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Bioconversion of Sugarcane bagasse to Xylitol by *Bacillus mycoides* (OM169281.1)

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Abstract

The agro-domestic wastes deposited on waste lands can be used to develop a value-added product by converting it into xylitol. Production of xylitol from ligno-cellulosic materials of agricultural wastes is currently being done since it is cost-effective and eco-friendly. Xylitol (1,2,3,4,5-pentol) is a pentahydroxy sugar alcohol widely used in food, agricultural, and pharmaceutical industries. The study was aimed to convert sugarcane bagasse waste into a useful bioproduct xylitol. The agricultural wastes were collected, hydrolysed to extract xylose using suitable microbial strain. Screening and identification of efficient xylitol producing microbial strain was done. Among the isolates *Bacillus* sp produced significant yield of xylitol under solid state fermentation. The isolated bacterial strain was identified as *Bacillus mycoides* (OM169281.1) on the basis of phylogenetic analysis of 16S rRNA sequencing.

Key words : Sugarcane bagasse, hydrolysed, screening, identification, phylogenetic tree.

An economical and environmentally beneficial method of producing xylitol is through microbial fermentation of low-cost ligno-cellulosic materials. Numerous studies have previously effectively assessed the potential for producing xylitol from a number of yeasts, including *Candida, Saccharomyces, Pachysolan,* and *Pichia.* But bacteria can only produce a limited amount of xylitol compared to filamentous fungus and yeasts. The essential enzyme xylose reductase can also be utilized to directly bioconvert xylose to xylitol, although it requires a pure substrate for this process. Process modification and other methods of reducing the industrial production cost can help overcome the market cost of pure xylitol and the byproducts emitted during processing that impede its global adoption. Technological developments are also required to maximize yield and reduce product loss,

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particularly with regard to substrate and purification techniques for bulk yield. For the bioproduction of xylitol, other substrates high in xylose can also be employed[17].

A major supply of raw materials for most businesses, including paper, pulp, food, textiles, and others, comes from agriculture. Industries not only use significant portions of raw materials, but they also discard a lot of leftover ligno-cellulosic biomass in various forms. If this biomass is not treated, it poses a serious threat to both the environment and the industry[2]. There is $200x10^9$ tonnes of plant biomass altogether, of which 10% is available as ligno-cellulosic waste[21].

Significant amounts of sugar moieties are present in ligno-cellulosic biomass, which can be effectively utilized to make a variety of useful products, such as xylitol, by employing distinct hyper-producer microbial isolates. With the aid of many enzymes, microbes also lessen the stages involved in the xylitol synthesis process. To produce xylitol from industrial waste, microbial isolates need to be hyperactive and tolerant. As a result, the search for hyper-producers that can withstand the severe environmental conditions encountered in industrial processes must be ongoing. The extraction of xylitol with maximal product recovery from fermentation broth is another crucial factor that requires careful consideration. Traditionally, the purification process after activated charcoal treatment involves concentration and crystallization. However, ion-exchange resins can be used as a single step purification system to shorten the time needed for xylitol recovery and minimize product loss[17,27].

The high cost of the market and disregard for health are two things that influence the usage of xylitol. In order to lower production costs and raise awareness so that it becomes a part of a healthy lifestyle, ongoing research and development activities are needed. In the present study, the sugarcane bagasse wastes dumped in the wastelands were hydrolyzed by microorganisms isolated from that and are converted to a value-added product xylitol.

Collection and pretreatment of the substrate:

The agro-domestic waste sugarcane bagasse was sun dried for about 48 h and then ground, sieved and used for further experiments. The powdered substrate was then added in 0.25 N hydrochloric acid to remove the microbial load that may be present on the substrate. After the pretreatment, the substrate were kept in hot air oven at temperature of 80ºC for few hours[11].

Preparation of substrate hydrolysate and detoxification :

The pretreated substrate were then added with concentrated Hydrochloric acid at ratio 8:48 and heated for 121ºC for 1 h. Then the pH of the solution was then adjusted to 2 by using dilute hydrochloric acid. This enhanced the production of hydrolysate. After heating for one hour the hydrolysate was filtered using Whatmann no 1 filter paper to remove the unwanted materials. The hydrolysate containing impurities and toxic substances were removed by adding 2.5% activated charcoal, which was directly added to the hydrolysate and filtered using Whatmann no 1 filter paper. The pure substrate hydrolysate was collected in sterile conical

flask and were stored in refrigerator for further use[11].

Collection of soil sample :

The soil samples were collected from the location rich in sugarcane bagasse dumped sites near Ettimadai farm, Coimbatore, India (10.898061 Latitude and 76.90031 Longitude). The soil samples were collected aseptically using sterile polythene bags and using sterile spatula. The soil sample collected was stored at 4ºC for further analysis.

Enrichment and isolation of microorganism:

To enhance the growth of several xylose reductase-producing microbial strains, soil samples were collected from different places and introduced (1%, w/v each) individually to presterilized enrichment media with pH 7.0 and 5.0 in separate flasks containing xylose (10.0 g/L) and peptone (5.0 g/L). For 24 and 48 hours, respectively, these flasks were incubated at 37°C and 30°C to encourage the development of microorganisms at the corresponding temperatures. To encourage the growth of xylose-utilizing microbes by xylose metabolism, xylose was added to the enrichment media[3]. Samples from the incubated enrichment medium were then scattered onto agar plates that contained agar (20.0 g/L), peptone (5.0 g/L), and xylose (10.0 g/L). These plates were then incubated for further 48 hours at 37°C and 30°C. After producing a pure culture by repeating the aforementioned streaking technique at least three times, well isolated colonies were selected and kept for additional screening.

Maintenance of discrete isolates :

Nutrient agar slants containing peptone (5.0 g/L), NaCl (5.0 g/L), beef extract (1.50 g/L), yeast extract (1.50 g/L), and agar (20.0 g/L) with a pH of 7.0 were used to maintain the pure bacterial isolates. For maintaining fungal isolates, potato dextrose agar slants containing potato extract (200 g/L), dextrose (20.0 g/L), and agar (20.0 g/L) with pH 5.0 were used. Subsequently, the cultures of bacteria, yeast, and mold were kept at 4±1°C until needed again. These cultures were routinely kept alive by aseptic sub-culturing every two weeks.

Inoculum preparation :

The isolated cultures were inoculated in various flasks with inoculum media (50 mL) containing peptone (3.0 g/L) and dextrose (5.0 g/L), with pH adjusted to 7.0 for bacterial cultures and 5.0 for yeast and mold cultures. This was done in order to prepare the inoculum. Bacterial cultures were cultured in flasks at 37°C, whereas yeast cultures were incubated for 24 hours at 150 rpm at 30°C. On the other hand, the mold cultures were incubated for 48 hours at 30°C in stationary conditions. For bacterial and yeast fermentations, the inoculum was employed at a concentration of 10% (v/v); however, for mold cultures, 10% (v/v) of harvested spore inoculum produced in saline solution (0.85%, w/v) was used.

Screening of bacterial isolates :
Primary screening :

Bial's test is a chemical test done to identify the presence of pentose sugar in the given sample. Since xylitol is a pentose sugar

alcohol, the bial's test was performed for the qualitative analysis of carbohydrate. Bial reagents components include orcinol, hydrochloric acid and ferric chloride. 2 ml of the test sample was added and to that around 3ml of bial's reagent was added in appropriately labelled test tubes. These tubes were heated at 100°C for 4 minutes and then cooled. The hydrolysate was compared with commercial glucose and xylose as negative and positive control respectively[1].

Secondary screening :

The organism was further screened using fermentation media with xylose (10.0 g/L) and peptone (5.0 g/L) with pH 7.0 and 5.0 taken in different flasks and sterilized at 121°C at 15 psi for 15-20 min. Xylose was sterilized separately at 110°C for 30 min to prevent undesired color formation in fermentation medium aseptically before inoculation. The medium was inoculated with 10% (v/v) culture and incubated at 37°C and 30°C for 48 h and 96 h at 150 rpm agitation for bacterial and yeast cultures respectively. For mold, stationary conditions were provided for incubation at 30°C for 96 h. The bacterial and yeast biomass fermentation was harvested by centrifugation of fermentation broth at 5000 g for 10 min. The mold biomass was harvested by filtration using Whatmann filter paper followed by centrifugation. As the enzyme xylose reductase is intracellular in nature, the biomass was washed with phosphate buffer of pH 7.0 (0.1 M) to remove any media components before subjecting them to cell disruption process to extract enzyme. Using sonication, cells were disrupted for the extraction of enzyme. The cells were then removed by centrifugation at 5000 g for 15 min and supernatant thus obtained was analyzed for xylose reductase activity[28]. Among all the isolates, the most efficient xylose reductase producing bacterial isolates were selected for further studies.

Identification of selected bacterial isolate:

The most efficient xylose reductase producing bacterial isolate (BX -5 was identified and characterized using standard protocols of cultural, morphological, physiological, biochemical, genotypic and phenotypic methods[4,9,13,14,19,22].

Morphological and Colony characteristics:

The morphological characterization of the bacterial cells of selected isolate was carried out by observing them under the microscope after Gram staining. The cultural characteristics were observed by quadrant streaking of isolate culture on the nutrient agar plate. Several characteristics such as colony color, surface, elevation and margin were observed after incubating the Petri plates for 24 h at 37ºC [12].

Biochemical characterization :

Several biochemical tests were performed like methyl red test, Vogues-Proskauer test, gelatin liquefaction, citrate utilization, starch hydrolysis, oxidase, catalase and sugar fermentation test such as glucose, sucrose, fructose, lactose, maltose, xylose and arabinose by using Bergey's Manual of Systematic Bacteriology (9th Ed.)[10].

Molecular identification of bacterial isolate:

DNA from the *Bacillus* genome was

extracted as per standard phenol- chloroform method. Bacterial cultures were prepared and suspended in Luria-Bertani broth (Hi-Media, India) and incubated at 37°C for 24 hrs. The 24-hr old bacterial cells were pelleted by centrifugation and this was suspended in lysozyme and saline EDTA, after mixing incubated at 37°C for 30 minutes. To this 150µl of 10% SDS was added and incubated at 65°C for 15 minutes. Phenol, Chloroform and Iso-amyl alcohol in the ratio of 25:24:1 were added and undergone centrifugation. The aqueous phase was precipitated by adding double volume of isopropanol and washed with absolute ethanol. The DNA was suspended in 30µl of TE buffer and visualized by 0.8% agarose gel electrophoresis.

PCR :

The 16S rDNA sequences were amplified by polymerase chain reaction (PCR) using universal primers of 27f (5l-AGAGTTTGATCCTGTGCTCAG-3l) and 1492r (5l - GACTTACCAGGGTATCTAATCC-3l).

Reaction mixture :

For the polymerase chain reaction (PCR), a total of 50 µl of PCR reaction mixture was prepared having 5 µl of 1X Taq buffer, 5 µl of 200 µM of each deoxynucleotide, 1.5 µl of 0.3 µM of each forward and reverse primer, 0.25 µl of 5U Taq DNA polymerase and 2 µl of genomic DNA extract. Amplification of DNA for 50 µL reaction was carried out under the following condition. The PCR conditions were set for 28 cycles with initial denaturation at 95°C for 5 min then final denaturation of 95°C for 1 min, annealing at 55ºC for 1 min and final extension at 72ºC for 2 min using Thermal cycler (AB, Applied Bio system Thermal Cycler, USA)

PCR products were electrophoresed on 1.5% agarose gel and documented. The PCR product was purified by using PCR purification kit (Genei, Bangalore, India). The nucleotide sequences of the PCR product was determined by using the automated DNA sequencer with forward and reverse primers (Bio-serve Bio Technologies Pvt. Limited Hyderabad, India). Sequence comparison with the databases was performed using Basic Alignment Search Tool (BLAST) through the National Centre for Biotechnological Information (NCBI), USA, and server. The isolate was identified as *Bacillus mycoides.*

Phylogenetic analysis of bacterial strain the 16S rRNA sequences of the bacterial strains were aligned with reference sequences showing sequence homology from the NCBI database using the multiple sequence alignment program of MEGA 11.0 (Tamura *et al.,* 2013). Phylogenetic analysis of gene sequence data were conducted using the neighbor-joining (NJ) method[15]. Nucleotide sequences were subjected to homology search using the nucleotide BLAST NCBI. The phylogenetic tree was constructed using MEGA 5 software, the partial 16s RNA sequences were submitted to the NCBI Gene Bank database.

Pretreatment and detoxification of substrate hydrolysate :

The agro-domestic waste sugarcane bagasse was collected and hydrolysed using acid hydrolysis in order to release sufficient amount of fermentable sugars. Pretreatment

not only liberates fermentable sugars but also weakens hydrogen bonding in-between glucan chains that enhances breakdown of fermentable sugars in the presence of xylose reductase enzyme.

Isolation of microorganism :

The different soil samples from sugarcane fields were used for isolation of xylose reductase producing microorganisms. A total of 58 microbial strains (6 bacterial, 13 fungal and 39 yeasts) were isolated using enrichment technique as mentioned in materials and methods. The cultures obtained were streaked several times on nutrient agar slants in order to obtain pure culture of the organism. About 32 isolates were obtained from sugarcane bagasse dumped sites. The isolate obtained was maintained on agar slants and used for further screening to identify the potent xylitol producing microorganism.

Screening of microbial isolates :
Primary screening - Bial's test :

Green coloration was observed in the tubes containing test samples which confirmed the presence of pentose sugar, xylose. It is then compared with commercial glucose and xylose as negative and positive control respectively is represented in table-1.

Table-1. Primary screening - Bial's test

Sample	Qualitative analysis (Color)	Xylose presence
YX -18	Green color	+
YX -29	Green color	+
YX -32	Green color	+
FX -7	Green color	+
BX -5	Green color	+
Positive control	Green color	+
Negative control	No green color	-

The microbial isolates obtained from different soil samples were screened for maximum production of xylose reductase after growing in production media. The cultures were observed for both extracellular and intracellular activity. The cells were centrifuged and the collected supernatant solution from respective cultures were used to analyze extracellular xylose reductase activity. The intracellular enzyme activity of cells was also estimated after cell disruption by sonication. The enzyme xylose reductase metabolizes xylose inside the cell, no extracellular xylose reductase activity was recorded for any cultures. Hence intracellular xylose reductase activity of isolated cultures were analyzed using coenzymes such as NADH and NADPH from the extract obtained after sonication followed by centrifugation. The xylose reductase activity of the best 5 isolates with both the coenzymes NADH and NADPH is represented in the table-2.

Table-2. Xylose reductase activity of best 5 isolates with coenzymes NADH and NADPH

Microbial Isolate	Xylose reductase activity with NADH (IU/mg of cells)	Xylose reductase activity with NADPH (IU/mg of cells)
YX -18	1.01	11.08±0.12
YX -29	Nil	16.26±0.14
YX -32	7.02	21.68±0.36
FX -7	Nil	22.12±1.3
BX -5	Nil	35.23±0.32

It is clear from the table that maximum xylose reductase activity of 35.23±0.32 IU/mg of cells was observed using coenzyme NADPH from bacterial isolate BX-5 isolated from sugarcane bagasse field. There is no NADH dependent xylose reductase activity was observed except in YX-32 isolate.

Identification and characterization of selected bacterial isolate :

The selected BX-5 isolate was identified using morphological, biochemical, genotypic and phenotypic methods.

Morphological and colony characterization:

The morphological and cultural characteristic of selected isolate was studied and the results are given in (Table-3).

The colonies of creamy white, irregular margin and convex colonies were observed when the culture was grown on nutrient agar plate (Figure 1). Red colored rod shaped cells were observed under light microscope after performing Gram staining and confirmed it is Gram positive rods (Figure 2).

Biochemical characterization :

The selected isolate BX -5 utilized glucose, sucrose, fructose, lactose, maltose, xylose, and arabinose as carbon source. It showed positive reactions for catalase, oxidase, methyl red, citrate utilization, nitrate reduction, urease, hydrolysis of starch, casein and gelatin, whereas the culture showed negative result for Voges-Proskauer test (Table-4).

Table-3. Morphological and colony characterization

S.No	Morphological tests	Observation	Results
1.	Gram Staining	Rod shaped cells in blue color	Gram positive bacilli
2.	Cultural characteristics	Creamy white, opaque, irregular margin and convex characteristically rhizoid colonies	Characteristics of colony resemble with growth of *Bacillus* sp

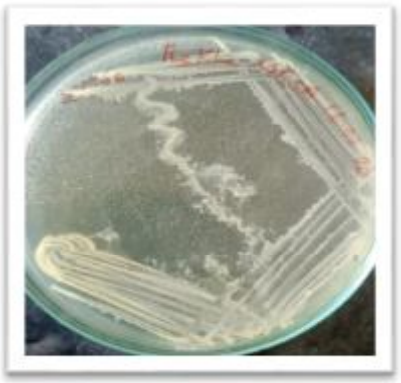

Fig. 1. Streaking of BX-5 isolate on agar plate

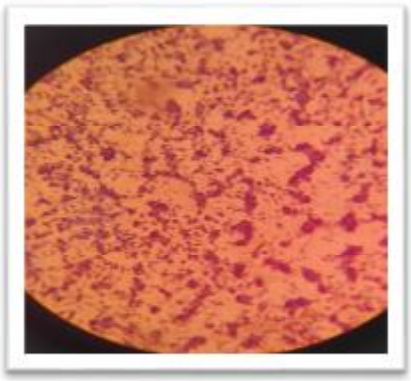

Fig. 2. BX -5 in Gram staining

Table-4. Biochemical characteristics of BX -5 isolate

S.No.	Biochemical Tests	Observation	Results
1.	Starch hydrolysis	Clear zone	Positive
2.	Casein hydrolysis	Clear zone	Positive
3.	Gelatin hydrolysis	Liquefaction	Positive
4.	Urease test	Pink Color change	Positive
5.	Catalase test	Slight effervescence	Positive
6.	Oxidase test	Purple color	Positive
7.	Citrate utilization	Prussian blue color	Positive
8.	Indole test	No cherry red ring	Negative
	Methyl red test	Bright red color	Positive
9.	Voges-Proskauer test	Pink-red color	Positive
10.	Nitrate reduction test	Red to pink color	Positive
Sugar fermentation			
11.	Sucrose fermentation	Red to yellow color, Gas formation	Positive
12.	Lactose fermentation	Red to yellow color, Gas formation	Positive
13.	Glucose fermentation	Red to yellow color, Gas formation	Positive
14.	Fructose fermentation	Red to yellow color, Gas formation	Positive
15.	Maltose fermentation	Red to yellow color, No gas formation	Positive
16.	Xylose fermentation	Red to yellow color, Gas formation	Positive
17.	Arabinose fermentation	Red to yellow color, Gas formation	Positive

Hence, from the preliminary tests based on morphological and biochemical characterization, it can be said that this isolate belongs to the genus *Bacillus*.

Molecular Identification of bacterial isolate:

The molecular characterization of the selected bacterial isolate BX -5 was carried out by estimating % G+C content and analyzing its 16S rRNA sequencing. The 16S rRNA sequence was deposited to Genbank under accession no. OM169281.1. The results represented in 16S rRNA sequencing of the isolate BX -5 clearly established its close relatedness to *Bacillus mycoides* (OM169281.1). The phylogenetic tree of the selected bacterial strain was constructed using neighbour joining method as shown in Figure 5. The position of selected bacterial isolate BX -5 in phylogenetic tree clearly shows its relationship with *Bacillus mycoides* (OM169281.1).

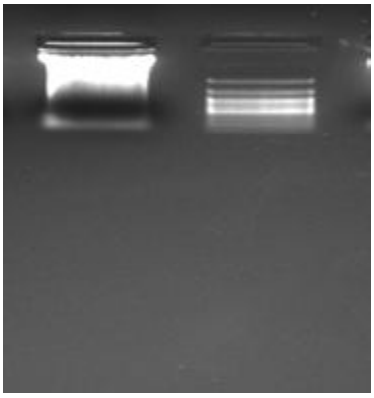

Fig. 3. Isolation of DNA

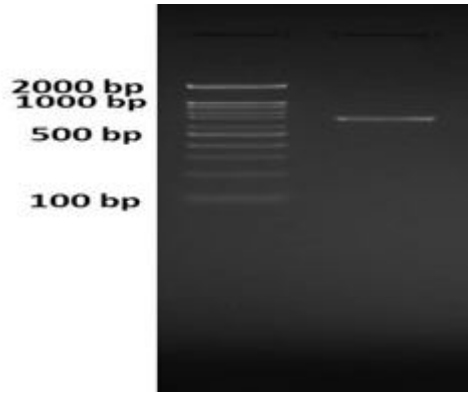

Fig. 4. PCR amplification

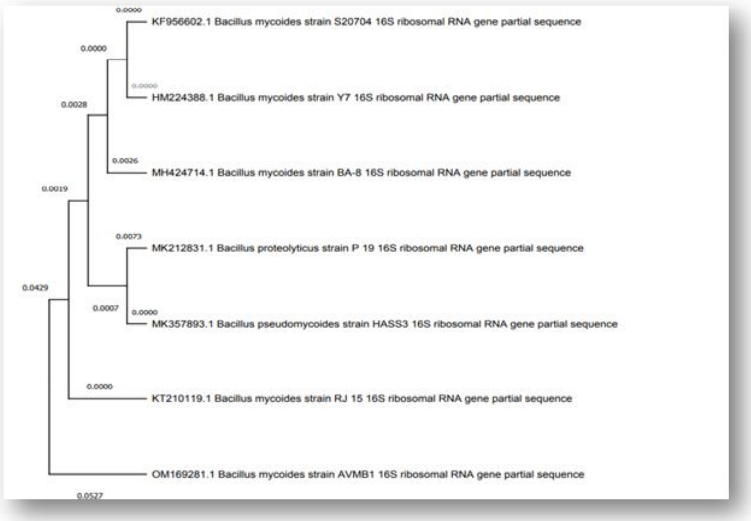

Fig. 5. Phylogenetic tree of *Bacillus mycoid*es (OM169281.1) using 16S rRNA sequencing

The agricultural wastes containing sugarcane bagasse were collected and hydrolysed to extract xylose. Sugarcane bagasse is a good source for producing xylose which can be used for xylitol production by acid hydrolysis of sugarcane bagasse. Detoxification of substrate hydrolysate by charcoal treatment increases release of fermentable monomers. Microorganisms were isolated from these wastes where screened and identified as suitable xylitol producers. The potent microbial strain was sequenced and identified as *Bacillus mycoid*es (OM169281.1) by 16S rRNA sequencing.

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