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Research Article

SCREENING, ISOLATION AND CHARACTERIZATION OF CYCLODEXTRIN GLYCOSYL TRANSFERASE PRODUCING BACTERIA FROM SOIL SAMPLES

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ABSTRACT

Cyclodextrin glycosyl transferase (CGTase), EC 2.4.1.19, is an extracelluar enzyme that converts starch into non-reducing, cyclic malto-oligosacchrarides called cyclodextrins (CDs). It is an important hydrolytic enzyme that carries out reversible intermolecular as well as intramolecular transglycosylation and performs cyclization, coupling and disproportionation of maltooligosaccharides. They are used in a wide range of application in food, pharmaceutical, cosmetic and agricultural industries. Soil samples from different environments were screened for the isolation of potent CGTase producing strains. 25 positive isolates were selected based on promising halozones. From this, six promising isolates, were further screened for CGTase activity. The isolate from the soil sample of rice mill waste at Rajamundry showed good CGTase activity and was selected for further studies and it was designated as TPR71H. The morphological, cultural and biochemical characteristics of TPR71H were investigated by using various media and biochemical reactions as recommended by the standard books. Based on the morphological, physiological and biochemical studies the selected strain was identified as *Bacillus* sp. Further confirmed with respect to species level, the genomic DNA of this strain was amplified and analyzed for molecular-based identification. Blast analysis denoted that the sequence of TPR71H has 99% similarity to the *Bacillus* family (*B. cereus, B. circulans*) and hence the newly isolated strain was designated as *Bacillus* sp. TPR71H. The present investigation reveals that newly isolated *Bacillus* sp. TPR71H may give good yield of CGTase.

INTRODUCTION

The success of an industrial fermentation process chiefly depends on the microbial strain used. An ideal producer or economically important strain should have the following characteristics;- (1) It should be pure, and free from phage (2) It should be genetically stable, but amenable to genetic modification (3) It should produce both vegetative cells and spores (4) It should grow vigorously after inoculation in seed stage vessels (5) Should produce a single valuable product, and no toxic by-products (6) Product should be produced in a short time, e.g., 3 days (7) It should be amenable to long term conservation (8) The risk of contamination should be minimal under the optimum performance conditions.

The first step in developing a producer strain is the isolation of concerned microorganisms from their natural habitats. Alternatively, microorganisms can be obtained as pure cultures from organization, which maintain culture collections, e.g., American Type Culture Collection (ATCC).

The microorganisms of industrial importance are, generally, bacteria, actinomycetes, fungi and algae. These organisms occur virtually everywhere, e.g., in air, water, soil, surfaces of plants and animals, and plant and animals tissues. But most common sources of industrial microorganisms are soils, and lake and river mud. Often the ecological habitat from which a desired microorganism is more likely to be isolated will depend on the characteristics of the product desired from it, and of process development.

A variety of complex isolation procedures have been developed, but no single method can reveal all the microorganisms present in a sample. The enrichment techniques are designed for selective multiplication of only some of the microorganisms present in a sample. These approaches however take a long time.

The main isolation methods used routinely for isolation from soil samples are: sponging (soil directly), dilution, gradient plate, aerosol dilution, flotation, and differential centrifugation. Often these methods are used in conjunction with an enrichment technique.

In the present study we searched for isolates of microorganisms which have a high CGTase activity from soils. CGTase production profiles were studied with different production media using soluble starch in a basal medium in order to optimize CGTase activity.

MATERIALS AND METHODS

Chemicals and media

All the chemicals and medium constituents used in this study were of analytical grade.Media constituents used in this study were procured from Hi-Media, Mumbai.

Sample collection

For isolation of active producers of cyclodextrin glycosyltransferase, six different samples were collected from various places located around coastal area of Andhra Pradesh, India and brought to laboratory; the samples were stored at 4°C till further use. All the samples were collected in the sterile screw capped tubes and care was taken to see that the points of collection had a widely varying characteristic as possible with regard to the organic matter, moisture content, particle size, colour of soil and geographical distribution.

Screening and Isolation of Bacteria

Soil samples were suspended in normal saline, serially diluted and then plated on a Horikoshi 11 agar plate containing (w/v) 1.0% soluble starch, 0.5% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 0.02% phenolphathalein, 1.0% Na_2CO_3 and 1.5% agar PH7.5 (Illias *et al*., 2002). Plates were incubated at 37°C for 24h. The bacterial colony that produced the largest clear and highest CGTase activity were selected.

In the preliminary screening of organisms for CG Tase production by the above procedure, 25 colonies that produced large clear halo zones were selected for further studies. These 25 isolates, having promising halo zones were transferred to a basal medium [1]. The inoculated medium was incubated at 30°C (250rpm) for 24h. After the incubation period these were tested for CGTase production.

Assay of CGTase

Assay of CGTase was carried out according to the method of Kaneko *et al.,* 1987 [2]. The method is described under Analytical Methods.

The amount of β-cyclodextrin produced was estimated from the standard graph of 0-500µg/mL β-CD concentration against absorbance. One unit of CGTase was defined as the amount of enzyme required to produce 1µmol of β-CD/min.

All the experiments were conducted in triplicate and the mean values were calculated. One of the isolates which showed significant CGTase activity was designated as TPR71H and selected for further studies.

Characterization of TPR71H

The morphological, cultural and biochemical characteristics of TPR71H were investigated by using various media and biochemical reactions as recommended by Bergey's Manual of Determinative Bacteriology (1957, 1974), Bergey's Manual of Systematic Bacteriology (1992), Mackie and McCartney Practical Medical Microbiology [3] and Laboratory Manual of Fundamental Principles of Bacteriology [4]. Uninoculated controls were always run for all the tests.

Micromorphology

For the microscopic study of cells, the cells were grown on nutrient broth. Shape of the cells, presence of endospores and motility of the cells were determined.

Cultural Characteristics

Culture characteristics were observed using Sodium azide medium [5], crystal violet blood agar medium [3], Litmus milk reduction test [6] nutrient agar medium, nutrient broth medium. The culture was inoculated into a tube containing the above said medium and incubated at 37°C for 48h and the growth pattern was recorded.

Growth of the cells at various temperature, pH, different sodium chloride concentration and heat tolerance were also determined.

Biochemical Tests

The different biochemical tests like methyred test, Voges Proskauer test, indole test, oxidase test, citrate utilization test, urease test, gelatin hydrolysis test and starch hydrolyzing test hydrogen sulfide test, catalase test and nitrate reduction test were carried out as per the standard procedures by employing the prescribed media for the taxonomic studies of the isolate.

16S rRNA Gene Sequencing

Isolation of DNA

The chromosomal DNA of isolated strain TPR71H was isolated according to Sharma and Singh, 2005. Four mL of microbial culture was taken and the cells were pelleted in Eppendorf tubes (2.0mL) by centrifugation at 5000rpm for 5min at 37°C. The cell pellet was resuspended in 200µl of TE [10mM Tris–HCl (pH 8.0), 1mM EDTA (pH 8.0)]. 50ng of RNase was added to digest the contaminating RNA. 400 µl of Solution I [(1%, wv-1) Sarkosyl, 0.5M NaCl, (1%, w/v) SDS] was added. The tubes were mixed properly and kept for 10min at 37°C with intermittent shaking for every 5min. Equal volume of PCI (phenol:chloroform:isoamyalcohol; 25:24:1) mixture was added immediately by inversion. The solution was centrifuged at 10,000rpm for 5 min at 37°C the supernatant was carefully transferred into a new Eppendorf tube. 0.1 volume of sodium acetate (3M, pH 5.2) and 0.6 volume of isopropanol were added and the contents were gently mixed by inverting. The DNA was precipitated in the pellet by centrifugation of Eppendorf tubes for 5min at 10,000rpm at 37°C. Pellet was washed with 1mL of 70 % ethanol and centrifuged at 10,000rpm for 3min at 37°C and the supernatant was removed and the pellet was air-dried. DNA was resuspended in 100µl of sterile deionized water and the suspension was stored at –20°C for further use.

Amplification and Sequencing of 16S rRNA Gene

In a typical procedure, 16S rRNA gene of the isolated strain, TPR71H, was amplified by PCR using 25ng of TPR71H strain chromosomal DNA, 25ρmol primers [27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500r (5'-AGAAAGGAGGTG ATCCAGGC-3') corresponding to the *Escherichia coli* numbering system] according to Brosius *et al*., 1978 [7]. PCR amplification was initiated by incubating at cycling parameters included denaturation at 94°C for 3min; followed by 30 cycles of denaturation at 94°C for 30sec, annealing at 58°C for 30sec and amplification at 72°C for 1min and final extension at 72°C for 5min using GeneAmp PCR system (Applied Biosystems). The amplicon was purified using Qiaquick PCR purification kit (Qiagen) and subsequently cloned into pGEM-Teasy vector system II (Promega, USA) in accordance with the manufacturer's instruction. Plasmid from the resulting clones, extracted by using the 'alkaline lysis method' [8] and the same was checked for the presence of 1.5kb inserts from Eco RI digestions prior to sequencing. Insert of recombinant plasmid p16S12 (1.5kb 16S rRNA amplicon in pGEM T) was amplified using T7 and SP6 primers and purified. Sequencing of 1.5kb insert of plasmid p16S12 was carried out with an ABI DNA sequenetor model 377a (Applied Biosystems) using Big-Dye Terminator kit (Applied Biosystems) using primers T7 (5' TAATACGACTCACTATAGGGC 3'), SP6 (5' ATTTAGGTGACACTATAG 3') and other universal 16S rRNA gene primers such as 518F (5' CAGCAGCCGCGGTAATAC 3'), 908F (5' AAACTCAAAGGAATTGACGG 3'), 1073R (5' ACGAGCTGAC GACAGCCATG 3'), 918R (5' CCGTCAATTCCTTTGAGTTT 3') and 361R (5'CCCACTGCTGCCTCCCGTAG 3')

Phylogenetic Analysis of the Strain

The 16S rRNA gene sequence of the strain TPR71H was used as a query to search for homologous sequence in the nucleotide sequence databases by running BLASTN program [9]. The high scoring similar to 16S rRNA gene sequences were identified from the BLASTN result and retrieved from GenBank database. The identified sequences were aligned using CLUSTAL-W software [10]. Distances among selected sequences were calculated according to methods of Kimura two parameter models [11]. Phylogenetic trees were inferred by using the neighbor-joining [12]. Bootstrap analysis with the help of MEGA 3.0 software package [13] based on 1000 resamplings.

Selection of optimal production medium for CGTase production

Selection of production medium

The isolated TPR71H strain was employed in the present study to determine the most suitable production medium for the fermentative production of CGTase by submerged fermentation process.For this purpose five different production media reported in the literature were chosen.

Composition of the media used is as follows:

Production Medium I [1]

Soluble starch1.0%

Yeast extract0.5%

Peptone0.5%

$Na_2HPO_4$0.1%

$MgSO_4$0.02%

Distilled water 100mL

pH7.0

Production Medium II [14]

Soluble starch 2.0%

Casein hydrolyzate 0.5%

Yeast extract 0.5%

Na_2HPO_4 0.5%

KH_2PO_4 0.2%
Water 100mL

pH 7.5

Production Medium III [15]

Soluble starch 2.0%

Yeast extract 0.5%

Peptone 0.5%

$MgSO_4$ $7H_2O$ 0.02%

K_2HPO_4 0.1%

Water 100mL

pH 8.5

Production Medium IV [16]

Soluble starch 1.0%

Peptone 0.5%

Yeast extract 0.5%

KH_2PO_4 0.1%

$MgSO_4$ $7H_2O$ 0.02%

Agar 3.0%

Phenolphthalein 0.03%

Sodium carbonate 1.0%

Water 100mL

pH 10

Production Medium V [17]

Beef extract0.5%

Polypeptone 1.0%

Sodium chloride 0.2%

Yeast extract 0.2%

Soluble starch 1.0%

Water 100mL

pH 7.2

For the preparation of inoculum, the growth contents of two days old slant culture was suspended into 5mL of sterile distilled water and transferred into 45mL of inoculum medium contained in 250mL EhrlenMeyer flask. The flask was incubated on the rotary shaker (220rpm) at 30°C for 24h. This suspension is used as an inoculum.

A 10% (5mL) of inoculum was transferred aseptically to 45mL of production medium. The flasks were kept on the rotary shaker (220rpm) at 30°C. The samples (5mL) were withdrawn for every 12h up to 72h. The samples withdrawn were centrifuged at 3000rpm for 10min and the clear supernatant was used for enzyme estimation.

RESULTS AND DISCUSSION

Isolation and screening for CGTase producing organisms

Soil samples from different environments were collected and used for isolation of bacterial strains producing CGTase enzyme. A total of 25 colonies were selected on the basis of their macroscopic characters, eliminating those that appeared close to each other (Table 1). These 25 colonies were sub-cultured and primarily screened for their CGTase production which was indicated by the formation of halozone (Table 2). It is evident from the data presented in the Table 2 that 6 isolates showed good formation of halozone, 4 isolates formed moderate zones and 7 isolates showed poor formation of halozone, while 8 isolates showed very poor formation of halozone. Six promising isolates, which showed good halozone diameter, were further screened for their CGTase activity by shake flask method comparing *Bacillus macerans* NCIM 2131 as a standard strain. The results of CGTase activity of the screened six isolates were presented in Table 3.

According to the data recorded in Table 3 it is clear that the isolate RM-9 exhibited maximum activity than the standard strain, *Bacillus macerans* NCIM 2131. The promising isolate RM-9 was selected for further studies and it was designated as TPR71H.

Identification and Characterization of Isolate TPR71H

The strain, TPR71H, was characterized for various physiological properties. The results indicated that this strain was Gram positive, rod shaped (Fig 1& 2) and produces endospores. The growth pattern (colony morphology) on the agar medium was observed to be irregular with undulate margins, smooth surface and opaque density (Table 4). The isolate's growth was studied at different temperatures (4°C to 42°C) and pH (5 to11). It was noticed that growth was seen in the temperature range of 15 to 42°C and in the pH range of 8 to 10. Further, the growth pattern of the selected strain was studied in the presence of different concentrations of NaCl. It was found that growth was seen up to 7% NaCl concentration (Table 5). The biochemical characteristics of the strain and ability to produce acid using carbohydrates were investigated according to Bergey's manual of Bacteriology and presented in Tables 6 and 7, respectively. Also in the case of sodium azide and crystal violet blood agar media, abundant, heavy & raised, good with smooth surface growth were observed respectively Table 8. The isolate exhibited negative litmus milk reduction test. The isolate hydrolyzed gelatin and starch and gave positive reaction on methyl red, nitrate reduction and catalase. Hydrogen sulphide, casein hydrolysis, citrate utilization, Voges proskauer, indole test, oxidase test and urease tests are negative. Based on the studies the selected strain was identified as *Bacillus* sp.

Characterization of isolate by molecular approach (16s Ribotyping)

Based on the morphological and biochemical characterization the strain TPR71H was identified as *Bacillus* sp. Further confirmed with respect to species level, the genomic DNA of this strain was amplified and analyzed for molecular-based identification. The amplicon was purified and used to determine the 16S rRNA gene. The gene sequence revealed that it contains 1425 base pairs consisting of Adenine – 25.6% (365 molecules), Cytosine – 22.8% (325 molecules), Guanine – 30.9% (440 molecules) and Thymine - 20.7% (295 molecules). Blast analysis denoted that the sequence of TPR71H has 99% similarity to the *Bacillus* family (*B. cereus, B. circulans*). Further the Phylogenetic tree was constructed by taking the sequences obtained in the blast search, using *Microbacterium indicum* (AM286267) as an outer group. Figure 3 depicts the constructed phylogenetic tree, from this it was observed that the isolate TPR71H belongs to the *Bacillus* family.

Based on morphological, biochemical and molecular characterization the strain TPR71H was identified as *Bacillus* sp. and hence, it is designated as *Bacillus* sp.TPR71H. The partial sequence of 16s rRNA gene was submitted to the GeneBank database and can be accessed under Genebank accession number FN993946.

Selection of optimal production medium for CGTase production

The optimal production medium for CGTase production by the newly isolated strain TPR71H was done by submerged fermentation method. For the present study, five different types of media were used and the enzyme activity was estimated by using modified assay of CGTase. The results were shown in Table 10 and Fig 4. The results indicate that medium number I was found to be the best, resulting in a yield of 9.66U/mL. This production medium was used for subsequent studies.

Table 1: Bacterial isolates from various samples

Sample	No. of isolates
RM	09
SM	05
FP	02
JI	04
PI	03
OI	02

Table 2: Primary screening for CGTase producing bacteria from soil samples

Isolate No.	Source	Diameter of halo zone (mm)
RM-1	Rice Mill	0.65
RM-2	Rice Mill	0.27
RM-5	Rice Mill	0.53
RM-7	Rice Mill	0.76
RM-8	Rice Mill	0.22
RM-9	**Rice Mill**	**0.98**
RM-10	Rice Mill	0.25
RM-11	Rice Mill	0.28
RM-12	**Rice Mill**	**0.92**
SM-4	Sugar Mill	0.58
SM-6	Sugar Mill	0.22
SM-7	**Sugar Mill**	**0.93**
SM-8	Sugar Mill	0.27
SM-9	Sugar Mill	0.54
FP-3	Fruit Processing	0.81
FP-4	Fruit Processing	0.45
JI-2	**Jaggery Industry**	**0.90**
JI-3	Jaggery Industry	0.54
JI-5	Jaggery Industry	0.44
JI-7	**Jaggery Industry**	**0.87**
PI-1	Paper Industry	0.48
PI-3	Paper Industry	0.72
PI-9	**Paper Industry**	**0.91**
OI-2	Oil Industry	0.14
OI-6	Oil Industry	0.12
Bacillus macerans NCIM 2131 (Reference)	NCIM	0.90

Table 3: Cyclodextrin glycosyltransferase production of selected isolates by shake flask method

Isolate No.	Cyclodextrin glycosyltransferase activity (U/mL)
RM-9	6.87
SM-7	5.05
RM-12	4.79
PI-9	4.66
JI-2	3.49
JI-7	3.30
Bacillus macerans NCIM 2131 (Reference)	5.32

Table 4: Morphological characteristics of newly isolated *Bacillus* sp.TPR71H

Tests	*Bacillus* SP.TPR71H
Colony Morphology	
Configuration	Irregular
Margin	Undulate
Elevation	Raised
Surface	Smooth
Colony Colour	Off-White
Opacity	Opaque
Gram's Reaction	+ve
Cell Shape	Rods
Motility	Motile
Spore(s)	+ve
Position	Central
Shape	Oval

Table 6: Biochemical characteristics of newly isolated *Bacillus* sp. TPR71H

Test	Reaction
Methyl red test	+
Voges Proskauer test	-
Citrate utilization test	-
Indole test	-
Gelatin hydrolysis	+
Starch hydrolysis	+
Catalase test	+
Hydrogen sulphide	-
Oxidase test	-
Urease test	-
Nitrate reduction test	+

(+): Positive; (-): Negative

Table 5: Physiological characteristics of newly isolated *Bacillus* sp.TPR71H

Tests	Growth
Growth at temperature	-
4°C	-
12°C	-
25°C	+
30°C	+
37°C	+
42°C	+
Heat Tolerance test (at 60°C)	-
Growth at pH	
pH 5.0	-
pH 6.0	-
pH 7.0	+
pH 8.0	+
pH 9.0	+
pH 10.0	+
pH 11.0	-
Growth on NaCl (%)	
2	+
4	+
6	+
10	-
12	-

Table 7: Acid Production from Carbohydrates by the newly isolated *Bacillus* sp. TPR71H

Acid production from	Reaction
Arabinose	-
Salicin	+
Galactose	+
Meso-Inositol	-
Mannitol	-
Raffinose	-
Fructose	-
Glucose	+
Xylose	-
Rhamnose	-
Sucrose	-

(+): Positive; (-): Negative

Table 8: Cultural characteristics of newly isolated *Bacillus* sp. TPR71H

Cultural characteristics	Growth
Nutrient agar	Abundant, smooth sufaced, non-viscid and non-glistening filiform growth
Nutrient broth	Turbid growth, creamy white
Sodium azide agar	Good growth
Crystal voilet agar	Good growth
Litmus milk reduction test	Negative

Table 10: Production of CGTase in different production medium by isolated TPR71H

Medium No	CGTase Yield (Units/mL)
Medium I	9.66
Medium II	8.16
Medium III	7.13
Medium IV	8.38
Medium V	5.66

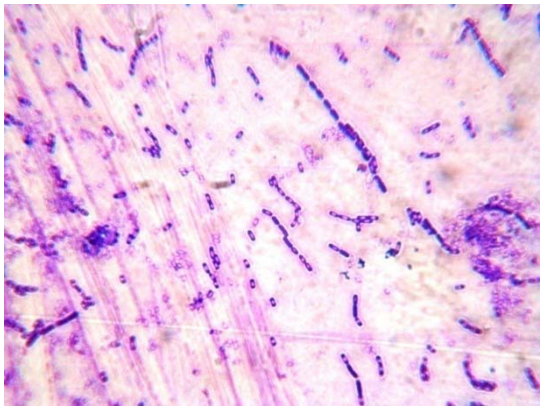

Fig. 1: Morphology of newly isolated *Bacillus* sp. TPR71H (100X)

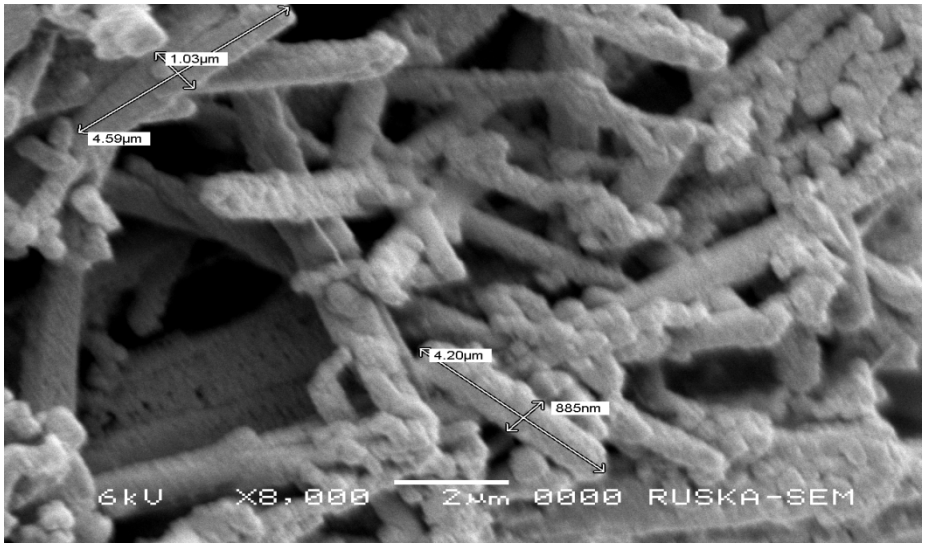

Fig. 2: Morphology of newly isolated *Bacillus* sp. TPR71H under Scanning Electron Microscopy

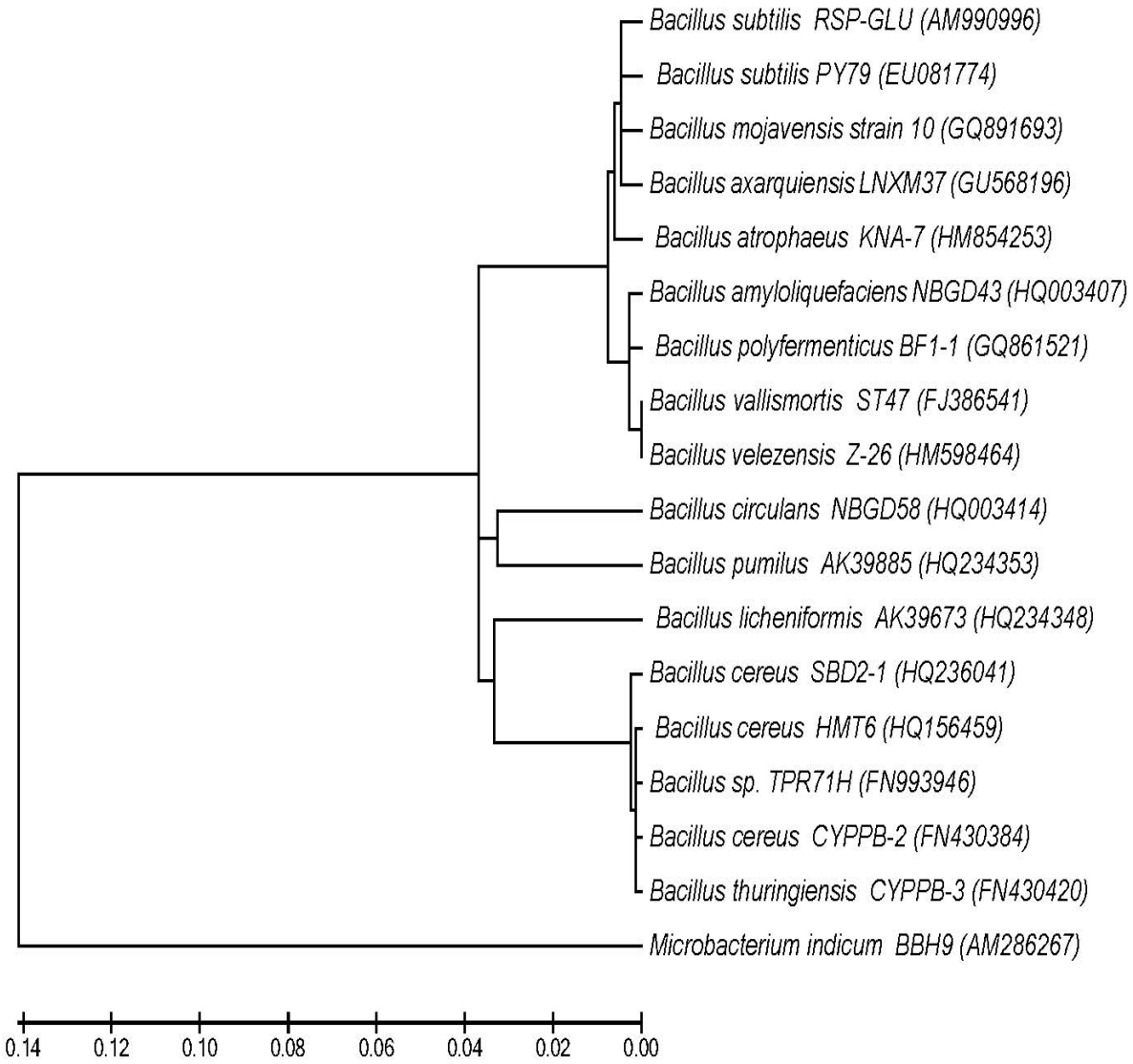

Fig. 3: Phylogenetic tree of newly isolated *Bacillus* sp. TPR71H

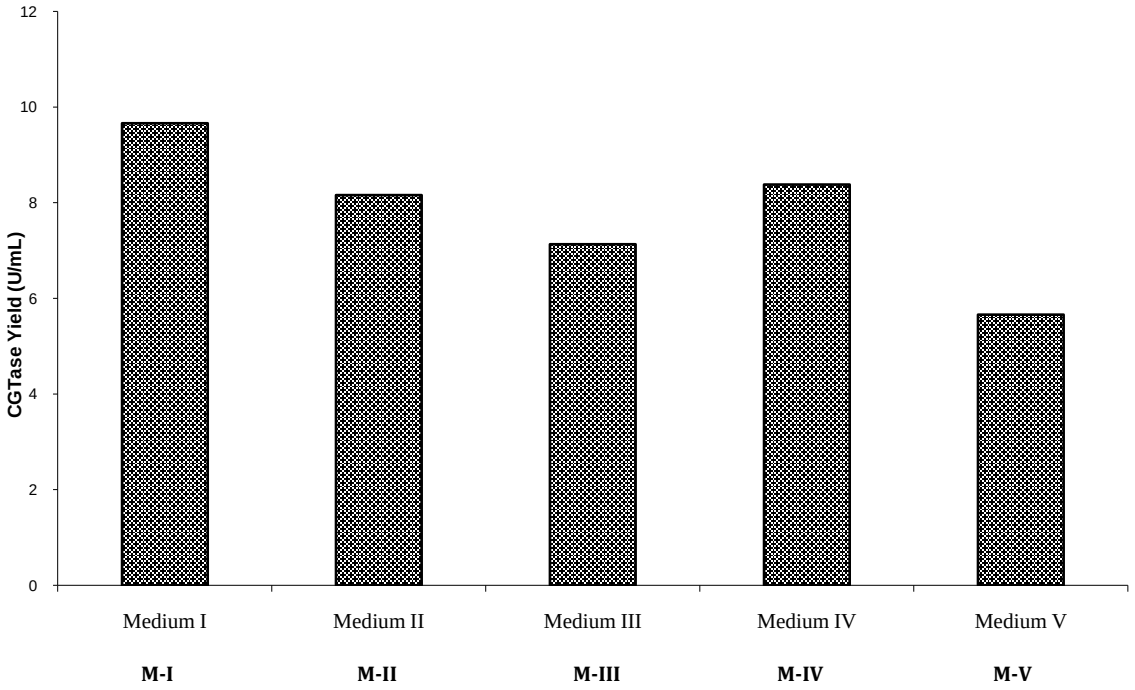

Fig. 4: Production of CGTase in different production media by isolated TPR 71H

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