



Identification of xylanase producing *Bacillus licheniformis* strain C1 and properties of crude xylanase

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Abstract

In the present investigation xylanase producing bacteria was isolated from compost. A total of 95 xylanolytic bacteria were isolated on oat spelt xylan agar medium and screened by the xylanolysis method. Out of these 95 isolates, only one bacterial isolates, strain C1 was selected for further study on the basis of zone of hydrolysis on xylan-congo red agar plate. This strain was identified by 16S rDNA analysis. The phylogenetic analysis using 16S rDNA sequence data showed that isolate C1 showed highest nucleotide identity of 98% with *Bacillus licheniformis* strain CICC 10181 (GenBank accession no. GQ375235) and identified as *Bacillus licheniformis* strain C1. *Bacillus licheniformis* strain C1 was gram positive and rod shaped. Morphology of *Bacillus licheniformis* strain C1 showed- smooth texture, medium size, opaque transparency, creamish-white colour and serrated margin. Maximal xylanase production for *Bacillus licheniformis* strain C1 was achieved at the incubation period of 48 h. Xylanase and cellulase activities were determined as 20.0 U/ml and 1.3 U/ml, respectively. The optimum pH and optimum temperature for xylanase activity was found to be 7.0 and 60°C, respectively. Xylanase was found to be thermostable at 60°C for 1h and retained 90% of its activity upto 6 h at this temperature. Approximately, 74% and 70% of its activity was retained at 70°C and 80°C respectively, after 6 h of incubation. All of these properties of the *Bacillus licheniformis* strain C1 xylanase make the suitability of this enzyme for its use in feed and baking industry.

Keywords: Feed and baking industry; phylogenetic analysis; 16S rDNA analysis; xylanase.

Introduction

Xylans are linear polysaccharides formed from β -1,4 -linked d-xylopyranoses. In cereals, xylans frequently contain side chains of α -1,2, α -1,3, or α -1,2 and α -1,3 linked L-arabinofuranoside. This substituted xylan is commonly referred to as arabinoxylans. Xylanases (endo-1,4- β -xylanase, E.C. 3.2.2.8) hydrolyze internal β -1,4-xylosidic linkages in xylan to produce smaller molecular weight xylo-oligomers. Xylanase can be used in animal feed, in baking and in brewing which are rich in arabinoxylans. The addition of xylanase to feeds (e.g. for monogastric animals, including poultry or swine) which contain cereals (e.g. barley, wheat, maize, rye, triticale or oats) or cereal by-products, improves the break-down of

plant cell walls which leads to better utilization of the plant nutrients by the animal. This leads to improved growth rate and feed conversion, also reduced the viscosity of the feeds containing xylan. The xylanase may be used as a supplement in animal feed by itself or in combination with vitamins, minerals, and other feed enzymes, agricultural co-products (e.g., wheat middlings or corn gluten meal). The xylanase may be used in monogastrics as well as in polygastrics. Diets supplemented with xylanase improve feed conversion ratio. Xylanases have also been used in bread making industry as bread improvers (Javier *et al.*, 2007), where it improves dough and bread quality leading to improved dough flexibility, machinability, stability, loaf volume and crumb structure (Polizeli *et al.*, 2005). Xylanases directly or indirectly improve the strength of the gluten network and therefore, improve the quality of bread (Baillet *et al.*, 2003). Xylans have an important role in bread quality due to their water absorption capability and interaction with gluten (Guy and

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Sarabjit, 2003). The hydrolysis of xylan using xylanase improves the dough properties, leading to a greater uniformity in quality characteristics (Gray and BeMiller, 2003). Xylanases make the dough soft, *i.e.* reduce the sheeting work requirements and significantly increase the volume of the baked bread (Dervilly *et al.*, 2002, Harbak and Thygesen, 2002). It significantly improves manufacturing conditions: made dough more 'machine-friendly' and it does not stick to the machinery parts (Nuyens *et al.*, 2001, Rouau *et al.*, 1994). Xylanases have gained much importance owing to their application in feed, food and fermentation industries (Rouau, 1993). Most commercial xylanases designed for feed applications are not very thermotolerant, especially when neutral or alkaline pH conditions are used. These xylanases are generally inefficient or inactive at temperatures higher than 60° C and often work under acidic and neutral conditions. The aim of present study was to isolate and identify new xylanase producing bacteria which secrete thermostable xylanase active under neutral and acidic condition and to make the applicability of this xylanase for feed and baking industry.

Material and Methods

A thermophilic bacteria was isolated from compost (MRDC, Pantnagar). This strain produced extracellular, thermostable xylanase on oat spelt xylan agar medium and screened by the xylanolysis method.

Isolation and screening

Sample suspensions in sterilized water were poured and spread onto nutrient agar plates. These plates were incubated at 60°C for 2 days and constantly observed for the appearance of bacterial colonies. Colonies found on these plates were transferred onto oat spelt xylan agar plates and incubated at 60°C for 2 days. This thermophilic bacterial strain was tested for their capabilities to produce xylanase by growing them on xylan-congo red agar medium. Bacteria was further screened by growing them in liquid medium and assayed for xylanase activity in cell-free culture supernatant fluid and cell pellet.

Culture media

The thermophilic bacteria were screened into nutrient agar media having pH 7.0. The screening of xylanase producing bacteria were done by growing them into following media: oat spelt xylan, 1%; yeast extract, 0.2 %; peptone, 0.5%; MgSO₄, 0.05%; NaCl, 0.05 %; CaCl₂, 0.015 % and agar,

2% at pH 7.0 (Cordeiro *et al.*, 2002). Qualitative analysis of xylanase positive isolates was done on xylan-congo red media consisting: K₂HPO₄, 0.05%; MgSO₄, 0.025%; congo red, 0.02%; oat spelt xylan, 0.5%; agar, 0.5% and gelatin, 0.2% having pH 7.0 (Hendrickset, 1995).

PCR amplification of the 16S rDNA and sequence determination

For the 16S rDNA sequence analysis, bacterial genomic DNA was extracted, then 16S rDNA gene was amplified by PCR using 5' AGAGTTTGATCCTGGCTCAG-3' and 5' AAGGAGGTGATCCAGCCGCA-3' as the forward and reverse primers, respectively (Edwards *et al.*, 1989). The amplification was carried out in 25 µl of reaction mixture containing 4.0 µl of DNA template, 2.5 µl of PCR buffer (10x) (Bangalore, Genei), 1.5 µl of dNTP (10 mM) (Bangalore, Genei), 2.0 µl of the primers FP (40 ng) and RP (40 ng) respectively, 1.5 µl of Taq polymerase (5 U/µl) (Bangalore, Genei) and 11.5 µl of autoclaved Milli-Q water (Millipore). The PCR program was run for 35 cycles in thermal cycler (Eppendorf, Germany). The following thermal profile was used for PCR: denaturation at 94°C for 1 min, annealing at 64°C for 1 min, extension at 72°C for 1min 30sec. Final cycle included extension for 10 min at 72°C to ensure full extension of product. The amplified PCR products were analyzed in a 1.0 % (w/v) agarose gel, excised and purified from the gel (by Spin gel extraction kit, Genei). The sequencing of purified PCR product was done by the Bangalore, Genei (India) using automated ABI 3100 Genetic Analyzer with fluorescent labeled dye terminators. The ABI's AmpliTaq FS Dye terminator cycle sequencing is based on Sanger's sequencing method. Databases (GeneBank) were searched for sequences similarity analysis of the 16S rDNA sequence obtained. The 16S rDNA gene sequence of the isolate was aligned with reference 16S rDNA sequences of the European Microbiological Laboratory (EMBL), GenBank (gb, Germany) using the BLAST algorithm (Altschulet, 1997) available in NCBI (National Centre for Biotechnology information) in internet.

Enzyme production

Growth media used for enzyme production contained: oat spelt xylan (OSX) 1%, yeast extract 0.2%, peptone 0.5%, MgSO₄ 0.05%, NaCl 0.05%, CaCl₂ 0.015%, pH 7.0. Media was inoculated with 10% of overnight culture and incubated at 60°C



with aeration in shaker at 200 r.p.m. for 2 days. Before assay the cells were separated by centrifugation at 10,000g and clear supernatant used as crude enzyme.

Enzyme assay

Xylanase activity was assayed by measuring the release of reducing sugar from oat spelt xylan. Reaction mixture consisted of 1% xylan in 0.1 M Tris-HCl buffer (pH, 7.0) and enzyme to give a final volume of 1.0 ml. After incubating for 10 min at 60°C, the release of reducing sugar was determined by Nelson-Somogyi method (Nelson, 1994 and Somogyi, 1952). The cellulase activity was measured under the same condition as described above using carboxymethyl cellulose as a substrate.

One unit of xylanase and cellulase is defined as the amount of enzyme required to release 1μmol of reducing sugar, xylose or glucose, per min under above assay condition.

Protein estimation

The protein content was determined by Lowry method using bovine serum albumin as standard (Lowry, 1951).

Incubation time on xylanase production

Bacillus licheniformis strain C1 was tested for xylanase producing ability in production media containing xylan. The active culture was inoculated into production medium and incubated under shaking at 60°C for 96 h. The enzyme activity and growth (at 600nm) were determined periodically after time intervals of 12h.

Effect of pH on activity of xylanase

The activities of xylanase at various pH values were measured by using oat spelt xylan (OSX) as substrate. The reaction pH was adjusted from 5.0 - 10.0 with various buffers by incubating xylanase with suitable amount of buffer solution containing xylan as substrate. The buffers used were 0.1M citrate-phosphate buffer for pH 5.0-6.0, 0.1M Tris-HCl buffer for pH 7.0-8.0 and 0.1M Glycine-NaOH buffer for pH 9.0-10.0.

Effect of temperature on activity and stability of xylanase

The effect of temperature on the enzyme activity was determined by assaying xylanase within temperature range of 40°C -80°C at pH 7.0 (0.1mM Tris-HCl buffer). Thermostability was determined by incubating crude xylanase at temperature ranging from 40°C-80°C for 1-6 hrs and residual enzyme activity was determined. The

residual activity was quantified, at optimum temperature i.e. on 60°C, using Nelson-Somogyi method at their optimum pH (7.0).

Results and Discussion

Screening of bacterial isolates

In the present study a total of 95 isolates were screened. Among these isolated strains good xylanase producers were screened by growing into xylan-congo red agar media. The large zone of hydrolysis was produced by *Bacillus licheniformis* strain C1 on xylan-congo red agar plates (Fig: 1). This strain was further tested for xylanase production by growing into oat spelt xylan agar medium.

Characteristics of bacterial isolate

Gram staining

Bacteria were identified by Gram stain reaction (Gerhardt *et al.*, 1994). Isolate *Bacillus licheniformis* strain C1 was rod shaped and gram positive (Fig: 2).



Fig 1: Zone of hydrolysis on xylan-congo red agar plate for *Bacillus licheniformis* strain C1



Fig 2: Gram staining of *Bacillus licheniformis* strain C1
Colony morphology of bacterial isolate

Bacillus licheniformis strain C1 was studied for their colony morphology-texture, size, transparency, colour and margin as shown in Table: 1.

Strain C1 identification by 16S rDNA sequence

In order to confirm the identification of strain C1, the 16S rDNA was amplified after PCR amplification of genomic DNA. The amplified product was analyzed in 1.0% agarose gel (Fig: 3). Sequencing result shows that amplified 16S rDNA sequence was 1502 bp (Fig: 4). The 16S rDNA analysis of isolate C1 shows its close phylogenetics to the genus *Bacillus* rRNA group. Isolate C1 showed highest nucleotide identity of 98% with

Bacillus licheniformis strain CICC 10181 (GenBank accession no. GQ375235). Therefore phylogenetic analysis confirmed that isolate C1 was one strain of *Bacillus licheniformis* and identified as *Bacillus licheniformis* strain C1 (Fig: 5).

Bacterial growth and xylanase production

Bacterial isolate was evaluated for their growth in terms of O.D. at 600nm, enzyme activity U/ml, enzyme activity U/mg and protein concentration after 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h and 96 h of growth. The highest xylanase activity for *Bacillus licheniformis* strain C1 was achieved after 48 h of incubation. Further incubation after this time cause decreased in xylanase production (Fig: 6).

Table 1: Colony morphology of bacterial isolate

S. N.	Isolates	Texture	Size	Transparency	Colour	Margin
1.	<i>Bacillus licheniformis</i> strain C1	Smooth	Medium	Opaque	Creamish White	Serrated

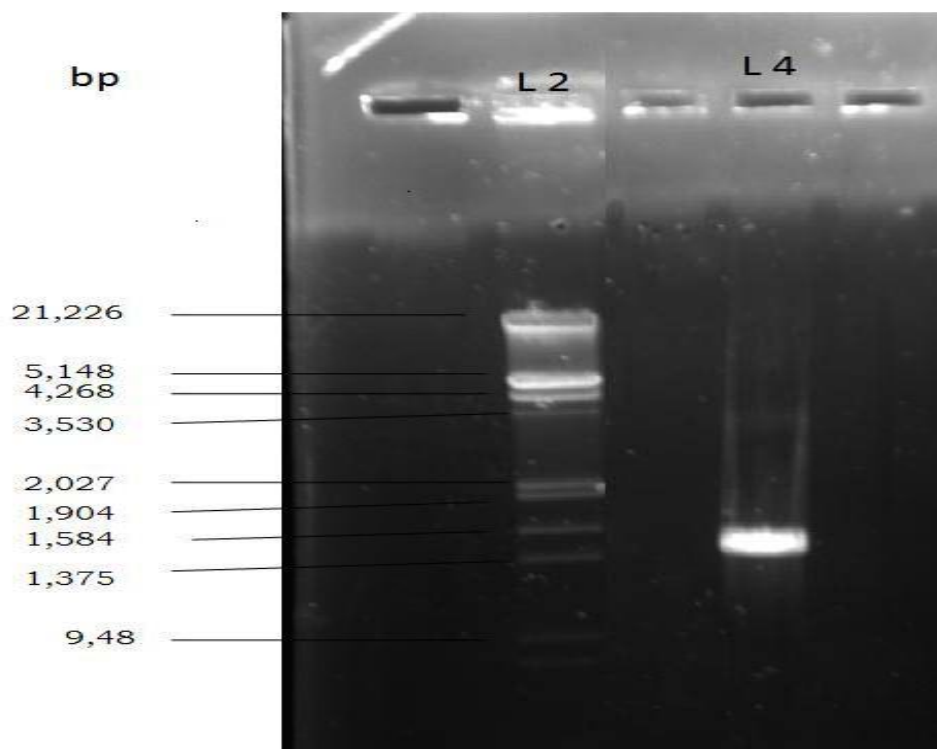


Fig 3: Agarose gel electrophoresis of amplified 16S rDNA product of *Bacillus licheniformis* strain C1. (1) Marker (Lambda DNA/Eco RI/ Hind III double digest, at lane 2) (2) amplified PCR product (16S rDNA, at lane 4).

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1  TGTTTTTTTG  TTAAGAGCGA  GTCCTATAAT  GCAGTCGAGC  GGACAGATGG  GAGCTTGCTC
61  CCTGATGTTA  GCGGCGGACG  GGTGAGTAAC  ACGTGGGTAA  CCTGCCTGTA  AGACTGGGAT
121  AACTCCGGGA  AACCAGGGCT  AATACCGGAT  GCTTGATTGA  ACCGCATGGT  TCAATTATAA
181  AAGGTGGCTT  TTACCTACCA  CTTACAGATG  GACCCGCGGC  GCATTAGCTA  GTTGGTGAGG
241  TAACGGCTCA  CCAAGGCAAC  GATGCGTAGC  CGACCTGAAA  GGGTGATCGG  CCACACTGGG
301  ACTGAAACAC  GGCCCAAAC  CCTACGGGAG  GCAGCAGTAG  GGAATCTTCC  GCAATGGACG
361  AAAGTCTGAC  GGAGCAACGC  CGCGTGAGTG  ATGAAGGTTT  TCGGATCGTA  AAACCTCTGTT
421  GTTAGGGAAA  AACAAGTACC  GTTCGAATAG  GGCGGTACCT  TGACGGTACC  TAACCAGAAA
481  GCCACGGCTA  ACTACGTGCC  ACCAGCCGCG  GTAATACGTA  GGTGGCAAGC  GTTGTCCGGA
541  ATTATTGGGC  GTAAAGCGCG  CGCAGGCGGT  TTCTTAAGTC  TGATGTGAAA  GCCCCCGGCT
601  CAACCGGGGA  GGTTTCATTGG  AAAGCTGGGG  AACTTGAGTG  CAGAAGAGGA  GAGTGGATTT
661  CCGCGTGTAG  CGGTGAAAGTG  AGTAGAGATG  TGGGAGGAAC  ATCAGTGAGG  AAGGCGTCTC
721  TTTGCTGTGT  AACTGACGCT  GAGGCGCGAA  AGCGTGGGGA  GCGAACAGGA  TTAGATACCG
781  TGGTAGTCCA  CGCCGTAAAC  GATGAGTGTT  AAGTGTTAGA  GGTTTTCCGC  CCTTTAGTGC
841  TGCAGCAAAC  GCATTAAGCA  CTCCGCCTGG  GGAGTACGGT  CGCAAGACTG  AAACCTCAAAG
901  GAATTGACGG  GGGCCCGCAC  AAGCGGTGGA  GCATGTGGTT  TAATTGCAAG  CAACGCGAAG
961  AACCTTACCA  GGTCTTGACA  TCCTCTGACA  ACCCTAGAGA  TAGGGCTTCC  CCTTCGGGGG
1021  CAGAGTGACA  GGTGGTGCAT  GGTGTGCGTC  AGCTCGTGTC  GTGAGATGTT  GGGTTAAGTC
1081  CCGCAACGAG  CGCAACCCTT  GATCTTAGTT  GCCAGCATTC  AGTTGGGCAC  TCTAAGGTGA
1141  CTGCCGGTGA  CAAACCGGAG  GAAGGTGGGG  ATGACGTCAA  ATCATCATGC  CCTTATGAC
1201  CTGGGCTACA  CACGTGCTAC  AATGGGCAGA  ACAAAGGGCA  GCGAAGCCGC  GAGGCTAAGC
1261  CAATCCCACA  AATCTGTTCT  CAGTTCGGAT  CGCAGTCTGC  AACTCGACTG  CGTGAAGCTG
1321  GAATCGCTAG  TAATCGCGGA  TCAGCATGCC  GCGGTGAATA  CGTTCCCGGG  CCTTGTACAC
1381  ACCGCCCCGTC  ACACCACGAG  AGTTTGTAAC  ACCCGAAGTC  GGTGAGGTAA  CCTTTTGAG
1441  CCAGCCGCCG  AAGGTGGGAC  AGATGATTGG  GGAAGTCGA  TCAAGAGCTG  GAAAAAAAT
1501  AT

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Fig 4: *Bacillus licheniformis* strain C1, 16S rDNA partial sequence (Length=1502)

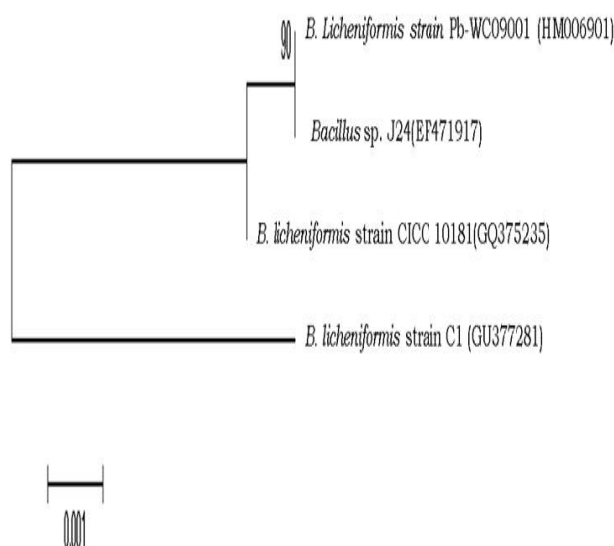


Fig 5: Phylogenetic analysis of partial 16S rDNA gene sequence of *Bacillus licheniformis* strain C1 and related microorganism, accession no. are given after species names. Bootstrap values (1,000 replicate runs, shown as percent)

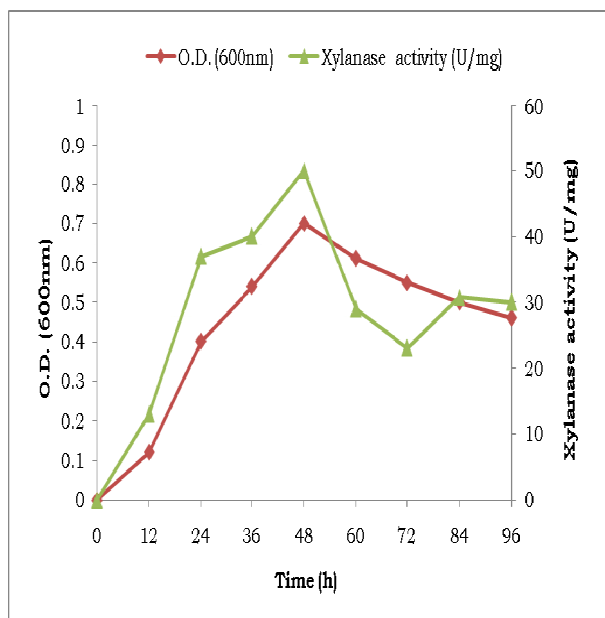


Fig 6: Effect of time of incubation on growth and xylanase activity for *Bacillus licheniformis* strain C1

Xylanase activity

The xylanase activity for *Bacillus licheniformis* strain C1 was found to be 50.0 U/mg or 20.0 U/ml. The xylanase activity of *Bacillus licheniformis* strain C1 may be compared with previously reported *Bacillus subtilis* strain (3.2 U/ml) (Khanongnuch *et al.*, 1998); *Bacillus subtilis* (12 U/ml) (Pereira *et al.*, 2002); *Bacillus* sp. AA3 (4.2 U/ml), *Cellulomonas* sp. CX 38 (5.1 U/ml) (Ten *et al.*, 2006) and *Bacillus* NT-9 (10.5 U/ml) (Fang *et al.*, 2004).

Cellulase activity

Cellulolytic activity of *Bacillus licheniformis* strain C1 crude enzyme preparations was obtained at pH 7.0 and 60°C. The cellulolytic activity was found to be 1.3 U/ml. Xylanases along with cellulase activity improve the properties of wheat bread and reduce staling during storage (Haros, 2002). Microbial xylanolytic enzymes in mixture with cellulase can be used for modification of baking products and for improvement of poultry diets. Xylanases and cellulase directly or indirectly improve the strength of the gluten network, this results in increased loaf volume, bread score and produce softer crumb and therefore, improve the quality of bread (Baillet *et al.*, 2003). Our results indicate that, xylanase produced by *Bacillus licheniformis* strain C1 could meet the requirement of baking industry.

Effect of pH on xylanase activity

Optimum pH of *Bacillus licheniformis* strain C1 xylanase was found to be 7.0 (Fig: 7). Xylanase from *Bacillus licheniformis* strain C1 active at pH 7.0 make it applicable for feed industry. Similar, optimum pH of 7.0 was reported by Choudhary *et.al.* (2006) for *Bacillus coagulans* xylanase and Kitamoto *et al.*, (1999) for *Aspergillus terreus* xylanase. Chadha *et al.*, (2004) isolated *Streptomyces* sp. having optimum xylanase activity at pH 6.0-8.0.

Effect of temperature on xylanase activity

Temperature optima for *Bacillus licheniformis* strain C1 xylanase was 60°C (Fig: 8), enzyme also showed good activity at 70-80°C. This optimum temperature value for *Bacillus licheniformis* strain C1 xylanase is similar or even somewhat higher than the optimal temperature reported by Durate *et al.*, (2000), Dhillon *et al.*, (2000), Morales *et al.*, (1993) Muthezhilan *et al.*, (2007) and Grabski and Jeffries (1991).

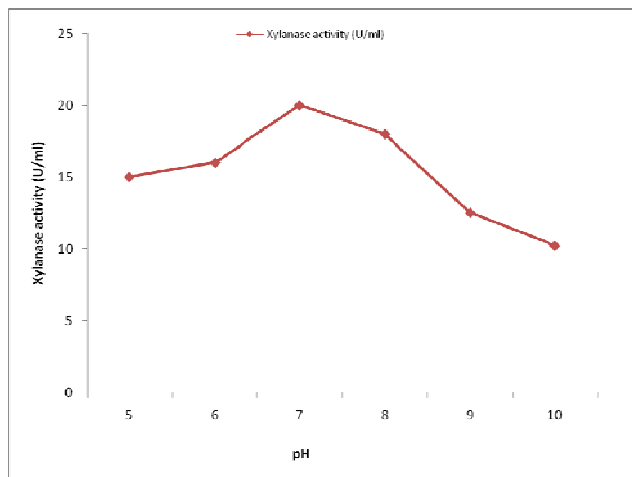


Fig 7: Effect of pH on xylanase activity.

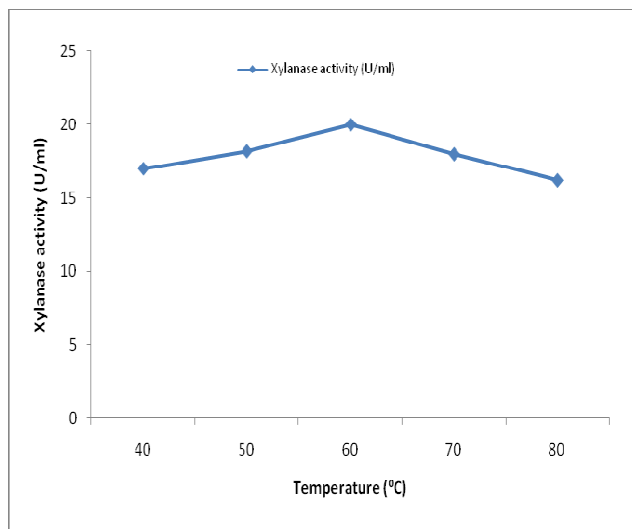


Fig 8: Effect of temperature on xylanase activity.

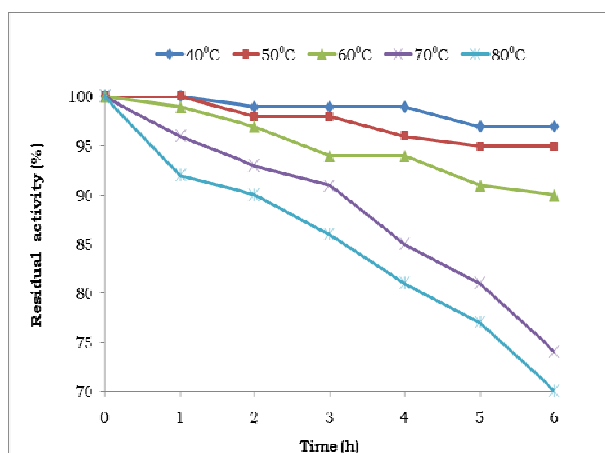


Fig 9: Thermostability profile of *Bacillus licheniformis* strain C1 xylanase

Thermostability of promising bacterial isolate

Xylanase thermostability was estimated at different temperature ranging from 40°C to 80°C for period of 1 to 6 h. Xylanase showed good thermostability at 60°C for 6h, it retained 99% of its activity after 1 h and 10% activity was lost after 6h of incubation at this temperature. Approximately, 74% and 70% of its activity was retained at 70°C and 80°C respectively, when incubated for 6 h (Fig: 9). Comparable to our results, Durate *et al.*, (2000) showed that xylanase from *Bacillus pumilus* 5₂ retained around 40% of their original activity, while 5₁₄, 4_a and 13_a retained 60% of its activity after 2 h of incubation. However, activity decreased gradually over time, with 30% of the activity remaining for strain 5₂, 50% for strain 5₁₄ and 40% for strains 13_a and 4_a after 6 h.

Thermal stability of xylanase is important property due to its potential applications in baking industry. Strains isolated by us could be a good source for its biotechnological applications.

Conclusion

In the present investigation we have isolated a bacterial source *Bacillus licheniformis* strain C1 that produced thermostable xylanase. Isolated xylanase is not only active at neutral pH but also exhibit broad range of thermostability at 60–70°C for upto 6 h. *Bacillus licheniformis* strain C1 xylanase also showed good cellulase activity of 1.3 U/ml. Xylanases along with cellulase activity improve the properties of wheat bread (Haros, 2002) and can be used for modification of baking products and for improvement of poultry diets. Enzymatic hydrolysis of highly viscous arabinoxylan in diets based on cereals results in improved poultry growth and increased feed efficiency. *Bacillus licheniformis* strain C1 xylanase active at neutral pH, temperature optima 60°C, thermostability at 70–80°C with 1.3 U/ml of cellulase activity fulfills the criteria of using this enzyme in feed and baking industry.

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