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Application of Xylanase produced by *Bacillus megaterium* in Saccharification, Juice clarification and oil extraction from Jatropha seed kernel

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Abstract:*Xylanases are enzymes that catalyze the hydrolysis of linear polysaccharide beta-1,4-xylan into xylose. The present study involves isolation of xylanase producing bacterium Bacillus megaterium from rice paddy soil followed by enzyme production in solid state fermentation and then its applications.*
After the isolation of xylanase producing organism, enzyme production was carried out and then crude xylanase was purified with 40-60% saturation of ammonium sulphate. Purified xylanase was then used to carry out saccharification of untreated, acid treated and alkali treated sugarcane bagasse. And the amount of reducing sugars produced by untreated bagasse (3200µg/ml) was found to be more as compared to acid treated (1460 µg/ml) and alkali treated (1760 µg/ml) bagasse.
Clarification of apple, pineapple and tomato juice were carried out using purified xylanase as these fruits contain more amount of hemicelluloses. Xylanase was found to efficiently clarify pineapple juice (44%), tomato juice (16%) and apple juice (20%).
Aqueous oil extraction from Jatropha seed kernels was enhanced when it was inoculated with the xylanase producing Bacillus megaterium. Oil extraction increased by 26.47 % as compared to control in which no organisms were added. Hence xylanase produced by Bacillus megaterium can be used in wide variety of applications which are of commercial significance.
Key words:*Xylanase, Bacillus megaterium, saccharification, Juice clarification, oil extraction.*

I. Introduction

Xylan is the most abundant hemicelluloses present in both hardwoods and annual plants and accounts for 20-35% of the total dry weight in tropical plant biomass. Xylan is found mainly in the secondary cell wall [1]. It is a heterogeneous polysaccharide consisting of β-1,4-linked D-xylosyl residues on the back bone but also containing arabinose, glucuronic acid and arabinoglucuronic acids side chains[2].Owing to its higher structural complexity, xylansaccharification involves two principal enzymes and several accessory ones. Endo-β-1,4-xylanases randomly cleave β-1,4-glycosidic linkages in the xylan main chain, releasing xylooligosaccharides, while β-xylosidases release xylose from xylobiose and xylooligosaccharides. Accessory enzymes, including α-L-arabinofurosidase remove side groups from the xylan main chain[3].

Xylanase are produced extracellularly by bacteria, yeast and filamentous fungi. The fungal genera Trichoderma, Aspergillus, Fusarium, and Pichiaare considered great producersof xylanases. Xylanases have been reported in Bacillus, Streptomyces etc.[4].

The technique of solid-state fermentation involves the growth and metabolism of microorganisms on moist solids in the absence or near absence of any free-flowing water. These fermentation systems, which are closer to the natural habitats of microbes, may prove more efficient in producing certain enzymes and metabolites [5].Moreover, the use of low cost substrates for the production of industrial enzyme is one of the ways to greatly reduce production costs. This can be achieved using solid agricultural waste materials such as wheat bran, rice husks, corn cobs etc as substrates.

In recent years, interest in xylan degrading enzymes have been increased due to their applications in various agro-industrial processes. The potential applications of xylanase include bleaching noncotton cellulosic fibres, melting hemp and linen fibres, clarifying fruit juice and wines and also increasing the brightness of pulp, breaking up the dough, etc [6]. The purpose of this study was to investigate applications of xylanase produced by Bacillusmegaterium in saccharification, juice clarification and oil extraction.

II. Materials And Methods

Isolation and screening of xylanase producing organisms:

In order to isolate xylanase producing organisms soil samples were collected. After enrichment, xylanase producing organisms were isolated on xylan agar plates, incubated at 37^{o}C for 24hrs.. Screening of isolates was performed for the quantitative detection of xylanolytic enzyme under submerged fermentation by xylan broth. After 72hrs of incubation, the aliquots of the medium were centrifuged at 1000rpm for 10mins and supernatant

was used as enzyme source. The organism producing maximum amount of xylanase was identified using Bergey's Manual.

Xylanase assay

The reaction mixture containing 0.9ml of 1%Birch-wood xylan prepared in 0.1 M sodium phosphate buffer (pH 7.0) as the substrate and 0.1ml of enzyme was incubated at 50°C for 10 min. The enzyme activity was determined by measuring the release of reducing sugars during the enzyme-substrate reaction using DNSA method[7].

Protein estimation

The soluble protein was determined by Folin's method using bovine serum albumin as standard. [8].

Xylanase production under Solid State Fermentation (SSF)

For production of xylanase by SSF, the isolate was grown on wheat bran (10g) plus mineral salt solutions. The mineral salt was added in such a way that the final substrate to- moisture ratio was 1:2. After sterilization of the media by autoclaving, the flasks were inoculated with 10% (v/w) 24 h grown inoculums. The enzyme was extracted with 0.1 M Sodium phosphate buffer, pH 7.0 and squeezed through muslin cloth. The enzyme extract was centrifuged at 4°C for 20 min at 10,000 rpm and cell free supernatant was used as crude enzyme for analysis[9].

Enzyme purification

The xylanase was precipitated with 40-60% saturation of ammonium sulphate. The precipitate was allowed to settle overnight at 4^oC. The precipitates were collected by centrifugation at 10,000 rpm for 15min. Pellet of enzymes were dissolved in phosphate buffer (0.1M, pH 7) and subjected to dialysis overnight against the same buffer[10].

Saccharification of Sugarcane bagasse

Sugarcane bagasse was washed, dried in oven and powdered. Then before subjecting it to saccharification, pretreatment of sugarcane bagasse was carried out. Two separate pretreatments were carried out. In one case pretreatment was given by preparing 10% (w/v) slurry of substrate in 1N NaOH and the second pretreatment was given by preparing 10% (w/v) slurry of substrate in 1N H_2SO_4 and was incubated for 12 h at room temperature. After pretreatment the substrates were washed with distilled water till neutrality and dried in oven at 80°C[2].

The reaction system for saccharification consisted of 20 ml 100mM sodium phosphate buffer (pH 7.0) containing 2.5 g% of substrates (untreated and pretreated) and 1600 U/g of xylanase enzyme. Controls were kept for each reaction in which the heat inactivated enzyme was added. The samples were removed at every 2hr and checked for total reducing sugars by DNSA method [2].

Qualitative sugar analysis from hydrolyzates obtained by enzymatic hydrolysis of pretreated and untreated lignocellulosic substrates was done on TLC plates of Silica Gel [2]. Suitable standards of Xylose (20mg/ml), Arabinose (20mg/ml) and Glucose (20mg/ml) were used. The solvent phase comprised of Acetonitrile and water (85:15) Sugars were detected by spraying 0.2% orcinol in the mixture of methanol and H2SO4(90:10) followed by heating at 100^oC for 10mins in oven.

Clarification of apple, tomato and pineapple juice.

Tomato (Lycopersiconesculentum), Pineapple (Ananascomosus) and Apple (Malusdomestica) was purchased from local markets. They were washed thoroughly with water, and macerated using a blender to form a smooth textured pulp[11] .

The pulps obtained were then clarified using the Xylanase enzyme obtained as under:

Pineapple juice: 10g pulp + 10 U/g ofenzyme [11].

Tomato juice: 10g pulp + 20 U/g of enzyme [12] .

Apple juice: 10g pulp + 20 U/g of enzyme [13].

The enzyme and pulp was incubated for 4hrs at 37^oC. After incubation, the enzyme was inactivated by heating the suspension in a boiling water bath for 5 min. The pulp was then cooled and filtered through muslin cloth. The filtrate was centrifuged at 10,000rpm for 15 min. The supernatant (juice) was used for determining juice clarity[13].

The untreated pulp for each of the fruit was kept as control.

Juice clarity was measured by measuring the absorbance of juice at 650 nm since at this wavelength other browning components do not interfere with the measurements[11].

Oil extraction from Jatropha seed kernel.

To prepare bacterial starter culture, 50ml Nutrient broth was inoculated with xylan degrading bacteria and incubated at RT on shaker. The nutrient broth medium was initially supplemented with 1.0% w/v Jatropha kernel before autoclaving [14].

J. curcas seeds were cracked and the husks (outer layer) were carefully removed. To prepare the oil seed slurry, 150g kernel powder was blended with 750g demineralized water for 5min using a home blender. Under constant stirring to keep the slurry homogenous, 30g of kernel slurry (equivalent to 5g kernel) was weighed out and used for the oil extraction [15].To extract the oil, 30.0 g of Jatropha kernel slurry was inoculated with 2.5ml of the bacterial starter culture. The mixture was incubated 37 °C for 24hrs. After incubation, the slurry was centrifuged at 7,400rpm for 15 min. The free oil on the surface of the liquid in the centrifuge tube was assayed gravimetrically [14]. Flask containing only Jatropha kernel slurry was kept as control.

Petroleum ether (PE 40-60 ^{O}C) was used to dilute the free oil after microbial extraction. The free oil on the surface of the liquid in the centrifuge tube was diluted with 3ml petroleum ether without shaking (to prevent oil extraction from emulsion) over a minimum of 6h. The top organic phase was carefully removed. The organic phases were collected in a pre-weighed dish and dried at 105^{o}C for 1.5-3h until constant weight was reached [15].

III. Results And Discussion

Isolation, screening and identification

After enrichment, xylanase producing organisms were isolated on xylan agar plates. Seven predominant cultures were obtained. After screening on Congo Red Assay agar plates, the organism producing maximum amount of xylanase was selected for further studies. This isolate was identified as Bacillus megaterium using Bergey's Manual.

Fig 1: Congo red assay

Enzyme purification

The crude xylanase was precipitated with 40-60% saturation of ammonium sulphate.

Enzyme	Total protein (mg)	Enzyme activity (U/ml)	Specific activity (U/mg)	% Yield	Purification Fold
Crude enzyme	20	256	12.8	100	1
Purified enzyme	17	620	36.4	85	2.84

Table :1 Enzyme Purification

Saccharification of Sugarcane bagasse

Effective utilization of lignocellulosic wastes require bioconversion to sugar followed by fermentation to bio alcohol. Xylan can be hydrolysed by xylanase into xylose and xylo oligosaccharides, which can be further converted to ethyl alcohol. Enzymatic hydrolysis of lignocellulosic biomass, such as agricultural residues, with subsequent fermentation of sugars into ethanol has long been recognised as an alternative to the existing starch and sucrose based ethanol production [16].

Before saccharification, sugarcane bagasse was pretreated with NaOH and H_2SO_4. Pretreatment of substrate reduces the lignin content ofthe agroresiduesand increases the porosity of the substrate.

Figure 2 : Untreated and pretreated sugarcane bagasse (After drying).

Untreated, alkali treated and acid treated sugarcane bagasse was subjected to saccharification for 8hrs. The reducing sugar content was determined after every 2hrs by DNS assay.

Time (hrs)	Conc of Reducing sugars (µg/ml)			Conc of Reducing sugars (mg%)		
	Untreated	Alkali treated	Acid treated	Untreated	Alkali treated	Acid treated
0	440	180	180	44	18	18
2	920	520	440	92	52	44
4	2080	800	600	208	80	60
6	2800	1140	880	280	114	88
8	3200	1760	1460	320	176	146

Table 2: Saccharification of Untreated, alkali treated and acid treated sugarcane bagasse.

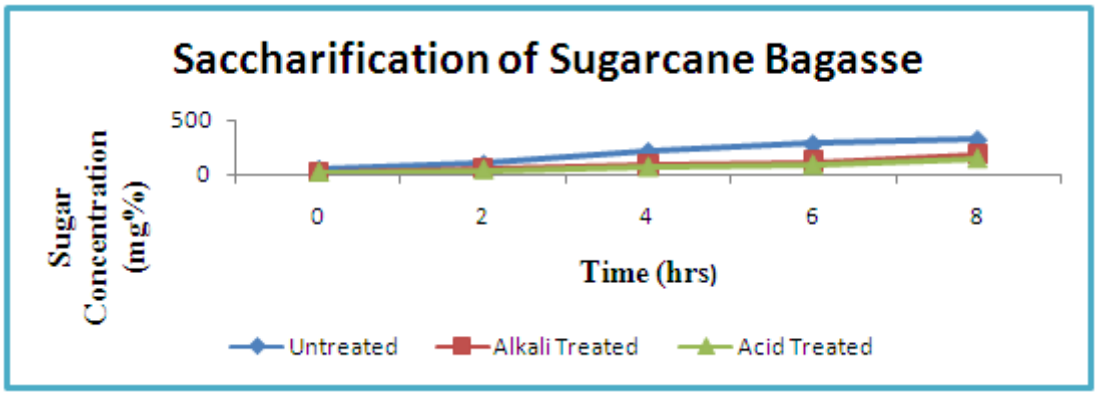

Fig 3 : Saccharification of Untreated, alkali treated and acid treated sugarcane bagasse

Saccharification of untreated, alkali treated and acid treated sugarcane bagasse was carried out for 8hrs. As the time increased, the amount of sugar produced from saccharification also increased. However untreated sugarcane bagasse produced greater amount of sugars as compared to the pretreated sugarcane bagasse. At the end of 8hrs, untreated sugarcane bagasse produced 3200µg/ml of sugar, whereas alkali treated produced 1760 µg/ml and acid treated produced 1460 µg/ml of sugars.

However in earlier studies, pretreated substrates such as wheat straw and wheat bran have produced greater amount of sugars[2,16]. But in this study the untreated substrate produced more reducing sugars, hence from this it can be said that xylanase produced by Bacillus megaterium does not require any pretreatment before efficient saccharification.

Qualitative sugar analysis from hydrolyzates obtained by enzymatic hydrolysis of pretreated and untreated sugarcane bagasse was done by TLC.

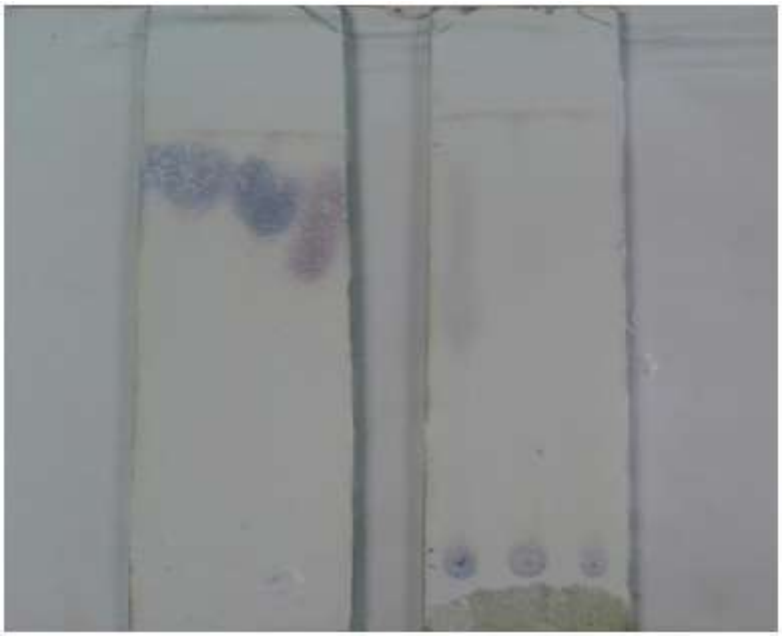

Fig 4: TLC of saccharified sugarcane bagasse**.**

Plate 1 Standards : Spot 1→ Arabinose; Spot 2 →Dextrose; Spot 3 → Xylose.
Plate 2 Test : Spot 1→ Untreated substrate; Spot 2 →Alkali treated substrate ; Spot 3→ Acid treated substrate.

SAMPLES	Arabinose	Dextrose	Xylose	Untreated	Alkali treated	Acid treated
Rf values	0.88	0.81	0.74	0.71	---	---

Table 3: Rf values in TLC

TLC of saccharified sugarcane bagasse was carried out after 2hrs of saccharification. Spot was developed in the sample containing untreated sugarcane bagasse. However no spot was found in the samples containing alkali and acid treated sugarcane bagasse. This may be because the concentration of reducing sugars present in them may be low. When the Rf value of the untreated sample was compared with that of standards, its Rf value was found to be similar to that of xylose.

Thus xylanase was efficiently able to carry out saccharification of sugarcane bagasse. The sugars obtained can now be used for production of bioethanol. As the cost of xylanase and substrate for the production of these sugar and bioalcohol plays a constraint in commercial production, usage of agricultural residues and microbial xylanase might be an easy solution not only for reducing the cost of sugar production but also for successful waste disposal.

Clarification of apple, tomato and pineapple juice

Raw fruit juices are cloudy, viscous and turbid due to the presence of polysaccharides (starch, pectin, cellulose, and hemicelluloses) and lignin. Mostly juices are pectin rich but fruits such as tomato (Lycopersiconesculentum), pineapple (Ananascomosus) and apple (Malusdomestica) contains a small amount of pectin but high hemicellulose content [11]. Since the major fraction of hemicellulose is composed of xylan, the application of xylanase in juice clarification was carried out.

In this study, apple, pineapple and tomato were incubated with enzyme for 4hrs and the clarity of juice was checked.

Juice	**Absorbance at 650nm**		**% Clarity**
	Control	**Test**	
Pineapple	0.16	0.09	44%
Apple	0.12	0.10	16%
Tomato	0.10	0.08	20%

Table 4: Juice clarification**.**

Xylanase produced from Bacillus megaterium was efficiently able to cause juice clarification. Maximum juice clarification was obtained in pineapple juice (44%) when it was treated with 10U/g of enzyme.

16% and 20% clarification was obtained in tomato and apple juice respectively. In study by[11] Pal et al., 2011 treatment of pineapple juice with xylanase produced by Aspergillusniger gave juice clarification of 64%. Also, xylanase produced by Bacillus pumilus, produced juice clarification of apple (22%), pineapple (19%) and tomato (15%)[13].

Oil extraction from Jatropha seed kernel.

J. curcas seed kernels have a high fat and protein content. The oil is used as biofuel, whereas the protein has been extensively studied for food and non-food application. In protease-assisted aqueous oil extraction from oilseeds, oil-bound proteins are hydrolyzed into smaller fractions, thereby altering their structure and functionality. If the protein structures are to be conserved to a large extent in the recovery of oil from oilseeds, the use of bacterial strains or enzymes liberating oil by other means than protein solubilisation is a reasonable choice[14].

Therefore use of xylan degrading bacteria which causes oil liberation without the degradation of protein was studied.

After inoculating Jatropha kernel slurry with Bacillus megaterium (xylan degrading bacterium) and incubating it for 24hrs, oil was extracted and was estimated gravimetrically. Total oil in Jatropha seed kernels was 0.47 kg/kg. The total oil content was taken as 100% recovery of oil while calculating the oil yield [15].

Sample	Oil extracted/ gram	% Yield
Control	0.026	5.53
Test	0.152	32

Table 5: Xylanase assisted oil extraction from Jatropha kernels.

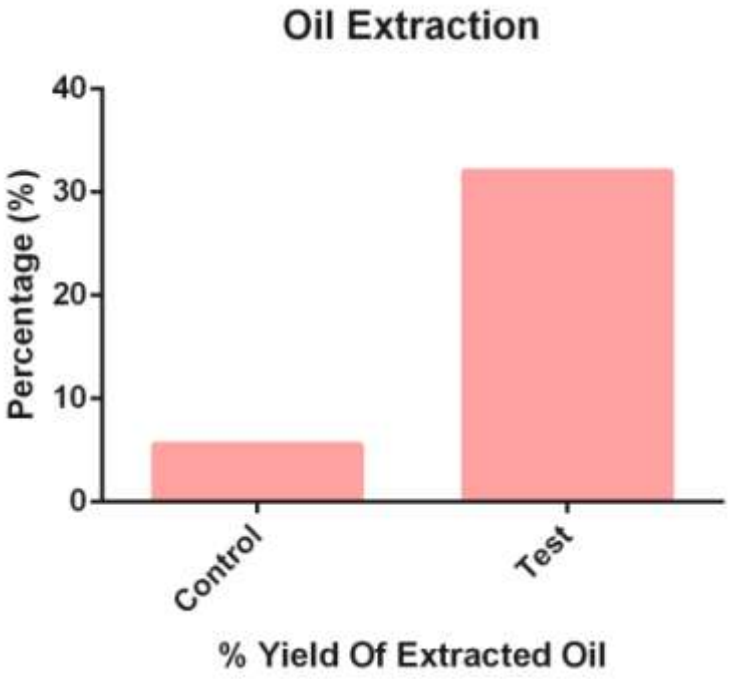

Fig 5: Xylanase assisted oil extraction from Jatropha kernels**.**

Fig 6 : Extracted oil (Control and Test).

During oil extraction, control was kept which contained only Jatropha kernel slurry without inoculating in it. Little amount of oil was liberated in the control, this may be due to grinding of kernels. However, oil liberation was enhanced by xylan degrading bacterium Bacllius megaterium.

Amount of increased oil liberation = Oil extracted in TEST – Oil extracted in CONTROL
= 32 – 5.53
= 26.47 %

Therefore the increased the oil extraction in the test was by 26.47 %. Hence it can be concluded that xyalanse secreted from Bacillus megaterium assisted in oil extraction from Jatropha kernels.

IV. Conclusion

Bacillus megaterium produced xylanase in high amount when grown on solid state conditions using cheaply available agroresidual substrate such as wheat bran. The use of agroresidues make the process of enzyme production environment friendly and subsequently reduces the cost of enzyme production. The results of this study demonstrated the potential of xylanase in production of sugar rich hydrolyzate which can be used for bioethanol production. The purified enzyme exhibited potential in clarification of fruit juices and hence could be exploited in food industry. Also, Bacillus megaterium facilitated oil liberation from Jatropha via degradation of hemicellulose. Hence xylanase produced by Bacillus megaterium can be used in wide variety of applications which are of commercial significance. However further research is needed to explore the full potential of enzyme production by this organism

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