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Production and Optimization of Xylanase Enzyme from *Bacillus subtilis* using Agricultural Wastes by Solid State Fermentation

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ABSTRACT

Background: Xylan, a major hemicellulosic polysaccharide found in the plant cell wall, represents up to 30-35% of the total dry weight of land plants. It is a heteropolysaccharide made up of a backbone of 1, 4-linked β-D-xylopyranosyl residues which can be substituted to varying degrees with glucopyranosyl, α-L-arabinofuranosyl, acetyl, feruloyl and/or p-coumaryl side chain groups. Hemicelluloses are used in various industries such as fuel, food, detergent, animal feed, agriculture, wine, beer, pharmaceuticals and cosmetics. The main objective of the study is to explore easy and cost effective method to produce the xylanase using sugar cane bagasse, wheat bran and rice bran as substrates, which is an agro waste. **Materials and Methods:** Xylanase producing bacteria were isolated from soil. Among the isolates, three strains show the maximum activity on xylan agar plates. Among the three strains, a strain was shows maximum enzyme activity, which was measured by Dinitro Salicylic Acid (DNS) method. The isolates were identified as *Bacillus subtilis* and which was identified by 16S rRNA gene sequence analysis. Optimization of the fermentation medium for production of maximum xylanase was carried out via solid state fermentation (SSF). **Results:** The highest production of xylanase was obtained on 3% xylose as a carbon source, 3% malt extract as a nitrogen source and 3% sugarcane bagasse at 40°C, pH 9, 3.5% Inoculum level at 48 hr. *Bacillus subtilis* are capable to produce the xylanase. **Conclusion:** The isolated strain *Bacillus subtilis* shows the maximum level of xylanase production at pH 9.0 and 40°C temperature on 48 hr incubation on 3% xylose, 3% malt extract and 3% sugarcane bagasse as a substrate during solid state fermentation.

Key words: *Bacillus subtilis*, Xylanase, Sugarcane bagasse, Wheat bran, Rice bran.

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INTRODUCTION

Xylanase is the most abundant noncellulosic polysaccharide. It is more heterogeneous polysaccharides and the second most abundant organic structure in the plant cell wall and is considered to be forming an interphase between lignin and other polysaccharides. *The* enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose. Hemicellulose plays a major role in micro-organisms thriving on plant sources for the degradation of plant matter into usable. The growth of agroforestry activity increases the agricultural and forestry waste which mainly consisted of lignocellulose. Lignocellulose is a sugar in non-degradable polymer form. About 25-40% of lignocellulose biomass consisted of xylan. Xylanase is a complex enzyme, includes endoxylanase (E.C.3.2.1.8), β-xylosidase (E.C.3.2.1.37), α-glucuronidase (E.C.3.2.1.139), α-arabinofuranosidase (E.C.3.2.1.55) and acetylxylan esterase (E.C.3.1.1.72).[1]Endoxylanases catalyzes the random hydrolysis of xylan to xylooligosaccharides, while β-xylosidase releases xylose residues from the non-reducing ends of xylooligosaccharides. Xylanase attracts attention of many researchers due its action to breakdown xylan into commercial product such as low calories sugar-xylose and L-arabinose[2] prebiotic.[3] Xylanases have numerous applications in the food and feed industries[4] and can be applied for the production of several useful economic products such as SCPs (Single Cell Proteins), sugar syrups and liquid and gaseous fuels.[5]

Apart from these, xylanases have found promising applications in dough softening, bleaching agent in pulp and paper industries and increase the effectiveness of detergent cleaning, debarking, production of acidic oligosaccharides, deinking-destaining process, fuel-alcohol production and in extraction and clarification of flavouring-agents, pigments and fruits juices.[6] Xylanases are produced by fungi, bacteria and yeast.[7] Bacterial genera, such as *Bacillus* spp, *Cellulomonas*, *Micrococcus*, *Staphylococcus*, *Paenibacillus*, *Arthrobacter*, *Microbacterium*, *Pseudoxanthomonas* and *Rhodothermus*[4,8] and some fungi species such as *Trichoderma* and *Aspergillus* spp.[9] Selection of potential isolates remains a tedious task especially when physiologically potential strains are *B. subtilis*, *B. stearothermophilus*, *B. amyloliquefaciens*, *B. circulans* and *B. pumilus*[10] to be obtained to achieve maximum enzyme production. Industrial production of enzymes on large scale is associated mainly with good source of substrate. The use of agriculture residues like sugar cane bagasse, wheat bran and rice bran as low-cost substrates for the production of industrial enzymes is a significant way to reduce production cost. The fermentation technique using solid state substrate has the great advantage compared with submerged fermentation due to absence of aqueous phase that provides natural habitat for growth of microorganisms. Microbial enzymes attract researchers because of their role in biological and commercial processes. At present, the largest part of the hydrolytic enzyme market is occupied by the xylanase. Aim of the study production and optimization of xylanase enzyme from *Bacillus subtilis* using agricultural wastes by solid state fermentation.

MATERIALS AND METHODS

Screening and Isolation of Xylanase producing microbes

The soil samples were collected from agriculture field in Rasipuram, Namakkal, Tamil Nadu. Collected samples were gently crushed and used

for isolation by serial dilution technique. Serially diluted samples were spread on xylan agar medium containing birch wood xylan (0.5%.). From (HiMedia) medium the inoculated plates were incubated at 37°C for 24 hr to 48 hr. After incubation to visualize the hydrolysis zone the plates were flooded with an aqueous solution of 0.1% Congo red for 15 min and washed with 1M NaCl. Degradation of hemicellulose was indicated by the presence of a clear zone around the bacterial colonies.

Identification of the isolated microorganisms

The isolated organisms were identified by morphological examination and biochemical characterizations. Morphological test, Gram's staining, Endospore Staining, Motility test. Biochemical test Indole test, methyl red test, Voges Proskar test, citrate utilization test, catalase test, oxidase test nitrate reduction test and Carbohydrate fermentation test by standards methods. 16S rRNA sequencing was carried out to identify the species.

Phylogenic Analysis

The partial 16S rRNA sequences was retrieved on NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLAST tool.

Maintenance of Bacterial Isolates

The isolated bacterial culture was maintained in Nutrient Agar slants and stored at 4°C to 7°C for future use. Sub culture was performed every ten days interval.

Production Media

The production medium containing, 0.5% birch wood xylan, 0.2% yeast extract, 0.25% NaCl, 1.5% KH_2PO_4, 3% NaH_2PO_4 0.5% NH_4Cl and 0.025% $MgSO_4$ $7H_2O$, pH 7.0[11] broth was prepared in 100 ml conical flask and pH was adjusted to 7.0. The broth was sterilized and allowed to cool. Following cooling the medium was inoculated with a loop full of selected isolate. Then flask was incubated at 37°C for 48 hr on a rotary shaker (120 rpm). After incubation the extra cellular enzyme separated by centrifugation at 10,000 rpm for 10 min at 4°C (REMI Cooling Centrifuge). The supernatant obtained served as the source of extracellular crude enzyme.

Xylanase enzyme assay

Xylanase activity was measured by Dinitro Salicylic Acid (DNS) method.[12] Add 3 ml of DNS reagent to 3 ml of enzyme sample in a lightly capped test tube. Heat the mixture at 55°C in water bath for 5-15 min to develop the red-brown colour. Add 1 ml of a 40% potassium sodium tartrate solution to stabilize the colour. After cooling to room temperature in a cold water bath, note the absorbance with a spectrophotometer at 575 nm.

Optimization of Xylanase Production by Isolated Bacteria

Production and optimization of xylanase from the isolates by using different physicochemical characters viz., incubation period, temperature, pH, carbon source, nitrogen source and inoculums level were studied to optimize the enzyme production.[13]

Substrates for Xylanase production

Agricultural by products rich in hemicellulosic and other nutrients can be exploited as cheap raw material for the production of industrially important enzymes. Various agro-wastes such as sugarcane bagasss, rice bran and wheat bran[14] which are cheaper can be used as substrates and thus reduce the cost of enzyme production. Xylanase production was carried out by solid state fermentation.[15]

RESULTS

Isolation and Screening of the Xylanase Producing Bacteria

The xylanase producing bacteria were isolated from agriculture field soil samples by serial dilution method and spread plating on xylan agar. Xylan agar is a selective media for growth of the xylanase producing bacteria can only utilize xylan as the carbon source. The screenings of the xylanase producing bacterial isolates were performed based on the clearing zone around the colony on the xylan agar medium. The appearance of clearing zone around the colony after the addition of 0.1% congo red solution was strong evidence that the bacteria produced xylanase in order to degrade hemicellulose.

The clear zone around the colony bacterial strains were identified as efficient xylanase producing bacteria and the isolate *Bacillus subtilis* has shown maximum clear zone in diameter (Figure 1) and its initial identification was done by gram staining, colony morphology and biochemical tests. The isolate *Bacillus subtilis* has been used for further studies in the enzyme production and their ability to degrade hemicellulose.

Morphological and Biochemical Characteristics of *Bacillus subtilis*

The isolate *Bacillus subtilis* purified by repeated sub culturing on the nutrient agar medium at regular intervals and stored at 4°C. The isolates were identified based on the morphological and biochemical characteristics result summarized (Table 1).

Phenotypic Characteristics

The 16S rRNA gene sequencing was carried out by using the primers, 8F (5'AGAGTTTGATCCTGGCTCAG3') and 1541R (5'AAGGAGGTGATCCAGCCGCA3'). The PCR product was purified and sequenced as described previously. The nucleotide sequence of isolates was aligned with selected sequences obtained from GenBank by using version MK249655.

Phylogenic tree

Phylogenic tree is depicted in Figure 2.

Effect of pH on Xylanase Production

The effect of initial pH of fermentation medium on the production of xylanase was studied by growing the *Bacillus subtilis* in culture medium having different pH (pH-6.0 to pH-9.0). It was found that the maximum xylanase production was achieved when medium pH was kept at 9.0 as shown in (Table 2).

Effect of Temperature on Xylanase Production

The optimum temperature for enzyme production with selected isolate of *Bacillus subtilis* was found to be 30, 37, 40 and 45°C. When the optimum temperature of, maximum enzyme production found at 40°C by *Bacillus subtilis* 9.5 (U/ml) (Table 2).

Effect of Incubation time on Xylanase production

When the time is taken for the maximum enzyme production was studied, maximum yield was found to be at 48 hr of incubation for enzyme produced by *Bacillus subtilis* 8.3(U/ml) (Table 2).

Effect of Inoculum level on Xylanase Production

The effect of inoculums level on enzyme production was studied by inoculating different concentration of inoculum ranges from 1.5% to 4.5% in xylan medium. The different concentration of initial inoculums plays

a critical role in enzyme yield in production media. The media was inoculated with different concentration of inoculums and incubated at 40°C for 48 hrs. The maximum production obtained in 3.5% (6.8U/ml) (Table 2).

Effect of Carbon sources on xylanase production

Results obtained showed that 3% Xylose brought the highest xylanase production (8.1 U/ml) compared to other carbon sources at 48 hr incubation (Table 3).

Effect of nitrogen source on Xylanase production

The Nitrogen source plays an important role in xylanase enzyme production. It effect on enzyme production by *Bacillus subtilis* was studied by supplementing different nitrogen sources such as peptone, meat extract and malt extract into production media. Different nitrogen tested individually at the different concentration of 1, 2 and 3% in production media and is incubated at 40°C for 48 hr. The maximum xylanase enzyme production observed in media supplemented with 3% malt extract (7.2U/ml) as nitrogen source. The organic nitrogen sources were more suitable for optimizing the xylanase production by *Bacillus subtilis* (Table 3).

Solid State Fermentation

Production of xylanase enzyme was carried out with different substrates viz., sugar cane bagasse, rice bran and wheat bran used under solid state fermentation. Different concentration of each substrate was hydrated with mineral solution and adjusted. The moisture content was adjusted to 60%. 1.5% to 4.5% inoculums (24 hr grown bacterial culture and 1×10^6 / ml) was inoculated and incubated at room temperature 30 to 37°C for 40 to 45 hr for bacterial cultures. After incubation, 22 ml of saline was added in each flask and incubated in rotary shaker for 15 to 30 min at 120 rpm. From these result, it has been observed that, 3% sugarcane bagasse broth has a maximum yield of enzyme from *Bacillus subtilis* 7.5 (U/ml) (Table 3)

DISCUSSION

The results of the present study revealed that *Bacillus subtilis* can produce xylanase using sugar cane bagasse, rice bran and wheat bran as a cheapest substrates in solid state fermentation. In this study, bacterial strains were used for production of xylanase, which has advantage of short period of growth as compared to the fungi. Our study indicated that nutrients and cultural properties played a pivotal role in enzyme production.

Similar findings were also reported by Ayishal Begam *et al.* maximum xylanase from *B. pumilus* and *B. cereus* was observed at pH 8.[16] The production of Xylanase from *Bacillus pumilus* MTCC8964 at pH 6 was reported.[17] *Bacillus arseniciselenatis* DSM 15340 was 100% xylanase activity for 2 hr incubation at pH 10.0. Stability at the high pH values may be due to charged amino acid residues. The enzymes stable in alkaline conditions were characterized by a decreased number of acidic residues and an increased number of arginines.[18] Temperature is also an important factor that influence the xylanase yield maximum enzyme production by *Bacillus* sps was most active at 50°C to 60°C. Kang *et al.* purified two xylanases which gave the highest activity at 50°C. They showed relatively high stabilities at 50°C.[19] Many workers have reported different temperatures for maximum xylanase production either in flask (or) in fermentation studies using *Bacillus* sps suggesting that the optimal temperature. Similar study by Sepahy, reported optimum temperature of 37°C for xylanase production by *B. mojavensis* AG137 in submerges fermentation.[20] Different strains of *Bacillus* sp. shows maximum yield of xylanase production at incubation temperature of 45°C and 55°C.[21] The enzyme production is depends on the depletion of nutrients in the medium which stressed the bacterial physiology resulting in the inactivation of secretary machinery of the enzymes most of the *Bacillus subtilis* are maintaining log phase from 3 to 12 hr of its growth. This variation of different phase timing is based on the nutrients present in the medium and the cultural condition of the organism. Same result founded by Kundu and Majumdar, the maximum xylanase production (1.9 U/mL) by free cells was recorded at 48 hr by *Bacillus pumilus*.[22] Gau and Tiwari, have reported that the maximum xylanase production 689.2 U/mL by *B. vallismortis* RSPP-

Table 1: Analysis of Biochemical Characters for *Bacillus subtilis*.

Biochemical Test	Results
Indole test	Negative
Methyl Red test	Negative
Voges Proskar test	Positive
Citrate utilization test	Positive
Urease test	Negative
Triple Sugar Iron agar test	K/A
Endospore Staining	Positive
Oxidase test	Negative
Catalase test	Positive
Carbohydrate test:	
Glucose	AG
Lactose	A
Sucrose	A
Manitol	A
Maltose	A
Motility test	Motile

K/A- Alkaline Slant Acid butt, AG-Acid Gas, A-Acid

Table 2: Different temperature, pH, incubation period and inoculums level on xylanase enzyme production.

Different Substrate	Enzyme Activity (U/ml)															
	Temperature (°C)				pH				Incubation period (hrs)				Inoculums level %			
	30	37	40	45	6	7	8	9	24	36	48	60	1.5	2.5	3.5	4.5
S cane bagasse	7.5	8.7	9.5	9.1	6.5	7.0	7.6	8.0	7.6	8.0	8.3	7.6	5.1	5.9	6.8	6.2
Rice bran	6.3	6.7	7.1	7.5	4.5	4.9	5.6	5.9	5.6	5.9	6.4	6.7	4.3	4.7	5.2	5.6
Wheat bran	6.5	6.7	6.9	7.2	5.3	5.8	6.3	6.0	5.1	5.6	5.9	6.2	3.8	4.2	4.5	4.9
MD	6.76	7.38	7.83	7.93	5.4	5.90	6.50	6.33	6.1	6.5	6.86	6.83	4.40	4.93	5.50	5.56
SD	0.64	1.15	1.44	1.02	1.00	1.05	1.01	1.18	1.26	1.25	1.26	0.70	0.65	0.87	1.17	0.65

Table 3: Different carbon source, Nitrogen source and agro wastes on xylanase production.

	Enzyme Activity (U/ml)								
	Agro wastes			Carbon sources			Nitrogen source		
Different concentration	SCB	Wheat bran	Rice bran	Glucose	Fructose	Xylose	Peptone	Meat extract	Malt extract
1%	6.1	5.2	4.7	6.1	5.3	7.0	4.9	5.7	6.1
2%	6.9	5.7	5.3	6.6	5.7	7.5	5.8	6.3	6.5
3%	7.5	6.4	5.7	6.8	6.3	8.1	6.4	6.7	7.2
MD	6.83	5.76	5.23	6.53	5.70	7.53	5.7	6.2	6.6
SD	0.70	0.41	0.50	0.36	0.50	0.55	0.75	0.50	0.55

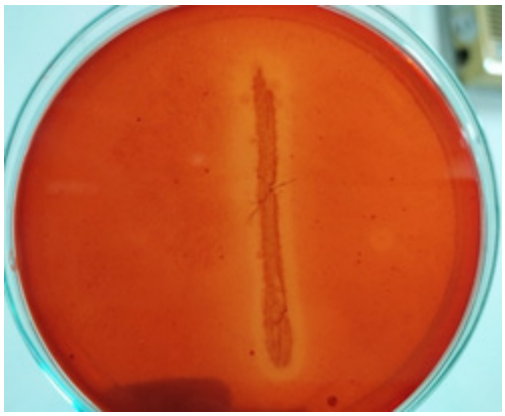

Figure 1: Congo red test for confirmation of Xylanolytic activity on Brichwood xylan medium by the isolated bacterial strain.

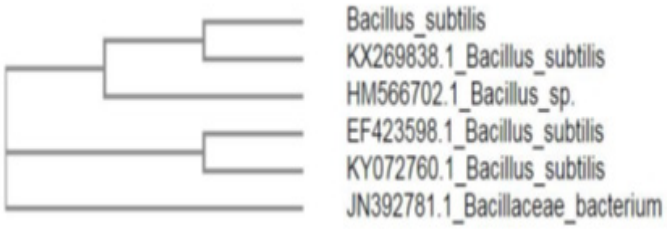

Figure 2: Phylogenic tree.

15 at 48 hr incubation.[23] Further increase in the incubation period did not increase the enzyme production but the stability of enzyme is 87% in 72 hr. Xylanase production was further enhanced by supplementing the fermentation medium with suitable additional carbon source. Sucrose and xylose at concentration of 0.5% were the best xylanase inducer by *B. subtilis* BS04 and *B. megaterium* BM07 in submerged fermentation respectively. Any other carbon sources like fructose, glucose, CMC (carboxymethyl cellulose) and arabinose were produce the less enzyme production.[24] Azeri *et al*. reported that different strains of *Bacillus* sp. and all exhibit maximum xylanase production by using birch wood xylan as a carbon source.[25] Saleem *et al*. reported that supplementation of sucrose to the fermentation medium significantly enhance the xylanase production by *B. subtilis*.[26] In 96 h of fermentation, wheat bran was best carbon source for xylanase production by *Streptomyces* sp.[27] In our study, we used malt extract as nitrogen source for media supplementation. Rahmani *et al*. studied nitrogen source were using cell growth on all of nitrogen source reach optimal at 96 hr fermentation, but casein give a greater cell growth than others.[28] All nitrogen sources not give the different activity until 72 hr, but urea mixed medium produce the higher activity than the others (4.06 U/mL) at 96 h fermentation. The results show that additional nitrogen sources in the media significantly can be increasing the activity of xylanase. Similar result were studied by Irfan *et al*. maximum yield of xylanase was observed at 2% with *Bacillus subtiltis* BS04 and 1.5% inoculum level with *B. megaterium* BM07.[24] Maximum yield of xylanase was observed at 2% with *Bacillus subtiltis* BS04 and 1.5% inoculum level with *B. megaterium* BM07. Increased level of inoculum reduced xylanase production in industrial fermentation process.[29] This may be due to the depletion of nutrients from the fermentation medium which resulted decline in enzyme synthesis. Sepahy *et al*. concluded that inoculum size of 2% was best for xylanase production by *B. mojavensis* AG137 in submerged fermentation.[20]

CONCLUSION

The isolated strain *Bacillus subtilis* shows the maximum level of xylanase production at pH 9.0 and 40°C temperature on 48 hr incubation on 3% xylose, 3% malt extract and 3% sugarcane bagasse as a substrate during solid state fermentation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVATIONS

DNS: Dinitro Salicylic Acid; **VP:** Voges proskauer; **rRNA:** Ribosomal Ribo nucleic acid; **U/ml:** Unit per milli liter; **SCB:** Sugarcanebagasse.

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