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Isolation and Characterization of Xylanase Producing Bacteria and Optimization of Parameters for Xylanase Production

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Abstract
A soil sample was collected from wood industry & Mangroove soil from Vasai creek (Maharashtra) for microbial and enzymatic study. Xylanase producing bacterial strain were isolated and after further screening, three bacterial strain were selected from the soil sample. All they are gram positive in nature. These three bacterial strains were screened as xylanase producers. The bacterial isolates were examined for Morphological, Physiological and Biochemical characteristics along with optimization of temperature and PH for improved xylanase activity. The Bacterial isolates were having potential to produce the xylanase enzyme were analyzed by enzyme assay. 62.39 IU/ml was the maximum enzyme activity found. The Purified fractions of enzymes were used to clarify the fruit juice, which revealed that in 6 hrs 46% clarification of juice can be obtained.

Keywords
Microbes, Xylanase, Microbial optimization, Enzymatic analysis and Juice Clarification.

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INTRODUCTION:
Xylan is the second-most-abundant polysaccharide in nature after cellulose and the most-abundant component of hemicellulose, though xylanse constitute a larger fraction of the hemicellulose in angiosperms than in gymnosperms. Xylan-solubilizing enzymes, xylanases, have been studied for their potential use as low-cost and environmentally friendly bleaching agents in the pulp and paper industry and as a component of cost-effective lignocellulose conversion in the emerging biofuels industry.

The three main components that constitute lignocellulosic substrates are cellulose, hemicellulose and lignin. Schulze first introduced the term 'hemicellulose' to represent the fractions isolated or extracted from plant materials using a dilute alkali. Hemicelluloses are composed of complex mixtures of xylan, xyloglucan, glucomannan, galacto glucomannan, arabino galactan or other heteropolymers.

Xylanases, a combination of hydrolytic enzymes, catalyze the hydrolysis of xylan, which are genetically single chain glycoproteins, ranging from 6–80 kDa and active at temperature between 40 and 60˚C. Xylanases are considered to be able to effectively hydrolyzexylan, the principal type of hemicellulose containing a linear polymer of β-D-xylopyranosyl units linked by (1-4) glycoside bonds which act cooperatively to convert xylan to its constituent simple sugars. These enzymes include β1,4-endoxylanases (xylanases; EC 3.2.1.8), which cleave

internal glycosidic bonds within the xylan backbone; arabinofuranosidase (EC 3.2.1.55), which hydrolyzes arabinose side chains; R-glucuronidase (EC 3.2.1.131), which removes glucuronic acid side chains from xylose units; xylanesterases (EC 3.1.1.6), which release acetate groups and finally xylosidase (EC 3.2.1.37), which hydrolyzesxylobiose to xylose.

Xylanases are produced by diverse organisms like fungi, bacteria, algae, protozoa, gastropods and arthropods. Bacterial genera like Bacillus, Cellulomonas, Micrococcus, Staphylococcus, Thermotoga, Paenibcillus, Arthrobacter, Microbacterium, Pseudoxanthomonas, Rhodococcus have been reported to produce xylanases.

MATERIALS AND METHODS

Sampling & Isolation of Bacteria:

A soil sample was collected from wood industry & Mangroove soil from Vasai creek (Maharashtra). The soil was collected from the surface to a depth using sterile spatulas and transported to the laboratory and stored at -20oC.

For the enrichment of the soil bacteria, 10 gm soil samples were added to 100 ml of sterile xylan broth and the medium was incubated at 37oC at shaker for 24 - 48 hours.

Take 1 ml of sample from xylan broth and perform serial dilution in 0.9 % Saline (NaCl). 100 µl of the liquid mixture were serially diluted until a dilution of 10^6. Then 100 µl of this from each dilution were plated on NA containing xylan by using spread plate technique. The plates were incubated at 37o C for 24 to 48 hrs.

Screening: -

After incubation, plates were stained with congo red solution (0.5%) for 15 min followed by repeated washing with 1 M NaCl for zone analysis. Colonies that showed clear zones around bacterial colonies were selected for xylanase production. Positive selected bacterial colonies stored (maintained) at NA slants containing xylan were kept at 4°C.

Microbiological Analysis: -

Different Morphological, Physiological and Biochemical test like Gram's staining, colony morphology, motility test and oxidation and fermentation test were studied.

Enzyme production & enzyme activity: -

Erlenmeyer flasks (250 mL) containing 50 mL of modified xylan medium were autoclaved at 15 psi for 20 min and cooled. The flasks were then inoculated with 2% (v/v) of 18 hr old culture and incubated at 37°C for 48 hrs under shaking at 200 rpm. Xylanase was harvested by centrifugation at 10,000 x g for 20 min at 4°C. The cell free supernatant was used as crude enzyme.

Dinitrosalicylic acid (DNSA method) was used. DNSA reagent appears to be yellow in colour due to its nitro group. One of its nitro groups in the reagent is reduced to amino group to yield amino-nitro-salicylic acid when the DNSA is boiled with reducing sugar to be estimated at 540 nm in a UV – Visible spectrophotometer.

Optimization of Enzyme Activity: -

In the characterization the xylanase enzyme involved determination of effect of different factors such as temperature, pH on its activity and stability was studied. Effect of temperature and pH was determined on Xylanase activity.

pH:

Effect of pH on xylanase activity and stability. The optimal pH for enzyme activity was determined by changing the assay reaction mixture pH using the following buffers (0.1 M): Sodium acetate (pH 5.0), Sodium phosphate (pH 6.0–7.0), Tris–HCl (pH 8), glycine–NaOH buffer (pH 9–10) and 1% soluble Birchwood as a substrate. To evaluate the stability of the enzyme at each pH, xylanase activity was measured after incubation the enzyme solution in pH buffers ranging from 4.0 to 10.0 at 25°C for 24 hours. Residual activity was determined under optimal assay conditions for each isolate.

Temperature: -

Effect of Temperature on xylanase activity and stability. The optimal temperature for enzyme activity was determined by ranging the temperature from 4, RT, 37 and 50.

Partial Purification of Enzyme: -

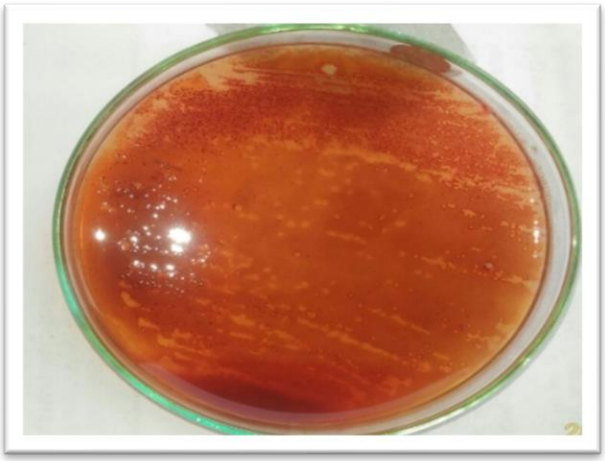
All steps of purification were performed at a temperature of 4oC using 100 mM sodium phosphate buffer, pH 6.5. Techniques used for the purification of xylanse enzyme were Ammonium sulphate precipitation, dialysis. The enzyme preparations at various stages of xylanase enzyme purification were analyzed for protein concentration and enzyme activity.

Application: -

Fruits (mausambi) were purchased from the local market and washed. Juices of fruits were extracted and filtered through four layers of muslin. Filtered juices were incubated at 85°C for 3 min. Fruit juices were treated with 12.5 U of xylanase/ml of fruit juice at 30o C for 30 and 60min. At the end of treatments, juices were centrifuged at 10,000 g for 5 min. Supernatants were analyzed for clarity, reducing sugar and yield.

The clarity of the juice was measured by determining the percent transmittance (T %) at a wavelength of 660 nm using UV-Vis spectrophotometer. The yield of juices was detected by measuring the volume of the fruit juice after centrifugation. Reducing sugars were determined using DNS method. The results were calculated as percentage of control. Untreated fruit juices were use as control.

RESULT AND DISSCUSSION:



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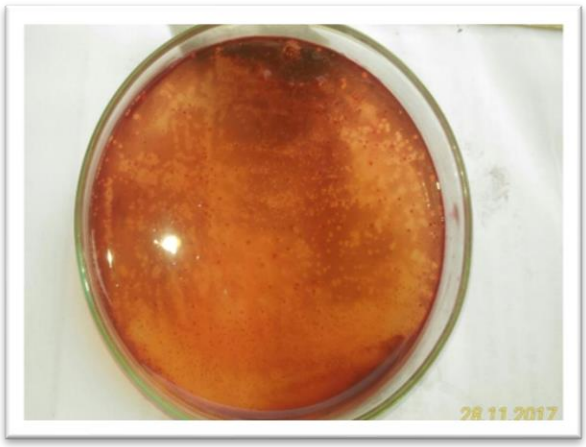
Isolate – 1
Isolate – 2
Isolate – 3

IMAGE – 1 (Three Positive Isolates)



The isolated three samples were characteristic analyzed and variable characters shown in Table 1.

Isolates / Character	IS 1	IS 2	IS 3
SIZE	Pinpoint	Pinpoint	Pinpoint
SHAPE	Circular	Circular	Circular
COLOUR	White	Yellow	White
MARGINE	Regular	Regular	Regular
OPACITY	Opaque	Opaque	Opaque
ELEVATION	Convex	Convex	Convex
CONSISTENCY	Mucoid	Mucoid	Mucoid
GRAM NATURE	+Ve Cocci	+Ve Cocci	+Ve Cocci

TABLE - 1

Sugar Fermentation analyzed and variable characters shown in Table 2.

Isolates / Character	IS 1	IS 2	IS 3
Sugar Fermentation: -			
1% Glucose	AG	AG	AG
1% Mannose	A	A	A
1% Lactose	A	A	A
1% Maltose	AG	AG	A
1% Xylose	AG	AG	AG
1% Sucrose	AG	AG	A

TABLE – 2

Biochemically analyzed and variable characters shown in Table 3.

Isolates / Character	IS 1	IS 2	IS 3
Indole	-Ve	-Ve	+Ve
Methyl Red	+Ve	+Ve	+Ve
Voges – Proskauer	+Ve	+Ve	+Ve
Citrate utilization	-Ve	+Ve	-Ve
Urease production	-Ve	-Ve	-Ve
Hugh Leifson (O)	Purple	Purple	Purple
Hugh Leifson (f)	Purple	Purple	Purple
Triple Sugar Iron Agar: -			
Slant	Acid	Alk	Acid
Butt	Acid	Acid	Acid
Gas production	+Ve	+Ve	+Ve
H2S production	-Ve	-Ve	-Ve

TABLE - 3

KEY: -

A: - Acid Production

AG: - Acid & Gas Production

-Ve: - Negative Test

+Ve: - Possitive Test

Enzyme Activity: -

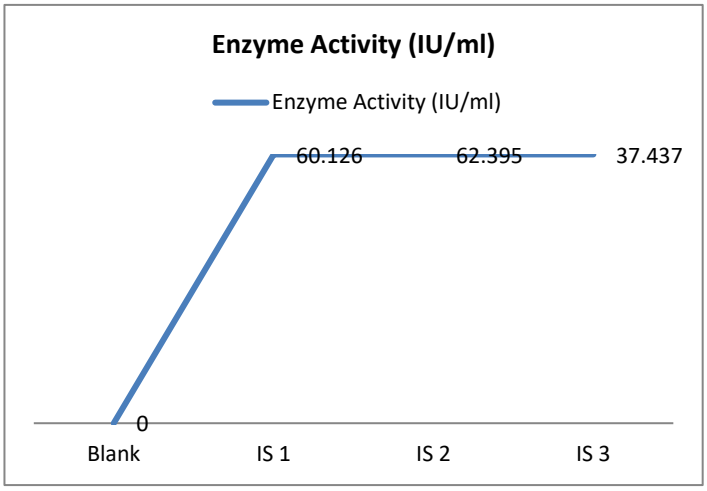
Three isolates were evaluated for xylanase activity using DNSA method. The reducing sugar obtained on degradation of xylan was estimated using DNSA.

Isolates	OD	Conc (ug/ml)	Conc (µmoles/ml)	Enzyme Activity (IU/ml)
Blank	0.0	0	0	0
IS 1	0.53	902.67	6.012	60.126
IS 2	0.55	936.73	6.239	**62.395**
IS 3	0.33	562.04	3.743	37.437

TABLE – 4 (XYLANASE ACTIVITY)

Graph - 1

Xylanase activities of the crude enzyme of selected three bacterial isolates were screened quantitatively by DNSA. It was found out that IS 2 bacterial isolate showed maximum Xylanase activity i.e. 62.395 IU/ml.



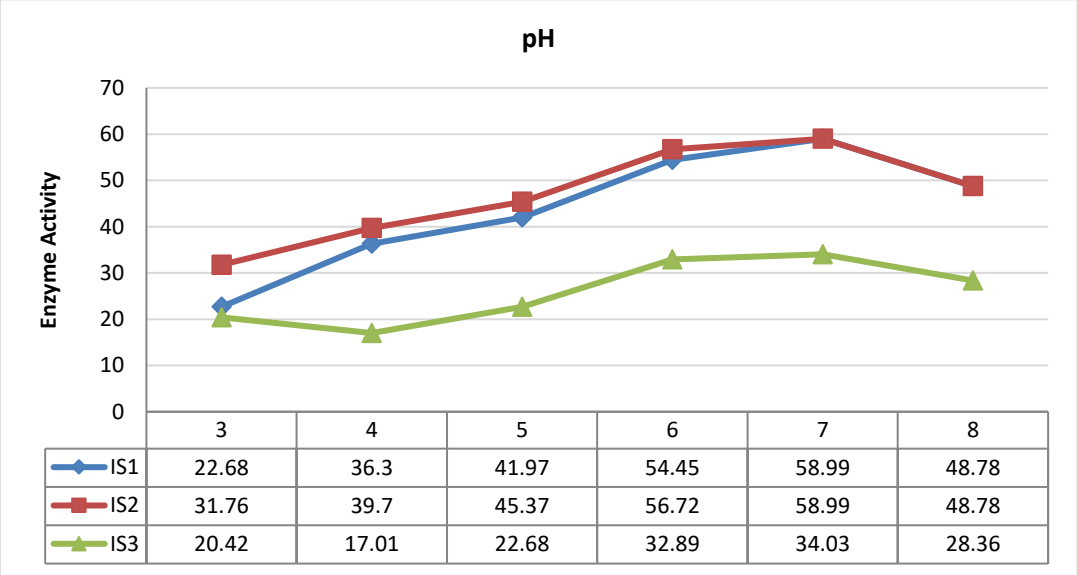
Optimization: -

pH: -

Highest Xylanase activity was observed for IS 1 and IS 2 at pH 7 i.e. 58.99 IU/ml.

pH	OD of IS 1	Enzyme Activity of IS 1 (IU/ml)	OD of IS 2	Enzyme Activity of IS 2 (IU/ml)	OD of IS 3	Enzyme Activity of IS 3 (IU/ml)
3	0.20	22.68	0.28	31.76	0.18	20.42
4	0.32	36.30	0.35	39.70	0.15	17.01
5	0.37	41.97	0.40	45.37	0.20	22.68
6	0.48	54.45	0.50	56.72	0.29	32.89
7	0.52	**58.99**	0.52	**58.99**	0.30	**34.03**
8	0.43	48.78	0.43	48.78	0.25	28.36

TABLE – 5 (Xylanase activity at different pH)



	3	4	5	6	7	8
IS1	22.68	36.3	41.97	54.45	58.99	48.78
IS2	31.76	39.7	45.37	56.72	58.99	48.78
IS3	20.42	17.01	22.68	32.89	34.03	28.36

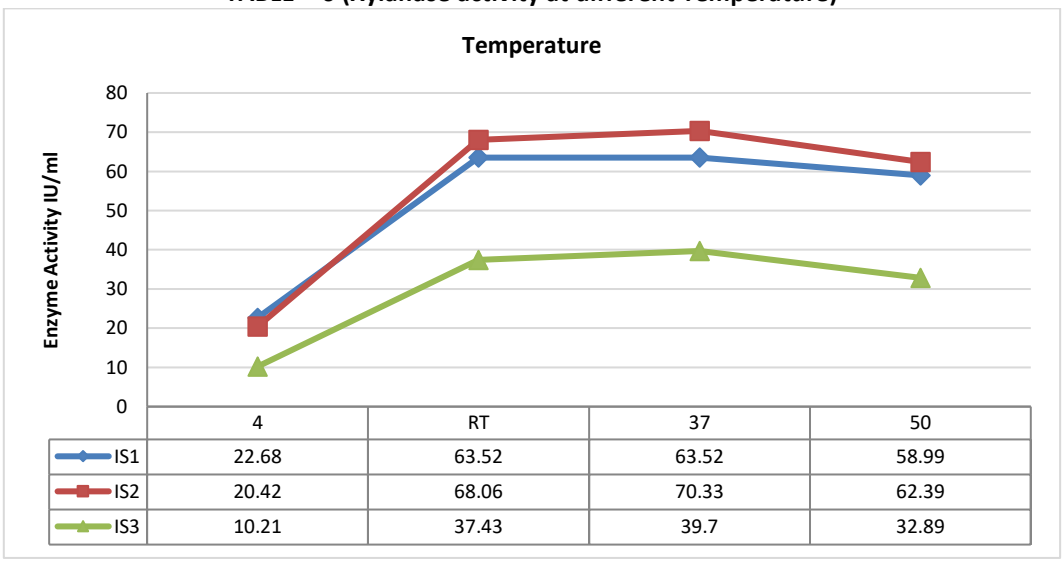
Graph – 2

Temperature: -

Highest xylanase activity was observed for IS 1 and IS 2 at temperature 37°C i.e. 63.52 IU/ml & 70.33 IU/ml.

Temp	OD of IS 1	Enzyme Activity of IS 1 (IU/ml)	OD of IS 2	Enzyme Activity of IS 2 (IU/ml)	OD of IS 3	Enzyme Activity of IS 3 (IU/ml)
4°	0.20	22.68	0.18	20.42	0.09	10.21
RT	0.56	**63.52**	0.60	68.06	0.33	37.43
37°	0.56	**63.52**	0.62	**70.33**	0.35	**39.70**
50°	0.52	58.99	0.55	62.39	0.29	32.89

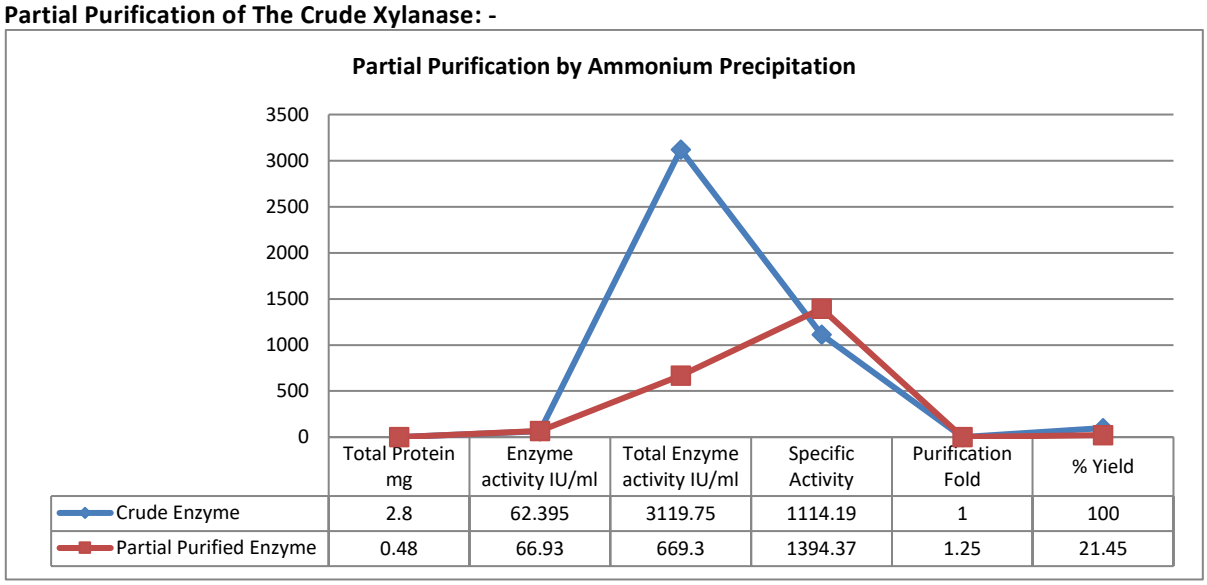
TABLE – 6 (Xylanase activity at different Temperature)



	4	RT	37	50
IS1	22.68	63.52	63.52	58.99
IS2	20.42	68.06	70.33	62.39
IS3	10.21	37.43	39.7	32.89

Graph – 3

Partial Purification of The Crude Xylanase: -



Graph – 4

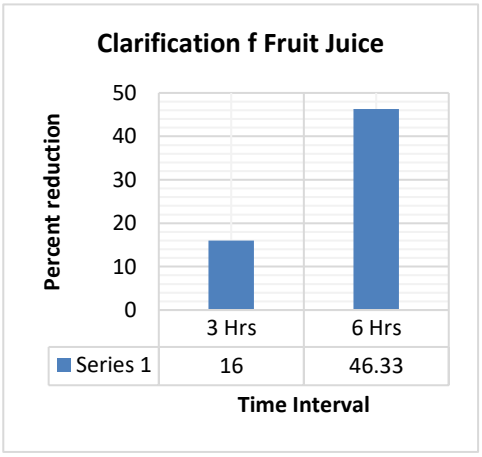
Total Xylanase Activity in sample (10 ml) was 66.93 IU/ml with 21.45% recovery and 1.25 purification fold with specific activity of Xylanase was 1394.37 U/mg Protein.

Application: -

Clarification of fruit juice (mausambi juice): -

Sr No	Juice Sample	Time hrs	Control	Test	% Clarity
1	Mausambi	3	0.12	0.10	16 %
2	Mausambi	6	0.12	0.07	46.33%

TABLE – 7 (JUICE CLARIFICATION)



Graph - 5

CONCLUSION:

Xylan being a hemicellulose with diverse structures due to the presence of many side chains and bonds need a complex enzyme called as xylanase for its hydrolysis to form monomeric subunits. It is also found in other microorganisms like bacteria, gut of termites, algae, gut of rumens etc. Juice clarification as an application of xylanase was also carried out. It estimates the amount of reducing sugar present in the sample. After 6 hrs of incubation at 30° C the clarification of mausambi juice was estimated to be 46.33%.

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