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Production, Purification and Characterization of Xylanase Enzyme from *Bacillus sp* in Solid State Fermentation

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Abstract Xylanases are extensively applied in paper and pulp industries as well as during preparation of baked products to improve their quality. Additionally, it is also used in coffee, oil and starch industries in order to increase their nutritional values. Soil samples were collected near saw mills in various localities of Bangalore urban to isolate organisms for the production of xylanase using solid state fermentation. Six organisms were isolated using selective media based on their morphological characters. Among them one organism showed maximum production of xylanase enzyme identified as *Bacillus sp* based on their biochemical test and 16s RNA sequencing. Solid substrate fermentation was carried out using various agro wastes such as sugar cane bagasse, saw dust, paddy husk, wheat straw and orange peel powder. Sugarcane bagasse showed maximum production of enzyme compared to other substrates with different physical parameters such as pH 8, temperature at 35 °C after 72 hrs of incubation. Trace element such as Mg^{++} enhances the production of enzyme more than 22 % compared with other metal ions like Ca^{++}, Mn^{++} and Fe^{++}. After production, enzyme purified by using three step methods such ammonium sulphate precipitation, dialysis, ion exchange and gel filtration. Fold purification was increased up to 12 fold, yield 36 % and molecular weight of enzyme was 62 KDa determined using SDS PAGE.

Keywords: *fermentation, ion exchange chromatography, SDS-PAGE, SSF, Xylanase*

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1. Introduction

Xylan is the major component of hemicellulose of plant cell walls and constitutes up to 35 % of total dry weight of higher plants in tropical biomass [1]. Moreover, 10 % - 12 % of solid waste is composed of hemicellulose on dry weight basis [2]. The enzymatic process can be used for the degradation of these materials involving the use of microbial enzymes that are less polluting, environmental approachable, energy saving and subordinate disposal glitches [3,4]. Microorganisms such as bacteria, fungi and actinomycetes are established to be the amusing cradles of xylanases [5]. Though many microorganisms have been isolated for enzyme production, there is silent requisite for novel strains accomplished in producing better pinnacles of enzyme [6,7]. β-1,4-Xylans are heterogeneous polysaccharides found in the cell walls of almost all plant parts. Hydrolysis of their characteristic backbone consisting of β-1,4-linked D-xylosyl residues, involves β-1,4-xylanases (1,4-p-D-xylan xylanohydrolase; EC 3.2.1.8) and β-xylosidases (1,4-p-D-xylan xylohydrolase; EC 3.2.1.37).

Xylanase is a biocatalyst degrades xylan into reducing sugars such as xylose and xylobiose. This enzyme has employed in the paper manufacturing to bleach paper pulp and increase the paper pulp illumination instead of using toxic and expensive chemicals [8]. It is used in numerous industrial uses such as paper, pulp, juice, bakery and beer industries [9]. Xylanase also used in the pre-treatment of lignocellulose biomass for bioethanol manufacture and used in handling of barley and wheat to progress the possessions of animal diet in animal feed productions [10]. Xylanase is produced by many bacteria and fungi and possesses a range of industrial and environmental applications [11,12]. Industries that have demand for xylanase are paper and pulp, baking for improving dough handling and quality of baked products, during extraction of coffee, plant oils, starch, for improvement of nutritional properties of silage and grain, and in combination with pectinase and cellulase for clarification of fruit juices. The enzyme finds applications in textile industry for degumming of plant fiber sources as well as to enhance

fiber quality [13,14]. Main source for the enzyme xylanase is microorganisms like bacteria and fungus, the two most active strains have been identified as *Streptomyces albus* and *Streptomyces chromofuscus* [15]. The host strain can be developed from the well-characterized nonpathogenic and non-toxigenic *B. Subtilis* wild type. Thermoalkalophilic *Arthrobacter* sp. produced extracellular xylanase, when wheat bran, rice husk, rice bran and bagassae were used as carbon source under solid state fermentation [16]. Solid state fermentation (SSF) can be performed on a variety of lignocellulosic materials like wheat bran, rice husk, rice bran, soya bran, ragi bran and bagassae etc are proved to be highly efficient technique in the xylanase production [17]. The fungal strain *Aspergillus niger* is also contains xylanase enzyme. In some higher plants and agricultural wastes, xylan constitutes from 20 % - 40 % of dry weight. So Wheat bran and rice husk were obtained from the local flour mill while rice straw and sugarcane bagasse can be used as a substrate.

Research on xylanase has markedly increased due to its potential applications in pulping and bleaching processes using cellulose free preparations, textile processes, the enzymatic saccharification of lignocellulosic materials and waste treatment. Interest in xylanases and other xylanolytic enzymes has grown markedly during recent years. One of the main reasons for this is the potential use of these enzymes in the pulp and paper industry, particularly in bleaching processes. This requires enzymes that are very specific for the hydrolysis of hemicelluloses in order to preserve the cellulose structure during biopulping process [18]. Xylanases are used as bleaching essences in handling of chemical or thermo mechanical pulps, it would be appropriate that the enzyme encounters the necessities of the progression rather than the process meeting the enzyme needs. Thus, perfect enzyme for a bleaching procedure must be vigorous at high temperature and pH [19]. The main problem faced by pulp and paper industry while using enzyme treatment is the availability and cost of the enzyme. About 30 % - 40 % of the production cost of many industrial enzymes is accounted by the cost of growth substrate. The use of low cost substrates for the production of industrial enzymes is one of the ways to greatly reduce production costs. This can be achieved using solid agricultural waste materials as substrates. Xylanase producers were mostly isolated from soil. Soil based isolation needs screening of many soil samples and microorganisms [20,21,22,23], culture enrichment steps [24,25] and serial dilutions [26]. The aim of this research work is to assess the potential of xylanase enzyme from soil samples collected from saw mills in Bangalore, India and isolated xylanolytic *Bacillus* sp. In addition, xylanase production level of isolates was determined using soil samples and characterized enzyme partially. The experimental conditions were pH of media, incubation period, temperature and metal ion concentration.

2. Materials and Methods

2.1. Culture and Growth Conditions

Augmentation procedure with xylan as a solitary source of carbon was used to isolate xylan exploiting bacteria. Using this technique bacterial cultures were isolated from thirty soil samples collected from saw mills of different localities in Bangalore, India. From these soil samples, one isolate that created comparatively more amount of extracellular xylanases was nominated for the current study [16].

2.2. Isolation of Organism

Soil samples were collected from 10 cm depth from the surface in a sterile polythene bags to the laboratory for isolation [27]. In the isolation process 1 gm of soil is dissolved in 10 ml saline used as inoculum. 1 ml of inoculum was poured into the sterile petriplate, above that xylan modified media (Xylan 0.5 %, NaCl 1 %, Yeast extract 0.5 % and Agar 2 %) was added and allowed for solidification. After solidification, plates were incubated for 48 hrs at 37 °C, next based on morphological characters, organisms were sub cultured on xylan modified media slants.

2.3. Screening the Organisms for Xylanase Production

Confirmation for the production of xylanase was carried out by zone of clearance method. In this process xylan modified media was poured into the sterile petriplate and allow it for solidification. After solidification, organisms were streaked on the media in zigzag manner and incubated for 48 hrs at 37 °C. Then xylanolytic activity was found after 1 % of solution was poured on the media and clear zone was observed surrounding the organisms.

2.4. Identification of Organism

The isolates showed maximum clear zone surrounding the organisms and more activity based on bioassay method. The pure isolates were characterized by means of conventional processes and isolates were identified on the basis of morphological, cultural, chemo-taxonomical characteristics [28] and 16S rRNA sequencing. Results were interpreted according to Bergey's Manual of Determinative Bacteriology [29] and NCBI database.

2.5. Xylanase Production in Solid State Fermentation

Selected strains subjected for the production of xylanase enzyme using various agro wastes such as sugar cane bagasse, saw dust, paddy husk, wheat straw and orange peel powder. In this process 25 gm of powder and 50 ml of minimal essential broth was taken in 500 ml conical flask and inoculated with 5 ml of culture, mixed thoroughly and incubated for 48 hrs at 35°C. After incubation solid substrate was removed and suspended in 250 ml of 100 mM Tris HCL buffer (pH 7) and agitated thoroughly for 1 hr to extract xylanase enzyme. Media was centrifuged at 12000 rpm for 30 min at 4 °C. Supernatant was filtered through Whatman No. 1 filter paper and the clear filtrate was used as crude sample for the estimation of xylanase enzyme activity.

2.6. Enzyme Assay

Xylanase activity was measured by modified method [30]. 1 ml of 1 % solubilized birch wood xylan solution and 3 ml 50 mM Tris HCL was added to a test tube and pre warm at 37 °C. 1 ml of enzyme (Crude sample) was added and incubated at 37 °C for 60 min. After incubation, 1 ml of DNS reagent was added and boil for 5 min, then allow to room temperature and absorbance was measured at 540 nm. The reaction was terminated at zero time in the control tubes, the standard graph was prepared using 100-500 µg xylose. One unit of xylanase activity was defined as the amount of enzyme that liberates 1 µM of reducing sugars equivalent to xylose per minute under the assay conditions described.

2.7. Effect of Physical Parameters for the Growth of Organism

Optimization of physical parameters such as incubation period, pH and temperature were carried out. During the process 25 gm agro waste, 25 gm of powder and 50 ml of minimal essential broth was taken in 500 ml conical flask and inoculated with 5 ml of culture, mixed thoroughly and incubated. For every 24 hrs, 5 gm of solid substrate was removed and subjected for xylanolytic activity, The effect of pH on enzyme activity was determined by incubating xylanase at various pH ranging from 6.0 to 11.0. To evaluate the stability of the enzyme at each pH, the purified enzyme was adjusted to SSF media incubated. Xylanolytic activity was measured spectrophotometrically after incubation. The optimum temperature for maximum xylanase activity was determined by the SSF media varying the reaction temperature from 25 °C - 45 °C.

2.8. Effect of Trace Element for the Production of Xylanase

Along with SSF media, 0.1 mg of various metal ions such as Mg^{++}, Ca^{++}, Mn^{++} and Fe^{++} were used for 1 hour at room temperature for the production of xylanase enzyme and in addition to this optimize the concentration of metal ions for the production.

2.9. Purification of Xylanase

The cell free media collected was subjected to different purification steps including ammonium sulphate $(NH_4)_2SO_4$ precipitation, dialysis, gel filtration and DEAE-cellulose ion exchange chromatography by using gradient elution buffer [31].

2.10. Molecular Weight Determination

The molecular weight of the purified xylanase was estimated by SDS-PAGE electrophoresis. SDS-PAGE was performed [32] using 4 % acrylamide stacking gel and 10 % acrylamide separating gel to determine the molecular mass and protein purity and staining was carried out with Coomassie brilliant blue.

2.11. Statistical Analysis

The experiment was conducted in triplicates and the results were statistically analyzed. All the standards were calculated by regression correlation values and Pearson's correlation is less than 0.05.

3. Results and Discussion

Isolation of organism: Xylan provided nutrition in the xylan modified media for the growth of organisms. The organisms have ability to degrade the xylan to xylose are simplest sugar molecules used as energy source. Fifteen organisms were isolated based on their morphology from the soil samples as shown in Figure 1. These organisms cultured on screening media for confirmation of the production, one organism showed maximum clear zone surrounding the other organisms and indicates that, organism has high xylanolytic activity as represented in Figure 2.

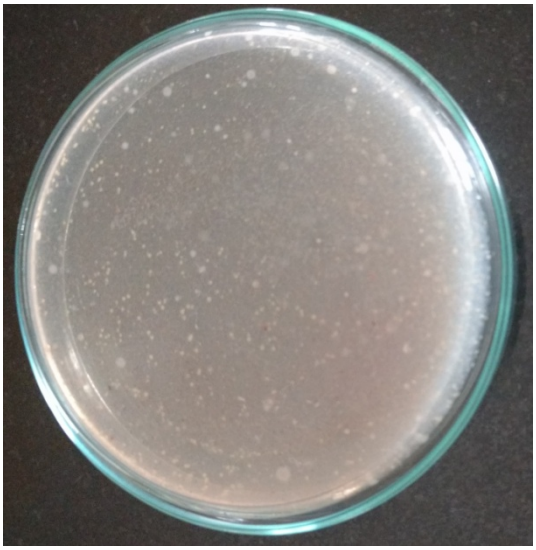

Figure 1. Isolation of organisms

Figure 2. Screening the organisms for xylanase production

Identification of organism: The organism showing more action confirmed through biological activity was identified by using 16S rRNA sequencing. The universal primers (27F´ and 1492R´) used for the amplification of 16S rRNA were able to amplify the region giving ~1.4 kb size fragment in isolated strain. Amplicons visualized on 1 % agarose gel with 1X Tris-acetate EDTA buffer at constant voltage (80 V). The absorbance ratios (A 260/280) of the preparations were in range of 1.80-1.85 by spectrophotometer. Based on 16S rRNA sequenced data, BLAST search showed that Bacillus sp. is highly resembled *Bacillus cereus* up to 99 % with *Bacillus cereus* strain as reported in Figure 3. Differentiation is considered based on biochemical test in species level and 16S rRNA sequencing is one of the best advanced processes for identification of bacteria. New *Bacillus* species similar to *Bacillus arseniciselenatis* DSM 15340 was isolated from soil sample collected at coastal areas of Mandovi, Goa, India [33]. *Bacillus arseniciselenatis* DSM 15340 and *Bacillus arsenicus were* isolated and reported from a bore well located in the chakdah region of West Bengal, India [34]. A novel moderately thermophilic, gram positive, endospore forming, rod shaped, motile and alkaline active xylanase producing bacterial strain D1021T was isolated from Kaynarca hot spring in the province of Izmir, Turkey. The isolated bacterial strain was identified as *Anoxybacillus kaynarcensis* on the basis of phenotypic characteristics, *rpoB* analysis and 16S rRNA gene amplification [35]. The isolation of a cellulase-free xylanase producing bacterial strain was examined and identified as *Cellulosimicrobium* sp. on the basis of morphological, physiological, biochemical characteristics as well as 16S rRNA sequencing by Microbial Type Culture Collection (MTCC) and gene bank. The bacterial strain was deposited as *Cellulosimicrobium* sp. MTCC 10645. Xylanase activity of 4,962 U/gds was obtained after optimization of xylanase production in SSF [8]. Four xylanolytic bacterial strains were isolated from Soda Lake (pH 8.2, salt concentration 9.9 %) on media encompassing xylan, categorized these on the basis of morphological, physiological and biochemical characters and the stain recognized were belonging to *Bacillus* genus [36]. *Bacillus* sp was isolated from soil sample from Rajshahi university campus. The isolated bacterium was an aerobic, gram-positive, spore forming, rod-shaped organism and was recognised as *Bacillus cereus* on the basis of 16S rRNA gene amplification, morphological properties and taxonomic characteristics [37]. Xylanase production from *B. pumilus* B20 was studied by using DeMeo's fractional factorial design. Xylanase production increased up to 3.4 fold under the optimized culture medium consisting of K_2HPO, NaCl, peptone, yeast extract and wheat bran. Among the different factors screened, wheat bran showed a positive effect in the first step of optimization and $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ had a negative effect [38]. SSF condition was optimized for xylanase production from *B. pumilus*, using paddy husk moistened with liquid fermentation medium (xylan, 20.0 g/L; peptone, 2.0 g/L; yeast extract, 2.5 g/L; K_2HPO, 1.0g/L; NaCl, 0.1 g/L; $(NH_4)2SO_4$, 2.0 g/L, $CaCl_2 \cdot 2H_2O$, 0.005 g/L; $MgCl_2 \cdot 6H_2O$, 0.005 g/L; and FeCl, 0.005 g/L) at pH 9.0. Highest xylanase activity was obtained after six days of incubation at 30 °C using the paddy husk to liquid fermentation medium ratio of 2:9. Production of the xylanase was increased by sucrose, fructose and arabinose but reduced by glucose, galactose and lactose [39]. The production of thermostable and cellulase-free xylanase was reported from *Streptomyces* sp. QG-11-3 in SSF using wheat bran and eucalyptus kraft pulp as solid substrates. The substrates were evenly mixed with mineral salts solution containing (g/l): KH_2PO_4, 1; NaCl, 2.5; $MgSO_4$. $7H_2O$, 0.1; $(NH_4)2SO_4$, 1; CaCl, 0.1; and soil extract, 2 ml (v/v) at pH 8.0. The maximum xylanase yield obtained using these two substrates were 2360 U/g and 1200 U/g dry solid substrate at substrate: moisture ratio of 1:3 and 1:2.5 respectively [40].

Xylanase production in solid state fermentation (SSF): Agricultural wastes like vegetables, dairy products and crops wastes are rich in polysaccharides such as hemicellulose, pectin, lignin and xylan and the degradation of these products is tedious. Microorganisms have the ability to degrade by producing hydrolytic enzymes, among them one such enzyme is xylanase. Several workers reported the suitability of sugar cane bagasse, saw dust, paddy husk, wheat bran and orange peel powder for xylanase production in SSF [41,42,43,44]. Commercial wheat bran consists of 30 % cellulose, 27 % hemicellulose, 21 % lignin and 8 % ash [45]. Sugar bagasse showed maximum enzyme activity based on xylanolytic enzyme assay compare to saw dust, paddy husk, wheat straw and orange peel powder as depicted in Figure 4. Xylanase production was optimized from *Arthrobacter* sp. MTCC 5214 in SSF using wheat bran as carbon source. Bacterial cultures were isolated by utilizing xylan from sediment sample collected from Mandovi estuary, west coast of India. Among these isolates, the bacterial culture was identified as *Arthrobacter* sp. MTCC 5214, produced highest xylanase at 30 °C using wheat bran as a substrate and moisture ratio 1:3 after incubation for 7 days [46].

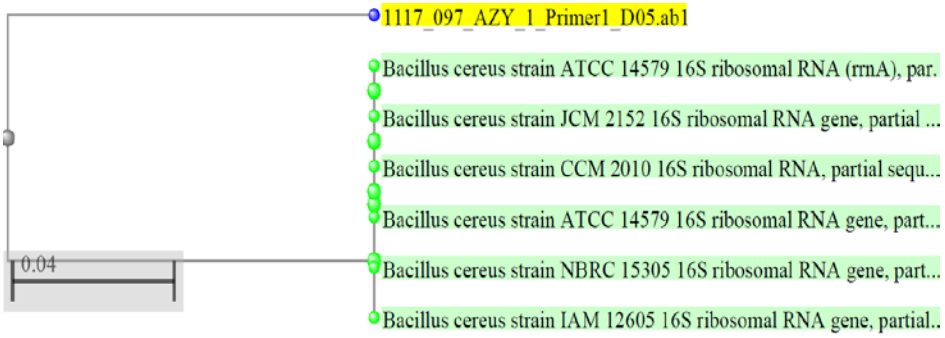

Figure 3. Phylogenetic relationship of 16S rRNA sequences of *Bacillus* sp

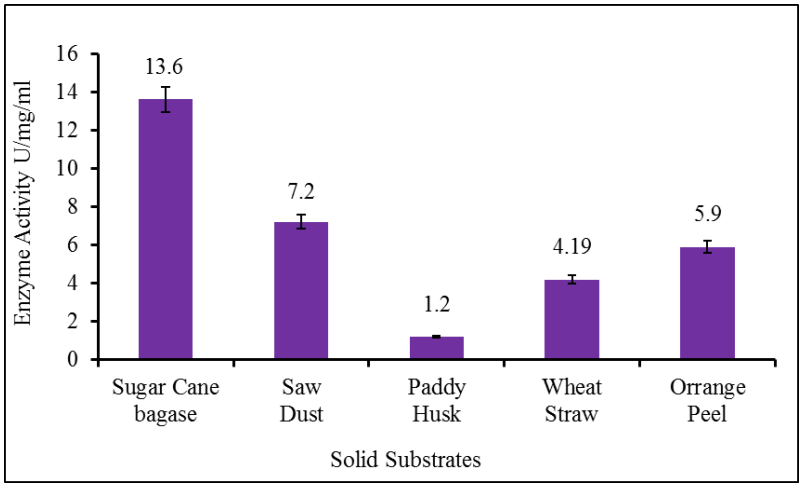

Figure 4. Enzyme activity of different solid substrate

Effect of physical parameters for the growth of organism: Physical parameters play a vital role in the organisms growth and optimal condition for their metabolism and growth. Multiplication of the organisms varies in time and it leads the production of particular metabolites required in different time intervals. Enzymes show their concentrated activity at their respective optimum conditions, divergences from the optimum origin a reduction in the action [47]. Maximum amount of xylanase production is at 72 hrs as depicted in Figure 5. pH stood the maximum significant factor to depict the enzyme. Xylanase from *Bacillus sp.* showed high level activity at pH 8.0 even though Optimum pH was 5.5 as recorded in Figure 6. At higher pH values also, activity was 95 %. With respect to stability, at all tested pH values xylanase activity was 100 % for 1 h. Likewise, two xylanases specifically xylanase N and xylanase A were purified from *Bacillus sp.* No. C-125. Between these xylanases, N displays full activity at pH ranging from 6.0 to 7.0, whereas xylanase A was vigorous at pH ranging from 6.0 to 10.0 and revealed certain activity at pH 12.0 [48]. Temperature influences all the physiological activities in living cell and is one of the important environmental factors to control the growth, microbial activities and normal functioning of enzyme [31]. The study of thermo stability showed that the enzyme was stable at temperature up to 40 °C. Maximum temperature showed at 40 °C for the production of enzyme and if the temperature increases or decreases, the production of enzyme is low as shown in Figure 7. Several forms of xylanases were purified from *Aeromonas sp.* and the properties of three xylanases were well characterized [49]. It was also reported that these three xylanases were most active at 50 °C - 60 °C [50]. Two xylanases contributed the maximum activity and relatively high stabilities at 50 °C temperature were purified and observed [51]. Xylanase production was boosted from *B. pumilus* SV-85S in SSF using wheat bran as substrate. Optimization of fermentation enhanced the enzyme production from 5300 IU/g to 73,000 IU/g [52]. The enzyme titre was highest after 48 h of incubation at 30 °C with 1:3 ratio of substrate to moistening agent using the inoculum level of 15 % and wheat bran as a carbon source [53]. Xylanase production was optimized in SSF from an alkalophilic *B. subtilis* using inexpensive agricultural residues. Among these agro-residues, wheat bran was found to be the best substrate. Xylanase production was highest (8,964 U/g) after 72 h of incubation at 37 °C and a substrate to moisture ratio of 1:2 (w/v). An inoculum level of 15 % resulted in maximum production of xylanase. Addition of nutrients such as yeast extract, peptone and beef extract stimulated the enzyme production. In contrast, addition of glucose and xylose repressed the production of xylanase [54].

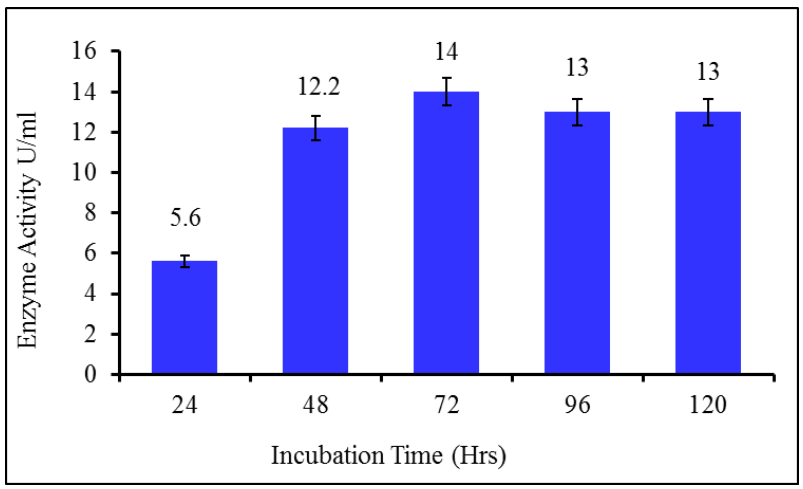

Figure 5. Effect of assay incubation time on xylanase activity

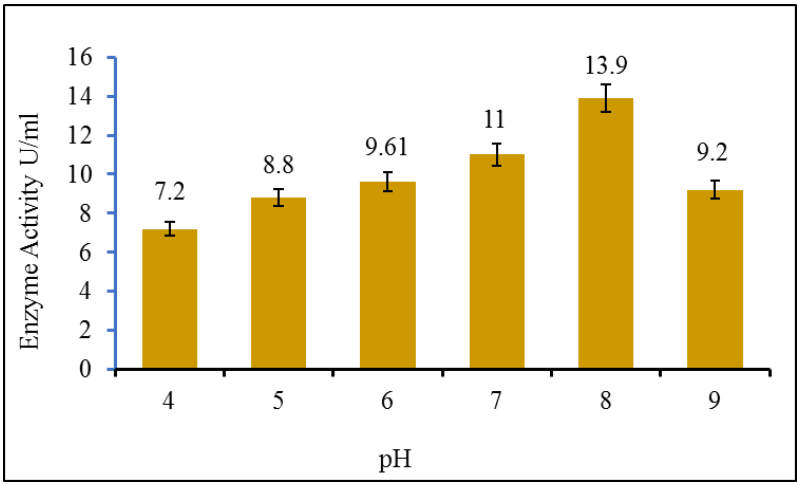

Figure 6. Effect of assay pH on xylanase activity

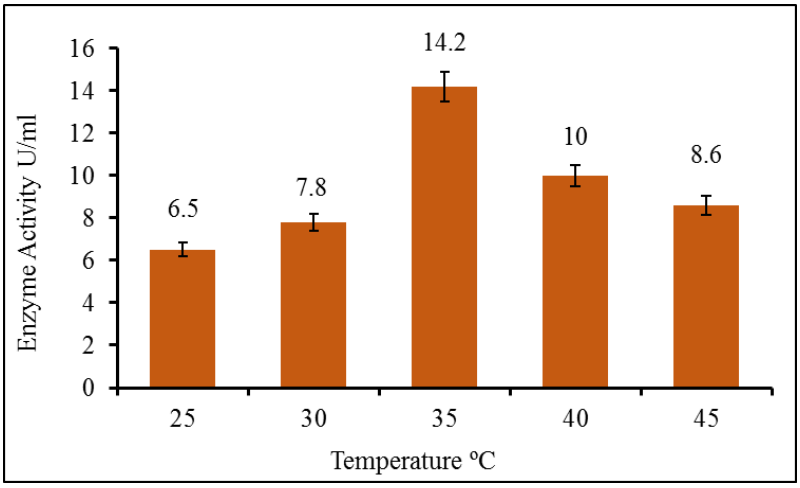

Figure 7. Effect of assay temperature on xylanase activity

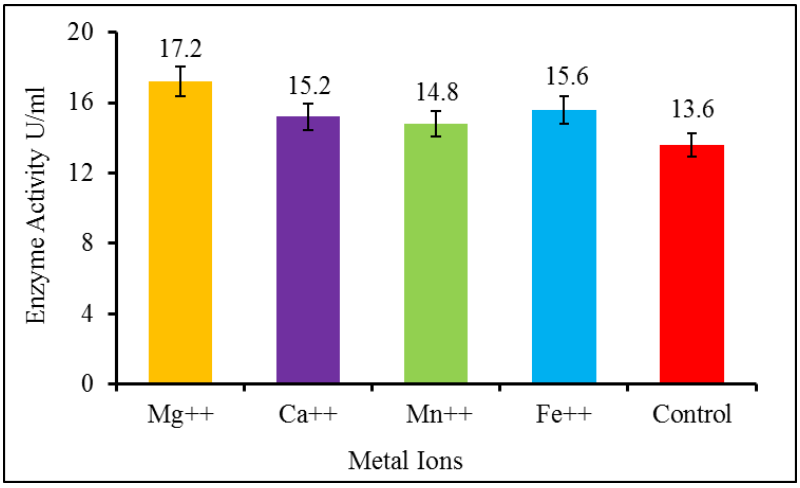

Figure 8. Effect of metal ions for the production of xylanase

Effect of metal ions for the production of xylanase: Metal ions acts as cofactor for the enzyme involved in the metabolism. It will enhance the organisms growth and leads to enzymes production. Results reveal that the production of enzyme enhances nearly 22 % by using metal ions Mg++ compare to control as predicted in Figure 8. *B. circulans* AB 16 was isolated from a garbage dump in Delhi, India. This bacterial strain produced 19.28 IU/ml of extracellular thermophilic xylanase when grown in basal medium containing 0.1 % KH_2PO_4, 0.1 % K_2HPO_4, 0.05 % $MgSO_4$, 0.1 % NaCl and 0.3 % oat spelt xylan. Optimum pH for the enzyme was 6.0-7.0, but it was stable over a pH of 5.0-9.0. The enzyme showed an optimum temperature of 80 °C [55].

Table 1. Purification of xylanase enzyme

Steps	Total volume (ml)	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Fold purification
Crude Enzyme	200	216	60	3.6	100
Ammonium Sulphate Precipitation	25	176	12	14.6	40
Ion Exchange	10	132	8	16.5	17
Gel Filtration	10	86	2	38	12

Purification of the enzyme: After production, the cell free media was taken for 70 % ammonium sulphate precipitation. The specific activity was increased up to 36 % and fold purification was 12 as recorded in Table 1. Similar results were observed as xylanase was purified 3.06 fold with a specific activity of 299.25 U/mg from *Bacillus Sp* [33]. Specific activity of xylanase produced by *Bacillus pumilus* was 298 U/mg [20].

Molecular weight determination by SDS page: A single band of monomer protein was obtained on 13 % of the SDS PAGE. The molecular weight of denatured xylanase estimated from the relative mobility of proteins on SDS-PAGE was approximately 62 kDa as depicted in Figure 9. Present results were supported by earlier works. Xylanase produced by *Bacillus sp.* strain BP-23 is of 32 kDa [56] whereas the second xylanase obtained from *Bacillus firmus* had a molecular weight of 45 kDa [57]. It was reported that the enzyme from a fungus *Plectosphaerella cucumerina* had a molecular weight of 19 kDa [58].

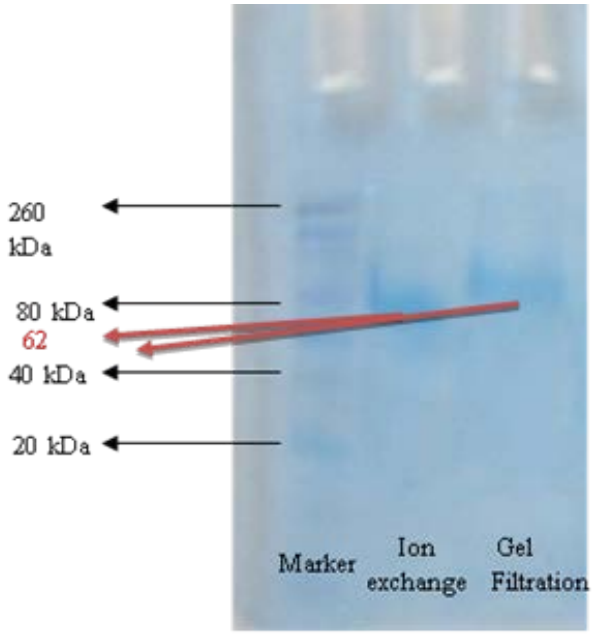

Figure 9. Molecular weight by SDS-PAGE analysis of purified xylanases from *Bacillus sp*

4. Conclusion

It has concluded that soil samples can be reflected as a source of xylanolytic microorganism and might assist as a basis for xylanolytic microorganism isolation. *Bacillus sp* is the best source for production of xylanase enzyme with cheaply available agro waste solid state substrate sugar cane bagasse. Mg++ are the best metal ions to enhance the production of xylanase, purified enzyme has strong ability degrading the xylan. After detailed characterization, such enzymes could be used in various applications in paper and pulp, textile, food and feed industries. Due to wide range of application area, isolation of new xylanase producers is of great value for both researchers and industry. This process can be used in large scale production to reach the present needs.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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