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Isolation and Identification of Xylanase- producing Novel Actinobacter *: Microbacterium hatanoins HAX-5.*

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ABSTRACT

Present investigation was done for screening microorganism with highly stable xylanase production. *In present days,* commercial microbial enzymes are increasingly replacing conventional chemical catalysts in many industrial processes. In current studies 56 actinomycetes were found positive for xylan-degradation. Among all isolates, best actinobacter found for xylanase production was rare species and less identified for any such properties. During studies *Microbacterium hatanonis* HAX-5 was found potential for xylanase production in submerged fermentation using agrowaste.

Keywords: Xylanase, *Microbacterium hatanonis* HAX-5, Submerged Fermentation

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INTRODUCTION

Enzymes have several advantages over chemical catalysts, including the ability to function under relatively mild conditions of temperature, pH and pressure. The real value of enzyme technology, is in enzyme-based processes, where operating costs can be typically reduced by anything from 9 to 90 % and are accompanied by savings in energy and raw materials (1) from available commercial enzymes, up to 75% are of the hydrolytic type (2)

Commercial microbial enzymes are increasingly replacing conventional chemical catalysts in many industrial processes. Enzymes have several advantages over chemical catalysts, including the ability to function under relatively mild conditions of temperature, pH and pressure. This results in the consumption of less energy and there is usually no requirement for expensive corrosion-resistant equipment. Enzymes are specific, often stereoselective, catalysts, which do not produce unwanted byproducts. Consequently, there is less need for extensive refining and purification of the target product. Also, compared with chemical processes, enzyme-based processes are 'environmentally friendly' as enzymes are biodegradable and there are fewer associated waste disposal problems.

In recent time, nearly all commercially prepared foods contain at least one ingredient that has been made with enzymes. Some of the typical applications include enzyme use in the production of sweeteners, chocolate, syrups, bakery products, alcoholic beverages, precooked cereals, infant foods, fish meal, cheese and dairy products, egg products, fruit juice, soft drinks, vegetable oil and puree, candy, spice and flavor extracts, and liquid coffee, as well as for dough conditioning, chill proofing of beer, flavor development, and meat tenderizing, in addition to cheese manufacturing - enzymes were used already in 1930 in fruit juice manufacturing.

Enzymes are also indirectly used in biocatalytic processes involving living or dead. This study concentrates on the use of isolated enzyme preparations in large scale and specialty applications and chemical manufacturing. The use of microorganisms as biocatalysts in chemical production, however, an interesting and growing field as shown in Table 1.

Table 1: Application of Microbial enzymes

Industry	Enzyme	Application
Pulp and paper	Xylanase	Bioleaching
Textile	Cellulase,Laccase	Microfibril removal color brightening
Animal feed	Xylanase Phytase	Fiber solubility release of phosphate
Starch	Amylase	Glucose formation fructose formation
Fruit juice	Pectinase,cellulase, xylanase	Juice clarification and extraction
Baking	Xylanase	Dough quality
Detergent	Proteinase, Lipase, Cellulase	Protein degradation,Fat removal color brightening
Dairy	Rennin,Lactase	Protein coagulation,Lactose hydrolysis

The discovery of a variety of thermostable enzymes has led to expanded growth in the industrial enzyme market. Interest in thermostable enzymes has grown mainly due to fact that most of the existing industrial processes are run at high temperature. The use of xylanase to breakdown hemicellulosic material has been extensively proved in paper industrial process. Xylanase is composed of endoxylanase and exoxylanase are major components of enzymatic consortium act in nature by depolymerizing xylan molecules into monomeric pentose units that are used by bacterial and fungal populations as primary carbon source.

Xylan is a potential significant resource for renewable biomass, which can be utilized as a substrate for the preparation of many products such as fuels, solvents and pharmaceuticals.

On the other hand, xylanases (E.C. 3.2.1.8) are needed for making use of hemicelluloses. For most bioconversion processes, xylan must first be converted to xylose or xylo-oligosaccharides. This can be done either by acid hydrolysis or by the use of xylanolytic enzymes Xylanase enzyme deconstructs plant structural material by breaking down hemi-cellulose, a major component of the plant cell wall. Xylanase are produced from many different actinomycetes, fungi and bacteria. Xylanase enzymes are used commercially industries (3) Most commercial xylanases are produced by *Trichoderma,Bacillus, Aspergillus, Penicillium, Aureobasidium,* and *Talaromyces sp* Hydrolysis of xylan is undoubtedly an important step toward proper utilization of abundantly available lignocellulosic material in nature.

Many studies have been performed on xylanases from bacteria and fungi, but actinomycetes have been explored to the lesser extent, especially regarding xylanase production although they are traditionally being considered as a rich source of primary and secondary metabolites.

In addition, although the fungal systems are excellent xylanase producers, they often co-secretecellulases, which affect pulp quality adversely.Actinobacteria are alignocellulose-degrading, heterogeneous group of Gram-positive bacteria, widely distributed in natural environments such as soil and compost that secrete a range of extracellular enzymes, including xylanases, cellulases and peroxidases (4 & 5). The major objective of the research was to explore a novel xylanase secreting bacteria and develop a production technique suitable for xylanase production using cheap and readily available agro-byproducts using simple technology.

MATERIALS AND METHOD

Source and Collection of Samples

Different locations were selected for the collection of xylanolytic organism from local area of Sabarkantha district, North Gujarat. Samples were collected from agricultural farms, garden, cow-

dung, hay, soil of paper mill and non-agricultural region soil. More than 35different samples were collected and selected for study. Even some samples were also collected from decayed wood materials like xylan rich-wood materials, which are potentially good sources of xylanase producing microorganisms. (6)

All selected sources were used for sampling. For collection of lignocellulosic waste containing soil sample approximately 3-4cm of soil from surface was removed and from a depth of 10-12 cm soil sample was collected. All samples were collected in sterilized vial and brought to the laboratory. Each sample was crushed, mixed thoroughly and sieved through a 2 mm sieve to get rid of large debris.The sieved sample was used to evaluate on the same day for the screening of potential xylanase producer

Enrichment and Isolation of *Actinomycetes*sp.

Collected samples were air dried at room temperature for 4-5h to reduce the proportion of bacteria other than actinomycetes (7). Before dilution for enrichment soil samples were mixed with CaCO3 for enriching number of actinomycetes and the soil incubated at 28°C for 7 days in a water bath(8)

Primary Screening of Xylanolytic Actinomycete

The medium used for primary screening was Berg's mineral salts (g/l)containing 0.3% $NaNO_3$, 0.05% K_2HPO_4, 0.02% $MgSO_47H_2O$,0.002% $MnSO_4H_2O$,0.002% $FeSO_4$ H_2O,0.1% $CaCl_2$ with agar 15-20%(**9)** and Besides 0.1% yeast extract ,0.1% peptone and 0.5% Birchwood xylan(Hi-media laboratories Pvt. Ltd., India) was added to the medium so that the colonies selected have the ability to grow using xylan by virtue of xylanase production. Medium pH was adjusted 7 and Plates were incubated at 37°C for 24-48 hours.

Qualitative test: Xylan hydrolysis reaction

The isolates of actinomycetes were tested for their ability to produce xylanase by growing on xylan agar medium. The plates were flood with Congo red solution (0.1%) and kept for 15 min, then detained with 1M NaCl solution (10). The xylanase producing microorganisms were selected by observing yellow zones around the colonies against the red background. The 45 bacterial isolates were selected on the basis of xylanase production coded HAX-1 to HAX-45. From 45 isolates five best actinomyceteisolates (xylanase positive) were selected for further studies.

The xylanolytic potential was estimated using the xylanolytic ratio (R/r), defined as the diameter of the hydrolyzation zone (R) divided by the diameter of the producing colony (r) **(11)**. According to the diameter size five strongest xylanase producing strains were selected All screened isolates were evaluated for purity by gram staining. Staining is used to differentiate isolates into gram positive or gram negative bacterial. The microscopic morphological characters were also studied.All screened

isolates were characterized by colony appearances. Colonies are discriminated according to their size, shape margin, elevation, texture and pigmentation etc.

Xylanase Assay by Dinitrosalicylic Acid Method

Xylanase hydrolyzes the polymer xylan into the xylose monomers. The free xylose units produced as a result of xylanase activity; react with 3-5 Dinitrosalicylic acid (DNS) reagent and form a colored complex that is measured by spectrophotometer at wavelength 540 nm. Greater the amount of xylose produced, darker will be the color of the enzyme-xylose complex and more will be light absorbed.(**12)**

Xylanase production by submerged fermentation

The selected five xylanase positive isolates were studied for xylanase production using agriculture residue as substrate under SmF.

Fermentation media:

The fermentation medium containing (g/l); wheat bran, 10.0; $NaNO_3$, 0.1; NH_4Cl, 0.1;KH_2PO_4,0.5;K_2HPO_4,0.5;$(NH)_2SO_4$,2,;$MgSO_4.7H_2O$, 0.03;$CaCl_2.2H_2O$, 0.1 and Tween 80, 0.2 mL (pH 7) were used for fermentation (**13).**

Fermentation process:

The activated cultures of the isolates (2%) were inoculated into production medium (100ml) contained in Erlenmeyer flasks (250 ml) after sterilized it in the autoclave at 121°C for 15 min.The flasks were placed in the rotary incubator shaker (200 rpm) at 37°C, pH 7 for 72hours. The enzyme activity was assayed periodically every 12 hours fermentation time. Crude enzyme was obtained by centrifugation of a suitable amount (10-15ml) of production(fermentation) broth at 10,000 rpm for 20 min at 4°C and supernatant/ Culture filtrate was considered equivalent to crude enzyme. Crude enzyme was estimated by DNS method for xylanase activity(12). All the experiments were carried out in triplicates and mean± S.E was considered for results.

Identification Of potential Strain HAX-5

Among all HAX-5 was found most promising for xylanase production and further identification was done using staining, biochemical test and genetic analysis. Results were cross checked with standard reference of the Prokaryotes(14) .The molecular characterization and identification of the isolated bacterial strain was carried out by one of the most precise method, i.e. Identification of 16 S rRNA gene sequence of the strain. This sequencing was carried out at MTCC Chandigarh.MTCC had given the identification according to their sequence data of Genbank. The results were also analyzed by using BLAST from the NCBI website for further interpretations regarding the identified *Actinomycete strain HAX-5*.

RESULTS AND DISCUSSION

Sources and sampling

By exploring different sources 35 soil samples were collected in sterile vials. All samples were kept in dry condition to maintain soil quality in our laboratory.From each selected samples serial dilutions upto 10^{-6} were prepared and spread on glycerol asparagine's agar and starch casein nitrate agar. Half of the Plates were incubated at 28°C for 5-6 days and remaining plates were incubated at 37°C for 24-48 hours.

Primary isolation

After incubation, well isolated colonies were randomly selected. Isolation was done to obtain pure cultures of organisms from natural sources. Colonies were checked for purity by gram staining result concluded with gram positive rods (10), cocci(5) and filamentous actinomycetes.(52) While certain gram negative short rods(03) were found

Primary identification concluded that most of colonies were belonging to *Streptomycetes sp, Microbacterium sp,Nocardia sp.,Bacillus sp and Xanthomonas sp Pseudomonas sp,Micrococcus sp* and *Aztobacter sp.*Primary isolation concluded that 70 different pure cultures were obtained from 125 colonies. . (figure 1)

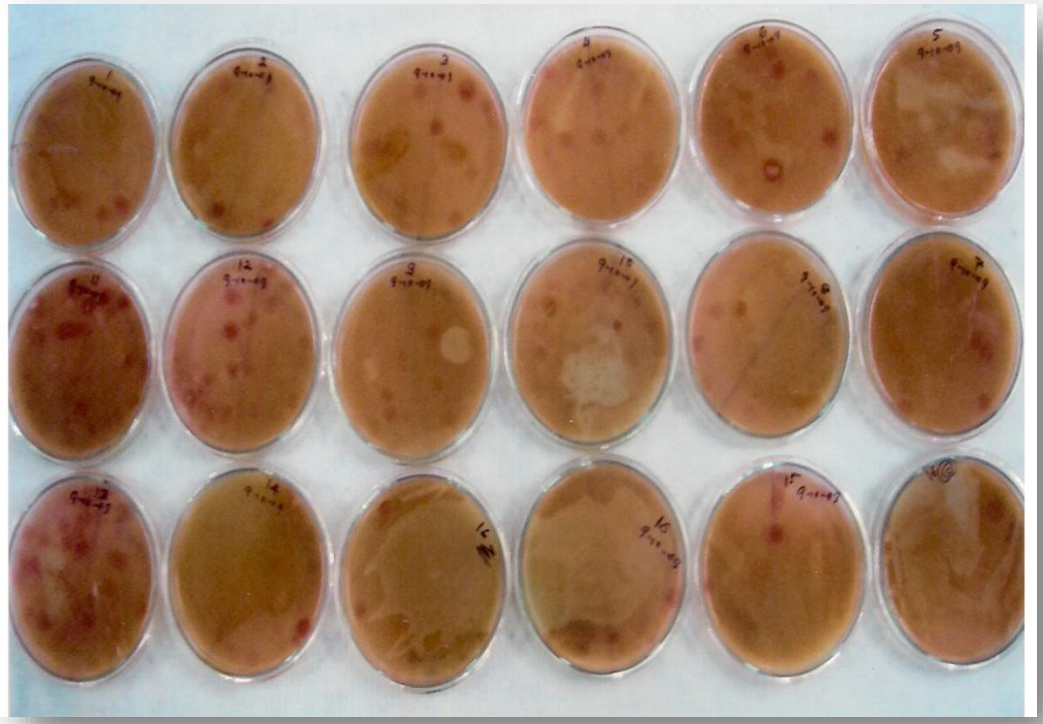


Figure:1: Primary screening of Xylanolytic organisms

Primary screening of xylanolytic organisms

Initial screening was done by plating isolates on selective medium i.e. xylan-agar plate as discussed in methodology.70 different colonies were grown on xylan agar plate at pH-7. Out of 70 isolates, 45 showed good growth on plate and so screened as xylanase positive organisms by clear zone of hydrolysis. All 45 primary screened isolates belongs to genus *Actinomycetes*.

According to the diameter of clear zone xylanolytic actinomycetes were classified into four different categories. (Table: 2 & 3).

Further, five most potential xylanolytic actinomycetes were screened by the help of qualitative test as in compare to other xylanase positive isolates. Potential strains were selected on basis of clear zone (zone of hydrolysis) observed on xylan agar plates. For further confirmation selected potential pure isolates were also spreaded on xylan agar plate to obtain clear zone and treated with 0.1% congored solution as discuss in methodology

Table: 2Screening of xylanolytic Actinomycetes by zone of hydrolysis

No of species	Clear-zone (xylanolytic activity)	Zone diameter(mm)
5	++++	3.5-2.6
14	+++	2.6-1.5
17	++	1.5-0.5
9	+/-	0.5-0.1

++++ = Highly degradable(>80%)

+++ =Moderate degradable(>40%)

++ = Less degradable(≤ 20%)

+/- = Non degradable(5%)

Table: 3Colony characteristics of xylanolytic actinomycetes isolates

Isolate code	Size	Shape	Margin	Consistency	Elevation	Texture	Opacity	Pigment
HAX-5	Small	Round	Entire	Moist	Convex	Shiny smooth	Translucent	Off-white
HAX-9	Medium	Round	Lobate	Dry	Flat	Rough	Translucent	Light grey
HAX-19	Medium	Round	Rhizoid	Moist	Raised	Smooth	Transparent	Yellow
HAX-23	Small	Round	Irregular /uneven	Dry	Umbonate	Rough	Translucent	Off white
HAX-34	Medium	Round	Even	Moist	Convex	Rough	Translucent	Whitish grey

Xylanase assay by DNS method

According to the International Union of Biochemistry, one international unit of Xylanase (1 IU) corresponds to the amount of enzyme required to release 1 micromole of reducing sugar (xylose) in

1 min under standard condition. Xylanase activities were calculated using reducing sugar concentration obtained by DNS method that were compared with standard xylose (Figure-2) Assay was repeated several times to obtain standard graph 1. To standardize estimation was done several times and mean±S.E. was used to plot data.

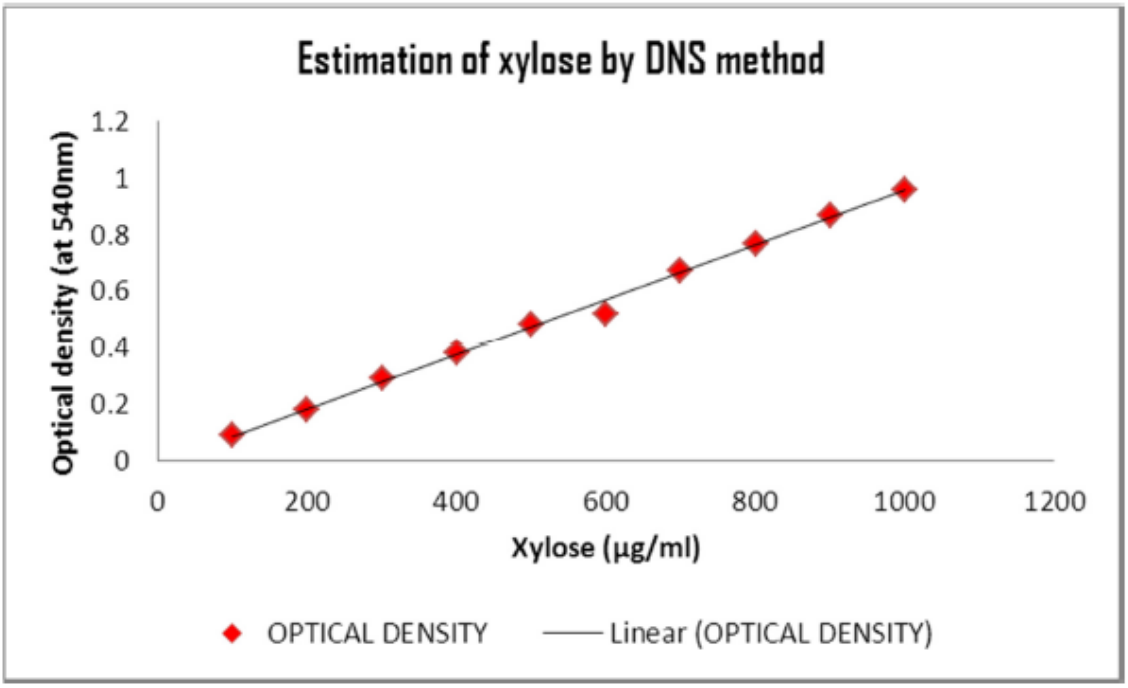


Figure 2: Estimation of xylose by DNS method

Xylanase production by submerged fermentation

SmF result indicated, HAX-5 showed maximum xylanase activity of 6.78 U/ml at 48 h incubation and HAX-9 showed maximum xylanase activity of 4.47 U/ml after 36h incubation. While HAX-19 and HAX-23 showed very low xylanase activities respectively 3.46 U/ml and 3.12 U/ml after 48 hours incubation period. While HAX-34 shown maximum xylanase activity 2.3U/ml after 60 hours of incubation (Figure-3)

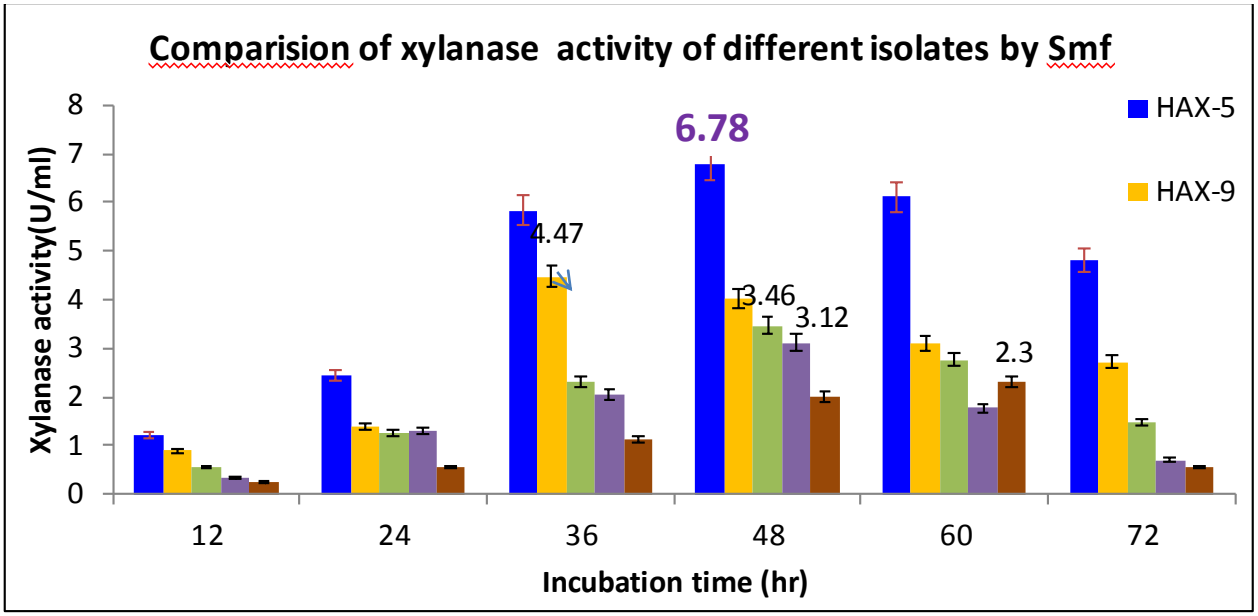


Figure 3: Xylanase production using different Actinomycetes strains by SmF

Identification of Strain:

Results obtained from SmF indicate HAX-5 strongest and potential xylan-degrading strain among all other isolates. Strain was further studied for all parameters. It includes from primary identification to industrial level production of xylanase. Primary identification include Gram-staining, colony characteristic as summarized in table-4 and figure4. While general biochemical reactions were carried out in microbiological laboratory and biochemical characteristics are described in table 4

Table: 4 Biochemical characteristics of isolate HAX-5

Biochemical characteristics of isolate HAX-5

Characteristic	HAX-5
Starch hydrolysis	+
Gelatin liquefaction	-
Casein hydrolysis	+
Nitrate reduction	-
Sugar fermentation	
Gluose	+
Arabinose	+
Maltose	+
Xylose	+
Lactose	±
Sucrose	±
Indole production	-
Methyl red test	-
Voges-Proskauer test	-

Oxidation(Hugh-Leifson) medium	+
Fermentation (Hugh-leifson medium	+
Lipid (Tributyrene) hydrolysis	-
H_2S production test	-
Urease activity	-
Ammonia production	+
Catalase test	+
Nacl tolerance	Upto 5%
Enzyme activities	
Alkaline phosphatase	-
Lipase	-
Xylanase	+
Cellulase	-

+ positive - negative ± acid positive gas negative

While Blast analysis result of 16 S r-RNA obtained from MTCC, Chandigard showed that the isolated strain is showing more than 97% similarity with four known strains that is *Microbacterium hatanonis* JCM14558(97.27%), Whereas isolated strain HAX-5 is nearly similar to Microbacterium *phyllospharaeDSM13468(97.24%) Microbacterium testaceum DSM20166(97.17%) and Microbacterium barkeri DSM20145(97.17%).*These suggested that the isolated xylanase strain is *Microbacterium hatanonis JCM14558*.(figure. 5)

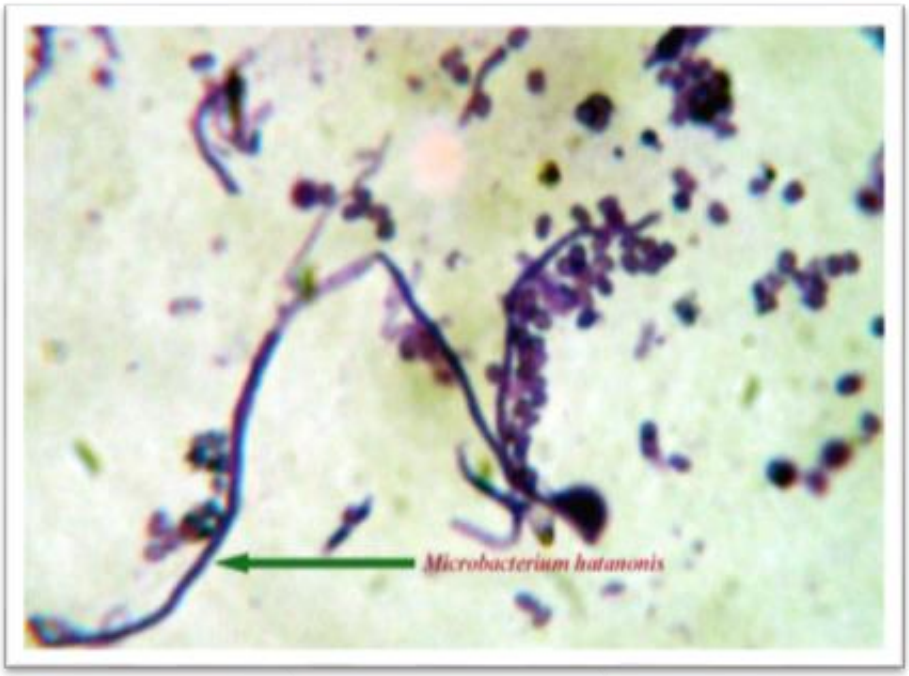
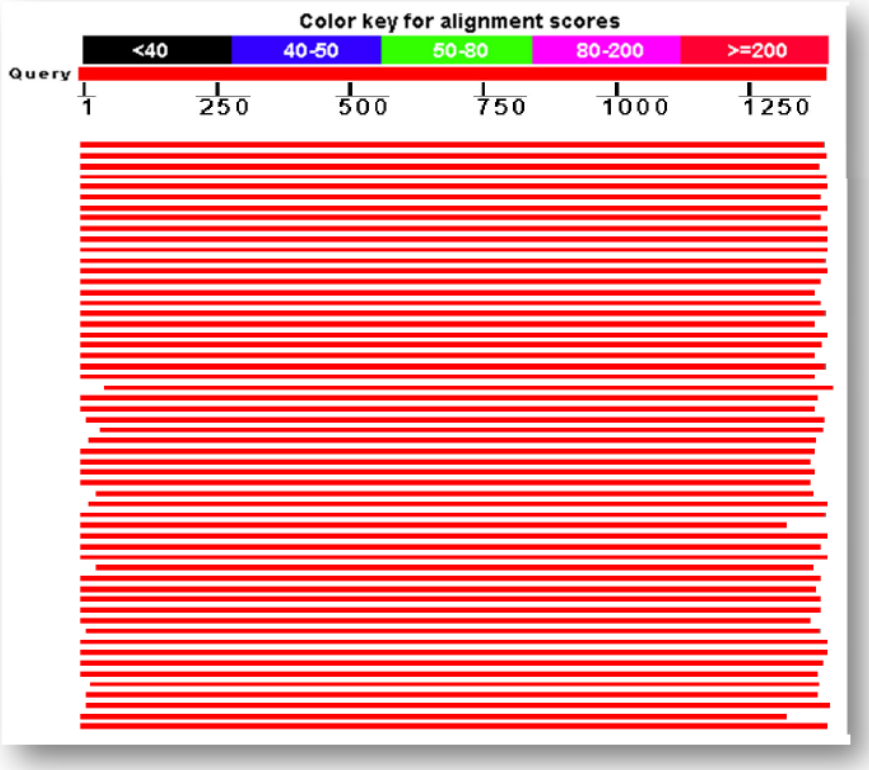


Figure: 4 Gram-staining of HAX-5 (Microbacterium hatanonis)

Microbacterium hatanonis.



dbj|AB274908.1| : *Microbacterium hatanonis* gene for 16S rRNA, partial sequence

Length=1484,

Score = 2451 bits (1327),

Expect = 0.0 Identities = 1425/1472 (97%),

Gaps = 8/1472 (1%) Strand=Plus/Minus

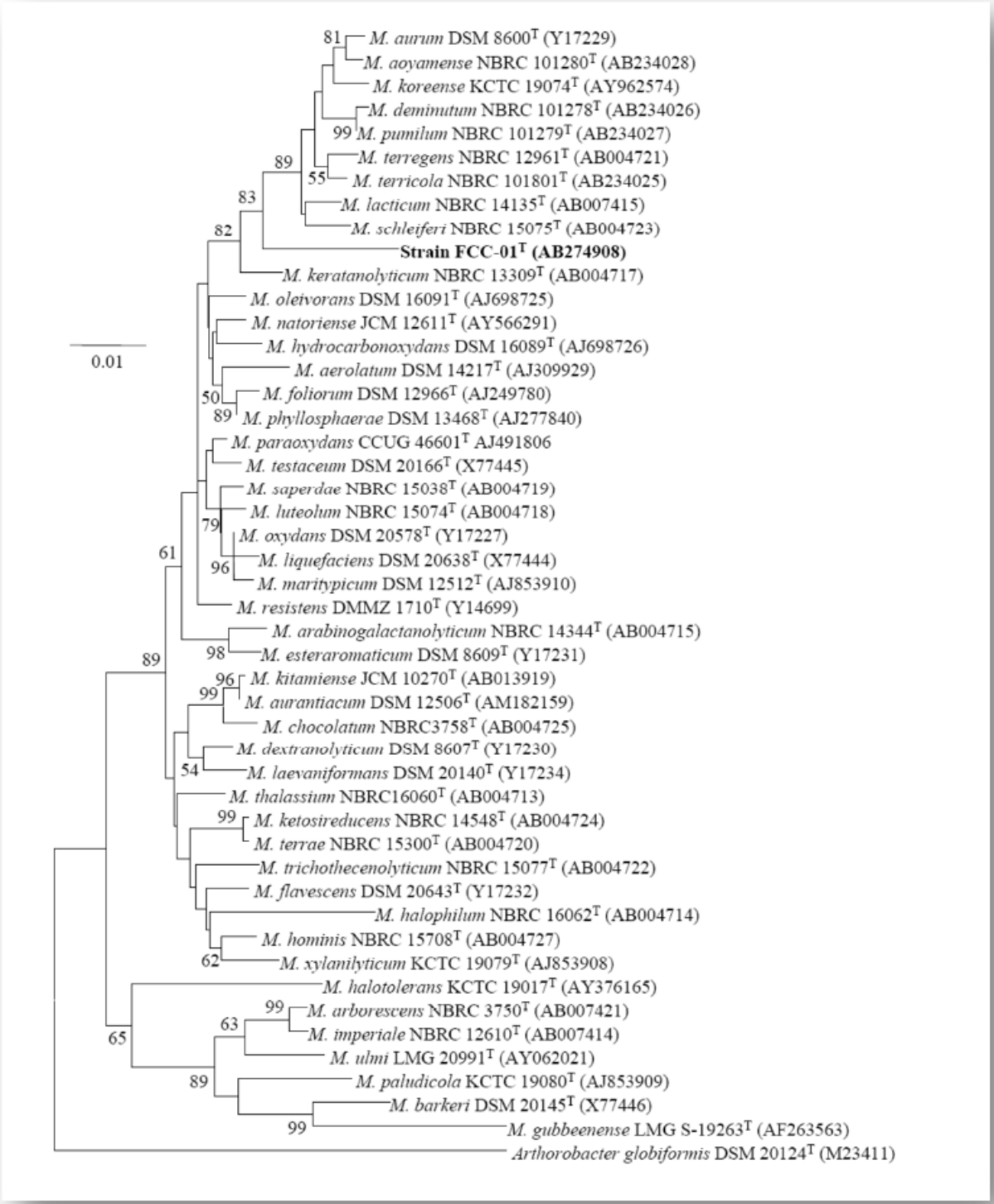


Figure 5: Minimum-Evolution Phylogenetic Tree, based on 16 S r-RNA gene sequences

The results discerned during the course of this investigation are summarized and concluded as below:

- Various dilute samples of soils from agricultural farms, garden, cow-dung, hay, soil of paper mill and non agricultural regions were used to isolate xylan degrading actinomycetes on glycerol asparagine agar medium and Starch casein nitrate agar medium because it favors the growth of various groups of *actinomycetes*.
- Among 45 isolates five isolates of actinomycetes were selected as positive xylanase producing strains. Plate assay showing zone of hydrolysis of xylan by xylanase producing actinomycetes were used to select most positive xylanase producing strains.
- 2-3 gram of sample was harvested from fermentation medium and added in 50mM phosphate buffer, then centrifuged at 10,000 rpm for 20 min at 4°C. Supernatant was used as crude xylanase. Xylanase activity was checked by DNS method.
- Among all screened isolates HAX-5 was found most potential strain for xylanase production. Quantitative test means submerged liquid fermentation was used to find potential xylanase producing strain. HAX-5 strain showed highest 6.78 U/ml xylanase activity after 48 hours of fermentation.
- A colony characteristic, staining techniques, biochemical characterization and 16S r-RNA sequencing was performed at MTCC, Chandigarh to identify the most potential xylanase producing bacterial isolate.
- Results of 16 S r-RNA sequencing and BLAST analysis of 16 S r-RNA sequence comparison from the NCBI Gen Bank was carried out at MTCC, Chandigarh confirmed HAX-5 as *Microbacterium hatanonis* FCC-01T with97.27% similarities. While Blast analysis report conclude that *M.hatanonis* is closely related to *M.phyllosphareae(97.24%)* and have similarity with *M.testuceum(97.17%), M.barkeri (97.17%), M.hydrocarbonoxydans(97.13%)* and *M.Profundi(97%)*.

CONCLUSION

Research explore a novel strain of Actinobacter capable for xylan degradation from natural resources like agriculture wastes, paper mill wastes and other solid wastes. On other side, research is also helpful in recycling of waste material. We conclude our technique of enzyme production an *'eco-friendly processes*. Technique reuses agriculture residues and other lignocellulosic waste as substrate for fermentation media. The results from phenotypic and genotypic characterizations suggested that the strain screened belongs to the genus *Microbacterium*, which was first described by Orla-Jensen

in 1919(15& 16) and more recently it has been emended by Takeuchi and Hatano also to unit the genera *Microbacterium* and *Aureobacterium.*(17). The *Microbacterium species* described in the literature have been isolated from milk products, various environmental sources, including soil, water and plants, and from clinical samples. The genus Microbacterium comprises more than 30 physiologically versatile species isolated from various environments (17) *Microbacterium paludicola* sp. nov., a novel xylanolytic bacterium isolated from swamp forest(18). *Microbacterium ulmi* sp. nov., axylanolytic, phosphate solubilizing bacterium isolated from sawdust of *Ulmus nigra* (19).*Microbacterium xylanilyticum* sp. nov., a xylan-degrading bacterium isolated from a biofilm.(20). Microbacterium is still less exploited asenzyme produces specially xylanase producer so this research work as far as my knowledge is new direction in enzyme technology

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