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rRNA Gene sequence (Genbank ID JX896668.1) analysis and biochemical characterisation of enzyme production by *Bacillus* sp. NO9

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Abstract

Newly isolated *Bacillus* sp. NO9 is unique in that it produced good growth as well as good clear zone in xylan agar plates. The 16S rRNA gene of this bacterium has been isolated, sequenced and submitted to GenBank in the nucleotide database with the ID No. JX896668.1. BLAST analysis using eubacteria database and Clustal analyses resulted in good multiple sequence alignment. The resultant phb file could create a phylogenetic tree with clear cut clade of *Bacillus* species. The new bacterium showed 90% similarity to *Bacillus atropheus* and *Bacillus subtilis* and has been named as *Bacillus* NO9. The bacterium could grow well in wheat bran liquid medium and xylan liquid medium. The xylanase production was a promising with an activity of 10.2 U/ml in wheat bran medium. Here the FPase activity was 3.01U /ml while CMCase activity was 2.98 U/ml. In xylan liquid medium the growth pattern was different with maximum growth by 48 hours. The bacterium was producing very low level of cellulases in the xylan liquid medium compared to wheat bran medium. The maximum FPase recorded was 1.15 U/ml and that of CMCase was 1.38. Both the cellulolytic enzymes were having an activity range less than 1.5 /ml in this medium.

Keywords: *Bacillus*, CMCase, FPase, 16S rRNA gene analysis, Xylanase, Carboxy methyl cellulose, FPase

Introduction

All living cells produce hundreds of enzymes catalyzing various pathways. In the case of microorganisms enzymes are the ultimate tool needed for their very existence. The microorganisms are producing a large number of enzymes, involved in hydrolysing, oxidizing or reducing reactions. However the kind and pattern of various individual enzymes produced vary markedly between species and even between strains of the same species. Therefore it is very essential to select strains producing specific enzymes suitable for commercial application. Such research is focused at the collection and identification of new and improved microbes capable of producing novel enzymes for industrial purposes.

Lignocellulosic is one of the most abundant natural complex organic carbons in form of plant biomass, which mainly consist of three major components ; cellulose, hemicelluloses and lignin. Cellulose is a homopolysaccharide of β-1,4 –linked polysaccharide in nature mainly consists of β-1,4 –linked xylopyranosyl residues. In addition to xyloglucan, xylomannan, galacto glucomannan and arabino galactan are important constituents of hemicelluloses (Collins *et al*., 2005). These enzymes have applications in food, animal feed, bioconversion, and textile and production of bioethanol as well as in paper and pulp industries (Subramaniyan and Prema *et al*., 2002).The most important industrial application of xylanase is in the preleaching of kraft pulp. This application allows for lower consumption of chemicals during the bioleaching process, and also results in a brighter product than can be achieved without the enzymatic treatment (Ninawe and Kuhad, 2006; Viikari *et al*., 1994). The main commercial use of xylanases is in bioleaching of paper and

pulp due to their bleach boosting properties (Li *et al*., 2010). Xylanases pretreatment of the pulp has been reported to lower bioleaching chemical consumption and to result in greater final brightness.

Materials and Methods

Isolated cultures from forest soil were spread onto xylan agar plates containing 0.5% xylan (Oat spelts xylan, Sigma Chemicals Co.). After six days of incubation, colonies that showed areas of clear zones with a minimum radius of 1 cm were selected for further screening in liquid medium. Two types of liquid media were used. In the first set of fermentation wheat bran was the carbon source while in the second set xylan served the purpose. Both the liquid medium contained Peptone (0.25%), Yeast extract (0.25%), K_2HPO_4 (0.1%) $MgSO_4.7\ H_2O$ (0.02%) (pH 7).

Isolation of 16 S rRNA gene - genomic DNA amplification

Genomic DNA was extracted through CTAB method (Wilson 1987). 16S rRNA gene was amplified with forward and reverse primers - EUB 341f (5'- CCT ACG GGA GGC AGC AG -3') and EUB 907r (5' CCG TCA ATT CMT TTG AGT TT-3')
(IDT, USA) under standard PCR conditions using a thermo cycler.

DNA sequencing and identification of bacteria up to genus level

The amplified DNA fragment was run in 1.5% agarose gel for size confirmation. It was purified subsequently and sequenced commercially (Scigenom, India). Approx. 550 bp long gene sequence obtained was compared with corresponding sequences of related organisms retrieved from GeneBank database with BLAST algorithm for identifying the isolated strain.

Biochemical Analysis

From the culture broth periodically taken from the growth medium, about 3 ml was subjected to centrifugation at 10000 rpm for 10 minutes. Cell free culture supernatant was decanted from the centrifuge tubes and subjected to xylanase enzyme studies and the sediment is dissolved in 3 ml of 0.1 ml N NaOH and it was taken for cell protein studies. Reducing sugar was estimated by Miller's Method (Miller, 1959) using Di-Nitro salicylic acid reagent. The concentration of reducing sugar was estimated against glucose standard by noticing the absorbance at 540 nm. The glucose stock solution and its different dilutions served as standards. Soluble protein of the cell free culture supernatant was periodically estimated using Lowry's method (1951).

Inoculum used for the fermentation studies

The inoculum was raised in the same medium under similar conditions in 250 ml Erlenmayer flasks and 18 hour old inoculum was used to initiate growth.

Production of hydrolases

Production of plant cell wall hydrolases from the selected isolate was studied in correlation with the growth profile of the cultures. Inoculated media were incubated in a rotary shaker at 120 rpm for a period of 144 hours. 4.0 ml of growth medium were taken at an interval of 24 hrs and after 144 hours all media were decontaminated and discarded. Cells were separated by each sample by centrifugation (10,000 g, 15 min, at 4^{O}C) and the cell free supernatant was used as the extracellular crude enzyme preparation.

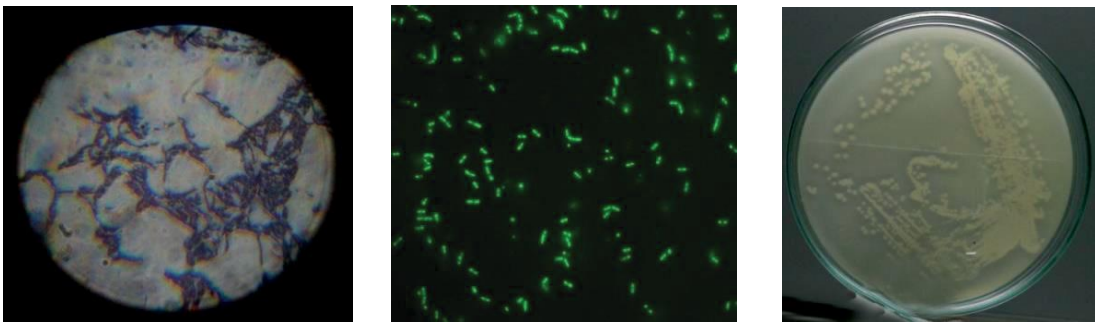
Xylanase assay was conducted using oat spelts xylan. The substrate xylan (2.0 gm) was homogenized in 0.2 M phosphate buffer of 500 ml ($NaHPO_4$ / Na_2HPO_4) and subjected to boiling point with thorough shaking. Reaction mixture containing 1.8 ml substrate and 0.2 ml suitably diluted enzyme were incubated at 50 ^{O}C for 10 minutes. The reaction was terminated by adding 3.0 ml of dinitro salicyclic and reagent, which was then kept in boiling water bath for 5 minutes. One unit of xylanase activity was defined as mols of xylose liberated per minute per ml of enzyme preparation. (Subramaniyan *et al*., 2001).

CMCase assay was conducted by using CMC. The substrate (1g) was dissolved in 0.2 M Phosphate buffer of 100 ml ($NaHPO_4$/Na_2HPO_4). The reaction mixture containing 0.5 ml of preincubated CMC solution and 0.25 ml of enzyme preparation was incubated at 50^{0}C for 10 minutes (Mandels *et al*., 1976). FPase Assay was conducted using Whatman No. I filter paper. The reaction mixture containing filter paper (6cm x 1cm) in 1 ml phosphate buffer PH-7, which was pre incubated at 50 ^{0}C for 10 minutes and 0.5 ml enzyme preparation was added to this and incubated at 50 ^{0}C for 10 minutes The reaction was terminated by adding 3 ml of DNS reagent which was then kept in boiling water bath for 5 minutes. The absorbance was measured at 540nm (Mandels *et al*., 1976).

Results and Discussion

The importance of xylanases in paper, pulp and food industries initiated the search for microorganisms producing higher levels of xylanases. There is major concern over the applicability of cellulases along with hemicellulases in the bioconversion of lignocellulosics. Only a few bacterial and actinomycete xylanases have been reported earlier with pH optima in the neutral or alkaline ranges (Nakamura *et al*., 1994; Duarte *et al*., 1999; Ratanakhanokchai *et al*., 1999). Various methods have been adopted for isolation of microorganisms degrading lignocellulosics. (Kluepfel, 1988). Bacterial culture used in the present study was isolated from forest soil samples (Fig. 1 C). The samples were subjected to serial dilution followed by plating on to the xylan agar plates.

The newly isolated bacterium no 9 was smeared onto a clean glass slide and subjected to Christian Gram's differential staining protocol. The bacteria were gram positive rods. Flourescent microscopy shows the rod shaped morphology. The actively dividing cells are present in chains. Fluorescent microscopy ((Fig. 1 A and B).) using 'SYBR Green II'showed the rod shaped morphology. The actively dividing cells are present in chains.



A B C

Fig 1. Morphology of newly isolated bacterium no 9. A. Gram staining of Sporulating bacterial isolate 9 at low resolution (X 400). Purple coloured Rods are clearly visible. B. Fluorescent (dye SYBR Green II) labelled cells of isolate number 9. C. Morphology of the bacterium xylan in agar medium.

From the Fig. 1 A and B it is evident that the spore forming rod shaped bacterium may be belonging to the genus *Bacillus*. However, confirmation is needed for the identification of such microorganisms. The earlier works on identification largely depended on the wet lab study that consumed more time and expenditure. A review on the present status of bacterial identification reveals that nucleic acid sequences and bioinformatics tools can be used as an alternative which is easier and a more reliable method (Li *et al*., 2010).

Isolation and sequencing of 16S rRNA gene

The sequence of the amplified 16SrRNA is given below.

TGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCTT
GACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG
GCT
CAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTA
G
CGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGC
TGAG
GAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AGTGT
TAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCA
GGACT GAAACTCAATGAATTGACGGA

Fig. 2 Sequence of the 16 S rRNA gene of isolate no9 in 'FAST A' format. Gene sequence submitted to GenBank nucleotide database with ID JX896668.1 (http://www.ncbi.nlm.nih.gov/nuccore/JX896668.1). [We acknowledge the Environmental Technology Section, NIIST-CSIR, TVPM, for the support extended for the work].

Gene sequence was then annotated and submitted to GENBANK database belonging to NIH USA, through NCBI server.

Analysis of 16S rRNA Gene Sequence

The gene sequence was compared in BLAST (Basic Local Alignment Search tool) web page. From the home page of NCBI (National Centre for Biotechnology Information) BLAST the link of microbes was selected. This led to a collection of all available bacterial genomes and gene sequences. Care was taken to select only the eubacteria databank. All the members of eubacteria were selected before pressing BLAST button. The BLAST search showed that the bacteria isolated was showing 99% similarity to the genes of *Bacillus.* Most closely related organisms are the species of the Genus Bacillus. Thus the new bacterium is identified as belonging to the Genus *Bacillus* and it showed 90% similarity to *Bacillus atropheus* and *Bacillus subtilis.*

Multiple sequence alignment with clustal X

The Blast sequence helped in finding the most suitable taxonomical position of the selected bacterial isolate no. 9. The 16 S rRNA gene sequence of blast results and other bacterial strains were used to construct Multiple sequence alignment (MSA). Clustal X 2.0.11 was used for MSA for 16S rRNA. Identification up to the species level was conducted by direct comparison. 16 S rRNA Gene sequences of different species of the genus Bacillus was downloaded in FASTA format. For this NCBI's ENTREZ search algorithm was used using the nucleotide section. The keyword searched were *Bacillus* and 16S rRNA.

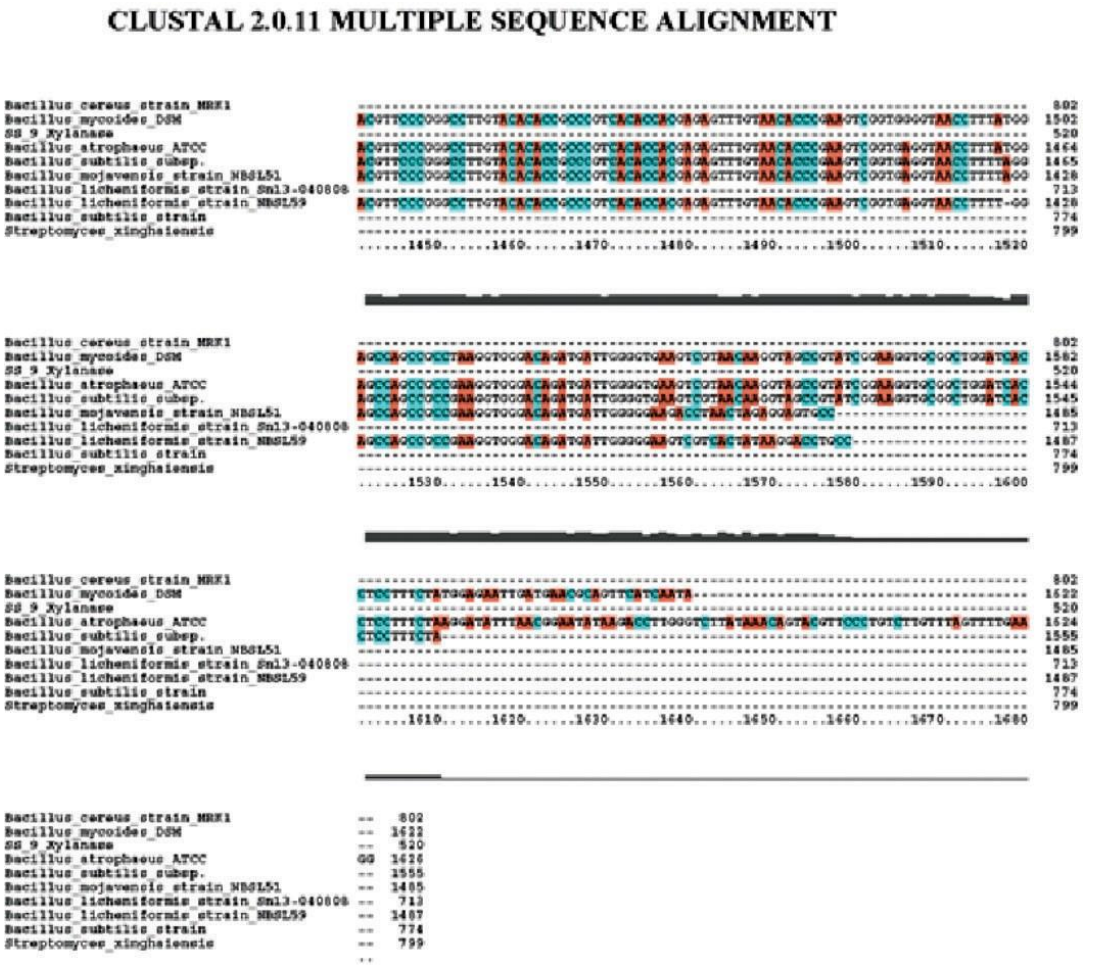


Fig. 3 A portion of the complete MSA of selected bacterial partial 16 S rRNA gene and that of other bacteria

In order to generate a phylogenetic group one distantly related taxonomic unit must be added to the test group. Here the 16S rRNA gene sequence of Streptomyces was used as the phylogenetic out group (Fig. 3). The gram positive bacteria under fircutes are well characterised by the gene sequence of 16 S rRNA. After the alignment the tree output was saved in .phb format.

Phylogentic tree creation

In the phylogentic tree is created with bioinformatics tool NJPLOT. The input file used is in the *.phb produced by the MSA with CLUSTAL X 2.0.11. The phylogram showed obvious grouping of the members of the genus Bacillus. Thus the systematic position of the newly isolated genus is *Bacillus* (Fig. 4).

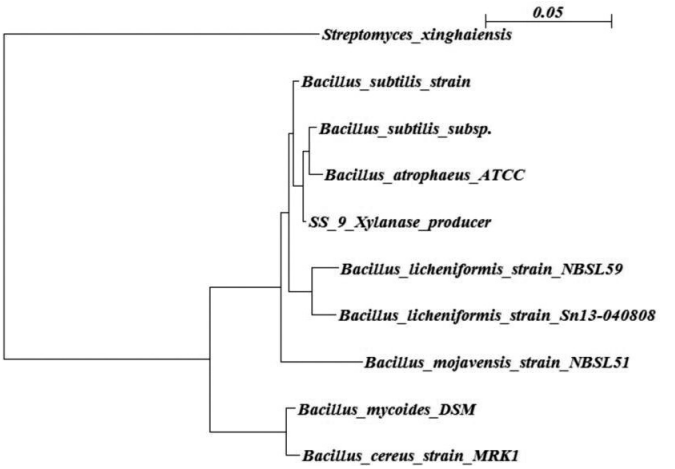


Fig. 4 Phylogram showing the phylogentic affinity of the new bacterial isolate - SS 9 Xylanase producer- with other bacteria.

The tree output was viewed in the tree viewing software NJ Plot (Fig. 4). It is clear that the new isolate no 9 is belonging to a clade of Bacillus, and it is showing 90 percent similarity to Bacillus subtilis and Bacillus atropheus. Further full length gene sequence is needed to confirm this result. The isolated bacterium will be submitted to Institute of Microbial Technology Chandigarh. Now onwards the new isolate is designated as *Bacillus* sp. NO9.

Biochemical Characterisation of Enzyme Production

The bacterial culture cultures *Bacillus* sp. NO9 was grown in liquid medium for a period of 144 hours. Culture broth, pH and biomass were monitored at every 24 hours and the cell free culture supernatant was used for the estimations of xylanase activity (Bailey *et al*., 1992), FPase and CMCase acticities (Mandels *et al*., 1976) soluble protein (Lowry *et al*., 1951) and reducing sugar (Miller, 1959).

Enzyme production pattern by Bacillus sp. NO9. in wheat bran medium

Bacterial isolate *Bacillus* sp. NO9 is unique in that it produced good growth as well as good clear zone in xylan agar plates (Fig. 1C). The culture was resulting in conspicuous optical density by 24 hour. It was also noticed that the wheat bran particle in the medium were solubilized by the growth of isolate no 9. This is an indirect indication of enzyme production potency. Cell free culture supernatant was prepared by centrifuging the culture broth at 4 ˚C at 10000 g.

The xylanase production was a promising one with an activity of 10.2 U/ml (Fig. 5A). This is highest among the other bacterial isolates. Cellulolytic enzyme production pattern was also observed. It is clear that the bacterium is producing low levels of cellulolytic enzymes. The FPase activity is only 3.0 1U /ml while CMCase activity is 2.98 U / ml (Fig. 5 B). There are several reports regarding the production of good xylanase producer with low levels of cellulose activity (Balakrishnan *et al*., 1992; Subramaniyan and Prema 2000; Joo *et al*., 2011).

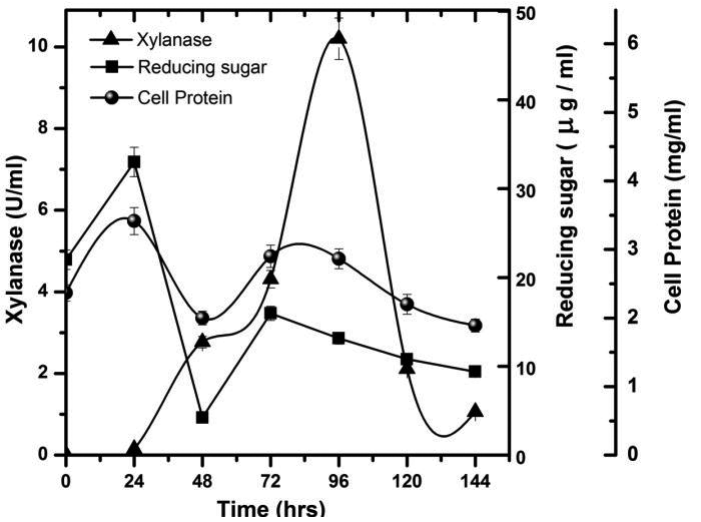


Fig. 5 A and B Enzyme profile by isolate no. 9 in wheat bran liquid medium

Fermentative production of enzymes in Xylan Liquid Medium by Bacillus sp. NO9

The *Bacillus* sp. NO9 turned to be the one with maximum xylanase activity. This was achieved by the growth of the bacterium for 96 hours in xylan liquid medium. The optical density showed a steep growth by 48 hours and later on entering the stationary phase. This stationary phase was interrupted by a dip at 96 hours of growth after which a slight increase. These are all pointing towards the diauxic growth. Earlier Nakamuara *et al*. (1993) and Subramaniyan *et al*. (1997) reported such type of growth in several *Bacillus* spp. Recently such observations were made by Joo *et al*. (2011). The diauxic growth was exemplified by the elevated occurrence of xylanase at 96 hours. The increased presence of xylanase at 96 hours was well documented (Joo *et al*., 2011; Li *et al*., 2010, Subramaniyan *et al*., 1997 and Nakamuara *et al*., 1993). The isolate *Bacillus* sp. NO9 which shows a good growth and clear zone in xylanase agar plates was also producing good xylanase activity compared with the other cultures.

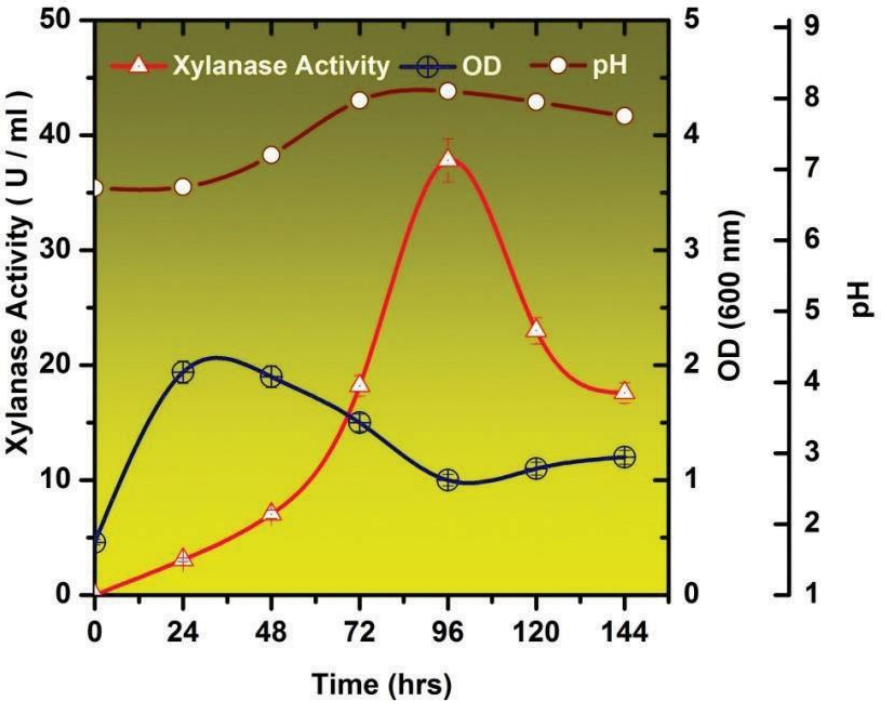


Fig 6. Xylanase production Profile by Bacillus sp. NO9 in Xylan Liquid Medium

The bacterium was producing very low level of cellulases in the xylan liquid medium as similar as in the case of Wheat bran medium. The maximum FPase recorded was only 1.15 U/ml while that of CMCase is 1.38. Both the cellulolytic enzymes were having an activity range less than 1.5 U.ml. This is comparatively a low level production. Similar to the culture number 9 there are several reports regarding the potent xylanase producer with low cellulolytic activity. *Bacillus stearothermophilus* strain T-6 was reported to produce low levels of cellulases (Shoham *et al*., 1992). One alakalophilic *Bacillus* sp. was nearly cellulose free in nature (Balakrishnan *et al*., 1992). *Bacillus* sp with highest xylanase activity (range near 400 U/ml) was reported by Ratto *et al*., (1992) and Subramaniyan (2012).

Conclusion

The newly isolated bacterium no 9 was growing well in wheat bran and xylan media. The rRNA gene sequence study confirmed the genus to be *Bacillus*. Further gene analyses are needed for species level identification. The bacterium up on biochemical analyses showed the good levels of FPase, CMCase and Xylanases in both Wheat bran and Xylan liquid media. It showeda diauxic growth in xylan medium. The 16 S rRNA gene sequence resulted in solving the systematic position of the newly isolated bacteria such as *Bacillus* sp. NO9.

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