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Isolation and characterisation of *Bacillus australimaris* P 5 and optimizing the parameters for xylanase production.

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Abstract: *Studies were carried out on the cultivation of a newly isolated bacterial strain Bacillus australimaris P5 for xylanase production. Production of xylanase strongly depends on optimised experimental parameters. In the present study, bacterial strain Bacillus australimaris P5 was isolated which produces cellulase-free crude xylanase of titre 104 U/ml. Carbon sources that favoured xylanase production were xylose, xylan, rice bran, molasses etc. Xylanase production was found best at an incubation period of 48 h, temp 35˚C and pH 8.The inoculum level best suited for highest titre was found to be 5% of 18 h grown culture. Addition of yeast extract enhanced xylanase activity followed by beef extract, peptone and ammonium hydrogen phosphate (NH_4HPO_4) in the production medium. Physiological characterization revealed that the bacterial strain is Gram positive, strictly aerobic strain. Scanning Electron Micrograph of the strain revealed the morphological features of the isolated organism.*

Keywords*: Bacteria, Isolation, Production, Optimization, Xylanase*

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I. Introduction

The north eastern state of India, Assam is an important biodiversity hot spots of the world. The microbial resource of this region can be considered as an enormous source of beneficial enzymes which can be utilized as organic catalyst in wide range of processes on an industrial scale. A wide range of enzymes has been studied and have found a significant place in the list of microbial enzymes. Out of various enzymes, an important enzyme is the xylanase enzyme.

Microbial xylanase have tremendous potential which have established their applications in various industries. Xylanase enzyme finds its application in clarification of fruit juices, improvement of the nutritional value of feed, bioethanol production, fruit and vegetable processing and most importantly in paper industry [1, 2]. Recently, the potential use of xylanase enzyme from microbes in pulping and bleaching processes using cellulase-free preparation has drawn a worldwide attention of various researchers. However, Viikari [3] was the first to demonstrate the use of xylanase in pulp bleaching. In response to the growing concerns for the environment, the enzymatic bleaching is considered as desirable, safer and economically viable method. The paper industries have given importance to xylanase which are thermostable, having alkaline pH and devoid of cellulase activity [4].Initially investigation has been done on fungal xylanase as a prebleaching agent but this enzyme source proved limited acceptance on industrial scale due to cellulase presence, low pH and less stability. Commercial applications require cheaper enzymes with thermostability and optimum pH [5] which can be attained only from bacterial sources because of their extensive environmental and biochemical adaptability. Xylanolytic bacterial species include *Bacillus, Micrococcus, Staphylococcus, Paenibacillus, Cellulomonas, Arthrobacter, Microbacterium, Pseudoxanthomonas* and *Rhodothermus* importance [6]. In xylanase production, the dominant genus is the *bacillus* [7]**.** Several *Bacillus* strains had been reported to produce cellulase- free xylanase at alkaline pH and high temperature [8].

In this context, the present study report the isolation of xylanase producing bacterial strain *Bacillus australimaris* P 5 from decaying bamboo samples, its biochemical characterization and optimization of xylanase production from the strain.

II. Materials and methods

2.1 Microbial Strain isolation:

The xylanase producing microbial culture P 5 was isolated from decaying bamboo samples collected from HPCL paper mill in Jagiroad, Assam, India. Its ability to produce xylanase was confirmed by inoculating the strain in Xylan agar plates containing xylan (1%) as a sole carbon source and incubating the plates for 48

hours at 37˚C [9]. The plates were flooded with Congo red solution (0.1% Congo red) for 15 min followed by washing with 1M NaCl solution. The orange digestion halos around the colonies were chosen as positive. The microscopic, morphological, physiological and biochemical tests were performed according to Bergey's Manual of Systematic Bacteriology [10].For molecular identification, genomic DNA was extracted followed by amplification of PCR product. The PCR product was sequenced and 16S rRNA gene sequence was compared with those in the gene bank using BLASTn program [11]. The 16S rRNA gene sequence was deposited in GenBank with the accession number KY697915 .The pure culture was stored at 4˚C and preserved in 20% glycerol for future reference.

2.2 Xylanase Production in SSF:

5% (v/v) 18 h grown bacterial suspension of P 5 was transferred to Erlenmeyer flasks (250 ml) containing 1% of xylose and 0.5% of yeast extract with 50mL of mineral salt solution (g/L:$MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4,0.4; pH 8.0). The flask were incubated at 35˚C under shaking condition (150 rpm) for 48 h. Crude enzyme extract was obtained after centrifugation at 10000 rpm for 10 min at 4˚C for enzyme assay.

2.3 Enzyme assay:

The xylanase activity was determined according to Miller [12] by measuring the release of reducing sugar during the enzyme-substrate reaction using 3, 5-dinitrosalicylic acid (DNS). The reaction mixture for enzyme assay contained 500 µL of (0.5%) xylan as substrate prepared in 50 mM phosphate buffer (pH-7.0) and 100 µL appropriately diluted enzyme solution. The reaction mixture was incubated at 60˚C for 30 min followed by addition of 3 mL of DNS solution. Finally, the mixture was put in boiling water bath for 10 min. A control was run simultaneously but the reaction was terminated prior to addition of crude enzyme. A blank was prepared without enzyme addition and zero was set against the blank in calorimeter. Absorbance was measured at 540 nm by spectrophotometer (Model: Perkin Elmer). Xylanase activity was calculated by calibration curve constructed by using D-xylose as a standard. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of one micromole reducing sugar in one min under specified conditions.

2.4 Parametric Optimization of Xylanase Production**.**

2.4.1 Effect of carbon sources and nitrogen sources:

Carbon sources such as glucose, maltose, xylose, CMC, starch and agricultural residuals such as rice bran, rice straw, bamboo powder and molasses were tested by replacing substrate in 50 ml production medium at the rate of 1%. Similarly, the effects of both inorganic and organic nitrogen sources on xylanase production at 0.5% concentration was examined by supplementing the basic medium with urea, ammonium sulphate, tryptone, potassium nitrate, ammonium chloride, ammonium nitrate, ammonium hydrogen phosphate, potassium phosphate as inorganic sources, where as for organic sources peptone, beef extract and yeast extract were used. The synergistic effect of various combinations of carbon and nitrogen sources was also examined for highest xylanase activity. The flasks were incubated for 48 h. The enzyme was extracted and assayed for activity as described earlier.

2.4.2 Xylanase production vs incubation period and inoculum size:

The impact of incubation time on enzyme production was assessed individually by incubating the bacterial isolate in production medium for different time intervals (24 hours, 48 hours, 72 hours, 96 hours, and 120 hours) and then measuring the enzyme activity.

Inoculum size was optimized by using 18 h old bacterial culture in production medium inoculated at a level of 1%, 2%, 5%, 7% and 10% .After incubation for 48 h, the enzyme was extracted from each set and assayed.

2.4.3 Effect of temperature and pH:

To study the effect of temperature on enzyme production, the organism was cultivated at different temperatures ranging from 25 to 55˚C for 48 h. Similarly, the effect of pH on enzyme production was evaluated by varying pH from 6 to 11 at an increment of 0.5. Extracellular enzyme was extracted and assayed.

III. Results and discussion

3.1 Microbial strain and its growth conditions

The isolated bacterial strain was an aerobic, gram positive, mesophilic (30-37˚C), spore forming rod bacterium (Fig. 2A). The strain was an neutrophile being capable of growing best at pH 8. The microscopic, morphological, physiological and biochemical characters of the isolate revealed that it belonged to genus *Bacillus* (Table 1). In Fig. 1, the ability of the strain to produce xylanase enzyme was confirmed when it formed yellow halo zone around the colony grown on xylan agar plates after treated with 0.1% congo red and washed

with 1M NaCl [9]. SEM analysis of the bacterial strain revealed its detailed morphological structure (Fig. 2B). The isolate was identified as *Bacillus australimaris* P 5(Gene Bank Accession no. KY697915).

3.2 Xylanase production in solid state fermentation

Under SSF conditions, xylanase enzyme was successfully produced, for which it was considered as one of the important industrial enzyme [13]. *Bacillus australimaris* P 5 produces 104 U/ml of xylanase under optimized solid state fermentation. The cellulase activity determined in the cell free supernatant was quite negligible (CMCase 0.0021 U/ml) indicating that xylanase was more or less cellulase free. Some studies were reported where xylanase yield from *B. licheniformis* 77-2 was only about 41 U/ml [14].

3.3 Xylanase Production vs. Incubation period

The maximum xylanase production was achieved when different experiments were conducted in solid state fermentation to study all the necessary optimum parameters.

Time course of enzyme production was monitored for 120 h of incubation. In the early stages of incubation, a low level of xylanase activity was detected and increases with time reaching a maximum of 104 U/ml at 48 h. After 48 h, enzyme yield gradually decreases (Fig. 3) which may be either due to the depletion of nutrients or proteolysis [15] or even due to the production of toxic metabolites that inhibits enzyme synthesis [16]. Two key elements on which incubation periods mainly depend are the microorganism growth rate and its enzyme production pattern. Due to the bacterial faster doubling rate, the time required for enzyme production was far less compared to that of fungal cultures [17].Similar reports on incubation periods has been reported from *B.subtilis* BS04 [18] , in an another study, *Bacillus* when grown in saw dust showed maximum enzyme production at 48 h of fermentation [19]. Sepahy [20] reported that *Bacillus mojavensis* AG137 produce maximum yield in fermentation period of 48 h.

3.4 Xylanase Production vs. carbon sources

Various carbon sources were used to study their effects on xylanase production (Fig. 4). Xylose was proven to be the best carbon source when *Bacillus australimaris* P 5 was grown in medium supplemented with 1 % xylose (w/v) followed by birchwood xylan, glucose and maltose. Among other carbon sources, CMC and starch supported negligible xylanase production. Different agro residues such as molasses, bamboo powder, rice bran, rice straw were tested to attain cost effective production. Supplementation of these substrates to the fermentation medium enhances the xylanase production to some extent, but the higher xylanase synthesis was only observed in the presence of xylose (104 U/ml).The reason may be due to the heterogeneous and complex nature of agro-residues which results in lower enzyme yield, possibly because the extracellular degradative enzyme system is not sufficient to degrade the xylans in the complex substrates [21]. Supplementation of xylose that acts as an inducer in xylanase production has also been reported in *Bacillus pumilus* GESF-1[22]. Thus, the results of the present study are in accordance with the previous reports.

3.5 Xylanase Production vs nitrogen sources.

The main mechanisms governing the extracellular enzymes formation are influenced by the availability of precursors for protein synthesis; hence the nitrogen concentration plays a vital role on enzyme production. During fermentation, the enzyme activity is significantly influenced by the pH of the medium which in turn is affected by the presence of nitrogen source. Xylanases with minimal cellulases can be produced while using low nitrogen to carbon ratio. In present investigation, 0.5 % of different inorganic and organic nitrogen sources are tested among which addition of yeast extract resulted in maximum xylanase activity followed by beef extract, peptone and ammonium hydrogen phosphate(NH_4HPO_4).However, no appreciable increase in enzyme activity was registered when the fermentation medium was supplemented with tryptone, KNO_3, $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, Potassium phosphate (Fig. 5).Earlier reports showed that higher activity was observed in enzyme production on supplementation of organic sources compared to other inorganic nitrogen sources [23] . Reports on the combination of yeast extract with other nitrogen sources such as yeast extract + tryptone and yeast extract+ NH_4NO_3 [20], yeast extract with soyabean meal and yeast extract with peptone for better stimulation of xylanase biosynthesis are also available.

3.6 Production vs. Temperature & pH

The fermentation temperature plays a major role in production of enzyme. Temperature is known to influence the metabolic rate of the organism involved in the process and in turn determines the amount of the end product. Various incubation temperatures (25-60˚C) were tested for maximum xylanase production. From the analysis of results, *Bacillus australimaris* P 5 in this study produces maximum xylanase titre (104 U/ml) at an optimum temperature of 35˚C at 48 h. Fig. 6 illustrates that, when temperature is altered below or above the

optimum, the activity decreases. Similar findings were reported by Poorna and prema [24] and Muhammad [25] showing optimum temperature for higher xylanase yield is 35˚C for *B.pumilus* and *B.subtilis* BS04, respectively.

Xylanase production by various bacteria and fungi considerably depends on pH. Fungal xylanases are favoured by lower pH [26] while alkaline pH favours bacterial xylanases [27]. The impact of pH markedly affects the synthesis and secretion of xylanase because substrate binding and catalysis are often dependent on charge distribution on both substrate and in particular enzyme molecules. In present study, pH efficiency on enzyme production was studied over a wide range of pH (6-11) at 0.5 increments. The optimum pH for maximum xylanase production (104.42 U/ml) was found to be at pH 8 followed by 91.65 U/ml at pH 9. Lower pH (6) and higher pH (11) of the medium retards xylanase production (Fig. 7) .Xylanase with pH value 7-9 makes them suitable for bleaching applications. This observation is in agreement with other bacillus sps., namely *B.mojavensis* AG137 [20], *Bacillus sp*.[21], *B.subtilis* BS04 and *B.megaterium* BMO7 [25] maximum xylanase titre was recorded at optimum pH 8.

3.7 Production vs inoculums size.

In this study, inoculum size of 1%, 2%, 5%, 7% and 10% were tested for enzyme production in SSF. Xylanase production was enhanced when a 5 % inoculum of 18h-old culture was used in the production medium. Initially, enzyme production was minimum when inoculum size was 1% but as the level increased the enzyme production was also increased. Inoculum size of 5% has found to be maximum in *Bacillus* P 5 strain (Fig. 3). Similar findings on xylanase production by *Streptomyces sp.*QG-11-3 have been reported with 5% inoculum size reported by Beg [28]. Inoculum size higher reduces the xylanase production due to exhaustion of macro and micro nutrients from the medium and thus, inoculum concentration greater than 10% was not preferred in industrial fermentation [23]. Decline in enzyme yield at larger inoculums size may also be due to the formation of thick suspensions and improper mixing of substrates in shake flasks. There are several reports on production of xylanase that showed its maximum activity at 10% (v/w) inoculums [24]**.**In contrast, a 2% inoculum was found sufficient for production of xylanase by *B. Mojavensis* AG137 [20] and *B.subtilis* BS04 [25].

IV. Conclusion:

From the above investigation, it has been observed that bacterial xylanase produced by *Bacillus australimaris* P 5 shows encouraging results and could be advantageous. The major significance of the optimization studies is to increase the yield of enzyme from the isolated bacterial strain from this part of India. The novelty of the stain to produce cellulase free xylanase can be explored for bleaching of pulp and paper industries leading to reduction in chemical consumption. Further work on characterization and purification of xylanase is currently underway.

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Table 1: **Morphology, Physiological and biochemical properties of *B. australimaris* P 5 KY697915**

Tests	**Characteristics**	**Tests**	**Characteristics**
Colony Morphology		Biochemical tests	
Configuration	Circular	Voges Proskauer	+
Colony color	Light yellow	Citrate	+
Surface	Moist	Indole	-
Opacity	Translucent	Gelatin hydrolysis	+
Gram's reaction	+	Starch hydrolysis	+
Cell shape	Rod shaped	Catalase test	
Size (µm)	0.6–0.8 µm in width 2–2.2 µm in length	Oxidase	
Spores	-	Nitrate	-
Motility	Motile	H_2S production	-
Growth	Strictly aerobic	Methyl red	+
	Morphological tests		
	Temperature (Range)	8-45˚C	
	Temperature (Optimum)	30-37˚C	
	pH (Range)	5-11	
	pH (Optimum)	6-8	
	NaCl (Range) (w/v)	0-12%	
	NaCl (Optimum) (w/v)	1-3%	

FIGURE CAPTIONS:

Fig. 1. Xylanase production by the bacterial strain *B. australimaris* P 5
Fig. 2. Gram staining (A) and SEM picture (B) of bacterial strain *B. australimaris* P 5
Fig. 3. Effect of different inoculums size on xylanase production in different time periods.
Fig. 4. Effect of different Carbon source on enzyme production
Fig. 5. Effect of different Nitrogen source on enzyme production.
Fig. 6. Effect of temperature on enzyme activity.
Fig. 7. Effect of pH on xylanase activity.

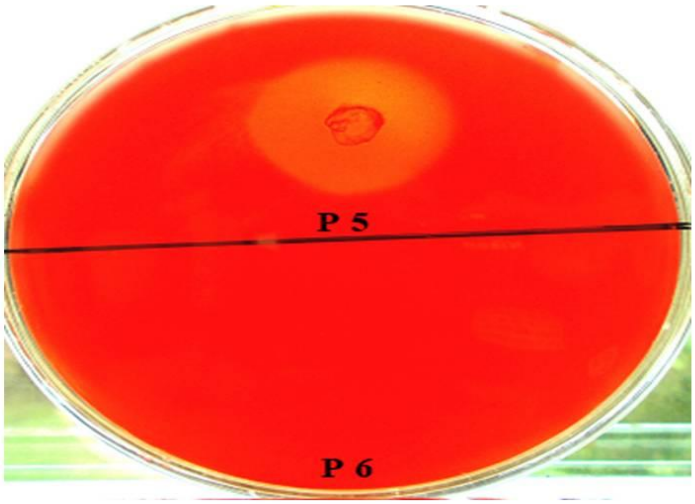

Fig. 1. Xylanase production by the bacterial strain *B. australimaris* P 5

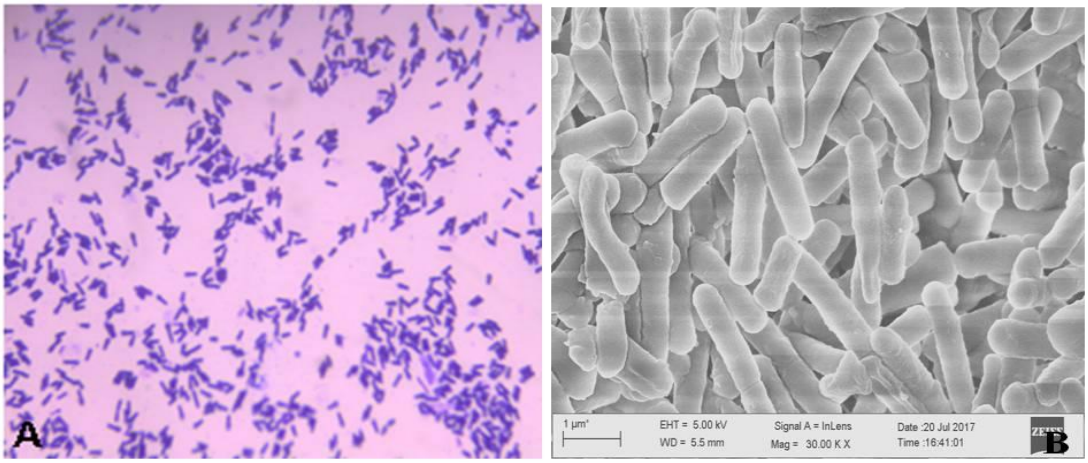

Fig. 2. Gram staining (A) and SEM picture (B) of bacterial strain *B. australimaris* P 5

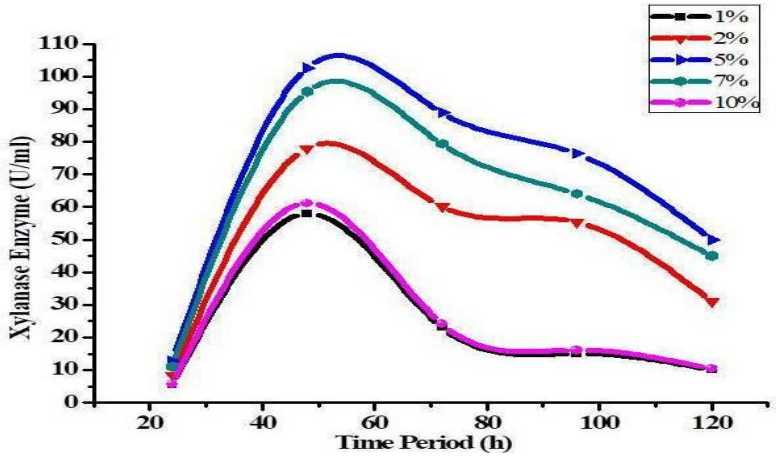

Fig. 3. Effect of different inoculums size on xylanase production in different time periods

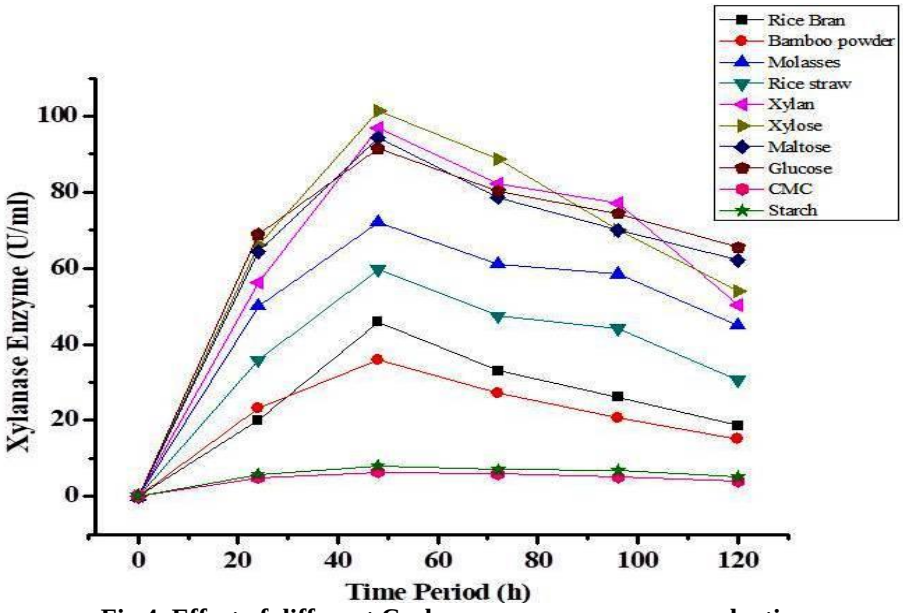

Fig.4. Effect of different Carbon source on enzyme production

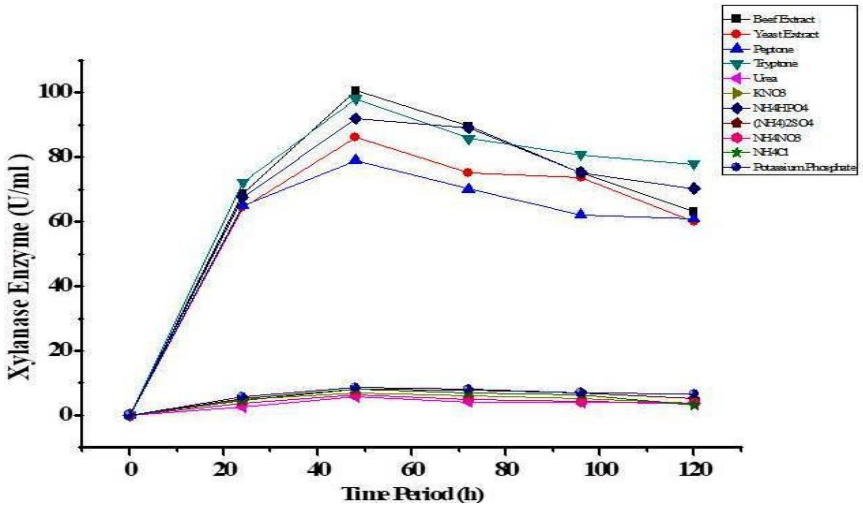

Fig.5. Effect of different Nitrogen source on enzyme production

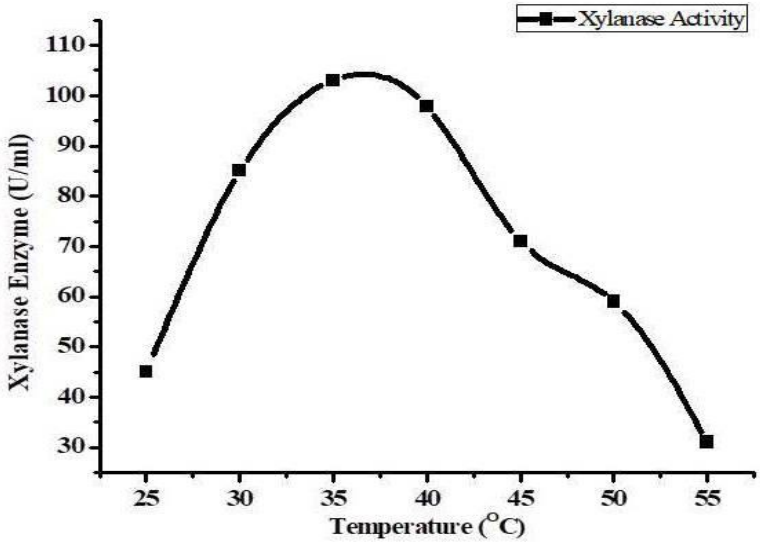

Fig.6. Effect of temperature on enzyme activity

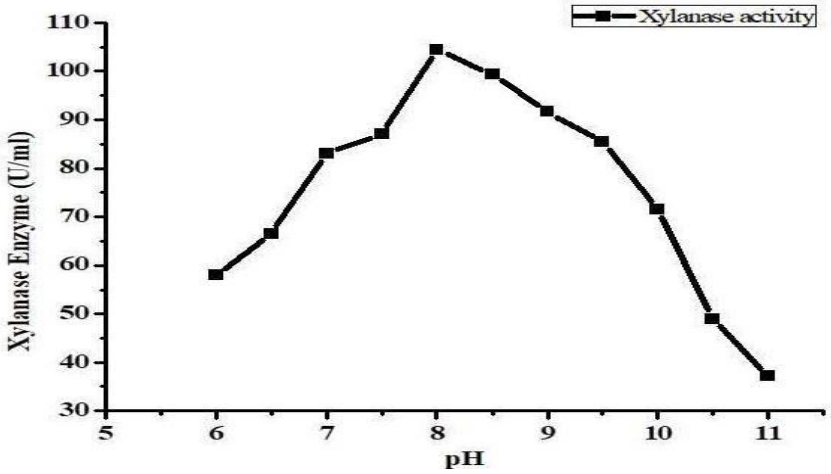

Fig. 7. Effect of pH on xylanase activity

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