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Research Article

PRODUCTION AND OPTIMIZATION OF ENZYME XYLANASE FROM *ASPERGILLUS NIGER* ISOLATED FROM DECAYING LITTER OF ORCHHA WILDLIFE SANCTUARY, M.P., INDIA

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ABSTRACT

Litter is the dead organic remains of plants and animals present in natural habitats. Soil micro-organisms are attached with litter and convert complex organic matter into simple inorganic compounds. Present study has been undertaken with a view to understand the biochemistry involved in the decomposition process occurring in the Orchha forest range of Madhya Pradesh. For this degrading samples have been collected from the forest and in all 17 fungal genera have been isolated from collected samples. All the identified strains have been subjected to test xylanolytic activity. Fungus *Aspergillus niger* has been found to have xylanolytic activity. Then after the growth parameters such as incubation period, temperature, pH, carbon and nitrogen sources have been optimized for optimum growth conditions for maximum enzyme production under liquid state fermentation (LSF). In the present study, wheat bran and corn cobs (powdered) have been used as substrates for enzyme production. Maximum xylanase production was observed at 7th day & 9th day of incubation, 4 mg/mL & 20 mg/mL of substrate, 0.3 mg/mL & 0.5 mg/mL of peptone,0.8 mg/mL & 0.9 mg/mL of yeast extract for wheat bran and corn cobs respectively. The optimum temperature and pH for each substrate was 30°C and 5.5. Enzyme activity was also optimized for certain parameters such as incubation period, temperature, pH and substrate (Oat spelt xylan) concentration.

Keywords: *Aspergillus niger*, Lignocellulose, Hemicellulose, Liquid state fermentation

1. INTRODUCTION

Lignocellulosic wastes are generated in bulk during agricultural practices, forestry and many other industrial processes related with agriculture and food industry. These wastes are removed by burning or left as such for decomposition. In both the processes several obnoxious gases and products are added in the atmosphere as well as lithosphere and causes pollution. These lignocellulosic materials are the source of inexhaustible renewable natural resources. Lignocellulose is the major structural component of plants, shrubs, herbs and trees. It consists of cellulose (35-50%), hemicelluloses (20-35%) and lignin (5-20%). These biomolecules are of enormous biochemical value.

Cellulose molecule is a homopolymer of glucose units linked with β-1, 4- glycosidic units and is the most abundant biopolymer on Earth [1]. Hemicelluloses are short chains of branched hetero-polysaccharides composed of hexoses and pentoses and are easily hydrolyzed [2]. The main constituent of hemicelluloses is xylan, second most abundant hetero-polysaccharide in nature. It consists of homopolymeric backbone of 1, 4-linked-β-D-xylopyranose units and short branches including- acetyl, 4-O- methyl- D- glucuronosyl and L-arabinofuranosyl residues [3]. Lignin is bound to xylans with an ester linkage to 4-O- methyl- D-glucuronic acid residues.

A large number of microorganisms such as fungi, bacteria and actinomycetes have been found to have ability to degrade hemicelluloses by secreting hemicellulases or hemicellulolytic enzymes [4].

Xylanases, a class of hydrolytic enzymes, have the capability to degrade linear polysaccharide β-1, 4-linked xylan into monomeric sugar i.e. xylose. Endo-xylanases are extracellular enzymes secreted by different micro-organisms such as bacteria, yeasts, filamentous fungi, actinomycetes etc. Generally filamentous fungi have been widely used to produce xylanase much higher than those produced by yeast or bacteria [5]. Xylanases can be produced by various fermentation technologies. Submerged fermentation have advantage over solid state fermentation due to more nutrient availability, high oxygen supply and it takes less time for fermentation. Liquid state

fermentation also allows the control of pH, temperature of the medium and several other environmental factors which are required for the maximum growth of microorganisms [6].

Xylanases are of many industrial applications in bakery industry, pulp and paper industry, bioethanol industry and in improving food quality of animals [7]. This paper focused on the production of xylanase from *Aspergillus niger* and its optimization of different growth parameters for maximum enzyme production by using comparatively cheaper agricultural by-products namely wheat bran and corn cobs.

2. MATERIAL AND METHODS

2.1. Collection of sample

Partially decomposed litter samples were collected from different areas of Orchha Wildlife Sanctuary, M.P. situated at 25° 21' 6.91" N and 78° 38' 25.19" E. Samples were brought to laboratory in sterilized plastic bags for further investigation and studies.

Fig 1: Location of Orchha Forest Reserve

2.2. Isolation of fungi

For isolation, serial dilution technique was used.[8] Litter samples were diluted upto 1:1000 times using sterilized distilled water. The diluted suspension was streaked over petriplates, containing Potato Dextrose Agar (PDA) medium (Potato-200.0 g; dextrose- 20.0 g; agar- 15.0 g; distilled water- 1000 ml; pH- 5.6) under aseptic conditions and plates were incubated at 30°C for appearance of fungal colonies.

2.3. Identification of isolated fungi

Isolated fungi were identified by preparing slides and mount with lactophenol cotton blue. These slides were examined under microscope (45×10X; Olympus). Standard texts and photographs were used for identification [9].

2.4. Screening of xylanolytic activity

Xylanolytic activity of *A. niger* was examined on Malt extract agar (MEA) medium (K_2HPO_4- 0.2 g; peptone- 5.0 g; Yeast extract- 5.0 g; oat spelt xylan- 1.0 g; agar- 20.0 g, distilled water- 1L and pH 5.5) [10]. Media was autoclaved at 121°C for 30 minutes. After solidification of medium, wells of 5mm diameter was prepared with the help of sterilized cork borer. Then after 1.0 ml spore suspension of *A. niger* was poured aseptically and petri plates were incubated at 30°C. After 5 days of incubation, if growth appeared on plates then such plates has to be flooded with 1% congo red solution for 10 minutes. Afterwards, to have clear vision of clear zone, plates were washed with 1M NaCl [11].

2.5. Xylanase production by liquid state fermentation

For production of enzyme through liquid state fermentation, Mandel's nutrient (modified) medium (KH_2PO_4- 2.0; $MgSO_4.7H_2O$- 0.3; $CaCl_2$- 2.0; $FeSO_4.7H_2O$- 0.5; $ZnSO_4.7H_2O$- 1.4; $MnSO_4.H_2O$- 1.6; peptone- 0.3; yeast extract- 0.75; distilled water- 1L and pH – 5.5) was used [12]. After autoclaving 1.0 ml of spore suspension was inoculated aseptically into flasks and was incubated at 30°C for growth. Flasks were stirred by shaker regularly at every 24 hours.

2.6. Optimization of Xylanase production

Different cultural conditions were optimized for enhanced xylanase production under liquid state fermentation conditions, including incubation period, temperature, pH, carbon source and nitrogen source.

2.6.1. Incubation period (days)

For maximum xylanase production broth have been incubated at 30°C and harvested after 72 hrs, 96 hrs, 120 hrs, 144 hrs, 168 hrs, 192 hrs, 216 hrs, 240 hrs and 264 hrs to check released sugar by *A. niger*.

2.6.2. Temperature (°C)

Temperature has been an important physical factor for fungal growth and metabolism. Temperature has also been optimized at different temperature range 20°C, 25°C, 30°C, 35°C and 40°C for optimized incubation period. Then amount of released sugar was measured spectrophotometrically by recording absorbance at 540 nm.

2.6.3. pH

The effect of pH on xylanase production was examined at different pH ranges 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0,

7.5 and 8.0. pH was adjusted by using 0.1 N HCl and 0.1 N NaOH with the help of electronic pH meter. Inoculated flasks were incubated at optimized incubation period and temperature and then released sugar was measured by spectrophotometer.

2.6.4. Carbon source

Wheat bran and corn cobs were used as carbon sources for enzyme production in their different concentrations (g/L): 2.0, 4.0, 8.0, 12.0, 16.0, 18.0, 20.0, 22.0, 24.0, 26.0, 32.0 and 40.0. Flasks were incubated at optimized incubation period, temperature and pH and released sugar was measured by spectrophotometer.

2.6.5. Nitrogen source

Peptone and yeast extract were studied as nitrogen source for the production of xylanase in following concentrations. Peptone concentration was taken as (g/L)- 0.1, 0.2, 0.3, 0.4, 0.5, 0.8, 1.0 and 1.2. Different concentrations of yeast extract were taken (g/L)- 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9 0.95, 1.0 and 1.2. Flasks were incubated at optimized incubation period, temperature, pH and carbon sources. Then measured released sugar by spectrophotometer.

2.7. Enzyme harvesting

After incubation, the broth was filtered through Whatman filter paper. The filterate was then centrifuged at 10,000 rpm for 10 min. Clarified supernatant was collected in sterile glass bottles and used for xylanase assay.

2.8. Xyalanse assay

Xylanase activity was assayed by measuring reducing sugar using 3,5-dinitrosalicyclic acid method.[13] The reaction mixture contained 1.0 ml of substrate solution (1% xylan prepared in buffer solution), 1.0 ml of crude enzyme and 1.0 ml of 0.05 M Sodium citrate buffer at pH 5.3 with the addition of 3.0 ml of DNS. The mixture was incubated at 55°C for 30 minutes that form coloured complex. The amount of sugar (xylose) was measured by spectrophotometer at wavelength 540 nm [14]. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µmol of xylose per minute under described assay conditions.

2.9. Xylanase activity optimization

Several parameters such as incubation period (minutes), temperature, pH and substrate (oat spelt xylan) have also been examined to assess the maximum activity of enzyme mixtures. For incubation period, absorbance was recorded at different time intervals *i.e.* 5min, 10min, 15min, 30min, 45min and 60 min. To find out thermostability, enzyme activity was performed at temperature ranging from 40°C to 65°C. Optimum pH was examined when reaction mixture was prepared in buffer solution at different pH (4.0 to 6.0). For substrate concentration, xylan was taken in reaction mixture at different concentrations (mg\ml): 1.0, 2.0, 5.0, 10.0, 15.0 and 20.0.

3. RESULTS AND DISCUSSION

A total of 17 fungal genera were Isolated and identified from the litter collected from Orchha Wildlife Sanctuary. They are- *Aspergillus, Rhizopus, Trichoderma, Acremonium, Chaetomium, Ceratocystis, Penicillium, Cladosporium, Curvularia, Cunninghamella, Fusarium, Paecilomyces, Mucor, Rhodotorula, Alternaria, Beltraniella,* and *Scopulariopsis.* Identification of fungi was done on the basis of morphological appearance during culture and microscopic examination with the help of standard texts. Further work was carried out with *Aspergillus niger* (Figure 2 a, b).

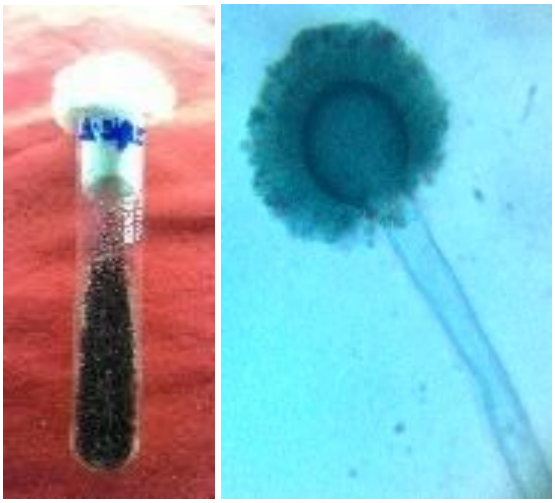

Fig 2: *Aspergillus niger* (a) Slant culture (b) Microscopic view

Xylanolytic activity of fungus was determined by well diffusion technique on MEA medium supplemented with 1% xylan. Fungus was found to metabolize xylan which has been clearly envinced by formation of a clear zone around the wells, indicating that fungus has ability to produced xylanase. This observation was later confirmed by congo red solution. At the end, the strain was also observed and confirmed under microscope (Figure 3 a, b).

Fig. 3: Zone of clearance produced by *A. niger* (a) Before Congo red treatment (b) After Congo red treatment

After confirming xylanolytic activity of *A. niger,* various growth parameters such as incubation period, temperature, pH, substrate concentration, peptone concentration and yeast extract concentration were optimized for maximum xylanase production using comparatively cheaper substrates i.e. wheat bran and corn cobs. Time period of fermentation on the xylanase production varies from organism to organism and substrate to substrate which is envinced from our results. In *A. brasiliensis* maximum production of enzyme occurred after 96 hours of incubation while in *A. niger* production reached a maximum at 144 hours of fermentation [15, 16]. In the present study, maximum xylanase production was observed from *A. niger* on 7th day (168 hrs) of incubation on wheat bran while on corn cobs optimum production was noted at 9th day (216 hrs) of incubation (figure 4). Further incubation enzyme production was decreased due to depletion of nutrients in the culture medium which reduces the growth of fungal strain [17].

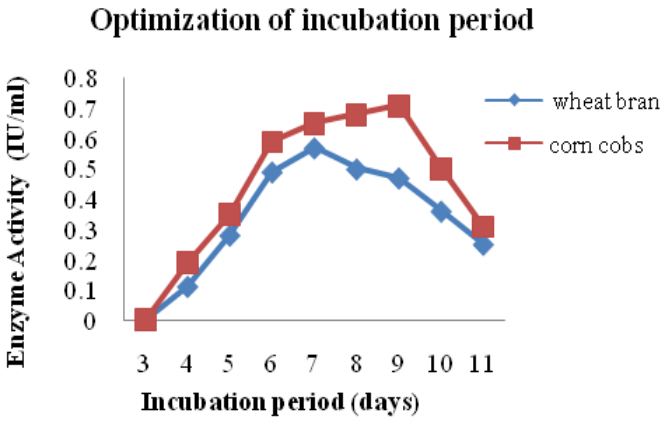

Fig. 4: Effect of incubation period on xylanase production by *A. niger*

Temperature is one of the important factors that determine the success of enzyme production under liquid state fermentation conditions. Previous study described that *Aspergillus flavus* exhibits maximum production of xylanase at 32°C in wheat bran [18]. But in our findings 30°C was the optimum temperature for xylanase production with both wheat bran and corn cobs as substrate. Above and below than the optimum temperature fungal growth was inhibited, hence the xylanase production decreased sharply.

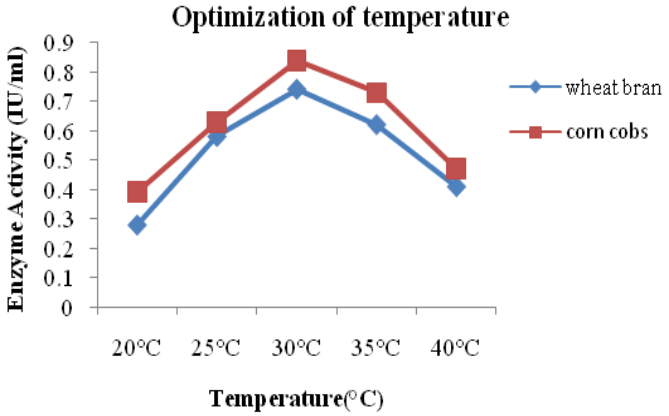

Fig. 5: Effect of temperature on xylanase production by *A. niger*

The pH of culture medium was maintained at different levels from 3.0 to 8.0. Maximal enzyme production was detected at pH 5.5 for both substrates i.e. wheat bran and corn cobs (figure 6). At pH 5.5, it was observed that *A. niger* produced 0.78 IU/mL xylanase on wheat bran and on cobs 0.9 IU/mL. Either increase or decrease in pH levels beyond the optimum value showed declination in xylanase production. Most fungi are able to grow at wide pH ranges of 5.0-8.0 [19]. Similar work with *A.*

niger was conducted for optimal pH conditions for xylanase production [20].

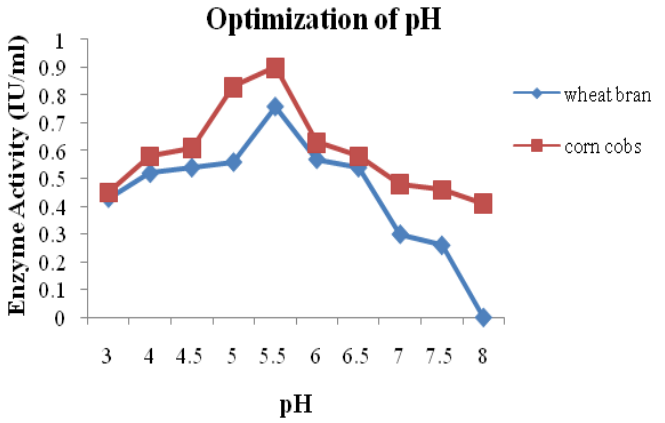

Fig. 6: Effect of pH on xylanase production by *A. niger*

Xylanase production was greatly influenced by addition of different substrates in the production medium. Several agricultural wastes like sugarcane baggase, paddy straw, wheat straw, rice bran and saw dust were examined as carbon sources for the growth as well as production of xylanase by *A. niger* [21]. In the present study oat spelt xylan was replaced by wheat bran and corn cobs. It has been found that *A. niger* produced maximum xylanase on corn cobs (0.92 IU/mL) as compared to wheat bran (0.84 IU/m L). Wheat bran and Corn cobs were produced maximum xylanase when they were present in the culture medium at concentration 4 mg/mL and 20 mg/mL respectively (figure 7).

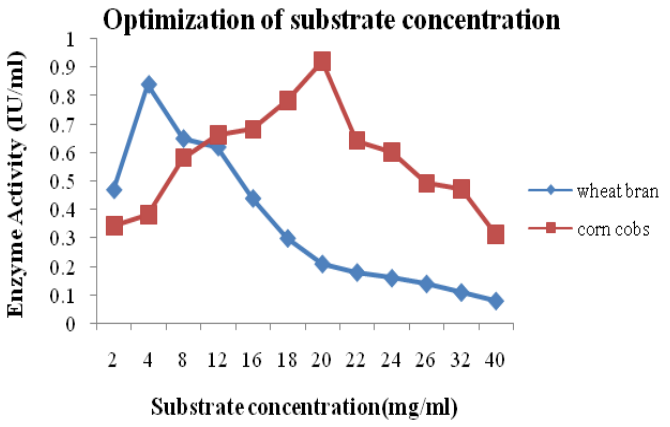

Fig. 7: Effect of substrate concentration on xylanase production by *A. niger*

Besides carbon, growth of microorganisms is also influenced by nitrogen sources in the culture medium. In the present study, peptone and yeast extract have been used as nitrogen sources. It has been showed that organic nitrogen sources were more effective for production of endo-1,4-β xylanase as compared to inorganic nitrogen sources [22]. From the figure 8, it has been concluded that maximum xylanase production (0.96 IU/mL) was found when peptone concentration was at 0.5 mg/mL for corn cobs and 0.3 mg/ml for wheat bran (1.02 IU/mL).

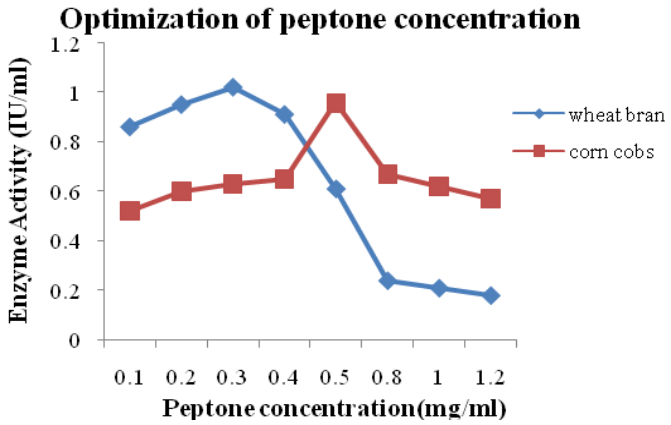

Fig. 8: Effect of peptone concentration on xylanase production by *A. niger*

With yeast extract, *A. niger* gave the maximum enzyme production (0.98 IU/mL) at 0.9 mg/mL concentration for corn cobs. In the case of wheat bran, it was noted when concentration of yeast extract was 0.8 mg/ml maximum xylanase was produced (1.1 IU/ml) (figure 9).

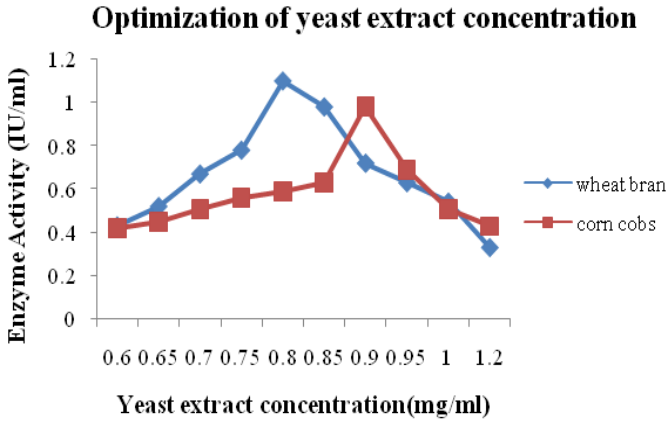

Fig. 9: Effect of YE concentration on xylanase production by *A. niger*

After optimization of growth parameters for maximum xylanase production under LSF (Liquid State Fermentation), certain parameters such as incubation period (min), temperature (°C), pH and substrate (xylan) concentration was used to assay maximum xylanase

activity. During investigation it has been found that the enzyme activity was maximum at 60 and 30 minutes of incubation (figure 10), and at 55°C and 60°C incubation temperature of reaction mixture (figure 11).The harvested enzyme has maximum activity at pH 4.5 and 5.5 (figure 12), and it requires 15 mg/ml and 10 mg/ml oat spelt xylan concentration (figure 13) produced with wheat bran and corn cobs respectively.

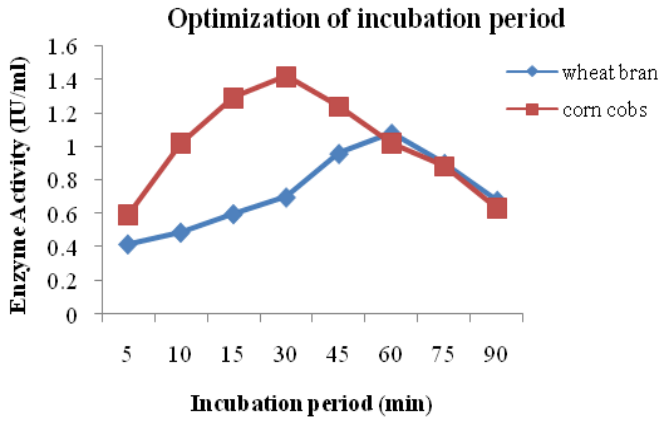

Fig. 10: Effect of incubation period on xylanase activity

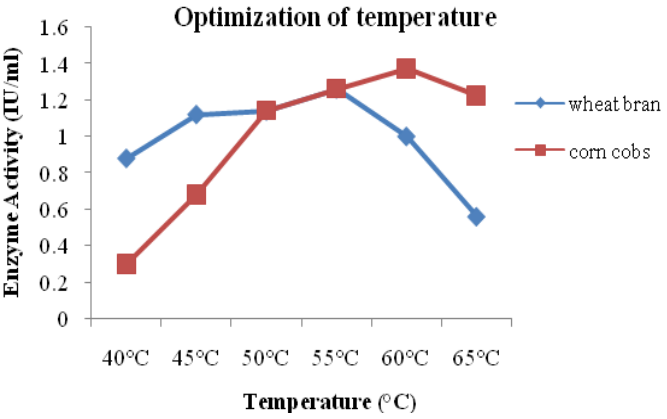

Fig. 11: Effect of temperature on xylanase activity

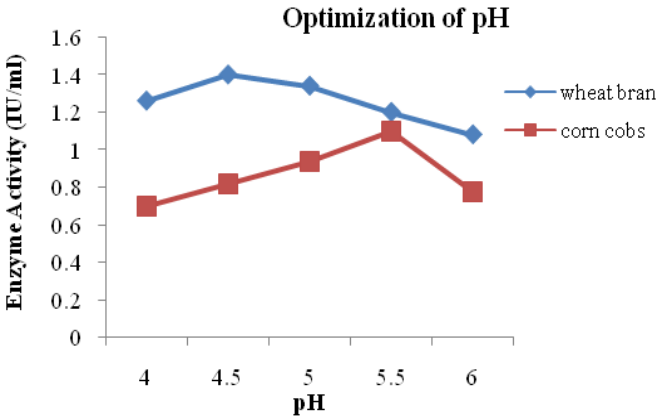

Fig. 12: Effect of pH on xylanase activity

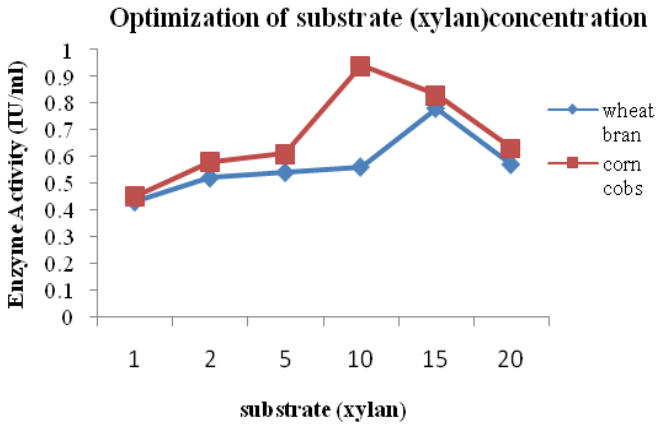

Fig. 13: Effect of substrate (xylan) concentration on xylanase activity

4. ACKNOWLEDGEMENT

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