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Optimization, Isolation and Characterization of Cellulase–Free Thermostable Xylanase from *Paenibacillus sp.*

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Abstract: Xylanases are hydrolytic enzymes that cleave the β-1,4-linkage ofwheat bran xylan. For screening of xylanase producing bacteria soil samples were diluted by serial dilution and cultured on selective wheat bran xylan agar media. Two bacterial strains showing clear transparent zone around the colony on xylan agar plate were selected as xylanase producing bacteria. The strain *Paenibacillus sp.* showed highest xylanolytic activity. The strain was thermophile and produced highly active cellulase free xylanase. The enzyme secretion was enhanced when the medium was supplemented with 0.5% wheat bran xylan, peptone and Ca^{2+} salt. The peak in xylanase production was achieved within 48-60 hours at temperature 50°-55°C and at pH 7.0.The cellulase free xylanase was partially purified by ammonium sulfate fractionation and heat treatment at 50°C. The xylanase was optimally active at pH 7.0 and 55°C; and showed high substrate activity to wheat bran xylan but no activity towards carboxymethylcellulose, cellulose and starch.In future we want to know the structure function relationship of the purified enzyme and also want to known the molecular biological study using highly purified xylanase. For this purpose we have to determine the N-terminal & C-terminal amino acid sequence.

Keywords: Xylanases, *Paenibacillus sp.*, Cellulose, Xylan, Thermophile, N-terminal & C-terminal Amino Acid Sequence

1. Introduction

Xylanase are the collections of glucanase and esterase enzyme [1]. Xylanase plays a key role for the degradation of xylan [2]. The xylosidic linkage of xylan is degraded by xylanase produce xylose with other mono-residues. The major component of xylanolytic system produced by bio-degradative microorganisms such as bacteria and fungi [5, 20-24, 26-31, 33, 54].

Xylanase activities are important for the carbon flow in the carbon cycle and thus biomass turnover in nature. Due to the abundance and the structural heterogeneity of xylan, xylan-degrading enzymes are diverse. For the recovery of D-xylose from β-1, 4 xylan molecules at least two discrete enzyme activities are necessary. Typical xylan degrading enzyme is endo β-1,4 xylanase (1,4 β-D-xylanohydrolase; EC 3.2.1.8) [3]. This enzyme is responsible for the cleavage of linkages with accumulation of xylobiose. Xylobiose is converted into D-xylose through the action of another β xylosidase (β xyloside xylohydrolase; EC 3.2.1.3.7). Some purified xylanase are able to cleave xylotriose but not arabinoxylotriose [4, 5]. So hydrolysis of branch xylan requires the auxiliary enzymes. It is becoming apparent that more than one xylanase are usually produced by individual microorganisms [6]. The most common auxiliary enzymes are phenolic acid esterase, acetyl esterase, α-glucuronosidase and α-L-arabinofuranosidase.

The importance of arabinose releasing xylanase [7, 8, 9],

acetyl esterase [10, 11] and α-glucuronidases [12] are widely recognized. Cooperative interactions between α-arabinosidase [13, 14], acetyl esterase, α-glucuronosidases and xylanase have demonstrated their functional significance in xylan hydrolysis. The xylan-degrading enzymes include xylanase (1, 4-β-D-xylanohydrolase; EC 3.2.1.8) and β-xylosidase (1, 4-β-D-xylan xylohydrolase; EC 3.2.1.38). Xylanase catalyze the hydrolysis of xylan to xylooligosaccharides and xylose, while β-xylosidases release xylose residues from the non-reducing ends of xylooligosaccharides.

Xylanases occur widely in bacteria and fungi. Microbial xylanase may show maximum activity at extreme condition (at high temperature or very low temperature and at high acidic or basic condition).

The vast majority of xylanases are excreted into the extracellular environment as the large size of the substrate prevents its penetration into the cell. In fact, the current belief is that xylanase production is induced by means of the products of their own action [15, 16, and 17]. It is believed that small amounts of constitutively produced enzymes liberate xylo-oligomers which may be transported into the cell where they are further degraded by β-xylosidases, or indeed by intracellular xylanases [7, 8, 9], and where they induce further xylanase synthesis.

Microbiology is the safer and environment friendly technology. Most of the organisms are beneficial because they are key components in photomass decay and play a key role in the recycling of compounds and elements in terrestrial ecosystems.

Other microorganisms are beneficial because they provide nutritional benefits to ruminants through symbiotic associations. More directly microorganisms are used for antibiotic synthesis, preparation of food beverages, large-scale production of fuel (e.g. ethanol), additives (e.g. citric acid) and chemicals [18].

Many bacteria and fungi synthesize and secreteindustrially useful enzymes into the surrounding medium. These enzymes include xylanase, cellulase, isomerase, amylase, invertase, protease and pectinase. Xylanase hydrolyze xylan. Xylanase are commonly isolated from diverse genera and species of bacterial and fungi strains that colonies aerobic and anaerobic ecosystem, enduring both mesophilic and thermophilic environments [19]. Some microorganisms excrete large amount of xylanolytic enzymes in culture media while other, although growing on xylan, excrete little or no enzymes into the medium.Thousands of bacterial species are xylanase producing (xylan degrading) that occur in nature [20]. Xylanase producing bacteria are *Bacillus agaradhaerens* [21], *Bacillus circulans* [22], *Bacillus subtilis* [23], *Bacillus coagulans* [24], *Geobacillus stearothermophilus* [25], *Streptomyces halstedii* [26], *Streptomyces lividans* [27], *Cellvibrio japonicus* [28] etc. A large number of fungi have been characterized to have xylanase activity. Xylanase producing fungi are *Aspergillus kawachii* [29], *Aspergillus niger* [30], *Trichoderma harzianum* [31], *Trichoderma reesei* [32], *Trichoderma viride* [33], *Penicillium simplicissimum* [34], *Penicillum junthinellum* [35] etc.

The main aim of this work is to search more active xylanase producing microorganisms (i.e. bacteria), which might be successfully and efficiently able to degrade the complex xylan. Although many bacteria have been studied for xylanase production such as *Streptomyces* sp.

(Sreenath H. K. *et al* 1982) [36]; *Streptomyces xylophagus* (Iizuka H. *et al* 1965) [37]; *Bacillus subtilis* (Bermire R. *et al* 1983) [38], *Clostridium* sp. (Berenger, J.F. *et al* 1985) [39]. In our laboratory *Aeromonas* sp. (Roy *et al* 2003) [40] and *Bacillus sp.* (Roy *et al* 2004) [41] were isolated for xylanase production.

But the production of thermostable cellulase free xylanase from *Bacillus sp.* hasn't been investigated. So, keeping the above view in mind, the present studies were under taken to screening, isolation and characterization of cellulase free thermophilic xylanase producing bacteria as well as partial purification of xylanase. At first screening, isolation and characterization of cellulase free thermostable xylanase producing bacteria were investigated from soil and at second optimization of cultural parameter for the production of xylanase were studied and finally xylanase was partially purified and characterized from *Paenibacillus sp.*infectious diseases, cancer and heart diseases [7, 8]. Recently, it has been found that date fruit might be of benefit in glyacemic and lipid control of diabetic patients [9] and have also been identified as having antioxidant and antimutagenic properties [1, 10].

However, the biochemical properties and nutritional values of the available dates in Bangladesh are still unknown. There is not detailed report of biochemical and nutritional investigations of available date fruits in Bangladesh. Therefore, we approached to establish a thorough analysis regarding nutrient contents in available date fruits in Bangladesh. Due to lack of modern facilities we stopped our research works as an immature stage. Further study on date fruits from Bangladesh can lead to the development of a new therapy for the treatment of various physiological disorders.

2. Materials & Methods

2.1. Bacteriological Media

For the growth of bacteria various media were prepared such as Liquid media including Xylan broth, Luria bertani (LB) broth, Glycerol broth, Peptone water (Broth) without NaC1and Solid media including Nutrient agar media, Xylan agar medium.

2.2. Bacterial Sample Collection & Screening

For the screening of enzyme producing bacteria soil waste were collected from the Rajshahi University, Natore, Harian, Mahercandi, and Benodpur village of Rajshahi. After collection of sample, Screening of the xylanse producing bacteria, Preparation of pure culture, Preservation of bacterial strains were carried out.

2.3. Isolation and Characterization and Identification of Bacteria

All the xylanase-producing bacterial strains were isolated by their growth on xylan agar media as clear zone xylanolytic properties and were characterized and identified according to the morphological studies and biochemical tests described in the "Bergey's Manual of Determinative Bacteriology, Eighth edition, [144]" Text book of "C. H. Collins, and Monica Cheesbrough"

2.4. Determination of Xylanase Activity

For the determination of xylanase activitycrude enzyme (Xylanase) extract were prepared.

Determination of xylanase activity by DNS method

The xylanase activity was determined according to the method of Mahadevan and Sridhar (1962).

2.5. Optimization of Cultural Parameters

For the optimization of Cultural Parameters were selected as follows

Selection of a suitable broth medium for the production of xylanase, optimum pH, optimum temperature, Carbon sources, % of Xylan N-sources, Incubation period, Metal salt.

2.6. Partial Purification and Characterization

2.6.1. Preparation of Crude Enzyme (Xylanase) Extract

Cultivation of xylanolytic bacteria to extract xylanase, 1000 m1 of xylan broth was taken in 250 m1 conical flasks and sterilized by autoclaving at 15 1bs./sq inch pressure and 121°C for 15 minutes. A single colony of the isolated bacteria was inoculated into the xylan broth media in the conical flask sterile loop and incubated at 37°C for 24 hours with slow shaking. When the strain grown vastly, this culture was transferred into centrifuge tubes and the clear supernatant was collected after centrifugation at 8000×g for 15 minutes at 4°C and stored at 4°C very rapidly. This supernatant was then used for the further experiment as the crude enzyme.

2.6.2. Test of Purity by Sodium Dodecyl Sulfate Polyacrylamide Slab Gel Electrophoresis (SDS-PAGE) Method

The protein pattern of the selected fraction was determined 10% SDS-PAGE according to the method of Laemmli (1970) [4] as modified by smith (1995) [5].

2.6.3. Determination of Xylanase Activity by DNS Method

Xylanase activity was determined by detecting the release of reducing sugars from the used substrate (wheat bran xylan) in 50mM phosphate buffer at pH 9 and 55°C. The amount of reducing sugar present was determined by Dinitrosalisylic acid (DNS) method (Miller, 1972). The enzyme activity is generally expressed in terms of units. An enzyme unit (IU) is that concentration of an enzyme that catalyzes the formation of 1 µmole of product per minute under defined assay conditions. The concentration of enzyme in crude biological preparation is expressed as unit/ml (U $m1^{-1}$).

2.6.4. Determination of Water-Soluble Protein

Water-soluble protein concentration was determined by the method of Lowry *et al* (1951) [42] using bovine serum albumin (BSA) as standard.

2.7. Characterization of Xylanase

2.7.1. Determination of Effect of pH on the Activity of Xylanse

The activity of purified xylanase obtained from strain S1 (*Paenibacillus sp*.) wasdetermined at different pH values (50mM sodium phosphate buffer) ranging from 5.0 to 11 by the DNS method as described in previous.

2.7.2. Determination of Effect of Temperature on the Activity of Xylanase

The activity of the purified xylanase enzyme obtained from the bacterial strain S1 (*Paenibacillus sp*.) was determined at different temperature ranging from 30°C to 80° C by DNS method as described in previous.

2.7.3. Determination of Substrate Specificity on Xylanase Activity

To determine the substrate specificity of xylanase towards substrates including wheat bran xylan, cellulose, carboxymethylcellulose (CMC), glucose, xylose and starch were used as substrates. Theactivity of xylanase towards substrate was determined by DNS method as described in previous.

3. Result and Discussion

3.1. Purification of Xylanase

For the purification of xylanase, the crude enzyme extract of bacterial strain S1 (*Paenibacillus sp*.)was pr prepared. It was seen that the specific activity of the xylanase after heat treatment was high than the activity obtained after saturation.

Table 1. *Effect of metal salt on xylanase production of strain S1.*

Metal salt (2m M)	Optical density at 590nm
NaCl (Control)	0.09
$CaCl_2$	0.119
$MgCl_2$	0.076
KCl	0.072
$FeCl_3$	0.04
$MnCl_2$	0.039

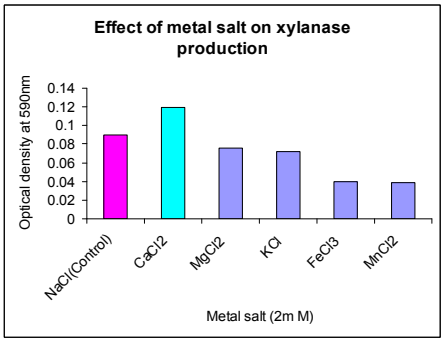

Fig. 1. *Effect of metal salt on xylanase productionof strain S1.*

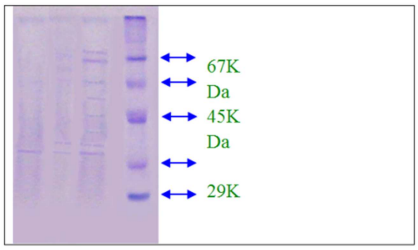

Fig. 2. *Check of purity on Gel Electrophoresis*

To check purity the heated sample was applied on slab gel electrophoresis and it was observed that there were three band of protein (Fig.-2). Thus at this stage xylanase was partially purfied.

L1 = Molecular mass markers: Bovine serumalbumin or BSA (MW. 67 KDa), Albumin fromegg white (MW. 45 KDa),Carbonic anhydrase(MW. 29 KDa), Trypsin inhibitor (MW. 20KDa) and Lysozyme (MW.14.6 KDa) as reference proteins.)

(L_2 = Crude enzyme extract, L_3 = PPT with ammonium sulphate, L_4 = Crude enzymeextract after heat treatment at 50°C).

3.2. Characterization of Xylanase

3.2.1. Effect of Temperature on the Activity of Xylanase

The effect of temperature on activity of xylanase against xylan obtained from strain S1 (*Paenibacillus sp*.) was examined in the temperature range of 30°C-80°C. The enzyme showed the best activity around 50°-55°C. But it was observed that optimum temperature of the activity of the enzyme was 55°C. With further rise of the temperature the activity of the enzyme was decreased more sharply and 15% of the activity was only retained at 70°C but destroyed at 80°C (Table-2 & Fig.-3).

Table 2. *Effect of Temperature on xylanase activity of S1.*

pH	Optical density at 590nm	Relative enzymeactivity orhydrolysis (%)
5	0.02	18.18
6	0.075	68.18
7	0.11	100
8	0.09	81.82
9	0.035	31.82
10	0.023	20.91
11	0.015	13.64

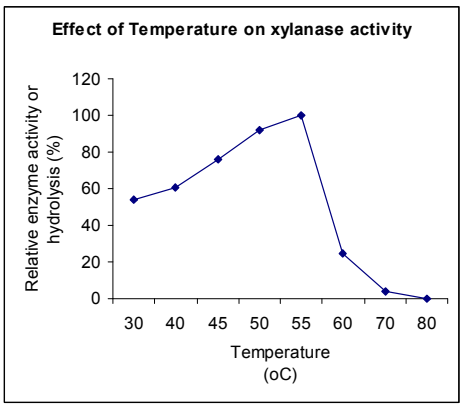

Fig. 3. *Effect of Temperature on xylanase activity of S1.*

3.2.2. Effect of pH on the Activity of Xylanase

To determine the optimum pH of the enzyme activity against xylan, 50mM sodium phosphate buffer at a range of pH 5-11 was used. The enzyme showed the best activity around the pH range 7 to 10, but the optimum pH of the enzyme activity was 7.0 (Table-3 & Fig.-4).

Table 3. *Effect of pH on xylanase activity of S1.*

pH	Optical density at 590nm	Relative enzymeactivity orhydrolysis (%)
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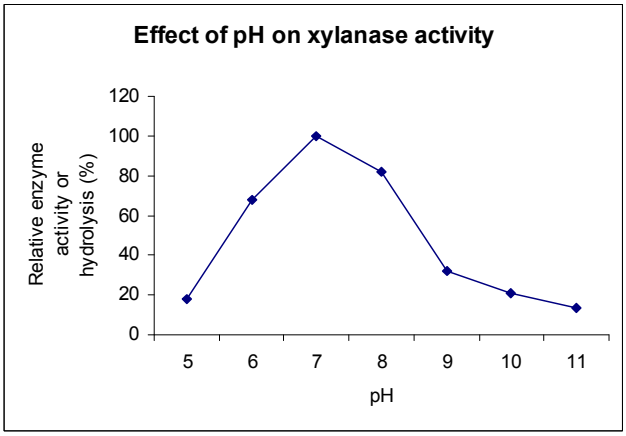

Fig. 4. *Effect of pH on xylanase activity of S1.*

3.3. Substrate Specificity of Xylanase

The substrate specificity of purified xylanase was studied using various polysaccharides as the substrates and the results obtained were summarized in the Table-4. From the table it was seen that purified xylanase was able to hydrolyze strongly wheat bran xylan but no activity towards cellulose, carboxymethyl cellulose and starch. Thus it was a true xylanase.

Table 4. *Substrate specificity of xylanase of strain S1.*

Substrate	Optical density at 600 nm	Relativehydrolysis (%)
Wheat bran xylan	0.13	100
Oat spealt xylan	0.09	69.23
Beech wood xylan	0.07	53.85
Starch	0.00	0.00
CMC	0.00	0.00
Cellulose	0.00	0.00

4. Conclusion

Xylanases are hydrolytic enzymes that randomly cleave the β-1,4- backbone of the complex plant cell wall. Xylanases have potential applications in a wide range of industrial processes, in the bioconversion of xylan containing agricultural and forestry

wastes for the production of fermentable sugars which can be subsequently utilized for the production the liquid fuel, chemical feedstock and food materials.In addition xylanases have been used in upgrading of low-grade jute, in textile industries, in food processing (e.g. clarification of juices and wines), in production of dissolving pulps in enzymatic beating to remove dark color of lignin from unbleached pulps and in the paper and pulp industry to increase the bio-bleachability of pulps. The uses of xylanases have also been proposed for extracting coffee, plant oils etc. The new major scale application area of xylanase in clearly in the pulp and paper industry in order to increase the bleach ability of kraft pulps.

For screening of xylanase producing bacteria soil samples were dilluted by serial dilution and cultured on selective wheat bran xylan agar media. Two bacterial strains (S1 & S2) showed clear transparent zone around the colony on xylan agar plate were selected as xylanase producing bacteria. The xylanase activity in each strain was again confirmed by measuring the amount of reducing sugar liberated from xylan by DNS method.

Depending upon morphological and biochemical test demonstrated that xylanase producing bacterial strains were *Paenibacillus sp*. (strain S1), and *Coccobacillus sp*. (Strain S2) but molecular level characterization in essential for identification and classification of these organisms. So further work is recommended to 16S rRNA gene PCR amplification and DNA sequences analysis for species confirmation. Between of them the strain *Paenibacillus sp*. showed highest xylanolyticactivity than other. All strains showed multidrug sensitivity.

In our study we emphasized on the cultural parameter for the production of xylanase. It was seen that the strain *Paenibacillus sp.* was thermophile and produced highly active cellulase free xylanase.

The optimization of cultural variables resulted in a marked enhancement in the secretion of cellulase free thermostable xylanase by an extreme thermophile *Paenibacillus sp*. The enzyme secretion was enhanced when the medium was supplemented with 0.5% wheat bran xylan, peptone, Ca^{2+} and Mg^{2+} salts. The peak in xylanase production was achieved within 48-60 hours at temperature 50°-55°C and at pH 7.0

The cellulase free xylanase was also partially purified from *Paenibacillus sp*. and characterized. The purification of xylanase was carried out by ammonium sulfate fractionation (80%) and heat treatment at 50°C. The xylanase was optimally active at pH 7.0 and 55°C as well as showed high substrate activity to wheat bran xylan but no activity towards carboxymethyl cellulose, cellulose & starch. Thus the strain *Paenibacillus sp*. isolated from soil was highly active cellulase free thermostable xylanase producing bacteria.

In addition, the bacterial strain S1 was tested to the production of antimicrobial compound.

In future we want to know the structure function relationship of the purified enzyme and also want to known the molecular biological study using highly purified xylanase. For this purpose we have to determine the N-terminal & C-terminal amino acid sequence.

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