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Isolation and Identification of Xylanase Producing Bacteria from Soil Samples of Kerala

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ABSTRACT
Microorganisms play a very vital role in the biological recycling of plant bio-matter. Xylan is the second most plentiful polysaccharide and is found as an interface between lignin and other polysaccharide in the secondary cell wall. When these polysaccharides are hydrolyzed by a suitable strain of bacteria it will become an end to many environmental issues and had many useful applications such as in paper and pulp industry, food and feed industry, bread quality improvement, agro waste treatment, seed germination and generation of biofuels. To isolate a suitable strain of bacteria which possess good xylanase activity several samples of soils were collected from various regions of Kerala and were undergone a three-step enrichment process. In the primary screening 85 bacterial strains were obtained. Twenty two isolates produce clear zone during plate assay using beech wood xylan. The isolates which possess more than 0.5 cm clear zone were undergone final screening process and the strain PSS1 showed the maximum enzyme activity of 112.38 U/ml.

Key words: Identification, isolation, screening, xylan, xylanase, 16SrRNA

INTRODUCTION

Enormous quantities of agronomic and industrial residues are released out every year in our country. To fulfill the needs of ever increasing human population it is necessary to make improvement in the field of agriculture. Even though crop improvement satisfies the various needs, there occurs a problem of unscientific disposal of agricultural wastes which leads to many environmental issues. Proper disposal of agricultural residues is a crucial problem facing now-a-days. The plant biomass in dry weight is composed of cellulose (40%), hemicelluloses (33%) and lignin (23%) (Biely 1993). When these cell wall constituents are hydrolyzed by suitable strains of microorganisms it will become an end for landfill problems and enzymes produced as a result of fermentation of these residues can be effectively employed for the well-being of mankind. Hemicellulosic content varies depending on the nature of plant body. It constitute about 30-35% of hard wood, 15-30% of graminaceous plants and 7-12% gymnosperms (Whistler and Richards 1970). Xylan, mannan, galactans and arabinans are the major classes of hemi celluloses. Since xylan is the most abundant hemicellulosic substance it can be satisfactorily converted to industrially useful products. Xylan is composed of D-xylose monomer units linked by β-1, 4, glycosydic linkage. It is present in the cell wall in between cellulose and lignin and constitute about one third of dry weight of wood and agricultural wastes (Biely 1985). The structure of xylan varies based on the nature of substituent present in them. Homoxylans consist of only xylose residues and are reported from few sources like esparto grass, tobacco stalk and guar seed husk (Chanda et al. 1950)

Microbial degradation of agricultural residues generates enzymes of economic importance. Microbes are important sources of various plant cell wall hydrolyzing enzymes. Several organisms like Bacteria, fungi, actinomycetes and yeast were reported as excellent producers of xylanase (Shanthi and Roymon 2014). Among them bacteria are preferred over other sources. This is due to rapid division of bacterial cells, high yield, low rate of contamination, medium simplicity and easy recovery (Kamble and Jadhav 2012). Soil enriched with hemicellulosic content are an excellent sources of xylan degrading microorganisms. To

isolate bacteria having potent xylanase activity a three step screening technique was followed (Subramaniyan et al. 1997)

MATERIALS AND METHODS

Collection of soil samples

The criteria used to collect sample was that soil mixed with decaying wood were considered primarly. They were collected under aseptic conditions using sterile spatula and gloves and were dispensed in to sterile polythene bags and brought to the laboratory.

Screening of soil samples

One gram of soil was weighed and added to 10 ml of sterile distilled water and mixed well and centrifuged at 5000 rpm for three minutes to separate the soil particles and agricultural residues. 1ml of the supernatant was taken for doing the serial dilution and added to 9 ml distilled water. This process was repeated until 10^{-6} dilution. In the primary screening 0.1 ml of 10^{-6} dilution was spread on to wheat bran extract agar containing (g/l): wheat bran, 10.0; yeast extract, 0.6; peptone, 0.6; $MgSO_4$, 0.2; K_2HPO_4, 1.0 and agar 20.0. The agar plates were incubated at 37^0C for two days. The bacterial colonies obtained were purified by repeated streaking techniques.

In secondary screening instead of wheat bran the purified cultures were spread on xylan agar plates containing 0.5% xylan (Beech wood xylan from Sisco Laboratories). The plates after an incubation period of three days were flooded with 1% Congo red and kept for 15 min and destained with 0.1N NaCl to visualize the clear zone around bacterial colonies. The bacterial colonies that showed a minimum of 0.5 cm radius clear zone were selected for tertiary screening using xylan liquid medium containing (g/l): xylan, 5.0; yeast extract, 0.6; peptone, 0.6; $MgSO_4$, 0.2; K_2HPO_4, 1.0.

Extraction of crude xylanase

The xylan liquid medium was inoculated with selected isolate and fermentation was carried out. Samples (5 ml) were collected at successive intervals of 24 hrs and crude enzyme and cells were separated by centrifugation at 7000 rpm for 15 min.

Xylanase assay

Xylan hydrolysing capacity of the selected strain was measured according to the method of Bailey et al. (1992) with some modification. D-xylose was used as the standard. 0.5% xylan in phosphate buffer of pH 7 was used as the substrate. To the pre incubated Beech wood xylan substrate (0.2 ml), suitably diluted crude enzyme was added. After 10 min of incubation the reaction was stopped by adding 3 ml DNS. Along with this a control was set up where the enzyme was added after adding DNS. All the reaction tubes were boiled for 5 min to stabilize the colour of DNS. After that it was cooled and the absorbance was measured by colorimeter at 540 nm against the blank. One unit of enzyme activity was defined as the amount of xylanase required to release 1 µmol xylose residues min^{-1} under controlled assay conditions.

Estimation of soluble protein

The protein concentration was estimated by the method of Lowry et al. (1951) and bovine serum albumin was used as the substrate.

Cellulase assay

To measure the cellulase activity DNS method (Miller 1959) was followed with some modification and D-glucose was the standard. Carboxy methyl cellulose (0.5%) in phosphate buffer pH 7 was used as the substrate. One unit of enzyme activity was defined as the amount of cellulase required to liberate 1 µmol of glucose min^{-1} under controlled condition.

Identification of the bacterial strain

The morphological, cultural, and biochemical characteristics of the isolated strain was studied on the basis of Bergey's manual of systematic bacteriology (Sneath et al. 1986). Phylogenetic analysis was carried out by 16S rRNA sequencing. Matrix Assisted Laser Dissorption Ionography–Time of Flight Mass Spectroscopy of PSS1was carried out for the legitimate identification of the microorganism up to the species level. It is a technique of high speed identification and taxonomic

classification of bacteria using high throughput MALDI-ToF MS.

Substrate specificity of crude xylanase

The substrate specificity of crude xylanase was checked out by using different sources of xylan. Beech wood xylan, Birch wood xylan, Corncob xylan and Oat-spelt xylan were used as the substrate.

RESULT AND DISCUSSION

A total of 17 soil samples were collected from various regions of Kerala and about 85 bacterial strains were obtained from them through primary screening on wheat bran agar extract medium. Since wheat bran is a complex polysaccharide, these bacterial strains cannot be considered as xylanase producers. To ameliorate the possibility of getting potent xylanase producers secondary screening was performed by using xylan agar extract. A total of 22 strains showed positive result on secondary screening. Congo red staining method was adopted for knowing the extent of clear zone around the bacterial colony. The strains which showed more than 0.5 cm of clear zone were undergone tertiary screening using xylan liquid medium. One of the isolate named as PSS1 (Fig. 1a) from the soil collected in the nearby regions of Bonacaud exhibit nearly 2 cm of clear zone (Fig. 1b, c) in congo red treatment and significant amount of xylanase activity during tertiary screening and was selected for further studies.

Xylanolytic activity and growth profile

The xylanase activity of the selected strain PSS1 was estimated by DNS method. Liquid medium containing 0.5% xylan as the carbon source was inoculated with the selected culture. Growth, pH change, soluble protein and reducing sugar found in the growth medium are correlated with enzyme activity. The culture attained stationary phase after 72 hrs of incubation (Fig. 2). The pH of the fermentation medium remained alkaline throughout the fermentation period (Fig. 2). This show that alkaline environment of the fermentation medium support the growth period. Several species of Bacillus having high yield of xylanase at alkaline pH has been reported. Generally in bacteria maximum xylanase activity is reported in alkaline pH (Khasin et al. 1993). *Bacillus circulans* produced a xylanase activity of 400 IU/ml at a pH of 7 (Ratto et al. 1992). Similarly xylanase activity of Bacillus SSP 34 reached maximum when the pH value ranges from 6-8 (Subramaniyan and Prema 1998).

It was observed that xylanase activity gradually increased during the initial hours of incubation. Even though significant enzyme production occurred from 24 hrs onwards a maximum of 112.38 U/ml was obtained at 72 hrs of incubation and a sufficient activity was obtained even in 96 hrs of fermentation (Fig. 3). Beyond 96 hrs xylanase activity decreased gradually. This may be due to the depletion of carbon source in the medium, interaction of xylanase and medium components, interaction of other enzyme like protease or may be due to the

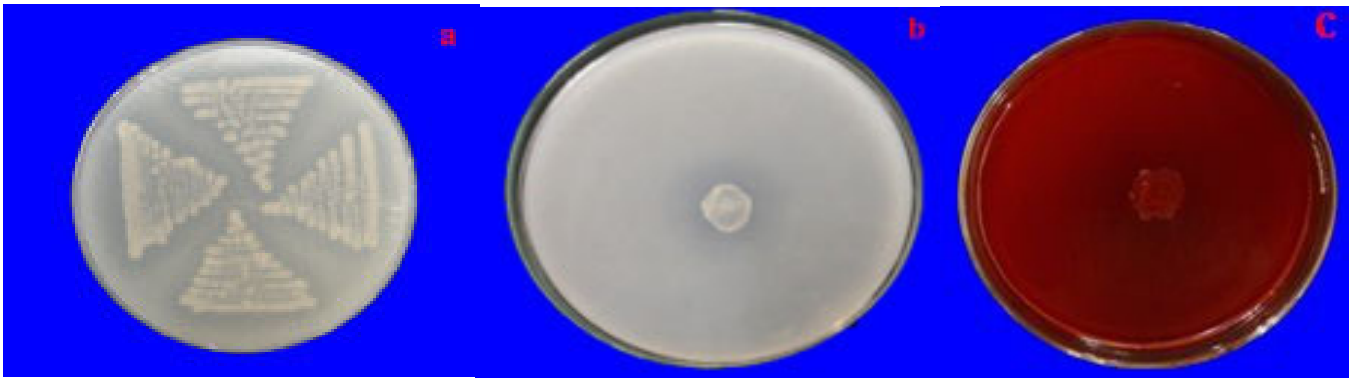

Figure 1. Culture plate showing (a) PSS1 (b) zone of clearance around the colony (c) visualised by Congo red method

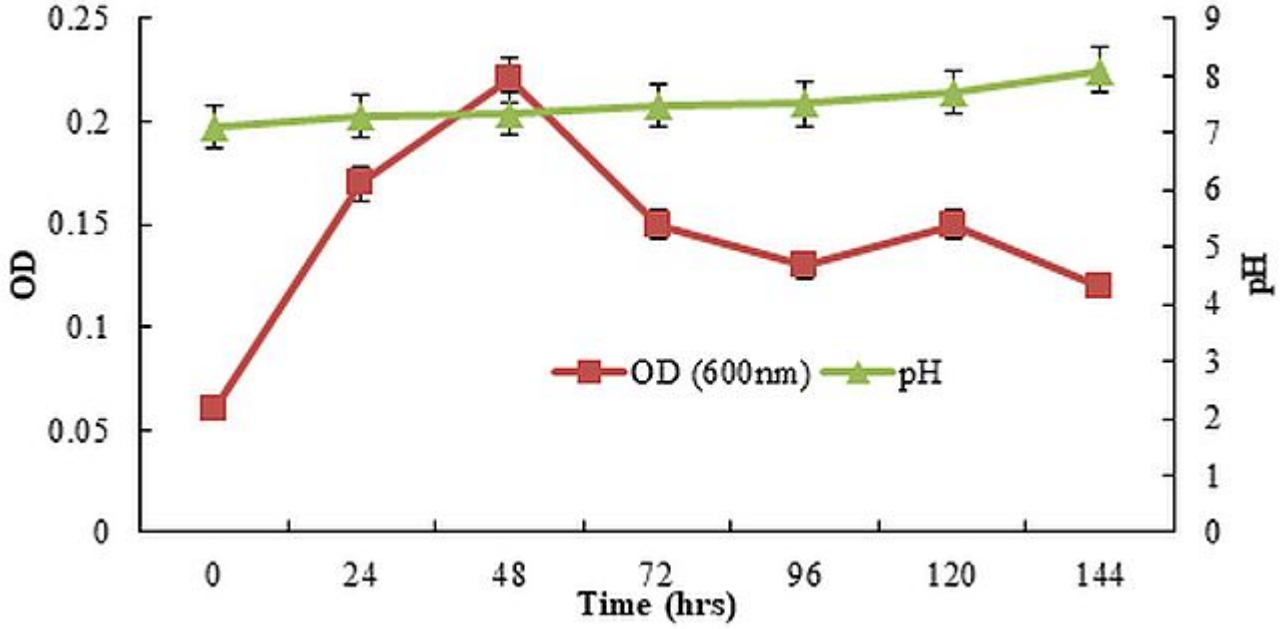

Figure 2. Growth and pH of PSS1 during fermentation period

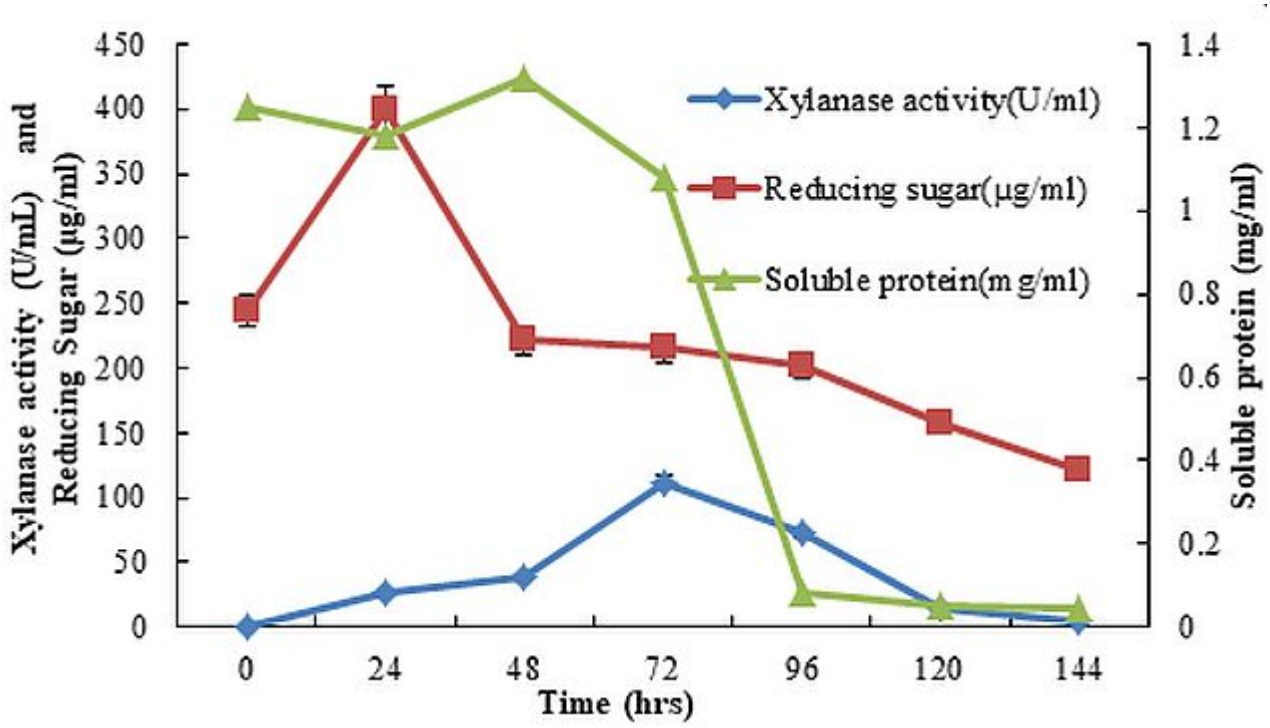

Figure 3. Xylanase activity, reducing sugar and soluble protein of PSS1

components secreted by the cell. Xylanase activity has been previously reported from similar *Bacillus* species. In a study conducted by Akhavan Sepahy et al. (2011) a potent xylanase (249.308 IU/ml) producing strain *Bacillus mojavensis* AG137 was reported from the cotton farm regions of Kashan-Iran under submerged fermentation condition. Similarly Gaur et al. (2015) isolated a .bacterial species *Bacillus vallismortis* which produced a maximum xylanase activity of 3,768 U/ml. Geetha and Gunasekaran (2017) reported a xylanase activity of 755.81 U/ml in *Bacillus pumilus.* In another study *Bacillus firmus* HS11 showed xylanase activity of 3.35 U/ml which is explored from Sikkim Himalayan

region (Phukon et al. 2020). Recently Guler and Ozcelik (2023) isolated a bacterial strain *Bacillus halotolerans* DSM 8802 and reported a xylanase activity of 23.47 U/ml.

The concentration of reducing sugar is the highest during early hours of growth (Fig. 3). The pre-inoculum contains a substantial amount of xylanase which is inoculated in to the production medium. This causes the hydrolysis of xylan and facilitates the release of reducing sugar in to the fermentation medium. As the growth of bacteria increases during the fermentation period, it will metabolise the substrate and a gradual decrease in the concentration of sugars occurs. In the hour of peak enzyme production reducing sugar is less as compared to the initial hours of incubation. Even though the highest soluble protein content is observed at 48 hrs of incubation (Fig. 3) the peak hour of enzyme secretion is 72 hrs. This may be due to the fact that all proteins are not enzymes.

Cellulase activity

Most of the xylanases are associated with cellulase activity. For industrial purposes xylanase without cellulase activity is preferrable (Bajpai 1997). In the case of PSS1 cellulase activity is negligible (0.207 U/ml). Even though fungi are chief producers of xylanase, presence of significant amount of cellulase activity and acidic pH make them unsuitable for paper and pulp industry. Similar to the present study Subramaniyan et al. (2002) reported that the cellulolytic activity of *Bacillus* SSP-34 is minute, which makes these bacterial xylanases more suitable for industrial scale applications.

Morphological and physiological characteristics

Cells from cultures were subjected to Grams staining and SEM analysis (Fig. 4a, b). They founded singly, in pairs and occasionally in the form of chains. Colonies have raised elevation and rough surface and was found to be gram positive.

Identification of the isolate

The phenotypic characteristics (Table 1) of the strain reveals that it is a gram positive, aerobic, spore producing bacteria having raised elevation and rough surface. Biochemical characterisation tells that the strain give positive result for casein test, starch hydrolysis, gelatine hydrolysis, nitrate reduction test, catalase test, Esculine hydrolysis, H_2S gas production test and negative result for growth on MacConkey agar medium, Indole test, Methyl red test, Voges Proskauer test, Citrate test, Oxidase test and Urease test (Table 1). The strain

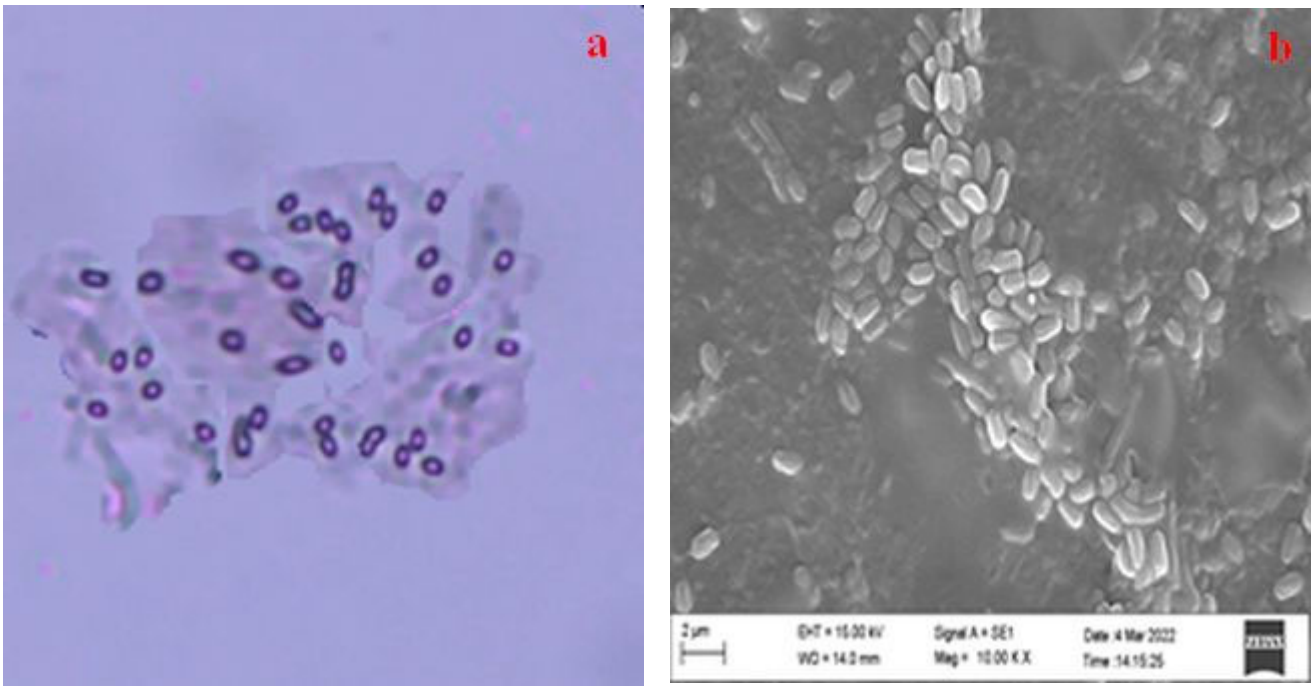

Figure 4. Microscopic images of the strain PSS1 (a) Grams staining, (b) SEM image of cells displaying the colonies

Table 1. Morphological, physiological and biochemical characteristics of PSS1

Sl.No	Test	Result
Colony morphology		
1	Configuration	Irregular
2	Margin	Irregular
3	Elevation	Raised
4	Surface	Rough
5	Density	Transluscent
6	Pigmentation	No pigment
7	Gram reaction	+ve
8	Spore, Shape, and Position	+ve, Oval and Central
Growth at different temperature		
9	10 ºC	-
10	15 ºC	-
11	25 ºC	+
12	30 ºC	+
13	37 ºC	+
14	42 ºC	+
15	50 ºC	+
16	55 ºC	-
Growth at different pH range		
17	pH 5.0	+
18	pH 6.0	+
19	pH 7.0	+
20	pH 8.0	+
21	pH 9.0	+
Growth at different NaCl (%) concentration		
22	2.0%	+
23	3.0%	+
24	5.0%	+
25	7.0%	+
26	8.0%	+
27	9.0%	+
Biochemical test		
28	Growth on MacConkey agar medium	-
29	Indole test	-
30	Methyl red test	-
31	Voges Proskauer test	-
32	Citrate test	-
33	Casein test	+
34	Starch hydrolysis	+
35	Gelatin hydrolysis	+
36	Nitrate reduction	+
37	Catalase	+
38	Oxidase	-
39	Esculine hydrolysis	+
40	H2S gas production	+
41	Urease	-
Acid production from carbohydrates		
42	Fructose	+
43	Raffinose	-
44	Cellobiose	-
45	Rhamnose	-
46	Mannitol	-
47	Xylose	-
48	Lactose	-
49	Galactose	-
50	Trehalose	-
51	Inositol	-
52	Sorbitol	-
53	Sucrose	-

PSS1 will come under the genus *Bacillus* according to the principles of Bergey's manual of systematic bacteriology (Sneath et al. 1986).

As described in the materials and methods section the 16S rRNA sequencing of the strain PSS 1 was carried out (Fig. 5). Based on the BLAST result a phylogenetic tree (Fig. 6) was constructed. From this tree it is clear the strain PSS1 form clade with *Bacillus mojavensis* and showed 99% similarity. The gene sequence was deposited in Gene Bank and accession number was SUB14458392.

Recently MALDI –ToF MS is a potential tool for the microbial identification. To authenticate the result of 16S rRNA sequence the strain is also identified by MALDI-ToF MS. It has the ability to measure peptides and other bio molecules in the presence of the ions. The simplicity of sample preparation and sample acquisition facilitate the advancement of MALDI-ToF MS in microbial taxonomy. The possibility of high throughput sample automation is a valuable nature of this technique. According to Bruyne et al. (2011) identification score obtained by analysis of spectral observation could legitimately identify the microorganism up to the species level. In the present study MALDI-Biotyper profiling of the culture PSS 1 resulted a score value of 2.075 which will be safely coming under the range of identification such as 2.000-2.2gg for genus and species identification. The highest peak darker near 2000 (Fig. 7) indicate that the strain belong to the genus *Bacillus* and Species *mojavensis.*

>Bacillus_PSS1 SR1535-RSF1_C05.ab1

ACCGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACC
GCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCA
CCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGA
AGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGT
CCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGG
GTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGA
AGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAAGCGTGGGGAAGCGAACAGGATTAGAT

Figure 5. 16 S rRNA sequence of PSS1

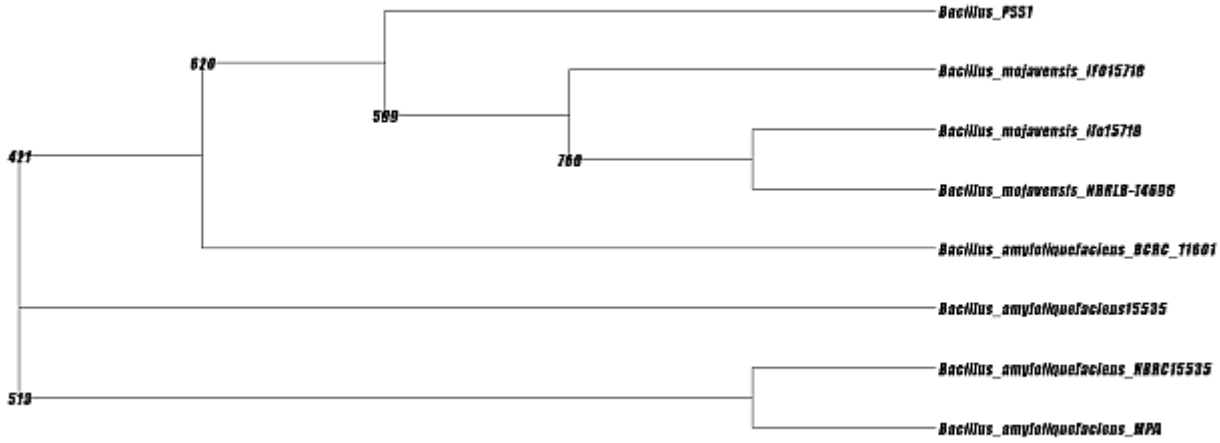

Figure 6. Phylogenetic tree

Substrate specificity of crude xylanase

The highest xylanase activity of 112.38 U/ml is produced when beechwood xylan was used as the substrate. A significant enzyme activity of (110.8 U/ml) was also obtained when oatspelt xylan was employed which is close to that produced by beechwood xylan. Likely a notable production of xylanase occurs when birch wood xylan (101.92 U/ml) and corncob xylan (98.6U/ml) were utilized as the substrate. Therefore crude xylanase strongly hydrolyzed all xylan substrates.

CONCLUSIONS

A total of 85 bacterial strains were obtained from soil samples collected from different forest regions of Kerala. Among them 22 strains showed positive response during secondary screening in xylan agar medium. The strain PSS1 produced a maximum xylanase activity of 112.38 U/ml during tertiary screening and selected for identification. Xylanase production from the newly isolated *Bacillus* sp. PSS1 is maximum at 72 hrs of growth. Morphological and biochemical characterization followed by 16s rRNA sequencing and MALDI-ToF MS successfully identified the strain as *Bacillus mojavensis* PSS 1. Present study showed that Beechwood xylan and oatspelt xylan are the best xylanase inducing substrates for *Bacillus mojavensis* PSS 1. Isolation of xylanase from *B. mojavensis* is rare. The strain *B. mojavensis* PSS1 isolated in this study is a potential candidate for xylanase production and further study by optimization process will certainly enhance the enzyme production.

Authors' contributions: All authors contributed equally.

Conflict of Interest: The authors declare no conflict of interest.

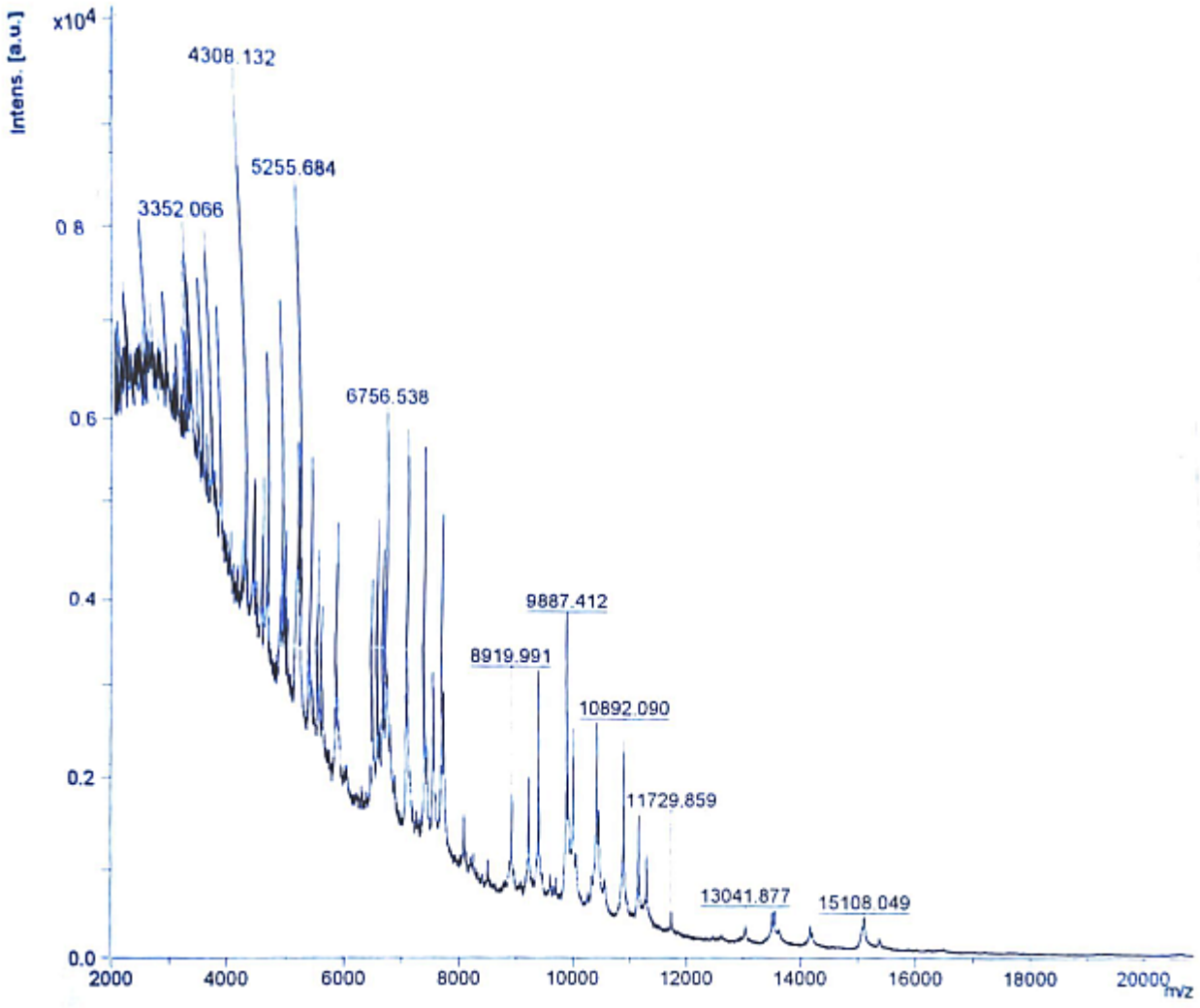

Figure 7. MALDI-ToF MS of PSS1

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