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Isolation, purification, and characterization of xylanase produced by three species of bacillus under submerged fermentation conditions

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Abstract: This study focuses on the screening and identification of bacteria, which can produce alkaline xylanase at alkaline pH and high temperature. Bacterial isolates from enriched decaying soil, capable of hydrolyzing xylan were screened. Selected and purified 13 bacterial colonies (*Bacilli and Kurthia*) grown on xylan- nutrient agar slants, were activated and transferred into the fermentation medium. Three highest xylanase producing isolates (*Bacillus badius, Kurthia gibsonii, Bacillus circulans)* were selected for further studies and the xylanase produced by them were screened for their kinetic properties. The optimum temperature for the activity of the xylanase from Isolates A was 50°C; and for Isolate B was 40°C, while that of Isolate C was 30°C. The optimum pH value for the xylanase from isolate A and B was 9.0. In addition, the xylanase was also capable of producing high-quality xylo-oligosaccharides, which indicated its application potential not only in pulp bio-bleaching processes but also in the nutraceutical industry.

Keywords: *Bacillus badius, Kurthia gibsonii,* Submerged fermentation, Xylanase

INTRODUCTION

The uses of enzymes (microbial origin) in medicine and the industry are remarkably diverse. The enzymes are responsible for the hydrolysis of plant polysaccharides, including cellulose, lipid, nucleic acid, starch and cell wall components (Kalia *et al.*, 2000). The high yield of these enzymes in cultural fluids and their robust properties, especially their tolerance to extremes of pH and temperature, led to their application in various industries particularly in starch and food processing industries, where they have replaced several unsatisfactory chemical processes and in household laundry detergents (Viikari *et al.*, 1994; Flores *et al.*, 1997).

Commercial production of these microbial enzymes involves use of a wide range of species of bacteria, fungi and yeasts (Archana and Satyanarayana, 1997). The microorganisms are preferred for the production of industrially important enzymes as these have higher growth and multiplication rate; can be easily modified genetically to increase the production of particular type of enzyme due to the following reasons: growth is high in bacteria, can produce enzymes on extreme conditions of environment such as high temperature and pH and have simple nutritional requirements (Gupta, 2008; Srinivasan, 1999).

The main purpose of isolating the thermophillic and alkalophilic bacteria is to produce enzymes which can be used in new processes and improve existing processes such as single step conversion of starch to glucose- maltose syrup using the glucosidase from Thermococcus hydrothermalis.

Xylanases are a group of hydrolytic enzymes that act on xylan which is second most abundant polysaccharide in nature (Gupta and Kar, 2008). Xylanases are of industrial importance, which can be used in paper manufacturing to bleach paper pulp, increasing brightness of pulp and improving the digestibility of animal feed and for clarification of fruit juices (Srinivasan and Rele, 1999; Viikari *et al.*, 1990). Applications of xylanase avoid the use of chemicals that are expensive and cause pollution (Chidi *et al.*, 2008). Microorganisms are the rich sources of xylanases, produced by diverse genera and species of bacteria, actinomycetes, and fungi. Several species of Bacillus and filamentous fungi secrete high amounts of extracellular xylanases (Gupta *et al.*, 2001; Azeri *et al.*, 2010; Sudan and Bajaj, 2007). Production of xylanase can be carried out using either submerged fermentation (SmF) or solid state fermentation. Submerged fermentation systems are extensively used for the production of costly material and for the study of biochemical and physiochemical aspects of the synthesis of microbial metabolites. The objective of the study was isolation, physiological and biochemical screening and the time profile of alkaline (xylanase) enzyme production under submerged fermentation conditions.

MATERIALS AND METHODS

Isolation of alkalophilic bacteria from soil sample:
Soil samples rich in organic matter (collected from different sources) were dried in an oven at 37°C for 24 hours. One gram of soil sample was added to 10 ml of

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normal saline (0.85% NaCl) solution. Samples were vortexed for two minutes followed by the contents to settle down. One ml of supernatant was taken and added to 100 ml of enrichment (nutrient agar and actinomycetes agar) mediums.

Isolation of the pure culture by gram staining and spore staining: The isolated colonies on nutrient agar and actinomycetes isolation agar plates were studied for purity by Gram's staining and Spore staining (Bergey *et al*., 1994). The smears were observed under oil emulsion.

Plate assay for enzyme production: Prominent selected isolate was identified on the basis of morphological, cultural, biochemical properties. Single colonies of the selected isolates grown on xylan nutrient agar plate were observed for morphological characters in terms of margin, colour, surface, opacity and shape. Pure cultures were streaked on basal xylan (0.25%) agar medium to check the production of xylanase enzymes. Bacterial cultures were incubated at 37^{o}C for 48 hours. The plates were stained with Congo red for 15 minutes and then destained with NaCl (1ml) solution. The yellow zone round the colony surrounded by red background indicated the presence of xylanase (Miller, 1959).

Biochemical identification of the isolates: Biochemical testing (Carbohydrate, Citrate utilization, Catalase, Indole, Nitrate reduction, Voges- proskauer and Mac Conkey agar test) was done to identify and characterize the bacterial strains (MacFaddin, 2000).

Xylanase production under submerged fermentation: The selected strain was further tested for their abilities to produce extracellular xylanase under submerged fermentation. The bacterial isolates were cultured on two different basal media for production of xylanase i.e. wheat bran and corncob pulp mediums were used as substrates. The enrichment was done by inoculation in Peptone water and the xylanase production was monitored periodically after every 24 hours. The assay was performed using a suspension of Birchwood xylan as substrate. The reducing sugars were estimated by DNS method (Miller, 1959).

For identification of the isolates, the results of biochemical tests, plate assays and colony morphology were compared with identified ones from Bergey's Manual of Systematic Bacteriology (Sneath, 1986).

Protein measurement: Protein concentration was measured by Lowry's method (Lowry *et al.,* 1951) using Bovine serum albumin.

RESULTS AND DISCUSSION

Isolation of xylose producing strains and screening for xylanase producing strains: Of the 13 bacterial strains isolated from soil collected at selected study site, three (Isolate A,B,C) formed clear halos around their colonies on xylan agar plates and were picked up for further studies. The strain that showed zone of clearance around the colony proved its xylanolytic ability. All the three isolates were Gram positive as indicated by Gram staining and Mac Conkey test but the spore formation was observed only in isolate A and C. Isolate A formed a creamish white colony and diffused morphology with serrate margin. The colonies of Isolate B were with round morphology and elevated margins. Whereas the Isolate C was identified with small rounded creamish white bacteria with round and elevated margins. Biochemical tests were carried out to confirm the genus of the isolate and to identify the species. The isolates B and C were good catalase producers in comparison with Isolate A. All the three strains showed good growth under aerobic condition but did not grow under anaerobic condition. This indicated that the strains are strict aerobes. The isolates did not utilize citrate as the carbon source, also did not utilize tryptophan. Any one of isolate could not produce indole, did not produce starch hydrolysing enzyme and did not produce nitrate (Table 1).

Identification of the selected isolate: The morphological, physiological and biochemical characters of the isolates were compared to the identified bacteria from Bergey's Manual of Systematic Bacteriology (Sneath, 1986). It was observed that the characters of the Isolate A based on Gram staining; its morphology, aerobic nature, biochemical characters and its ability to grow at temperature 50^{o}C and pH > 7 were most closely related to the species *Bacillus badius*.

Likewise, the Isolate B with morphological characters (short Gram positive aerobic rod, yellow colony without spore formation) and ability to lipase digestion and casein hydrolysis with growth temperature 40^{o}C was identified as *Murtha gibsonia*.

In similar manner, as the Isolate C had the ability to grow at pH> 7 and at temperatures > 30^{o}C and other characters were similar to *Bacillus circulans* and it was identified as *B. circulans.*

Optimization of xylanase production in SmF: When the strain was grown in two different mediums i.e. wheat bran and pulp for 48 hours of incubation at pH 9.0 and 50^{o}C, maximum xylanase production was observed after 48 hours in pulp enriched medium (45IU) whereas; maximum production of 35.8 IU was observed after 24 hours in wheat bran medium by Isolate A. A similar pattern was observed in case of Isolate B (Figure 1). Several workers also reported the suitability of wheat bran for xylanase production in solid state fermentation (Kamble and Jadhav, 2012). But our study suggests that corncob is the better source for production of xylanase enzyme.

Effect of temperature on xylanase production: For xylanase from both the Bacillus sp., activity was observed to be gradually increasing with the increasing temperature and found significantly declined at 80°C. 50°C was found to be the most favourable for enzyme activity. Stability of the enzyme was the most important factor in studying characteristics. It was found that these xylanases were most active at 50°C to 60°C.

Table1. Morphological and biochemical characteristics of the selected bacterial colonies.

Test	*Isolate A*	*Isolate B*	*Isolate C*
Gram staining	+	+	+
Spore staining	+	-	-
Colony			
Morphology	*Diffused*	*Round*	*Small round*
Margin	*Serrate*	*Round*	*Round*
Elevation	*Flat*	*Elevated*	*Elevated*
Colour	*Creamish White*	*Yellow*	*Creamish white*
Xylanase	+	+	-
Tannase	-	-	-
Lipase	-	+	-
Amylase	-	-	-
Cellulase	-	-	-
Protease	+	+	-
Carbohydrate test			
Starch	-	+	-
Sucrose	-	+	-
Maltose	-	+	-
Lactose	-	-	+
Dextrose	-	+	-
Catalase	+	+++	++
Citrate Utilization	-	-	-
Nitrate reduction	-	-	+
Indole test	-	-	-
MR-VP test	-	-	-
Mac Conkey test	+	+	+
Temp.	*50°C*	*40°C*	*30°C*
pH	*>7*	*>7*	*>7*

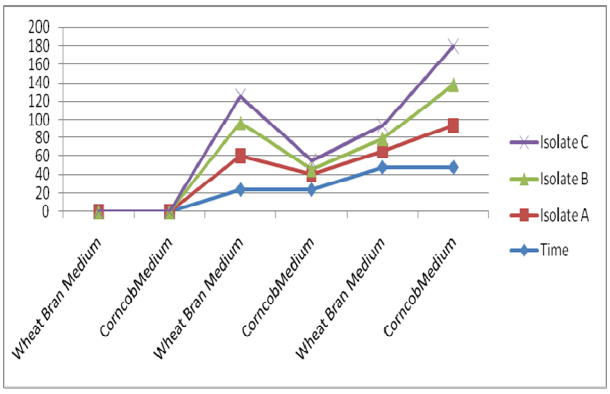

Fig. 1. *Comparative analysis of xylanase production in two different mediums at different temperatures.*

Kang *et al*. (Kang *et al*., 1996) purified two xylanases which gave the highest activity at 50°C and showed relatively high stability at 50◦C temperature. The similar observations were reported by Mahilrajan *et al*. also (Mahilrajan *et al.*, 2012). The bacterial strains from this study were also followed the similar pattern and the enzyme activity was prominent at and above 50°C. It has been previously reported that there are large number of alkali stable xylanase producing bacteria (*B. subtilis, B. halodurans* PPKS2, Bacillus AP4, AS11 and F) (Annamalai *et al.*, 2009; Prakash *et al*., 2009; Dholpuria *et al*., 2015). The purified xylanase also was capable of producing high-quality xylo-oligosaccharides, indicating its application potential not only in pulp bio-bleaching processes but also in the nutraceutical industry. *B. badius* and *K. gibsonii* produced thermoalkalophilic cellulose-free xylanase in greater amount when grown in submerged conditions using cheap and abundantly available agro-residual substrate like corncob and wheat bran. Hence, it can be used for large-scale production of xylanase using such agro-residual substrates.

Conclusion

Three high xylanase producing bacterial strains identified and isolated were *B. badius, M. gibsonii* and *B. circulans*. The ambient temperature for growth was 50°C for *B. badius, 40°C for M. gibsonia and 30°C for B. circulans* and the optimal pH was >7. The production of xylanase was observed in wheat bran and pulp enriched medium and the temperature for growth was 50°C. For xylanase from both the Bacillus sp., activity was observed to be gradually increasing with the increasing temperature and found significantly declined at 80°C. 50°C was found to be the most favourable for enzyme activity. Stability of the enzyme was the most important factor in studying characteristics. It was found that these xylanases were most active at 50°C to 60°C. *B. badius* and *K. gibsonii* produced thermoalkalophilic cellulose-free xylanase in greater amount when grown in submerged conditions using cheap and abundantly available agro-residual substrate like corncob and wheat bran.

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