–agar on the immobilization of endo-β-1,4-xylanase

The effect of agar–agar concentration was studied from 1.0% to 5.0%. The polymer solutions were prepared in phosphate buffer (25.0 mM, pH 7.0) by vigorously heating in water bath at 100 °C.

### 2.5. Effect of reaction time on the activity of free and immobilized endo-β-1,4-xylanase

Optimum reaction time of free and immobilized endo-β-1,4-xylanase was determined by performing the enzyme assay for different time intervals ranging from 5.0 min to 60 min at a constant temperature (50 °C), pH (7.0) and substrate concentration (2.0%).

### 2.6. Effect of temperature on the activity of free and immobilized endo-β-1,4-xylanase

The impact of different incubation temperatures was investigated on the catalytic activity of free and immobilized enzyme by measuring the enzymatic activity at different temperatures ranging from 30 °C to 80 °C at a constant pH (7.0), reaction time (5.0 min and 30 min for free and immobilized enzyme, respectively) and substrate concentration (2.0%). Immobilized beads were incubated without buffer at aforementioned temperatures and remained stable at all temperatures.

### 2.7. Effect of pH on the activity of free and immobilized endo-β-1,4-xylanase

The optimum pH of free and immobilized enzyme was determined by measuring the enzyme activity in different pH buffers ranging from 4.0 to 10.0 with same ionic strength (25.0 mM). Buffers used were phosphate–citrate buffer (pH 4.0–6.0), potassium phosphate buffer (pH 7.0–8.0) and glycine–NaOH buffer (pH 9.0–10.0).

### 2.8. $K_m$ and $V_{max}$ of free and immobilized endo-β-1,4-xylanase

The Michaelis–Menten constant ($K_m$) and maximum velocity ($V_{max}$) of free and immobilized enzyme were calculated using Lineweaver–Burk Plot. Xylan was employed as a substrate (1.0–3.0%) dissolved in phosphate buffer (25.0 mM, pH 7.0).

### 2.9. Thermal stability of free and immobilized endo-β-1,4-xylanase

Thermal stability of free and immobilized endo-β-1,4-xylanase was determined by pre-incubating them at different temperatures (40–80 °C) for different time periods (30–120 min). Enzyme aliquots were retrieved after every 30 min time interval and enzyme activity was calculated in terms of residual activity.

### 2.10. Scanning electron microscopy (SEM)

The scanning electron microscopy was used to analyze the surface morphology of agar–agar matrix before and after entrapment of endo-β-1,4-xylanase in the polymer. Agar–agar beads with and without immobilized enzymes (control) were allowed to dry and then coated with gold. These gold coated samples were subjected to scanning electron microscope (JSM 6380A Jeol, Japan) for morphological studies at different magnification range (1000–10,000×).