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ISOLATION, PRODUCTION, CHARACTERIZATION AND PURIFICATION OF ALAKALINE XYLANASE FROM PAPER AND PULP WATSE WATER OF SANGLI KUPWAD MIDC(MH).

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ABSTRACT

In the present research, 30 xylanase producers were isolated by serial dilution and spread plate method from waste water samples of paper and pulp industry, Kupwad MIDC, Sangli (MH), India. This isolates were named as AG1, AG2 up to AG30. This all 30 isolates were screened for xylanase production by primary and screening. In the primary screening, AG12 showed 1.8mm zone of hydrolysis therefore AG12 were selected for Solid State fermentation at 25℃. AG12 showed highest xylanase production on 5th days of incubation period with 44.12 µg/ml/Mol activity. Further, xylanase were extracted and purified by Ammonium sulphate precipitation and dialysis with 20 Mol/ml/min and 12.04 (U/ml) respectively. The crude Xylanase were characterized by using different parameters such as temperature ranges from 10-50°c (optima-30°c), pH ranges from 4-10 (optima-pH 8.0).

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Introduction

Plant cell walls have three major polymeric constituents: cellulose (Insoluble fiberss of 1,4-glucan), hemicellulose (non-cellulosic polysaccharides including glucans, mannans and xylans) and lignin (A complex poly phenolic structure). Xylan is the major hemicellulose in wood from angiosperms but is less abundant in the wood from gymnosperms. Structurally Xylan is a homopolymer of D-xylopyranose residues in β (1 → 4) linkages with a degree of polymerization ranging from 150 to 200. This backbone is substituted by some of the sugars and organic acids, such as Arabians, glucuronic acid, Ferulic acid, etc. Xylans is broadly classified into four major groups based on its substitutes, which is homoxylan, Arabinoxylans, glucuronoxylan, and glucuronoarabinoxylan. Homoxylans contain xylose residues only, and can be either linear or branched. Arabinoxylans consist of a (1 → 4) -β-Xylan main chain, but is substituted with α-arabinosyl residues. The β-(1 → 4) -linked D-xylopyranosyl residues are substituted with one α-(1 → 2) -linked 4-*O*-methyl-D-glucuronic acid in the case of glucuronoxylan; while in glucuronoarabinoxylan, the same backbone is linked to arabinofuranose and uronic acid (Gröndahl ***et al.*** 2003; Bergmans ***et al.*** 1996).

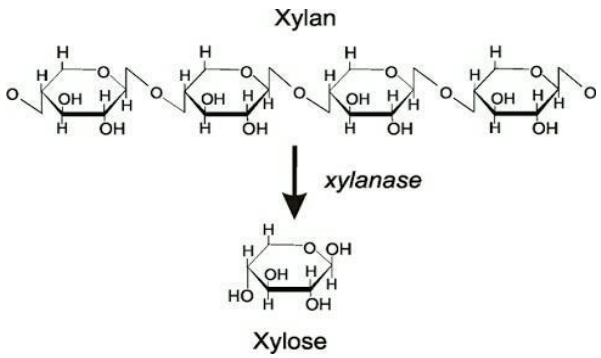

Figure 1. Structure and action of Xylanase

The side chains determine the solubility, physical conformation, and reactivity of Xylan molecules with other home cellulosic components, and, hence, greatly influence the mode and extent of enzymatic cleavage. In terrestrial plants Xylan is composed of a backbone of glycosidically 1,4- linked xylopyranose but in marine algae -1,3-linked back bone are found (Dekker & Richards 1976). Softwood contains 10-15% Xylan as arabino-4-0 methyl glucuronoxylan. This material, which is not acetylated, contains D-xylopyranose, 4-0-methyl D

Glucuronic acid and L-arabinose in a ratio of 1000:20:13. 000:20:13. The O-acetyl groups present at C2 and C3 positions of xylosyl residues inhibit xylanase from completely degrading acetyl Xylan probably by stearic hindrance. So the synergistic action of acetyl Xylan esters and Xylanase is necessary for the complete hydrolysis of acetyl Xylan.

Xylanase is the name given to a class of enzymes which degrade the linear polysaccharide β-1, 4-Xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls. Xylanase are produced extracellularly by bacteria, yeast and filamentous fungi. The fungal genera *Trichoderma*, *Aspergillus*, *Fusarium*, and *Pichiaare* considered great producers of xylanases. Microbial xylanases have important applications in the degradation of Xylan. Substrate xylan, a biopolymer comprising of D-xylose monomers linked through β1 4glycosyl bond, is found abundantly in lignocellulosic biomass. It can be classified as endo and exoxylanases (Min.Jen et al., 2002). Exoxylanases (ß D-xylopyranosidase) is sometimes referred to as extracellular xylanase. Xylanases have been classified in at least three ways: **based on the molecular weight and isoelectric point (pI) ,** the crystal structure and kinetic properties, or the substrate specificity and product profile. A wide variety of Microorganisms live in nature at various temperatures for their survival. They not only survive at high temperature but also carried out their activities according to maintained conditions. Many enzymes such as chitinases, amylase, protease, lipases, Xylanases etc. are industrially important.

Xylanolytic enzymes are inducible. It is produced in high amount during the growth on Xylan, the synthesis of enzyme are easily metabolized carbon source such as glucose or xylose. Therefore Xylan cannot enter into the cell, the oligosaccharide produced, but the hydrolysis of Xylan in the medium by small amount of xylanases produces continuously. Induction of various synthetic alkyl and aryl one β-D xylosides.

Xylanases can be produced using two main methods.

1. Solid state fermentation 2. Submerged fermentation

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Most of the researchers used submerged cultures, which allows control over the degree of aeration, pH and temperature of the medium, and control over other environmental factors required for optimum growth of microorganisms.

Interest in hemicellulytic enzyme has increased remarkably during recent years. This is mainly due to new areas of application of these enzymes within the pulp and paper industry. Among those the most promising seems to be utilization of hemicellulose, especially xylanases to increase the bleachability to craft pulps. This is partly due to the greatest potential of an environmental safe method. The main enzymes needed in aided which have been belong to group of endo, beta xylanases. Xylanases act mainly on the located.

Recipted xylan on the surface of pulp fibrus. enzymetic hydrolysis of this specific type of xylans renders the structure of fibres more permeable allowing. Enhanced extraction of residual lignin from fibres.

The hydrolysis of hemicellulses in the inner fiber layers.may also enhance the bleachability. The main goals in enzymes added bleaching of kraft pulps have been a reduction of congestion of chlorine chemicals in bleaching process and consequent lowering of the action of the effluents.

In the production of totally chlorine free pulp, enzymes have been successfully used for increases the brightness of pulp of the other suggested enzymatic modification of fibres are in at improved drainage in the paper machine, improvement of fibres properties of production of dissolving pulps.

The present research study was carried out in the investigation of novel xylanases from bacteria under standard and maintained conditions by offering following materials and methods.

2. Methods and Materials:

2.1. Sample collection

The waste water sample of paper and pulp industry of Kupwad MIDC, Sangli (MH), India was collected in bottles and brought to the laboratory for further procedure.

2.2. Isolation of micro organism

The collected samples were serially diluted and the highest dilution (10^{-7}) was spread on a sterile nutrient agar plate and incubated at 37^0C for 24 hrs. The plates were observed in isolated colonies of microorganisms.

2.3. Primary screening of xylanase producers

Isolated microorganisms were spot inoculated on sterile Mendel and Reese agar medium containing 1% xylan and these plates were incubated for 48 hours at 37 ^{0}C. After 48 hours of incubation period, the plates were flooded with a 1%Congo red solution.

2.4. Xylanase Production

Solid-state fermentation was employed for production of xylanase. 50 g of Rice bran and 20ml distilled water were transferred to the seven individual 500 ml cotton plugged Erlenmeyer flasks. The flasks were autoclaved at 15 lb/inch2 pressure and 121ºC for 15 min., and cooled the medium at room temperature. The xylanase positive microorganisms were inoculated in SSF and incubated for 7 days for xylanase production.

2.5. Xylanase Extraction

After 14 days of incubation period, 30 ml of 0.05 M Phosphate buffer (pH 6.0) was added to the fermented substrate in first flask. The contents of the flask were crushed with the help of a glass rod and flasks were rotated on a rotary shaker at 120 rpm for 1 h at 30ºC afterwards the fermented medium filtered through whatman filter no. 1 filter paper. After filtration, the contents were centrifuged at 5,000 RPM for 10 min at 4ºC and clear supernatant from each of the tubes was collected for further studies and tubes was stored at 4°C until used. This whole procedure was repeated for remaining six SSF flasks.

2.6. Xylanase Activity

Xylanase activity was assayed by using DNS method to measure the amount of reducing sugar liberated from Xylan. The crude enzyme protein was used for measuring the activity at pH 6 (phosphate buffer, 0.5) at 37^0c for 30 min.

2.7. Xylanase unit activity

According to the International Union of Biochemistry 1 enzyme International unit has been defined as amount of enzyme required to release 1µmol of reducing sugar in 1 min. at 40°c and at atmospheric pressure.

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IJAMSR

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2.8. Characterization of crude Xylanase

The obtained crude xylanase enzyme was used for further characterization.

2.8.1 Effect of temperature on Xylanase activity

The effect was carried out by adjusting reaction mixture at different temperature ranges from 4ºC to 60 ºC.

The reaction mixture was incubated at different adjusted temperature, such as 4ºC, 32ºC, 50^{0}C, 60ºC, .For 15 minutes and xylanase activity was measured using above mentioned protocol at absorbance 540nm.

2. 8.2 Effect of pH on xylanase activity

The effect of PH was carried out by adjusting the reaction mixture at different pH ranges on xylanase activity of crude xylanase enzyme from 5,6,7,8,9,10.using pH of phosphate buffer [0.1 M] were used during the study of the effect of pH.

2.8.3 Partial purification of xylanase enzyme

The fermented broth by solid state fermentation in which the broth is taken out. The fermented broth was centrifuged to remove cell debris. The supernatant was taken for ammonium sulphate precipitation. The proteins are commonly present in supernatant which neutralized by addition of salt. The amount of salt used for precipitation depends on volume of supernatant taken as well as a percentage of the salt precipitation.

The 80% salt precipitation was done and ammonium sulfate salt was added pinch wise in the crude enzyme at 4ºC on a magnetic stirrer.

Dialysis of crude enzyme

The semipermeable membrane like dialysis bag was used for removing ammonium salt from the crude enzyme by desalting.

Protein Determination

The protein concentration was estimated according to the method of Lowry *et al* using Bovine serum albumin as the standard.

Results:

Isolation, Production of Xylanase

30 isolates were obtained from the paper and pulp industry of Kupwad MIDC, Sangli (MH), India. They were labelled as AG1, AG2 up to AG30 and sub cultured on sterile nutrient agar slants in the duplicates and preserved at 4 ℃. The results were shown in table I. In the primary screening of Xylanase production AG12 showed highest zone of hydrolysis at 1.8 cm after flooding with 1% Congo red. This same AG12 were selected for solid state fermentation on rice bran with Xylan as a substrate for 7 days. The results were shoed in table II. AG12 showed highest xylanase production on 6th days of incubation period under static conditions, therefore xylanase were partially purified by using a solvent extraction method, ammonium sulfate precipitation and dialysis respectively.

Table I: Colony size and zone of hydrolysis showed by isolates.

Sr. No.	Isolate No.	Colony size (mm)	Hydrolysis Zone (mm)
1	AG1	0.3	0.4
2	AG2	0.2	0.6
3	AG3	0.3	0.8
4	AG4	0.4	-
5	AG5	0.2	0.6
6	AG6	0.3	0.4
7	AG7	0.1	-
8	AG8	0.1	0.9
9	AG9	0.3	0.7
10	AG10	0.5	1.2
11	AG11	0.4	15
12	AG12	0.3	1.8
13	AG13	0.3	-
14	AG14	0.2	1.4
15	AG15	0.4	-
16	AG16	0.2	1.5
17	AG17	0.1	1.2
18	AG18	0.4	-
19	AG19	0.5	0.4
20	AG20	0.2	0.9
21	AG21	0.1	0.8
22	AG22	0.2	0.6
23	AG23	0.4	0.4
24	AG24	0.3	1.2
25	AG25	0.3	1.3
26	AG26	0.3	1.2
27	AG27	0.2	0.6
28	AG28	0.3	0.4
29	AG29	0.3	0.4
30	AG30	0.1	-

Table II. Selection of AG12

Isolate No	Colony Size (mm)	Hydrolysis zone (mm)	Xylanase activity µg/ml/Mol
AG12	0.3	1.8	6.08

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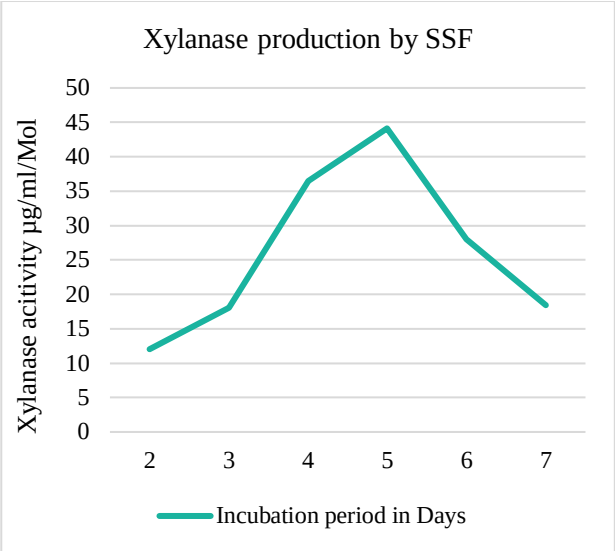

Figure 2: Xylanase production by SSF

Purification and characterization of xylanase

In brief, extracted crude xylanase were precipitated by ammonium sulfate salt by 80% and further protein concentration were determined by its specificity. Enzyme activity was observed as well as protein estimation was done by using the Folin Lowry method.

Table 3; Activity of precipitated Xylanase

Sample	**Enzyme activity (U/ml)**	Protein Concentration µm/ml/min.	Enzyme specificity Mol/ml/min
80%	16.05	700	20

This same precipitated xylanase were submerged in dialysis for partially purification and the results were shown in table 4.

Table 4: Activity of partially purified Xylanase

Sample	**Enzyme activity (U/ml)**
Partially purified enzyme	12.04

Such partially purified xylanase were further characterized at different temperature and pH.
According to xylanase activity and graph it seems that, Xylanase was active at temperature 25℃ and pH 8.

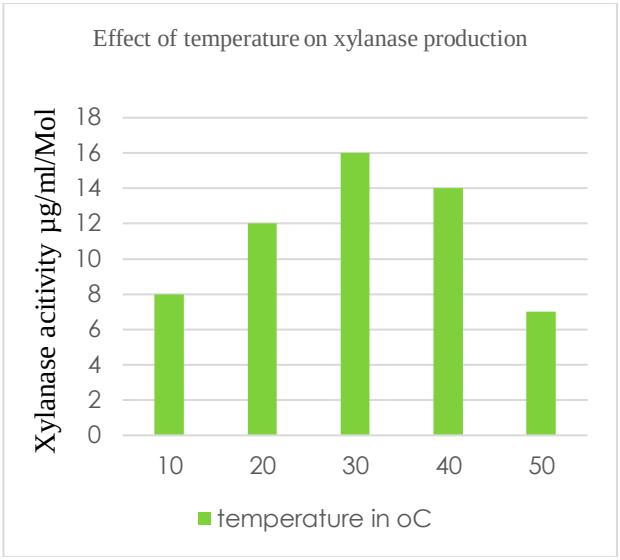

Figure 4; Effect of temperature on Xylanase production

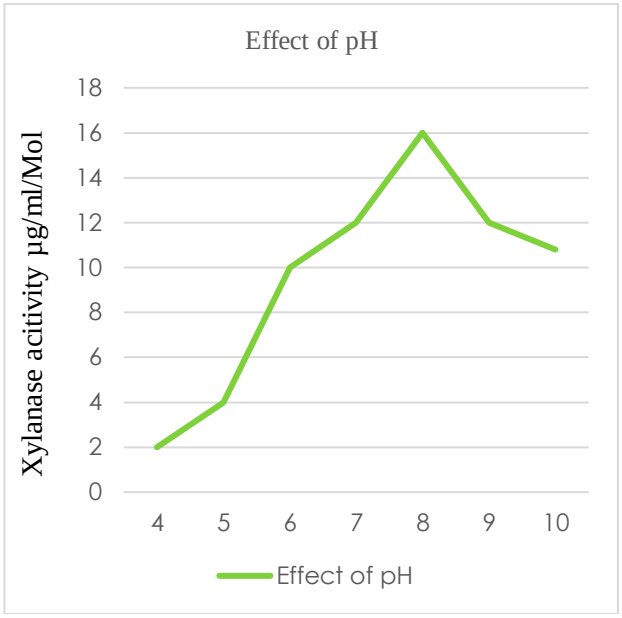

Figure 4: Effect of pH on xylanase production

Discussion:

Xylanase production, microbial study is rare in literature from pulp and paper industrial waste water.

F. A. Alebious worked on the production of xylanase from fungal isolates by solid state fermentation using sugar cane baggase. As agricultural residue studied on

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IJAMSR

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the optimum condition of temperature and pH a range from 30^0C - 80^0C and pH- 3 to 9. Comparative studies of the enzyme for its activity as produced by XM, XD and XC were 3000U/ml, 2800U/ml and 3400U/ml respectively.

Scientist N. Balamani *et al*., (2008) also worked on -xylanase production from *Pseudomonas putida* and *Staphylococcus aureus*. wheat bran supported maximum xylanase production in both species. The culture medium with pH-6.5 and tempetrature-40^0C in *Pseudomonas putida* and pH-6 and temperature-45^0C in *Staphylococcus aureus* promoted the growth and maximum xylanase production.

Hiremath. K.S et al., (2011) worked on-isolation, production and characterization of alkaline thermostable xylanase from newly isolated *Bacillus spp*. -application on the paper and sugar industrial waste are used to screen and produce the xylanase. The industrial waste of paper industry shows the maximum synthesis of xylanase at pH-9-12 and temperature 35-70^0C for 18-24 hrs. The produced alkaline thermostable xylanase by *Bacillus* Species show the most Xylanolytic activity at pH>10 at 70^0C.

Karunakaran *et al*., (2014) worked on-xylanase production from *Aspergillus Niger*. -by submerging fermentation studied on agricultural soil, decayed bread and waste material. The maximum concentration of xylanase at 25^0C and pH of 6.

In our project we used 1% Xylan as a substrate and the enzyme activity 12.04 U/ml at pH 7 and temperature 50^0C.

Conclusion:

The production of xylanases widely applied in the field of chemistry, biomedical, biotechnological, agricultural and environmental protection. Wide scope for extensive research to achieve industrial scale production from the xylanase by Solid State Fermentation.

Therefore present xylanase is novel in nature.

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