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Research Article

SCREENING OF β-MANNANASE PRODUCING MICROORGANISMS FROM NATURAL SOIL SAMPLES

***R. A. Bhaturiwala and H.A. Modi**
Department of Life Sciences, School of Science, Gujarat University, Ahmedabad-380001
**Author for Correspondence*

ABSTRACT
β-Mannanase (3.2.1.78) is largely extracted from microbes present in natural environments. A majority of bacteria, actinomycetes and fungi are listed to be mannan degraders. β-Mannanase is widely use in industry such as paper, pulp, detergent, textile, food and hydrolysis of coffee extract. Twenty five soil samples were collected for the screening of β-mannanase producing microorganisms from different natural habitats of Gujarat. During primary screening one hundred two isolates were screened for β-mannanase producers and clearing zone diameter ranging from 0.3 mm to 22.0 mm obtained on solid agar medium. In secondary screening ten strains were selected for β-mannanase production under submerged fermentation using minimal medium composition and crude enzymatic activity was determined by Enzyme assay method. During the incubation period isolate RA14 showed highest activity of 83.50 IU/ml followed by 68.86 IU/ml for RF54 and 45.34 IU/ml for RA30, after 120 hours, at 30 °C in agitation condition. The lowest activity 0.50 IU/ml was recorded for RA35 in static condition. The analysis of 16s rRNA gene sequence (KX656177) and phylogenetic analysis of RA14 revealed that RA14 strain belonging to *Streptomyces sp.*

Keywords: *β-Mannanase, Streptomyces sp, Screening*

INTRODUCTION
The β-Mannanase (EC.3.2.1.78) is a group of exotype carbohydrases catalyzing the main chain of beta-mannopyranose residues of linear mannan which is the structural component and omnipresent in the cell walls of plants and some types of plant seeds such as ivory nuts (*Phytelephas spp.*), green coffee (*Coffea spp.*), coconut kernel (copra) and the cell walls of some algae (*Codium spp.*).
The breakdown of mannan occur in nature to utilize D-mannose as a source of energy by particular microorganisms.
β-Mannanase as an single enzyme is not capable of debranching the mannan polymer. The other enzymes are involved and synergetic action of endo-1,4-beta-mannanases (E.C 3.2.1.78, mannan endo-1,4-beta-mannosidase) and exo acting beta-mannosidases (E.C 3.2.1.25) is required. Also side chains attached to the mannan are removed by beta- glucosidases (EC 3.2.1.21), alpha-galactosidases (EC 3.2.1.22) and acetyl mannan esterases (Tenkanen, 1995).
In addition, Breakdown of mannotriose sugar by β-Mannanase has also been reported at a much lower rate (Harjunpaa *et al.,* 1995). Mannibiose and amannotriose are often the end-products as β-Mannanase acting on mannan (Stålbrand *et al.,* 1993), also in some cases mannotetraose and mannose are produced in minor amount (Torto *et al.,* 1996).
In pulp industries, β-Mannanase can be used as effective agent to increase the brightness of pulp according to bleaching experiments (Montiel *et al.,* 2002). In food and feed industries, β-mannanase have been shown to be effective and used for the preparation of fruit juices and soluble coffee (Gubitz *et al.,* 2001), and also in the preparation of poultry diets (Jackson *et al.,* 1999). In oil and gas production, β-Mannanase have also been shown to have a strong potential as viscosity reducers of hydraulic fracturing fluids used (McCutchen *et al.,* 1996).
Furthermore, β-mannanase have potential applications in treating wastes of copra and coffee (Regalado *et al.,* 2000). Recently, the role of β-Mannanase active and stable in the neutral pH range and resistant to proteolysis, particularly to pepsin has been suggested as a fish feed additive (Li *et al.,* 2008; Yang *et al.,* 2009).

MATERIALS AND METHODS

Sample Collection

Different samples were collected from different locality of Gujarat region containing the lignocelluloses compost waste (peat hills in Gujarat university, Ahmadabad), Vegetable wastes (Agricultural Produce Market Committee Ahmadabad), Cotton farm waste (farm of Naroda, Ahmadabad), Mud compost (Gumasan village of Mehsana). All collected samples were bought to the labs in sterile polythene bags.

Isolation Procedure

1.0 gm from each sample was individually mixed with 10 ml of phosphate buffer (pH7.0) with vigorous shaking for 30 minutes.

The filtration was carried out to remove macro particles. 1 ml of the homogenate was added to 9 ml sterile physiological saline and serially diluted. From this 1.0 ml was plated in nutrient agar and incubated at 30°C for 48 hours.

After the incubation period individual colonies were picked and transferred to Nutrient agar plate at sufficient distance (2cm - 3cm) from each other and incubated at 30°C for up to 120 hours.

Primary Screening

The isolates were screened for β-Mannanase producing ability by inoculating them in a sterile medium containing 0.7% Guar gum, 0.3% Glucose, 0.1% yeast extract, 0.1% peptone, 0.1% $(NH_4)_2Po_4$, 0.14% Na_2HPo_4 , 0.02% $MgCl_2$, and 2% Agar (Abe *et al.,* 1994). The plates were incubated at 30°C for up to 96 hours.

After the incubation, plates were incorporated with congo red dye. The β-Mannanase producing isolates were recorded based on clearing zone formed. By comparing the area of clearing zone the potential isolates were preserved on slant culture at 4°C.

Secondary Screening

The secondary screening was carried out with submerged fermentation process. β-Mannanase activity was evaluated by preparing minimal salt medium containing 1.0% guar gum as a sole source of carbon, 0.2% NaCl, 0.1% K_2HPo_4, 0.1% KH_2Po_4, 100ml distilled water and pH adjusted at 7.0 in 250ml volume flask. Twenty flasks were prepared to measure effect of static and agitation condition. Flasks were inoculated with 1 ml of freshly prepared 24 hour old seed culture of selected isolates and incubated at 30°C up to 144 hours of incubation period. Agitation rate was adjusted at 120rpm using orbital shaker incubator. Crude enzyme activity was measured at the interval of every 24 hours for 6 days.

Crude Enzyme Extraction

After incubation, the fermentation medium was harvested at every 24 hour up to 6 days by centrifugation at 4000 rpm for 30 minutes at 4°C. The supernatant was used to assay for β-Mannanase activity.

Enzyme Activity Assay

β-Mannanase activity was assayed in the reaction mixture composing of 0.5 ml of 50mM potassium phosphate buffer (pH 7.0) and 1% Locust Bean Gum (LBG) with 0.5 ml of supernatant at 45°C for 60 min (modified method of El-Naggar *et al.,* 2006). Amount of reducing sugar released was determined bydinitrosalicylic acid reagent (DNS) (Miller, 1959). One unit of β-Mannanase activity was defined as amount of enzyme producing 1 micromole of mannose per minute under the experimental conditions.

16S rRNA Sequencing

The genomic DNA was isolated from the culture and PCR amplification was carried out using actinomycetal 16S rRNA primers ACTF243 (SGMNGADCHCGCGGCCTV) and ACTR1378 (CGGTDTGYACAMGSCCHGGGVACG). PCR product was sequenced using ABI 3500 XL genetic analyzer (Applied Biosystems) at Chromous Biotech Pvt. Ltd., Bangalore.

Phylogenetic Analysis

For phytogenetic analysis, strains for the reference were chosen from NCBI blast search (Altschul *et al.,* 1997). Multiple sequence alignment, phylogenetic analysis and construction of phylogenetic tree were carried out under the software MEGA version 6 (Tamura *et al.,* 2013). Neighbor joining (NJ) phylogenetic tree was generated using Kimura 2-parameter model including bootstrap method of phylogeny with 100 No. of replication.

RESULTS AND DISCUSSION

Isolation

After the incubation period total one hundred two colony were observed and sub-cultured to obtain distinct colony (pure culture). From the obtained isolates thirty isolates were observed as bacteria, forty two isolates were observed *actinomycetes* and thirty isolates were observed as fungi as per preliminary cultural and microscopic studies. These isolates further subjected to primary screening for mannan hydrolyzing activity.

Primary Screening

Total one hundred two isolates from the soil sample were checked for mannan hydrolysis on agar plate. Fifty six numbers of isolates showed positive result for mannan hydrolysis ranging 0.3mmto 22.0mm zone diameter.

A vast range of microorganisms appeared as mannan degraders from bacterial, *actinomycetes* and fungal genera. Among them *actinomycetes* and fungal strains were more potent to produce β-Mannanase. *Actinomycetes* species cultivated from cotton waste and fungal species cultivated from lignocellulotic as well as agricultural waste showed more dominances in β-Mannanase production as compare to other isolates.

Results obtained from the primary screening is shown (Table 1) illustrating different clearing zone diameter and isolates number. Isolates having zone diameter above 14.0 mm were selected for secondary screening.

Table 1: Isolates and Range of Mannan Hydrolysis Zone (RA: *Actinomycetes* Strain, RB: Bacterial Strain and RF: Fungal Strain)

Diameter of Zone Clearance (mm)	Name of the Isolates
(Insignificant)	RA100, RA15, RA18, RA3, RA37, RA44, RA47, RA63, RA66, RA67, RA70, RA74, RA87, RA96,RB12, RB17, RB19, RB2, RB20, RB25, RB28, RB29, RB31, RB4, RB42, RB45, RB49, RB5, RB51, RB52, RB7, RB71, RB78, RB79, RB91, RB92, RB98, RB99,RF1, RF102, RF23, RF50, RF64, RF69, RF73, RF75
0.3--7.0	RA10, RA101, RA11, RA13, RA21, RA34, RA46, RA55, RA57, RA59, RA6, RA61, RA8, RA80,RA84, RA86, RA9, RA94, RA95, RA97, RB32, RB83, RB89,RF16, RF27, RF41, RF48 , RF53, RF58, RF60, RF56, RF72 ,RF76, RF82, RF85, RF88, RF90
7.0 – 14.0	RA24, RA36, RA93, RB22, RB62, RF33, RF40, RF43, RF68
14.0–20.0	RA14, RA30, RA35, RA77, RB81, RF54, RF38
20.0 – 27.0	RF26, RF65, RA39

Secondary Screening

During the secondary screening ten isolates were selected based on preliminary analysis of zone clearance obtained from primary screening. Isolates were grown using minimal salt medium with static and agitation condition using submerged fermentation.

After completion of 24 hours there were no significant increase in the enzyme activity in static and agitation condition.

After the completion of incubation period, seven isolates were showing higher activity in agitation condition compare to static condition (Figure 1).

The lowest activity 0.50 IU/ml was reported with RA35 isolate in static condition, highest activity was observed 83.50 IU/ml forRA14 isolate, followed by 68.86 IU/ml for RF54 and 45.34 IU/ml for RA30after 120 hours of incubation with agitation condition.

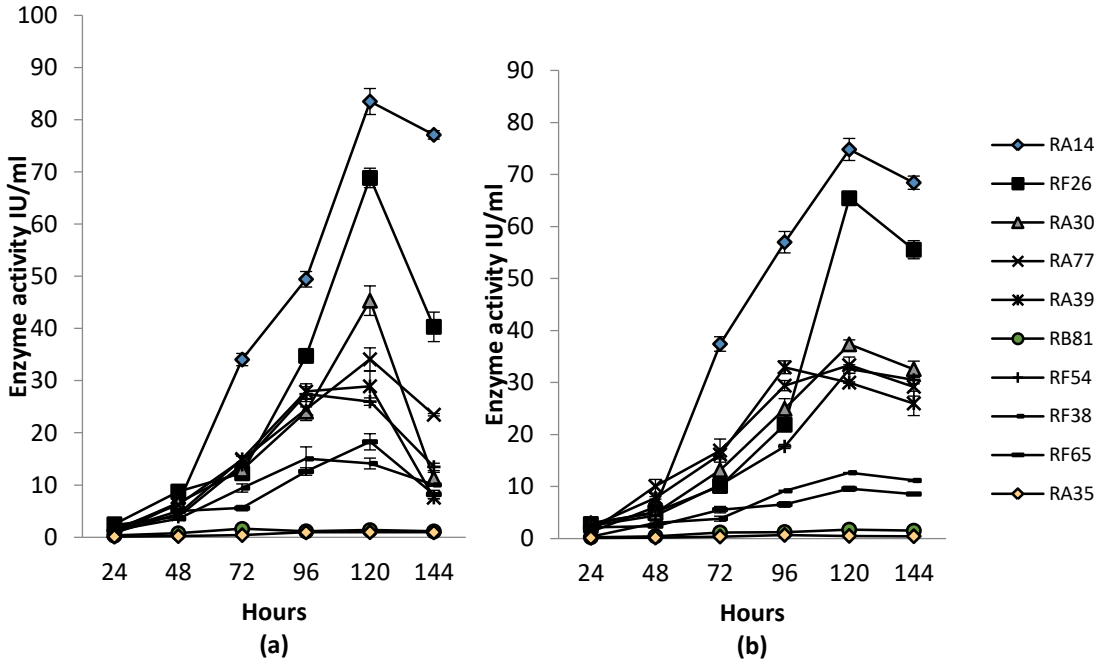

Figure 1: Enzyme Activity of Different Isolates Every 24 Hours a) Agitation Condition b) Static Condition

16S rRNA Sequencing and Phylogenetic Analysis

The nucleotide sequence of the 16S rRNA gene of strain RA14 was determined. Nucleotide sequence of the 16S rRNA gene (888 bp) of strain has been assigned GenBank (EMBL) under accession number KX656177 with the strain code RDA1496. This sequence was subjected to similarity searches against sequences of public databases and phylogenetic tree was established (Figure 2).

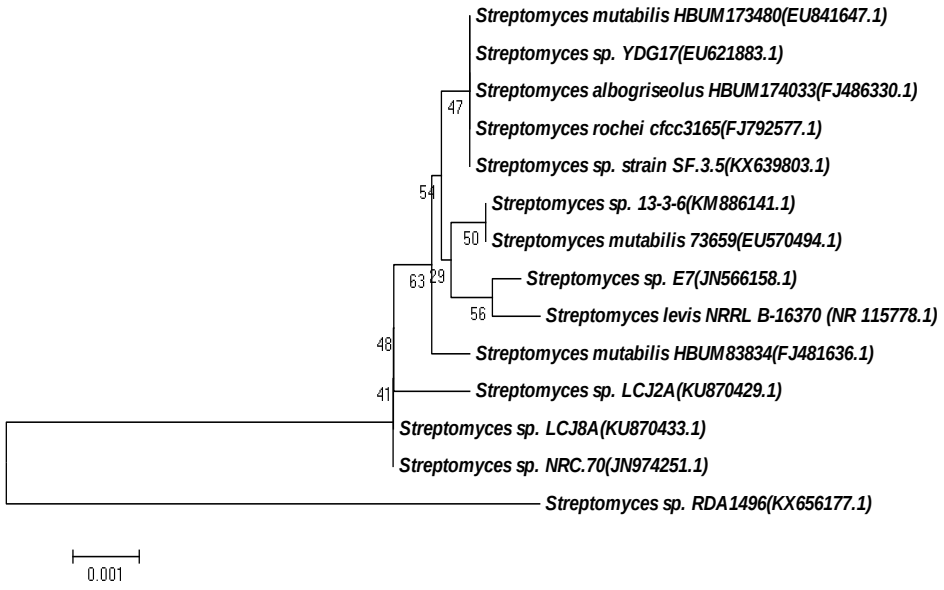

Figure 2: Phylogenetic Trees of *Streptomyces sp.* RDA1496 Strain

Conclusion

In view of results obtained, we are able to establish that natural soil sample accommodate β-mannanases producing microorganism. During this study we found that isolate RA14 having the highest activity belonging to *Streptomyces* genera and phytogenetic analysis indicate that there are genotypic differences between RA14 and neighboring *Streptomyces* species' neighbours and may have distinct industrial application. The RA14 (RDA1496) isolate will be subjected to optimization study to increase the enzyme yield.

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