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Advances in Applied Science Research, 2012, 3 (1):242-250

ISSN: 0976-8610
CODEN (USA): AASRFC

Screening and identification of a thermophilic and alkalophilic bacterium producing xylanase

SubajiniMahilrajan, Sandrasegarampillai Balakumar and Vasanthy Arasaratnam*

Department of Biochemistry, Faculty of Medicine, University of Jaffna, Sri Lanka

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ABSTRACT

This study focuses on screening and identification of bacteria, which can produce alkaline xylanase at alkaline pH and high temperature. Bacterial isolates from corncob decaying soil, capable of hydrolyzing xylan were screened. Selected and purified 108 bacterial colonies grown on xylan- nutrient agar slants were activated and transferred into the fermentation medium. Six highest xylanase producing isolates were selected for further studies. Isolates CS_1[132.0(±0.09)], CS_{27}[121.3(±0.11)]& CS_{88} [124.8(±0.44) UmL^{-1}] showed highest xylanase production at pH 8.5 while isolates CS_{52}[124.4(±0.01) UmL^{-1}], CS_{93}[113.0(±0.48) UmL^{-1}] and CS_{104}[110.1(±0.54) UmL^{-1}] showed at pH 8.0 and 45°C. Therefore isolates CS_1, CS_{27} & CS_{88} were selected and the xylanase produced by them were screened for the kinetic properties. The crude enzymes of the isolates CS_1, CS_{27} & CS_{88} showed zero order kinetics up to 4 min. The optimum temperature for the activity of the xylanase from isolates CS_1 and CS_{27} was 55°C, while that of isolate CS_{88} was 60°C. The optimum pH value for the xylanase from isolate CS_1 and CS_{88} was 8.4 and that of isolate CS_{27} was 8.0. Based on the kinetic properties of xylanase, isolates CS_1 and CS_{88} were selected and characterized and found to be belonging to genus Bacillus. As Bacillus CS_1 produced highest xylanase activities, 16S rDNA was analyzed and identified as Bacillus pumilus and selected for further studies to produce xylanase at 45°C and pH 8.5.

Key words: Xylan, Xylanase, Isolation, kinetic properties, Screening and Characterization.

INTRODUCTION

Xylan is the most abundant of the hemicelluloses. It has a linear backbone of β- 1, 4 linked D-xylopyranose residues [1]. Biodegradation of xylan requires action of several enzymes, among which xylanases play key role [2]. Xylanase can be produced by a number of microorganisms including bacteria, yeasts [3], Actinomycetes [4] and filamentous fungi [5]. Xylan degrading bacteria such as*Bacillus pumilus* [6], *Bacillus firmus* [7] and*Bacillus halodurans*[8]have been reported. Xylanases have applications in baking [9 & 10] textile [11], paper and pulp treatment [12], and animal feed [13]. Owing to the increasing biotechnological importance of thermostablexylanases, many thermophilic bacteria have been examined for xylanase production [14].Tolerance to higher pH and temperature are desirable properties of xylanase for effective use in pulp treatment. Due to their huge potential,xylanase-producing bacteria with novel properties must be isolated. In this study higher titer of xylanase producing thermophilic and alkalophilic bacterial isolates from corncob decaying soil were isolated, screened and identified.

MATERIALS AND METHODS

Materials

Birchwood xylan (Roth, Germany), Phusion enzyme, GC buffer &DiMethylSulfoxide (DMSO) (Fermentas, Germany), peptone & yeast extract, Peptone water (Oxoid, UK) Gram's Iodine, Kovac's reagent, tetramethyl-p-phenylene diaminedihydrochloride, H_2O_2, I_2/KI (BDH Laboratory Supplies, UK), primers andQUAquick gel extraction kit (Sigma Chemical C, USA), Sequencing kit (Applied Biosystem)were used.

Culture Media and culture conditions
The Xylan Nutrient Agar plates and slants containing(gL^{-1}) nutrient agar 28.0 and Birchwood xylan 20.0 at pH 7.0 were used for the storage of the strains and incubated at 40^{o}C for 24 h.

The activation medium contained (gL^{-1}) xylan, 20.0 and nutrient broth, 25.0 at pH 7.0.The bacterial cultures grown on the slant were transferred to 100mL conical flask containing 10mL of activation medium (1 loop/10mL) and incubated in an orbital shaker water bath at 42^{o}C and at pH 7.0, 120 rpm for 18 h.

Fermentation medium contained (gL^{-1}) xylan, 20.0; peptone, 2.0; yeast extract, 2.5; $CaCl_2.2H_2O$, 0.005; $MgCl_2.6H_2O$, 0.005; $FeCl_3$, 0.005; K_2HPO_4, 2.5; KH_2PO_4, 1.0; NaCl, 0.1 and $(NH_4)_2SO_4$, 2.0 at the pH values required. Fermentation medium was inoculated with the activated culture (20%, v/v) and incubated at appropriate temperatures based on the experiment.

Xylanase activity assay
Assay mixture consisted of 0.5mL of diluted enzyme solution and 0.5 mL of 20gL^{-1}xylan in 0.01M phosphate buffer, (pH 7.0). After incubation at 60^{o}C for 4 min, the increase in reducing sugars was determined by Dinitrosalisylic acid (DNS) method [15] with xylose as standard.

One unit of xylanase activity is defined as the amount of enzyme that releases one µmol of reducing sugar equivalent to xylose per minute at 60^{o}C and pH 7.0 with 20gL^{-1} xylan.

Sample collection
Soil samples on decaying corncob were scraped off and pooled together in a sterile bottle.

Isolation of xylan utilizing strains
Soil sample (1g) was suspended in 10mL of sterile saline (9gL^{-1}NaCl), mixed uniformly, and allowed to settle. The serially diluted samples were plated on xylan nutrient agar plate. Single colonies were chosen among those, which gave clear zone [16] and purified.

Screening for xylanase producing bacteria
Purified bacterial isolates were activated and transferred to the fermentation medium and incubated in a shaker water bath at pH 7.0, 42^{o}C and 120 rpm for 24h. The spent medium centrifuged at 3000 rpm for 20 min and the cell free filtrate was used as xylanase source.

Effect of temperature on xylanase production
The bacterial isolates CS_1, CS_{27}, CS_{52}, CS_{88}, CS_{93} and CS_{104}were activated at pH 7.0 and at different temperatures (42, 45, 50 & 55^{o}C) and transferred into the fermentation medium and incubated at respective temperatures (120 rpm).

Effect of pH on xylanase production
The selected bacterial isolates were activated at 45^{o}C and at different pH values (7.0, 7.5, 8.0, 8.5 & 9.0) and inoculated into the fermentation medium with respective initial pH values.

Kinetic properties of the crude enzyme
Xylan solution (20 gL^{-1}, 0.25 mL, pH 8.4) was mixed with 0.25 mL of diluted crude enzyme from CS_1, CS_{27}& CS_{88}at 60^{o}C and the amounts of xylose produced were monitored and the effects of temperature and pH on the xylanase activities were determined.

Identification of the selected isolates
Morphological studies
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.

Microscopic studies
Selectedisolateswere subjected to gram staining and motility test by hanging drop method [17].

Biochemical tests
Biochemical tests such as oxygen requirement, catalase test, oxidase test, citrate utilization test, indole test, Voges-Proskauer (Vp) test and production of urease were carried out [17].

Differentiation of selected species

Intergenic Transcribed Spacerpolymerase chain reaction (ITS-PCR) mixture was prepared with 5µL of dreamTaq buffer, 1µL of dNTP, 2.5µL of each primer, 0.5µL ofDreamTaq DNA polymerase and 10ng of DNA template of the isolates. The PCR reaction was performed in 50µL volumes for 35 cycles of 4.5 min at 95^{o}C, 30 seconds at 45^{o}C, and 1 min at 72^{o}C [Applied Biosystems, USA]. Additional extension was carried out for 10 min at 72^{o}C**.**

Confirmation of selected species

To confirm the species of the isolates, genomic DNA was extracted and purified, and its purity was assessed. The 16S rDNA gene fragment was amplified using two specific primers. The sequences of these primers were AGAGTTTGATCCTGGCTCAG (Forward) and GGTTACCTTGTTACGACTT (Reverse). The polymerase chain reaction (PCR) mixture consisted of 10µL of CG buffer, 1µL of dNTP, 2.5µL of each primer, 1.5µL of DMSO, 0.5µL of fusion enzyme and 10ng of DNA template. The PCR reaction was performed in 50µL volumes for 35 cycles of 4.5 min at 98^{o}C, 30 seconds at 60^{o}C, and 1 minute at 72^{o}C. Additional extension was carried out for 10 minutes at 72^{o}C. PCR product was purified using Qiagen PCR purification kit according to its protocol. The purified product was sequenced in both directions with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit following the manufacturer's protocol. The 16S rDNA sequence was compared with the sequences available in public database National Center for Biotechnology Information (NCBI). Based on the sequence BLAST phylogenetic tree was established.

RESULTS

Isolation of xylose producing strains and screening for xylanase producing strains

Among the 108 isolates, 30 isolates did not produce xylanase at pH 7.0, 60^{o}C and at 24 hours, while 72 isolates gave less than 5.0UmL^{-1}xylanase activity, and 6 strains named as CS_1 (17.1UmL^{-1}), CS_{27} (7.1UmL^{-1}), CS_{52} (6.1UmL^{-1}) & CS_{88} (12.8UmL^{-1}), CS_{93} (9.7 UmL^{-1}) and CS_{104} (9.3UmL^{-1}) producedxylanase activity above 5.0 UmL^{-1} in the fermentation medium (Table 1) and were selected for further study.

Effect of temperature on xylanase production

In order to select the isolates which can produced xylanase at high temperature, the range of 42-55^{o}C was selected. All six isolated strains produced highest xylanase activity at 45˚C and highest xylanase activity [75.0(±0.01) UmL^{-1}] was produced by the isolate CS_1. At 50˚C isolates CS_{27}, CS_1, CS_{93} and CS_{104} produced xylanase while isolate CS_{52} and isolate CS_{88}did not produce xylanase. All these six strains did not produce xylanase at 55˚C (24 h) (Table 2).

Table1: Xylanase produced at 24 h, 42^{o}C, pH 7.0 and 120 rpm by 108 bacterial isolates isolated from corncob decaying soil.

Activity range (UmL^{-1})	Name of the isolates
0.0	CS_4, CS_8, CS_{17}, CS_{21}, CS_{38}, CS_{45}, CS_{46}, CS_{49}, CS_{55}, CS_{57}, CS_{59}, CS_{60}, CS_{62}, CS_{63}, CS_{64}, CS_{65}, CS_{66}, CS_{67}, CS_{68}, CS_{69}, CS_{72}, CS_{75}, CS_{80}, CS_{85}, CS_{91}, CS_{94}, CS_{95}, CS_{103}, CS_{107}, CS_{108}
0-0.1.0	CS_7, CS_9, CS_{10}, CS_{12}, $CS1_3$, CS_{14}, CS_{15}, CS_{16}, CS_{18}, CS_{19}, CS_{20}, CS_{22}, CS_{23}, CS_{24}, CS_{25}, CS_{26}, CS_{28}, CS_{30}, CS_{31}, CS_{33}, CS_{34}, CS_{36}, CS_{37}, CS_{38}, CS_{39}, CS_{40}, CS_{42}, CS_{43}, CS_{44}, CS_{47}, CS_{56}, CS_{58}, CS_{61}, CS_{64}, CS_{65}, CS_{70}, CS_{71}, CS_{73}, CS_{74}, CS_{76}, CS_{77}, CS_{78}, CS_{79}, CS_{81}, CS_{82}, CS_{83}, CS_{86}, CS_{87}, CS_{90}, CS_{97}, CS_{98}, CS_{99}, CS_{100}, CS_{101}, CS_{102}, CS_{106}
1.0-2.0	CS_2, CS_3, CS_{11}, CS_{32}, CS_{51}, CS_{89}, CS_{96}, CS_{105}
2.0-3.0	CS_{29}, CS_{84}
3.0-4.0	CS_{54}, CS_{92}
4.0-5.0	CS_5, CS_6, CS_{41}, CS_{50}, CS_{53}
≥ 5.0	CS_1, CS_{27}, CS_{52}, CS_{88}, CS_{93}, CS_{104}

Effect of pH on xylanase production

When the initial pH of the fermentation medium was 8.5 isolateCS_1 [132.5(±0.09)], isolate CS_{27}[121.3(±0.11)]& isolate CS_{88} [124.8(±0.44) UmL^{-1}] produced highest xylanase activity while isolate (CS_{52}) [124.4(±0.01) UmL^{-1}], isolate CS_{93}[113.0(±0.48) UmL^{-1}] and isolate CS_{104}[110.1(±0.54) UmL^{-1}]produced the highest xylanase activities at pH 8.0. Further increase in initial pH of the fermentation medium decreased the xylanase production by all the six isolates (Table 3). As the isolates CS_1, CS_{27} and CS_{88} produced highest xylanase activity at 45^{o}C and at pH 8.5; they were selected for further studies.

Kinetic properties of the crude enzymes

Xylanase obtained from the isolates CS_1, CS_{27}& CS_{88} showed a linear relationship between the time and xylanase production up to 4 min. Therefore, the reaction time was fixed as 4min for all the three crude xylanase samples obtained from the three isolates (Figure 1).

The crude xylanase from three isolates showed highest activity at 55°C. The enzyme produced by the isolateCS_1 gave 65 and 25% of its maximum activity at 40 and 65°C respectively. While xylanase produced by the isolateCS_{27} showed 75 and 17% of its maximum activity at 40 and 65°C respectively. Likewise xylanase from*B.pumilus*CS_{88} gave 56 % of their maximum activity at 40°C and 19% of the maximum activity at 65°C respectively. All three xylanases showed broad activity between 50 to 60°C and there were sudden drop in the activity above 60°C (Figure 2).

Table 2: Effect of temperature on the production of xylanase from isolated species.

Temperature (°C)	CS_1	CS_{27}	CS_{52}	CS_{88}	CS_{93}	CS_{104}
42	71.2 (94.9)	61.1 (91.9)	60.1 (99.7)	60.9 (95.2)	55.8 (79.2)	56.0 (82.8)
45	75.0 (100)	66.6 (99.9)	60.3 (100)	64.0 (100)	70.4 (100)	67.6 (100)
50	26.5 (35.4)	41.6 (62.5)	3.4 (5.6)	0.9 (1.5)	11.4 (16.2)	6.8 (10.0)
55	1.8 (2.4)	0.5 (0.7)	0.3 (0.6)	0.6 (0.9)	0.6 (0.9)	0.6 (0.9)

The optimum pH values for the enzymes from isolates CS_1 and CS_{88} were 8.4, and that isolate$CS_{27}$was 8.0. The xylanases from three isolates showed broad activity between the pH ranges of 7.5- 8.4. Xylanase from isolateCS_1 showed 57% of its maximum activity at pH 9.0 while Xylanase from isolateCS_{27} showed approximately 50% of its maximum activity. Xylanase obtained from isolateCS_{88} showed 82% of their activities at pH 7.0 and 28% at pH 9.0 respectively (Figure 3). As the isolates CS_1 and CS_{88} were able to grow at 45°C and produced highest xylanase activity, with maximum activity at pH 8.4 and 55°C, they were selected for further studies.

Identification of the selected isolate

Morphological studies

Therefore morphological (Table 4) characteristic was used to identify the genus of the strain. Among the selected two isolates,strains CS_1showed circular, convex, entire yellow colour, moist and shiny colonies. The colony of the strain CS_{88} was circular, flat, irregular, yellow colour, dry, rough and the diameters of the colony and the clear zone was 16 and 26mm respectively.

Table 3: Effect of pH on the production of xylanase from isolated species

pH	CS_1	CS_{27}	CS_{52}	CS_{88}	CS_{93}	CS_{104}
7.0	110.5 (83.5)	115.9 (95.5)	89.9 (72.2)	100.3 (80.3)	86.6 (76.6)	98.6 (89.6)
7.5	118.2 (89.2)	110.7 (91.2)	103.4 (83.1)	108.6 (86.9)	98.5 (87.1)	103.7 (94.2)
8.0	120.1 (90.6)	106.3 (87.6)	124.3 (100)	119.8 (95.9)	113.0 (100)	110.0 (100)
8.5	132.5 (100)	121.3 (100)	116.9 (93.9)	124.8 (100)	107.3 (94.6)	108.6 (98.6)
9.0	78.2 (59.0)	108.9 (89.8)	110.4 (88.7)	108.1 (86.6)	105.5 (93.3)	101.6 (92.3)
9.5	64.3 (48.5)	101.13 (83.3)	109.2 (87.8)	91.5 (73.3)	102.7 (90.9)	85.0 (77.2)
10.0	56.7 (42.8)	97.8 (80.6)	106.8 (85.8)	76.2 (61.0)	85.6 (75.8)	77.2 (64.8)

Microscopic studies

Boththe strains were stained as blue- violet rods with spores indicating that they areGram- positive rods. These strains moved rapidly across the microscopic field with twisting and this indicated the true motility. The strains were non-branching, spore forming rods. The results indicated that both the strains were belonging to the Family Bacillaceae [18 & 19].

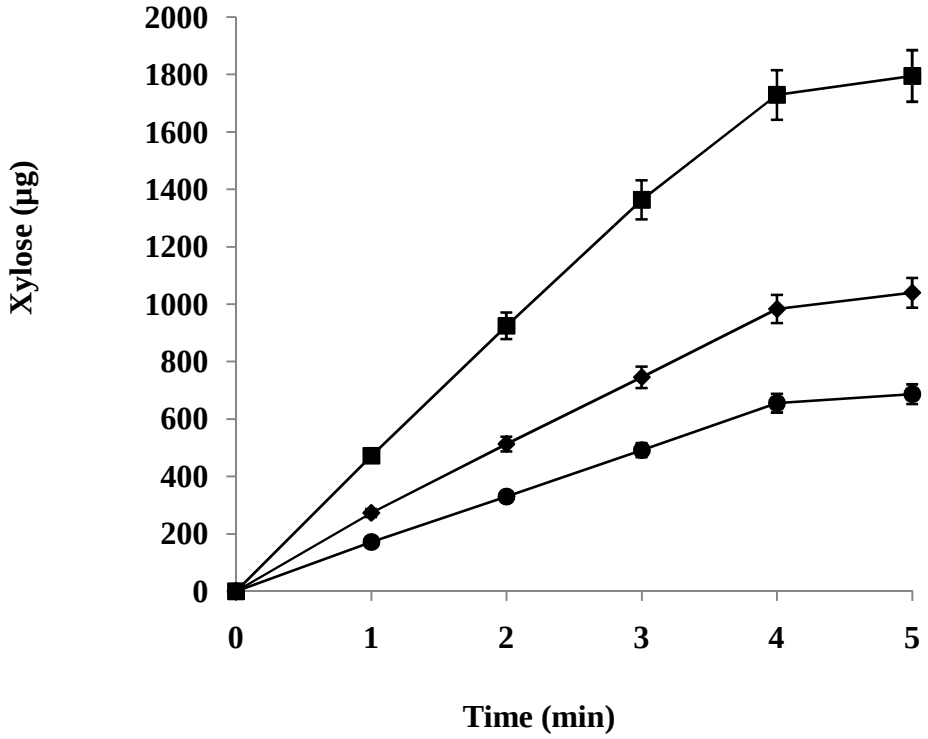

Figure 1: Xylose produced by the xylanases obtained from the isolates (◆) CS_1, (■) CS_{27} and (●) CS_{88}fromxylanat 60°C and pH 8.4 as a function of time.

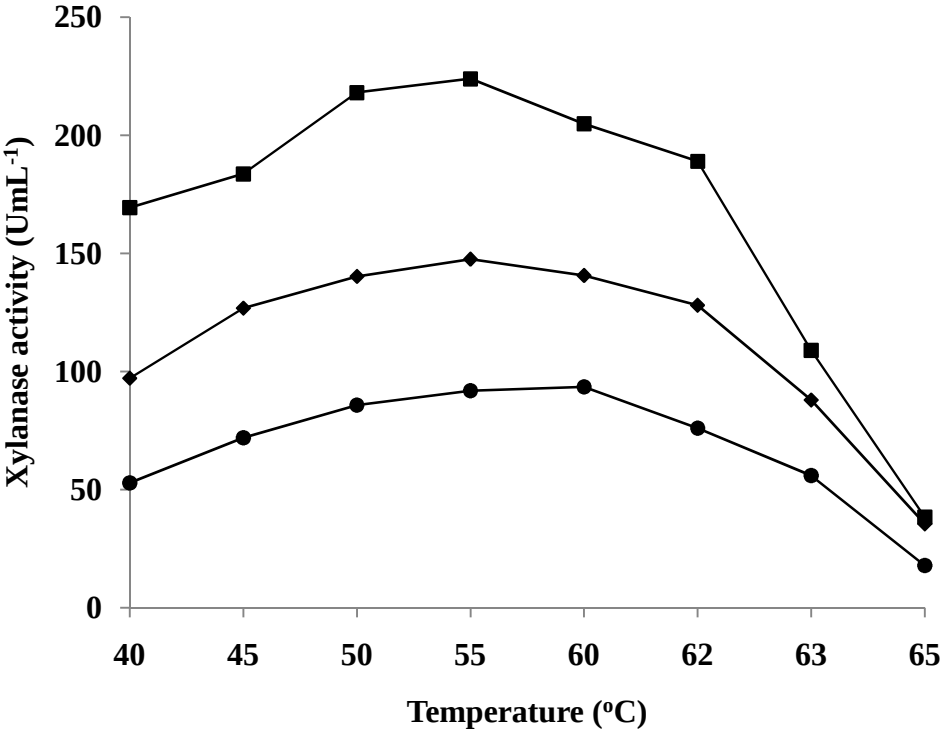

Figure 2: Effect of temperature on activities of xylanases producedby thestrains (◆) CS_1, (■) $CS_{27}$and (●) CS_{88} with xylan at pH 8.4.

Table 4: Morphological and Biochemical characteristics of the selected bacterial isolates.

Characters	Strains	
	CS_1	CS_{88}
Gram Staining	(+)ve	(+)ve
Shape of vegetative cell	Rod	Rod
Spore formation	(+)ve	(+)ve
Motility	(+)ve	(+)ve
Growth in air	(+)ve	(+)ve
Indole production	(-)ve	(-)ve
VogesProskauertest	(+)ve	(+)ve
Methyl Red test	(-)ve	(-)ve
Catalase production	(+)ve	(+)ve
Citrate production	(-)ve	(-)ve
Oxidase production	(-)ve	(-)ve
Urease test	(+)ve	(+)ve
Starch hydrolysis	(-)ve	(-)ve
Form	Circular	Circular
Elevation	Convex	Flat
Margin	Entire	Irregular
Diameter of colony after 24h (mm)	8	16
Diameter of clear zone after 24h (mm)	20	26
Colour	Yellow	Yellow
Surface	Moist Shiny	Dry Rough

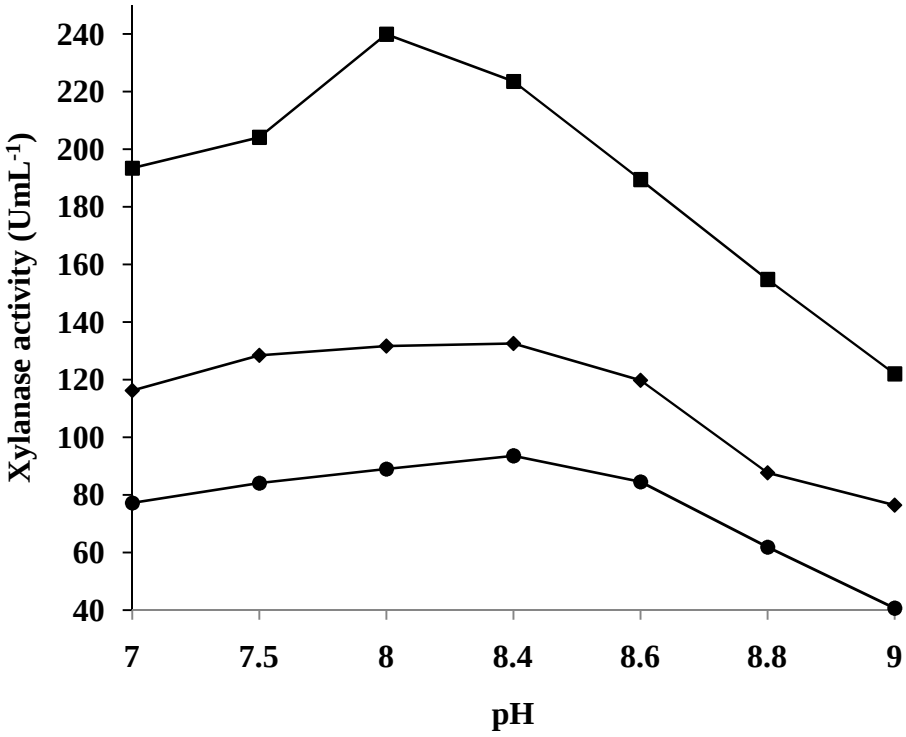

Figure 3: Effect of pH on the activities of xylanases producedby thestrains ( ) CS_1, ( ) CS_{27} and ( ) CS_{88}, with xylan at 60°C

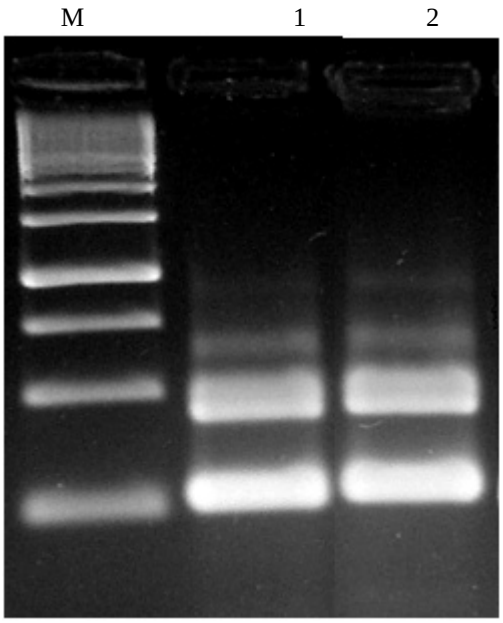

Figure 4: **Gel electrophoretic pattern of ITS -PCR amplification products of two selected isolates Lane M, DNA marker; Lane 1, isolate CS_1; and Lane 2, isolate CS_{88}.**

Biochemical studies

Biochemical tests were carried out to confirm the genus of the isolate and to identify the species. Both the strains have shown good growth under aerobic condition but did not grow under anaerobic condition. This indicated that the strains are strict aerobes. The isolates CS_1 and CS_{88} are catalase producers. Both isolates did not utilize citrate as the carbon source, also did not utilize tryptophan and could not produce indole, did not produce starch hydrolysing enzyme and did not produce urease (Table 4).

Based on the morphological, microscopic and biochemical results the selected two isolates belong to genus *Bacillus*, and on species level the isolate CS_1may belong to *B. pumilus* or *B.sphaericus*or *B.laterosporus*. The isolate CS_{88}may belong to *B.cereus*or*B.laterosporus*. These isolates did notshow clear characteristicson species level. Therefore genotypic characterization was studied to confirm the species of the selected *Bacillus* isolates.

Differentiation of the selected species

Based on the microscopic and morphological characteristics, the two selected strains may be included in the same species level. ITS PCR is used to differentiate the isolates (CS_1 and CS_{88}) showed same banding pattern, and hence they may be included in same species level (Figure 4). Therefore the isolates were selected for 16S rDNA studies to confirm the *Bacillus* species.

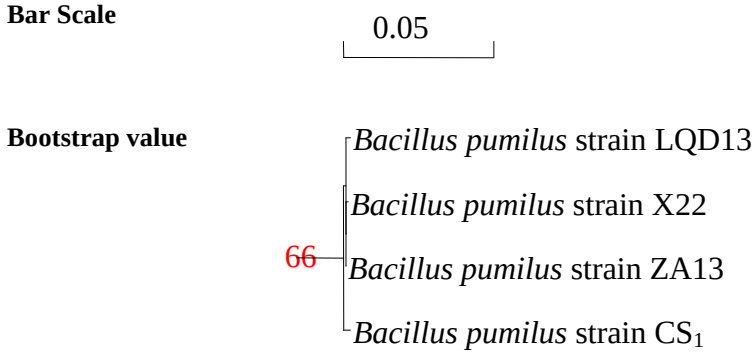

Figure 5: Phylogenetic tree based on the 16S rDNA sequence data indicating the position of isolate among representatives of the species of the *Bacillus pumilus.*

Confirmation of selected species

Amplification of the 16S rDNA gene of selected *Bacillus* CS_1resulted in about 1500 bp DNA fragment. According to the BLAST search of the rDNA gene sequence against sequences in public databases, the phylogenetic tree was established. Based on the microscopic, biochemical and genotypic characterization *Bacillus* $CS_1$was identified to belong the species *pumilus*(Figure 5), while they should differ at subspecies level because of the variation in the crude enzymesproduced.

DISCUSSION

Members of genus Bacillus produce large variety of extracellular enzymes, of which xylanases have particularly significant industrial importance [20]. Xylanases having activities at the alkaline pH is most preferable for industrial applications. In this study bacterial strains were isolated from corncob decaying soil for xylanases that are thermostable and alkali tolerant. Corncob is a rich source of xylan (upto40 g/100 g)[21] and it can be a good source for the isolation of xylanase producers.Sharma, *et al*.[22] was isolated an alkalophilicxylanase producing *B. Pumilus*strain MK001 from sanitary landfill and maintained on xylan-agar plates.

In the present study, six alkalophilic strains, which are capable of producing xylanases at 45^{o}C was selected [23]. Several alkaline tolerant xylanase producing bacterial strains have also been characterized recently [8 & 16].

There are numerous reports regarding the xylanase-producing bacteria. But only a few *Bacillus* sp. have been reported to produce xylanases that are active at alkaline pH and are thermostable [24]. These properties are needed for the application of xylanase in the paper and pulp industry [24 - 26]. Based on the alkalophilic nature Strain CS_1 and CS_{88} were selected for identification strain CS_{27} was eliminated.

ITS PCRis used to differentiate the isolates (CS_1 and CS_{88}) mainly at the species level. The 16S and 23S genes are separated by internal spacer regions (ITS), which exhibit a large degree of sequence and length variation at the levels of genus and species. The size of the spacer may vary considerably for different species. Based on the results $CS_1$and CS_{88} could be included in same species level. Therefore CS_1 was selected for 16S rDNA studies.

16S rDNA analysis has become the reference method for bacterial taxonomy and identification. It provides suitable phenotypic data that can be used to determine both close and very distant relationships between the species [27]. The sequencing of rDNA has successfully been used for identification of *B. subtilis* and *B. pumilus* in other studies [28 – 30]].Among the *Bacillus sp*, *B.pumilus* strain which is mainly reported as a protease producer [31] also produces lipase [32], xylanase [33], pectatelyase [34], etc.Isolate CS_1 revealed 99% sequence identity to *Bacillus pumilus.* The construction of phylogenetic tree based on 16S rDNA sequences resulted in two stable clades (Figure 5). One clade consist of bacterial species identified as *Bacillus pumilus strains* LQD13, X22 and ZA13. All members in this clade possessed identical sequences. The *Bacillus pumilus strain* CS_1 appeared to form second clade that was more closely related to the clade one.

CONCLUSION

A total of 108 bacterial strains which can grow on corncob were isolated and purified. Among the 108 bacterial strains,78 strains were identified as xylanase producers and six strains as potential xylanase producers. Strain CS_1 was selected and identified as *B.pumilus*. Selected strain produced highest xylanase at 45^{o}C and pH 8.5. The xylanase produced showed optimum activity at 55^{o}C and at pH as 8.4.

Acknowledgements

The authors thank Sida/SAREC and International Science Program of chemical sciences, Sweden for the financial support.

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