Research Paper

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Isolation, Immobilization and Characterization of Xylanase From A New Isolate Bacillus Atrophaeus E8

Biotechnology

KEYWORDS : Bacillus atrophaeus E8, 16s rDNA sequencing, xylanase production, immobilization, characterization

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ABSTRACT

Xylanase is an industrially important enzyme which degrades xylan randomly and produces xylo-oligosaccharides, xylobiose and xylose. It has a role in bio-bleaching of paper pulp, increasing pulp brightness, additive in animal feedstock to improve its nutritional value and can be used for ethanol production. A new isolate E8 was isolated from degraded saw dust on potato dextrose agar plates at pH 5.2 at 30°C. This isolate was identified by using 16s rDNA amplification and sequencing and subsequent analysis using BLAST as Bacillus atrophaeus E8. The 16S rDNA gene sequence Bacillus atrophaeus E8 was deposited in GenBank with the accession number JX658789. Free xylanase showed optimum activity at pH 5.0 and 35°C temperature. Immobilized xylanase showed higher pH (6.0) and temperature (55°C) stability then free enzyme. Immobilized enzyme can be reused 2-3 times under assay conditions.

INTRODUCTION

After cellulose, hemicellulose is the second most abundant natural polysaccharide present in plant fraction and waste residues contain up to 40% hemicellulose formed by pentose sugars. Xylan, a complex of polymeric carbohydrates is the major part of hemicellulose and is found in hardwoods (15-30% of the cell wall content), softwoods (7-10%), and annual plants (< 30%)[6,18,28]. Xylan has a backbone chain of a homopolymer of 1,4-linked β-D-xylopyranosyl units. Xylanases (E.C. 3.2.1.8) have been reported to be produced by a variety of microorganisms including bacteria, yeasts and filamentous fungi and catalyzes the endohydrolysis of 1,4-β- D-xylosidic linkages in xylans yielding various 1,4-β-D-xylooligosaccharides[4,7,20,23]. The simpler oligosaccharide obtained after the enzymatic hydrolysis of xylan (a highly branched heteropolysaccharide) have great area of interest , due to possible applications in animal feedstock, fuel, chemical production, paper and pulp industry, baking and fruit and vegetable processing, etc[5-7,15]. Other applications include; extraction of coffee, plant oils and starch, improvement of nutritional properties of agricultural silage and grain feed, and clarification of fruit juices in combination with pectinase and cellulase, and recovery of fermentable sugars from hemicelluloses[13,17,30].

Immobilized enzyme has some advantages compared to free enzyme. The immobilized enzymes are more stable at higher temperatures and are active over a wide pH range[12]. For industrial application, the enzymes immobilized on solid support can offer several advantages i.e. repeated usage of enzyme, ease of product separation, improvement of enzyme stability, can stop the reaction rapidly (or vice versa), can minimize effluent problem[2,27]. In present study an extra-cellular xylanase produced by a new isolate Bacillus atrophaeus E8 was selected for immobilization and characterization.

MATERIALS AND METHODS

Isolation and screening of bacterial culture

The bacterial cultures were isolated from Degrading saw dust (mixture of Deodar, Pine, Oak etc), collected from two different timber saw machine market situated in Shimla, Himachal Pradesh, India. The cultures were maintained on Potato Dextrose Agar (Himedia, India) slants. Pure colonies obtained were streaked on to PDA plates supplemented with 0.5% (w/v) birch wood xylan (Himedia, India) and incubated at 30^{0}C. After 48 h, the plates were flooded with 0.1% Congo red (kept for 15 min) and were destained with 1 M NaCl for 10 min. The zone of clearance around the bacterial colony on the plates indicated xylanase positive results. Based on zone of clearance the E8 strain was selected for further study.

Bacterial identification and phylogenetic analysis

The morphological, cultural, and physiological characteristic of the isolate E8 was studied according to Bergey's Manual of Determinative Bacteriology[11]. For molecular identification using a 16S rDNA sequencing technique, genomic DNA was extracted by the standard chloroform-isoamyl alcohol method.[1] PCR amplification of the 16S rRNA was performed using the following forward and reverse primers: 8F: 5′ AGA GTT TGA TCC TGG CTC AG 3′ and 1492R: 5′ ACG GCT ACC TTG TTA CGA CTT 3′ respectively[31]. The polymerase chain reaction (PCR) mixture consisted of 7.50 µL of DNase-RNase free water, 12.50 µL (1X) of 2X PCR master mix, 1.0 µL (10 pmole) of each primer and 30ng of DNA template. The PCR reaction was performed in 25µL volumes for 30 cycles at 95°C, 30 seconds at 52°C, and 1 minute at 72°C. Additional extension was carried out for 10 minutes at 72°C. Amplified DNA was examined by horizontal electrophoresis in 1.2% agarose with 5 µL aliquots of PCR product. The purified PCR product was sequenced and the 16S rDNA gene sequence was compared with database in the GenBank using the BLASTn program[3]. The 16S rDNA gene sequence was deposited in GenBank with the accession number JX658789. The phylogenetic analysis was done by MEGA 5.2 software and the tree was constructed using the neighbor-joining method[29, 25].

Production of xylanase

Xylanase production was carried out with isolate E8 by inoculating (35v/v) seed culture to PDA production medium supplemented with 0.5% (w/v) birch wood xylan (Himedia, India) and incubated at 30^{0}C for 48h. The supernatant obtained after centrifugation of fermentation broth at 5000 rpm for 15 min at 4°C was used as a crude enzyme. Xylanase activity was determined by measuring the reducing sugar with dinitrosalicylic acid (DNS) method using D-xylose as a standard [22]. The enzyme assay was carried out at 30°C using 0.5% (w/v) birch wood xylan (50mM Acetate buffer, pH 5.2) as a substrate. One unit of xylanase activity was expressed as 1µg of reducing sugar (xylose equivalent) released/ml/min under assay conditions.

Optimization of xylanase production and activity

The culture was inoculated to 50 mL of enzyme production medium, containing birch wood xylan (in g/L; xylan 5.0, Yeast extract 5.0) 1% (v/v) inoculum at different pH (4.0-10.0) for 48h under standard shaking conditions to assay the enzyme produced. Xylanase production was studied upto 120 hrs in production medium supplemented with 0.5% birch wood xylan using 1% (v/v) inoculum of 24h old culture (OD at 540 nm = 1.0) at 30°C in a incubator shaker at 150 rpm.

Immobilization of crude xylanase enzyme

Crude enzyme was immobilized onto glass beads by physical adsorption binding. The silanization must precede immobilization reaction to introduce reactive groups onto inert glass surface to increase the accessible surface area for immobilization. Therefore activation of glass beads was done.

Activation of glass beads

Activation of glass beads (1mm x 1mm) was carried out as described by Gabel et al.[9] Glass beads (10g) were heated in 40ml (5% HNO_3) for 1hr at 80-90^{0}C. Glass beads was then washed three times with distilled water and transferred into a polyethylene vessel to avoid silanization and immobilization of the enzyme to the walls of the flask. 10ml of distilled water and 3ml of 3-aminopropyl-triethoxysilane was added and pH is adjusted first with concentrated and finally with 2M HCl to pH 3-4. The pH is determined using litmus paper strips. This reaction mixture was incubated at 65^{0}C for 12 hrs. Glass beads were washed on suction filter (five times) with distilled water and three times with acetone (20ml each). Finally the glass beads were dried at 55^{0}C for 20 min. The activated glass beads were used for further studies.

Immobilization of crude xylanase enzyme on glass beads

Immobilization was carried out as described by D'Souza et al.[8] Activated glass beads (2 g) was drenched in 20 ml of polyethylenimine and kept at room temperature for 2 hours. It was again washed with 0.05 M phosphate buffer; five ml of crude enzyme added to it and the mixture was incubated at room temperature for one hour. The glass beads were again washed thoroughly with buffer and activity of the immobilized enzyme was determined by using 1 gm of immobilized glass beads in assay condition. The activity yield and other characteristics of immobilized enzyme were compared with that of free enzyme and % yield of immobilized enzyme was calculated using the formula:

Assay and reusability of immobilized enzyme

The free and immobilized xylanase activity was assayed at different pH of buffer (0.1M) ranging from 4.0-8.0 and at various temperatures (35-65°C) to determine the optimal activity under reactions conditions. The immobilized xylanase was tested for its reusability using 1 gm of immobilized support repeatedly up to four times and percent relative activity determined.

RESULT AND DISCUSSION

The bacterial culture E8 used in the present study was isolated from the degraded saw dust sample collected from Shimla, Himachal Pradesh, and was a gram positive bacterium (Table 1). When flooded with 0.1% Congo red, it formed a very prominent and clear zone on xylan agar plates. For 16s rDNA study the DNA of strain E8 was isolated and amplified with the ribosomal primers mentioned in the methodology section. The isolate produced a single band of about 1,500 bp (Figure 1). This size corresponded to the expected size of the 16S rDNA genes among bacteria[31].

Table 1. Some morphological and biochemical characteristics of xylanase producing isolate E8

Morphological Tests	Results
Colony colour	Cremish
Colony appearance	Opaque, Smooth, Circular
Gram reaction	Gram Positive
Shape	Rods
Biochemical Test	
Catalase	+
Oxidase	+
Indole	-
Carbohydrate	+
Amylase	+
Xylanase	+

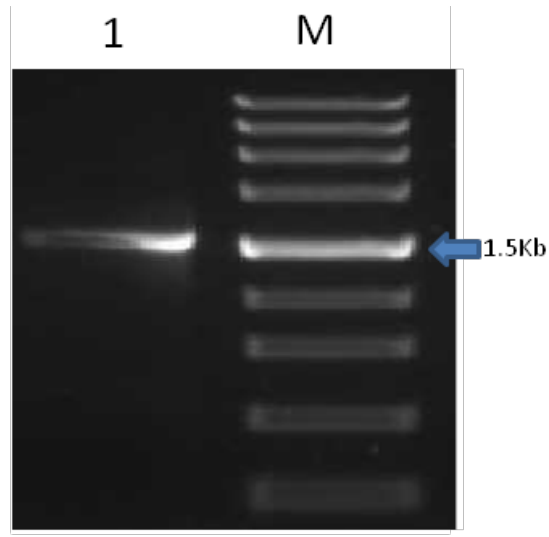

Figure 1: Gel electrophoretic of PCR amplified 16s rDNA product of isolate E8. Lane 1, PCR amplified product of approx. 1.5kb ; Lane M, DNA marker

This amplified 16s rDNA was sent for sequencing to Excelris, Ahemedabad. The amplified sequence of the isolate E8 so obtained was subjected to BLAST analysis and the BLAST search has shown 99% homology with the Bacillus atrophaeus strain 16S rDNA gene sequence. Therefore the isolate E8 is identified as Bacillus atrophaeus. The phylogenetic tree (Figure 2) shows the taxonomic position of the isolate E8.

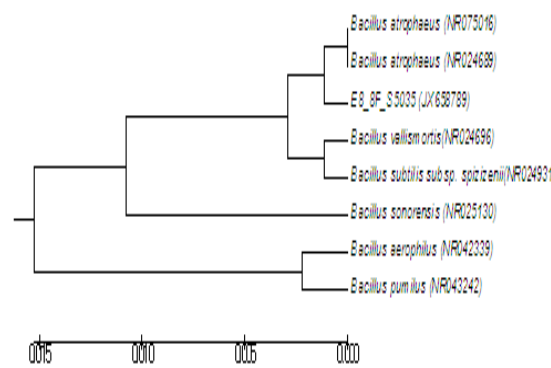

Figure 2: Phylogenetic tree showing the taxonomic position of Bacillus atrophaeus E8. Code within brackets represents the GenBank accession number of isolate E8 and other microorganisms.

The organism secreted xylanase under broad range of pH (4.0-10.0) for the hydrolysis of birch wood xylan (Figure 3). The isolate had shown maximum growth and xylanase production at pH 7.0 (21.09 U/ml). Kuhad et al. observed similar results of maximum xylanase production by Bacillus sp. RPP-1 at pH 7.0 whereas; the optimum activity was also recorded earlier at pH 6.0[14,16].

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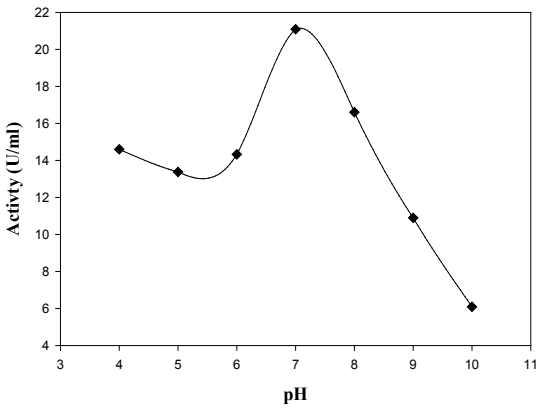
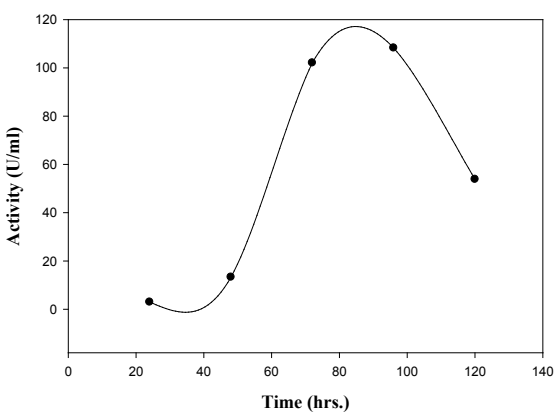
Figure 3: Effect of different pH of medium on xylanase production by Bacillus atrophaeus E8 at 30 ºC for 48 h.



The time course of xylanase production was studied in shake flasks under optimized condition. Enzyme was assayed after every 24h. Xylanase production increased exponentially up to 92 h (108.28 U/ml) with optimum production (Figure 4). Thereafter, enzyme production showed sharp decrease in production.

Figure 4. Time course of xylanase production by Bacillus atrophaeus E8 at 30°C.



Enzyme activity was assayed in the presence of different range of buffers. After immobilization the enzyme activity yield was found to be 1.184 % for glass beads. The enzyme exhibited a broad pH profile (4.0-8.0) for the hydrolysis of birch wood xylan (Figure 5). The optimum pH for the activity of free and immobilized xylanase enzyme was 5.0 (180.761 U/ml) and 6.0 (2.142 U/ml) respectively. The loss of enzyme activity after immobilization is normal phenomenon[24].

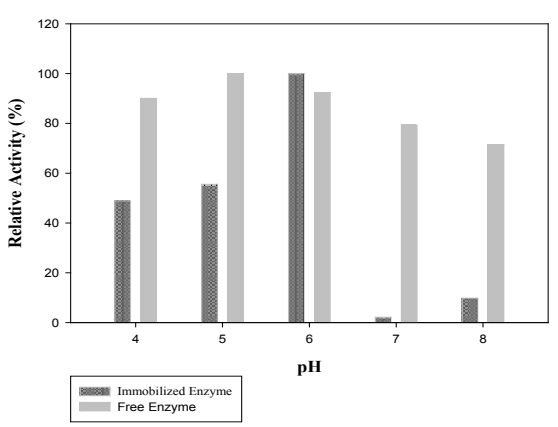
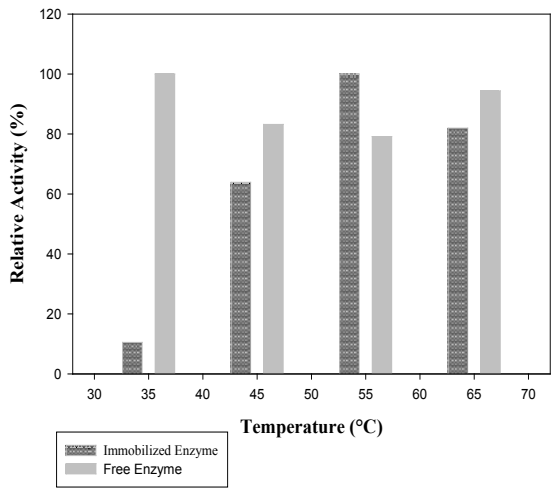


Figure 5. Effect of pH on relative activity of free and immobilized xylanase of Bacillus atrophaeus E8 (pH 4-6, 0.1M acetate buffer, pH 7-8, 0.1M phosphate buffer)

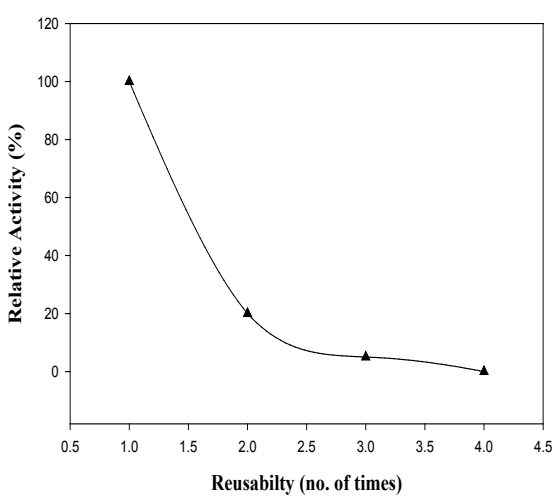
Effect of assay temperature on xylanase activity is shown in Figure 6. The Optimum activity of immobilized xylanase was achieved at 55°C (100%) and decreased 83% at 65°C. The optimum activity of free xylanase was observed at 35°C. Gaur et al. have also reported optimum activity of immobilized xylanase at 55–65°C[10]. The stability of immobilized enzymes at higher temperatures is one of the important improvements that have been achieved with immobilized enzymes on different supports[19]. The enhancement in activity and stability of immobilized enzymes is important for their industrial applications[24].

Figure 6. Effect of temperature on activity of free enzyme and immobilized xylanase of Bacillus atrophaeus E8



The immobilized xylanase of Bacillus atrophaeus E8 was quite stable and could be reused 3-4 times (Figure 7). The gradual loss in activity was observed on every new usage. This may be due to abrasion of supports during repeated use[24]. Earlier Meryandini et al.21 and Sardar et al.[26] have also reported decrease in activity during reusability of enzyme.

Figure 7: Reusability of xylanase of Bacillus atrophaeus E8 immobilized on glass beads



CONCLUSION

In conclusion, the results obtained from the present work indicates significant amount of xylanase production from a new isolate Bacillus atrophaeus E8 using selective growth conditions. Further xylanase was immobilized on glass beads as a carrier

by adsorption method and this method shows good loading efficiency. The enzyme immobilized glass beads can be reused repeatedly to minimize the enzyme loss in industrial applications. Further detailed characterization of the enzyme and applications is in process.

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