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ISOLATION, SCREENING, CHARACTERISATION AND PARTIAL PURIFICATION OF XYLANASE ENZYME FROM TWO DIFFERENT SOIL SAMPLES

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Abstract - *Xylan is the major component of hemicellulose that is found mainly in secondary cell wall of plants.β-1, 4-Xylans are heterogenous polysaccharides that are made up of xylosyl units connected by β-1, 4 glycosidic bonds, often branched with acetyl, arabinofuranosyl and glucuronic side chains attach to xylose backbone. Degradation of Xylan involves action of many enzymes among which Xylanase play an important role. Xylanase have many important applications in pulp Biobleaching but they are also extensively used in dye decolourisation, clarification of fruit juice and in baking industry. Endo-1, 4-β-Xylanases attack on the glycosidic bonds and hydrolyze the backbone structure of β-1,4Xylan to produce xylooligosaccharides, xylobiose and xylose. In this study the attempt is made to Isolate, screen, identify and characterize the Xylanase producing Bacteria from soil samples. To determine the maximum Xylanase activity in various media and the effect of pH, temperature optima on Xylanase enzyme production. Identification of microorganisms was done by gram staining and Bio-chemical test. DNS assay method was carried out to study the maximum activity of Xylanase. Crude extract was fractioned by Ammonium sulphate precipitation followed by dialysis. The partially purified enzyme was subjected to protein estimation and further used for the activity. Molecular weight of the partially purified Xylanase was determined by SDS-PAGE.*

Key Words: Xylan, Xylanase, Bacillus subtilis, DNS Assay, Ammonium sulphate precipitation, Dialysis

1. INTRODUCTION

Xylanase is an extracellular enzyme which hydrolyses β-1,4-D-xylosidic linkages of highly polymerized and substituted β-1, 4 linked D-xylobiose, xylotriose and glucucoronosyl residues. The enzyme holds potential for the degradation of plant cell wall materials [10]. The Xylanase enzyme molecular weights vary from source to source. Xylanase consists of 190 amino acids. Xylanase readily crystallizes in ammonium sulphate and sodium potassium phosphate buffer pH 5 to 9. Xylanase are important in the bioconversion of hemicelluloses into their constituent sugars [5]. Xylanase enzymes hydrolyze the Xylan polymer that exists within pulp fibres. Xylan are intimately linked to cellulose and lignin, thus it follows that disruption of the Xylan backbone affect their separation during bleaching. Xylanase was also shown to increase fiber wall swelling and in turn increase the speed of diffusion through the walls. Microbial Xylanase (β-1, 4-D-xylan xylano hydrolase, EC 3.2.1.8) are being used in various industries including food, feed, textile and paper processing industries. In food and feed, they liberate the nutrients by hydrolyzing the non degradable hemicellulose fibers thus make the nutrients available [8].These are the fundamental elements for biochemical processes and utilized in a number of food processing industries [3]. Sugars like xylose, xylobiose and xylooligomers can be prepared by the enzymatic hydrolysis of Xylan. The depolymerisation action of Xylanase results in the conversion of polymeric substances into xylo-oligosaccharides and xylose [4].Microbial Xylanase have commercial applications in agriculture, industry and human food production [7]. Xylanase is useful in bioconversion of lignocellulosics to fuel and chemicals, to improve silage for better digestion by ruminants, to improve quality of detergent, and also used for clarification of fruit juices, in flour improvements for bakery products and in controlling environmental hazards through bio pulping. Xylanase used in pulp pre-bleaching process remove the hemicelluloses, which bind to the pulp. The hydrolysis of pulp bound hemicelluloses releases the lignin in the pulp, reducing the amount of chlorine required for conventional chemical bleaching and minimizing the toxic, chloroorganic waste. Therefore, Xylanase from alkalophilic bacteria and actinomycetes have been studied widely. Thermostable Xylanase active at alkaline pH are of great interest for application in the pulp and paper industry to decrease the consumption of chlorine chemicals. Xylanase have been reported from bacteria, Fungi and actinomycetes. However, large-scale cultivation of fungi and actinomycetes is often difficult because of their slow generation time, coproduction of highly viscous polymers, and poor oxygen transfer. *Bacillus* sp. is used more extensively than other bacteria in industrial fermentations, since they secrete most of their enzymes.

2. MATERIALS AND METHODOLOGY

2.1 SAMPLE COLLECTION

Soil samples were collected from two different regions in and around Chennai. One sample from Paddy field at Chengalpattu and other sample from Garden at Chennai.

2.2 ISOLATION OF ORGANISM

10gm of soil from each place has been taken in a zip cover and brought to laboratory from which 1g of soil was weighed and mixed with sterile saline. The collected samples was serially diluted up to 10^{-5} dilution using sterile saline as a blank and the diluted samples 10^{-2} and 10^{-4} of garden and paddy field soil up to 20µl were plated into the sterile nutrient agar using L-rod by spread plate method. The nutrient agar was incubated at 37ºC for 24 hours. The pure culture was inoculated into sterile nutrient agar slants and nutrient broth for further use.

2.3 SCREENING FOR XYLANASE PRODUCING ORGANISM BY PLATE ASSAY

The isolated pure strains were inoculated into 50ml of nutrient broth and kept for overnight incubation at 37ºC in the incubator. After incubation the broth was centrifuged at 5000rpm for 10 minutes. The supernatant is the crude Xylanase enzyme since Xylanase is the extracellular enzyme. The production of Xylanase enzyme using screening medium contains Birch wood Xylan as a substrate [6].The plate assay was performed using agar plate amended with Birch wood Xylan. The Agar plates were prepared by mixing 1% of Birchwood Xylan with 1.7% Agar. After solidification of Agar, The wells were cut aseptically by gel puncher around 10mm diameter and the culture filtrate was poured to the well then the plates were kept for overnight incubation at 37ºC. The observation was seen as the substrate utilized the zone was formed around the well. For the better appearance of the zone around 0.1% of Congo red solution was over layered on the medium and kept for 15 minutes. Destaining was made using 1M NaCl to make the zone visible and clear [1]. The better zone formed strain was taken for further study, which is capable of liberating 1Mm equivalent of Xylose in one minute [2].

2.4 IDENTIFICATION OF MICROORGANISM

The bacterial isolate that showed high activity was selected for the further study. The microorganism was identified and confirmed by standard biochemical tests. Such as gram staining, oxidase test, methyl red test, vogues-proskauer test, lysine utilization test, beta hemolysis test,H_2S production test, glucose test, lactose test ,indole test, citrate utilization test, catalase test, urease test, nitrate reduction test.

2.5 OPTIMIZATION OF THE ORGANISM (DNS ASSAY)

The activity of Xylanase was measured by determining the increase in concentration of reducing sugars formed by enzymatic hydrolysis of Birchwood Xylan [9]. The reaction mixture consisted of 100µl of crude enzyme and 500µl of mixture of 0.05g of Xylan dissolved in 5ml of sodium citrate buffer. The mixture was incubated at 50º-60ºC for 10 minutes. The reducing sugar generated was quantified using D-Xylose as a standard. Then the reaction was arrested by the addition of 3ml of DNS and the mixture was incubated at 50ºC-60ºC for 10 minutes. 1ml of Rochelle salt was added to the mixture just before measuring the concentration of reducing sugar at absorbance 540nm by calorimetric estimation. Xylan without enzyme was taken as control to eliminate the possibility of substrate having any reducing sugar residues. One unit of Xylanase is defined as the amount of enzyme,

2.6 PARTIAL PURIFICATION OF ENZYME

2.6.1 AMMONIUM SULPHATE PRECIPITATION

For initial purification step the cell free supernatant was precipitated with 40% and 80% for overnight at 4ºC. The precipitated samples were taken for dialysis.

2.6.2 DIALYSIS

4.0 gm of Sodium carbonate was dissolved in 200ml of distilled water. The sodium carbonate solution was heated to boil and the dialysis membrane was kept in the boiling solution for 10-15 minutes. This will activate the membrane. The bags were removed by the forceps and washed with the distilled water. This membrane is used to dialysis the samples after ammonium sulphate precipitation. The membrane was tied with a twine at one end and the sample after ammonium sulphate precipitation was loaded inside the membrane and twined at the other end. This bag was treated with 100mM Tris-HCl buffer, to remove salts from sample. This was carried out for 24 hours while at an interval of 12 hours the buffer was changed. With each change of dialysis buffer additional unwanted substances are removed and substances inside the membrane are further purified by the volume difference between two compartments. The samples were then removed from the dialysis bag and were stored at 15ºC. This partially purified enzyme was used for further analysis.

2.7 PROTEIN ESTIMATION

Protein estimation was done by Lowry's method. Bovine serum albumin (BSA) was used as a standard. Five standards, a blank and test samples were taken. To the five standards (S1 to S5), 0.2ml, 0.4ml, 0.6ml, 0.8ml &1.0ml of bovine serum albumin was added respectively. To these tubes 5 ml of alkaline copper reagent was added followed by 0.5 ml of folin's phenol reagent was added to all the test tubes and made up to 6.5ml with distilled water. Incubation at dark room temperature was done for 30 minutes. Absorbance was measured in colorimeter at 660nm.

2.8 SDS-PAGE:

Molecular weight determination of Xylanase was done by using sodium-do-decyl sulfate polyacrylamide gel electrophoresis using the kit. The 5ml of separating gel mix provided in the kit was poured in the gap formed by placing spacers at the end of the glass plates which were tightly sealed previously. The gel was allowed to polymerase and

water is added to remove any heat that may evolve during polymerization. After polymerization, the water was removed and 5ml of constituents of stacking gel were added and a Teflon comb was inserted to form the wells. It was then allowed to polymerase for about 30 minutes, after which the gel is placed into electrophoresis tank, filled with 1X running buffer. The 20µl of sample was mixed with 5µl of CBB dye and carefully loaded into respective wells whereas the first well was loaded with molecular weight marker and the electrophoresis apparatus was connected to the power supply. The gel was allowed to run at 50 V until the dye reaches the stacking gel and then the voltage was increased to 150 V until the dye reaches the bottom of the gel. After electrophoresis the plates were separated and gel was removed using clean spatula and then subjected to staining. Gel was then removed and then subjected to coomasive brilliant blue staining using staining solution provided in the kit for overnight and then destained by using destainer for about 20 minutes. The gel was then observed for bands using white illuminator.

3. RESULTS AND DISCUSSION

3.1. Sample collection

The 2 different soil samples were collected from Chennai (Anna nagar) and Chengalpattu (Sembakkam).

3.2 Isolation of organism

Colonies of isolates of nutrient agar medium were showing four morphologically different colonies. These were identified in 10^{-2} of paddy field serially diluted plate. These colonies were further screened for the production of Xylanase enzyme in screening medium with Xylan as substrate. The Xylanase producing organisms were screened based on the zone of clearance which is formed in the plates. This can be visualized by staining with 0.1% Congo red solution giving red colour to the plates and destained with 1M NaCl solution, which removes unstained Congo red in the plates showing zone of clearance.

3.3 SCREENING FOR XYLANASE PRODUCING ORGANISM BY PLATE ASSAY

In plate assay the Xylanase enzyme activity was identified by a clear zone when compared with control.

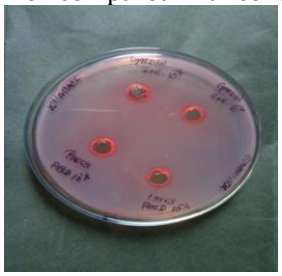


Fig 1: Screening for microorganism producing Xylanase.

3.4 IDENTIFICATION OF ORGANISM

The morphological characters of the bacterial isolate were observed and the results were noted.

3.4.1 GRAM STAINING

The slide was examined under the microscope where purple colour rods were formed and it was identified as gram positive bacteria.

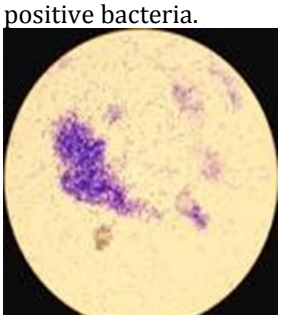


Fig 2: Gram staining

3.4.2 OXIDASE TEST

There is no colour change in the medium that indicates the negative result.

3.4.3 METHYL RED TEST

No acid production by bacteria. The yellow colour formation indicates negative result.

3.4.4 VOGUES-PROSKAUER TEST

Formation of red colour after adding VP reagents indicates the presence of acetoin.

3.4.5 LYSINE UTILIZATION TEST

There is no pink colour formation. The media remain yellow in colour thus indicates that there was no lysine utilization by organism.

3.4.6 BETA HEMOLYSIS TEST

There was formation of double zone indicates the β-hemolytic pattern of bacteria.

3.4.7 MOTILITY TEST

Random movement of bacteria was observed under light microscope at 40X.Thus the bacteria was found to posses motility.

3.4.8 H_2S PRODUCTION TEST

There was no formation of black coloured precipitate which indicates the negative result.

3.4.9 GLUCOSE TEST

The formation of yellow colour indicates the glucose fermentation.

3.4.10 LACTOSE TEST

There is no colour change to yellow colour in the medium. The absence of colour change indicates that there is no lactose fermentation.

3.4.11 INDOLE TEST

There is no cherry red or pink colour formation on the top of the solution. No colour change indicates the absence of Indole.

3.4.12 CITRATE UTILIZATION TEST

No colour change that indicates negative result.

3.4.13 CATALASE TEST

The bubble formation after addition of H_2O_2 indicates the presence of catalase enzyme.

3.4.14 UREASE TEST

There was no colour change which indicates negative results.

3.4.15 NITRATE REDUCTION TEST

Red colour formation in the media indicates the reduction of nitrate to nitrite after addition of sulphalinic acid and N,N-dimethyl-1-naphthylamine.

Table 1: Biochemical test for identification of the organism

BIO CHEMICAL TEST	RESULTS
Oxidase test	-
Methyl red test	-
Vogues-proskauer test	+
Lysine utilization test	-
Beta hemolysis test	+
Motility test	+
H_2S production test	-
Glucose test	+
Lactose test	-
Indole test	-
Citrate utilization test	-
Catalase test	+
Urease test	-
Nitrate reduction test	+

The Biochemical test was done and the organism that produces Xylanase from soil was found to be ***Bacillus* sp**.

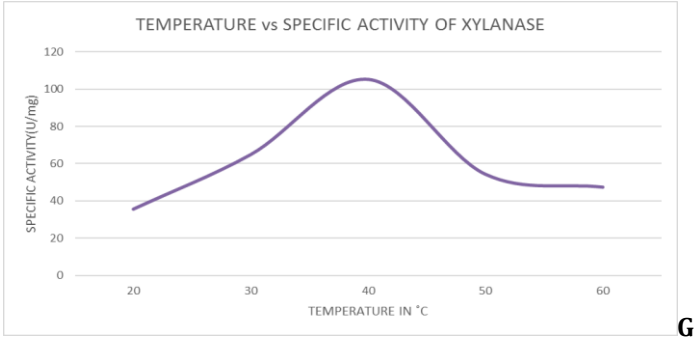
3.5 OPTIMIZATION OF THE ORGANISM (DNS ASSAY)

The DNS assay was performed to find the organism that shows maximum activity. Blank contains 0.5ml of 1% Xylan with 0.5ml of sodium citrate buffer. Xylose is taken as standard.

3.5.1a) TEMPERATURES OPTIMIZATION BY DNS ASSAY

The Temperature optimization was done by DNS Assay method. The test (T) corresponds to the Temperature from 20˚C to 60˚C. The maximum activity of Xylanase was found to be at 40˚C.

b) SPECIFIC ACTIVITY OF XYLANASE AT DIFFERENT TEMPERATURES

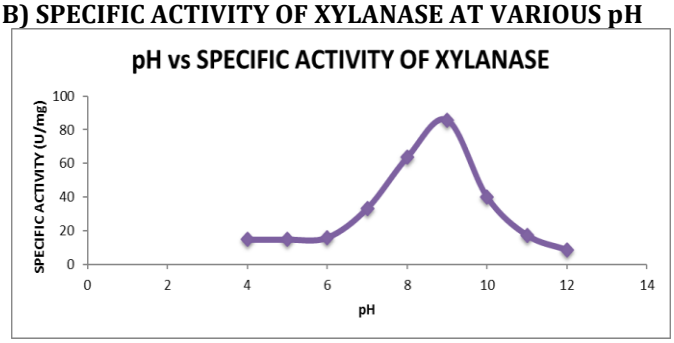


Graph 1: Specific activity of Xylanase at different temperatures

3.5.2 A) pH OPTIMIZATION BY DNS ASSAY

The pH optimization was done by DNS Assay and The Test (T_1-T_9) Corresponds to the pH 4-12. The maximum activity of Xylanase was found to be at pH 9.

B) SPECIFIC ACTIVITY OF XYLANASE AT VARIOUS pH



Graph 2: specific activity of xylanase at different pH.

Specific activity of Xylanase is determined to be high at pH 9 by the Graph 2

3.6 PARTIAL PURIFICATION OF XYLANASE ENZYME

Partial purification of Xylanase was carried out by Ammonium sulphate precipitation and dialysis.

3.6.1 AMMONIUM SULPHATE PRECIPITATION

20% and 80% of Ammonium sulphate precipitation was carried out with crude sample.

3.6.2 DIALYSIS

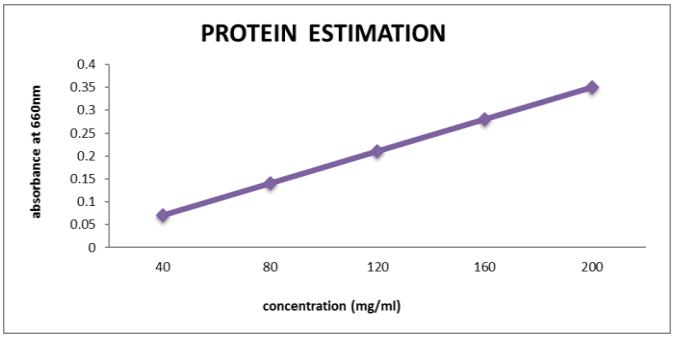
The crude enzyme after ammonium sulphate precipitation was subjected to dialysis to remove the excess salt present .DNS Assay and protein estimation was carried out to calculate specific activity of Xylanase.

3.7 PROTEIN ESTIMATION BY LOWRY'S METHOD:

BSA is taken as standard. Crude and Partially Purified enzyme samples are taken for Protein estimation.

3.7.1 PROTEIN ESTIMATION BY LOWRY METHOD

T_1 - Crude sample; T_2 – Partially Purified enzyme A; T3 Partially Purified enzyme B.



Graph 3: Standard graph for protein estimation.

Concentration of protein was determined through this standard Graph 3

3.7.2 SPECIFIC ACTIVITY OF XYLANASE IN CRUDE AND PARTIALLY PURIFIED ENZYME

Table 2: Specific Activity of Xylanase in Crude and Partially Purified enzyme

Samples	Total protein (mg/ml)	Specific activity of Xylanase(U/mg)	Purification fold
Crude enzyme	0.760	105.263	1
Partially Purified enzyme A	0.346	693.64	6.5
Partially Purified enzyme B	0.267	914.885	8.7

The specific activity of Xylanase was high in the Partially Purified enzyme B than A and Crude.

3.8 SDS-PAGE

The molecular weight of the enzyme was determined by using SDS-PAGE. It was found to be around 43KDa.

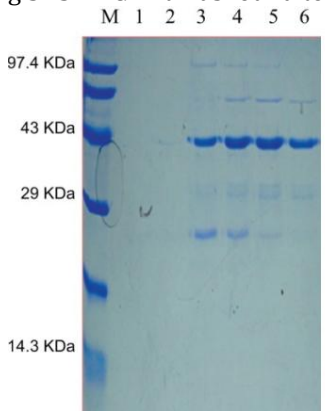


Fig.6: SDS-PAGE for partially purified XylanaseM-Molecular weight marker, Well -3, 4, 5, 6 - Partially Purified Xylanase

3.9 DISCUSSIONS

Xylanase depolymerizes Xylan molecules to xylose units, a primary carbon source for bacteria and fungi. Xylanases occur widely in bacteria and fungi. Many reports on Xylanases from *Bacillus* sp., *Clostridium* sp., *Streptomyces* sp.,*Aspergillus* sp., *Tricoderma* sp., and other microorganisms are available. In our present study, Xylanase producing organisms were isolated from two different soil samples and its maximum activity was found by DNS assay method. The isolate which had given the maximum activity was found to be *Bacillus sp*. Optimization of the isolate was carried out with various temperature and pH and the maximum activity was found in pH 9 at 40° C. The Xylanase from the optimized isolate were partially purified by ammonium sulfate precipitate and by dialysis process. Amount of protein present in the crude and partially purified enzyme estimated by using Lowry's method. The protein was estimated and the molecular weight was determined using SDS –PAGE around 43KDa

4. CONCLUSIONS

Xylanase producing organisms were isolated from two different soil samples and activity was determined. The isolate which have shown maximum activity was selected and further used. The isolate obtained was subjected to standard biochemical test results showed the presence of *Bacillus sp* in the isolates. The organism identified was optimized and the maximum enzyme activity was observed at pH 9 and temperature 40^{O}C.Xylanase was partially purified by ammonium sulphate precipitation method followed by dialysis and the protein estimation was done by Lowry's method. The molecular weight of partially purified Xylanase enzyme was determined by using SDS-PAGE. And the molecular weight was around 43 KDa.

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