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Research Article

An overview of Screening and Tentative Optimization of Microbial Xylanase from soil Samples Collected from Chittoor Paper Industry

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ABSTRACT
The development of microbial enzymes for commercial use is a specialized field which requires. Meticulous screening of new and improved strains from potential sources and Scaling up of enzyme production by optimizing conditions of fermentation. Xylanase is the name given to a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose. These days, xylanase are the enzymes on demand in pulp and paper industry Enzyme application improves pulp fibrillation and water retention, and selective removal of xylan from dissolving pulps. Three-quarters of the market is for enzymes involved in the hydrolysis of natural polymers, microbial exploitation with regard to xylanase production is a spectacular advancement with scientific progress in commercial scale of manufacturing of paper. Microorganisms are rich sources of xylanase enzymes. In our research study we have screened *Actinomycetes* which produce extra-cellular xylanase. The structural diversity associated with the xylan component from different plants gives us concept for requirement of various xylan-degrading enzymes which can utilize different lignocellulosic substrates those are being used as raw materials for the paper industry. In order to use xylanase for pulp treatment, it is required that enzymes should not have any additional cellulolytic activity, since the quality of paper will be adversely affected by cellulase. To overcome this problem, screening and tentative optimization of microbial xylanase was carried out by collecting soil samples from Chittoor paper industry waste.

Key Words: Xylanase, Lignocellulosic substrate, Paper industry, Cellulosic activity

INTRODUCTION
The enzyme named xylanase deconstructs plant structural material by breaking down hemicellulose, a major component of the plant cell wall. Plant cell walls are necessary to prevent dehydration and maintain physical integrity. They also act as a physical barrier to attack by plant pathogens. In nature, some plant consumers or pathogens use xylanase to digest or attack plants. Many microorganisms produce xylanase, but mammals do not. Some herbivorous insects and crustaceans also produce xylanase. Xylanase is the name given to a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose , thus breaking down hemicelluloses, one of the major components of plant cell walls. Since last few years, there has been a growing interest for microbial xylanases as they have important applications in the degradation of xylan. Substrate xylan, a biopolymer comprising of D-xylose monomers linked through 1, 4-glycosyl bond, is found abundantly in lignocellulosic biomass. Xylanases can be classified as endo- and exo-xylanases. Xylan is the non-cellulosic polysaccharide found abundantly in tropical plants and forms 20–35% of their total dry weight. It is the main constituent of the secondary cell wall and forms an association between lignin and other polysaccharides. Xylan molecules, which are linked covalently with lignin phenolic residues, are found to have interaction with polysaccharides, such as glucan and pectin. Xylans are linear homopolymers which have d-xylose monomers linked through β-1, 4-glycosyl bonds. Naturally, they are partly substituted by acetyl 1-arabinofuranosyl and 4-o-methyl-d-glucuronosyl residues, forming complex heterogeneous and poly-dispersed polymers (Kormelink *et. al.*, 1993). Due to problem with the isolation of xylans from natural raw materials, many features related to xylan structures are still not revealed. Various genera and species of bacteria, fungi are important sources of xylanase enzymes. Extracellular xylanase are secreted by numerous *Actinomycetes* species (Suneetha, 2011), but xylanase secretions are often accompanied by cellulolytic enzymes like in species of *Trichoderma*, *Penicillium* and *Aspergillus*. Although fungi are prolific producers of extracellular xylanases they are not free from cellulase. The microbial xylanase production has been broadly reviewed by several groups (Balakrishnan *et. al.*, 1992, Suneetha, 2011). Thus effort should be made to screen and identify suitable xylanolytic enzymes (free from cellulase) that are well-suited for pulp manufacture; i.e. Importance of cellulase-free alkali-stable xylanase

enzymes, mainly for developing feasible technologies (Beg *et. al.,* 2000) for proper pulp bleaching. Paper industry mainly makes use of chlorine for increasing pulp brightness but with advancement of this technology the use of toxic chlorine compounds in the paper industry can be reduced (Jones *et. al.,* 1961). *Actinomycetes* and *Streptomyces* which are highly suitable for producing thermostable xylanolytic microorganisms, particularly for the production of thermostable xylanase, which is free from cellulase, were obtained from the soil samples of Javadu Hills and Elagiri Hills by Maheswari *et. al.,* 2000 and Suneetha, 2011. Thus it is clear that in future thermostable xylanase which can work at high alkaline pH will be the subject of high interest. So focus will be on developing process technologies to manufacture such enzymes suited for pulp biotechnology at commercial-scale level (Adney *et. al.*, 1994). Currently, effort has been made for screening thermostable xylanases from Actinomycetes, process optimization ,(simplification, cost reduction of enzyme and its application in pulp industry.

MATERIALS AND METHODS

CSPY- ME Medium (Casein Starch Peptone Yeast Extract –Malt Extract)

It was prepared by using 0.5g of K_2HPO_4, 3.0g of Casein, 10g of Maize starch (soluble) , 1g of Peptone, 1g of Yeast extract, 10g of Malt extract and 25g of Agar. Final volume was made up to 1000ml with water, resulting in pH of 7.8.

Aqueous Medium

It was prepared by using 7g of $NaNO_2$, 1g of K_2HPO_4, 0.5g of $MgSO_4$ and 5g of Yeast extract. Final volume was made up to 1000ml with distilled water.

Production Medium (Kim J.H *et. al.,* 1985)

Production medium was prepared using 0.5g of peptone, 0.3g of Urea, 0.2g of K_2HPO4, 0.3g of $Cacl_2$, 0.2g of Tween 80, 1.4g of $(NH_4)_2SO_4$, 0.3g of $MgSO_4$. Final volume was made up to 1000ml with distilled water.$7H_2O$ and 1% of Xylan(Sigma Chemicals).

Designed Medium (Suneetha, 2011)

It was prepared using 0.3g of Urea, 0.1g of $MgSO_4$, 0.2g of K_2HPO_4, 0.3g of $CaCl_2$, 0.2g of Tween 80, 1.4g of $(NH_4)_2SO_4$, 0.3g of $mgso_4.7H_2O$ and 1% of Xylan(Sigma Chemicals). Final volume was made up to 1000ml using distilled water.

Screening of Actinomycetes

Soil is a natural source for microbes.Soil samples collected from Chittoor paper industrial waste, were used for screening of *Actinomycetes*. The potential xylanase producing *Actinomycetes* were isolated from culture plates and streaked on CSPY-ME medium to obtain pure cultures. Screening of xylanolytic *Actinomycetes* was initially carried out on medium supplemented with gelrite substrates. Moisture-saturated plastic bags were used for incubation at 45^oC to reduce desiccation due to the high incubation temperature. The tubes were analyzed weekly to see the utilization of the substrates. Xylanolytic activity was confirmed by repeating baiting techniques. The xylanolytic organisms were maintained on yeast extract, malt extract medium at 4^oC with periodic transfer into fresh media and the stock cultures were maintained at -20^oC. The thermophilic *Actinomycetes* was maintained on malt agar (MA) medium at 25^oC. Fresh malt agar slants were made and inoculated with stock culture and incubated at 45^OC in moisture-controlled incubator for 6-9 days until maximal growth was obtained. The slants were then maintained at $20\ ^oC$. A spore inoculum was used to initiate growth in Erlenmeyer flasks using various substrates at 1% (w/v) in either production media medium. The flasks were incubated at $45\ ^OC$ for 96 hours to 240 hours until maximal xylanase activities were obtained. The cultures were filtered out through a Whatman glass fiber filter and those filtrates were used for characterization and enzyme assays (Okazaki *et. al.*, 1984).

Effect of Media

Aqueous medium, production media and Designed medium, was used to see the maximum production of xylanase enzyme. All experiments, which were carried out in duplicate, were repeated thrice for confirmation.

Assay of Xylanase

Xylanase enzymatic activity was determined by incubating 1 ml of an appropriately diluted enzyme preparation (crude *Actinomycetes* culture filtrate) with 10 mg of oat-spelt xylan in 1 ml of 0.1M citrate phosphate buffer (pH 5.0) at 50^oC for 30 minutes. The reaction was terminated by the addition of 3ml of 3, 5-dinitrosalicylic acid reagent. One unit of xylanase activity was defined as 1 µmole of xylose equivalents released per minute.

Optimization of Xylanase Production

Xylanase production depends on physical factors like pH and temperature. The pH optimization was performed in 0.05M citrate phosphate buffer adjusted to initial pH values of 4.5 to 10. The reaction mixtures, containing oat-spelt xylan (0.5%, w/v) and an appropriately diluted enzyme preparation (crude culture filtrate), were incubated at 50^oC for 30 min. Temperature optimization was similarly carried out in citrate phosphate buffer at pH 5.0 and assayed for reducing sugars released after incubation at various temperatures for a period of 30 min.

Effect of pH

pH of the medium has an intense influence on the overall metabolic activity of the microorganisms. The effect of ph on xylanase production was studied by adjusting the ph of the media between 4.5 and 10. The temperature of incubation was maintained at 37 °C and the procedure for xylanase assay, described earlier, was followed.

Effect of Temperature

Temperature is one of the important physical parameter that influences metabolic activities of microorganisms. Temperature change affects the metabolism and thus product yield. Therefore, it is essential to study the temperature effect on the fermentative production of xylanase enzyme. This was carried out by inoculating the organisms in production media and incubating the samples at six different temperatures namely 28 °C, 37 °C, 45 °C, 50 °C, 60 °C and 70 °C. The pH of the medium was maintained at 7.8. The samples were assayed for the xylanase activity at various intervals.

Thermostability Studies

Thermostability of the enzyme activities was determined by incubating the crude *Actinomycetes* culture filtrates at 70 °C, 60 °C, and 50 °C for various durations ranging from hours to weeks. The enzymatic activity of the treated filtrates were then assayed by incubating an appropriately diluted aliquot of the treated sample with the assay substrate in citrate phosphate buffer at pH 5.0 and 60 °C for 30 min, as described in the previous section.

Stability of the Enzyme

Stability of the enzyme was studied by storing the enzyme for a period of 15 weeks at room temperature.

RESULTS AND DISCUSSION

Actinomycetes Species with xylanolytic activity from 100 soil samples (Table 1) were screened from Chittoor soil samples . Characterization was done, based on xylan degrading ability, into highly degrading (>90%), moderately degrading (>60%), and non- degrading (0-30%) *Actinomycetes* strains .Four strains which were showing the best xylanolytic activity were selected(named as **X1, X2, X3 and X4)** for further studies such as characterization, xylanase assay, effect of media and optimization of physical factors like pH and temperature. Optimal xylanase activity was observed at pH 8-9 and 60^0C-70^0C. Xylanase activity was determined to be thermostable with half-life (i.e., the time of incubation at a particular temperature during which 50% of the original enzyme activity is lost) at 70 °C. Xylanase activity was found stable at 60 °C for at least 13 weeks.

Effect of media

Media plays a significant role in product formation. In our study (Table 2), the four strains (**X1, X2, X3** and **X4)** were exhibiting xylanase units in the range of 15-23 XU/ml in Designed media. We made comparative studies on three different media like aqueous, production and Designed media. From the first two media, we got 2-7 XU/ml and 5-9 XU/ml.

Figure 1. Screened xylanase producing Actinomycetes (From left to right are X1, X2, X3 X4 strains in 4 test tubes respectively)

Effect of pH

pH of the medium has major influence on the overall metabolic activity of the microorganisms. The comparative study (Table 3) of the effect of pH ranging from 4.5 – 10.0 on four different strains X1, X2, X3 and X4 of *Actinomycetes* was found to be 14-17 XU/ml, 19-28 XU/ml, 18-24 XU/ml, and 23-29 XU/ml respectively.

Effect of Temperature

Temperature is another important physical factor influencing metabolic activities of microorganisms. The effect of temperature on four different strains (Table-4) X1, X2, X3 and X4 at temperatures 28 °C, 37 °C, 45 °C, 50 °C, 60 °C and 70 °C was observed to have the xylanolytic activity ranging from 13-26 XU/ml, 18-28 XU/ml, 17-29 XU/ml and 22-29 XU/ml respectively Indicating the thermophilic nature.

Thermostability Studies

Thermostability studies indicated the stability of xylanase enzyme up to 60 °C (Table 5).

Stability of the Enzyme

The stability study (Table-6) on the four different strains X1, X2, X3, and X4 was done for a period of 15 weeks. From the study it was found that the enzyme is stable for 13 weeks and started showing decrement in the xylanolytic activity by the end of 15th week.

Table 1. Screening of xylanase producing Actinomycetes

No. of Species	Zone of inhibitions (Xylanolytic activity)
4	+ + +
19	+ +
27	+

+ + + : *Highly degradable (>50%)*
+ + : *Moderate (>25%)*
+ : *Non degradable (<10%)*

Table 2. Effect of media on xylanase producing Actinomycetes

Types of media	Strains			
	X1	X2	X3	X4
	Xylanolytic activity XU/ml			
Aqueous	2	5	4	7
Production	5	7	6	9
Designed media	15	21	19	23

Table 3. Effect of pH on xylanase producing Actinomycetes

pH	Strains			
	X1	X2	X3	X4
	Xylanolytic activity XU/ml			
4.5	14	19	18	23
5.5	15.1	22	19	24
6.5	16	24	21	25
7.0	16.4	25	22	26
8.0	16.7	26	23	27
9.0	17	28	24	29
10.0	16.6	21	21	24.5

Table 4. Effect of temperature on xylanase producing Actinomycetes

Temperature (°C)	Strains			
	X1	X2	X3	X4
	Xylanolytic activity (XU/ml)			
28	13	18	17	22
37	15	21	19	23
45	17	25	23	26
50	21	26	25	26
60	26	26	27	28
70	23	24	21	24

Table 5. Thermo stability studies of xylanase enzyme

Temperature (°C)	Strains			
	X1	X2	X3	X4
	Xylanolytic activity (XU/ml)			
50	21	26	25	26
60	26	26	27	28
70	24	23	21	25

Table 6. Stability of the xylanase enzyme

No. of weeks	Strains			
	X1	X2	X3	X4
	Xylanolytic activity XU/ml			
3	17	26	23	25
6	16	26	23	25
9	16	26	23	23
11	15	25	22	23
13	15	23	21	22
15	4	9	7	11

RESULTS AND DISCUSSION

This Xylanase technology is one of the beneficial tools for pulp and paper industry which can produce good quality paper using this enzyme. Designed media (Still we are optimizing) used in our study reported an alkalophilic *Actinomycetes* species producing cellulase free xylanase that was stable in the pH range of 5 to 9.5 and active above ph 9.0 (alkali-tolerant), and retained its activity even at 60 °C. We can even go for further strain improvement that can improve quality and reduce the cost. Application of biotechnology to pulp and paper industry operations is no longer an academic or potentially useful alternative proposition for the future. Thus novel approaches for the screening of new xylanolytic microbial strains **(**like, **X1, X2, X3** and **X4** also **s**hown in Fig. 1) which can work in different environmental conditions, the use of above Xylanases , was stable in the pH range of 5 to 9.5 and active above pH 9.0 (alkali-tolerant), and retained its enzyme activity even at above 60 °C., this wonderful enzymes from Actinomycetes sps improves pulp fibrillation and water retention, and selective removal of xylan from dissolving pulps in paper manufacturing can be eventually exploited commercially. We can even go for further strain improvement that can improve quality and reduce the cost. Application of biotechnology to pulp and paper industry operations is no longer an academic or potentially useful alternative proposition for the future.

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