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Research Article

ISOLATION AND IDENTIFICATION OF L-GLUTAMINASE AN ANTILEUKEMIC ENZYME PRODUCING MICO-ORGANISM FROM GODAVARI RIVER BANK SOILS IN ANDHRA PRADESH

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ABSTRACT
Considering at the potential application of the L-glutaminase in pharmaceutical, food industries and chemical industry, a L-glutaminase producing micro organism was isolated from the soil samples of Godavari river bank of Andhra Pradesh, India. Among several isolates, one isolate (RSP-GLU) exhibited the highest L-glutaminase activity. The isolate RSP-GLU was identified based on the morphological, physiological and biochemical properties. Further 16S rDNA sequence (GenBank Accession Number AM990996) based phylogenetic analysis conformed that the isolated strain RSP-GLU as *Bacillus subtilis*.

KEYWORDS: L-Glutaminase, Isolation, Identification, 16s rDNA gene, Biochemical characterization, Godavari river bank

INTRODUCTION

L-glutaminase (EC 3.5.1.2) is amidase enzyme which catalyzes the L-glutamine to L-glutamic acid and ammonia. L-glutamine has a significant role in nitrogen metabolism at cellular level[1-4]. L-glutamine and L-glutamate are one of the important amino acid for the production of proteins and nucleic acids in biotic metabolism. L-glutamine can either be produced within the cell by an enzyme called glutamine synthetase or can be obtained from the food sources. Tumor cells, more specifically lymphatic tumor cells unable to synthesis the L-glutamine unlike the normal cells hence require huge amounts of glutamine to keep up their rapid growth. Thus these tumor cells are dependent on the exogenous supply of L-glutamine for its growth and survival. Therefore, the use of amidases deprives neoplasms for essential nutrients and causes selective death of L-glutamine-dependent tumor cells by depriving of L-glutamine[5].

High content of L-glutamate gives pleasant and palatable tastes of oriental fermented foods like soy sauce, miso and sufu. Hence hydrolysis of L-glutamine by L-glutaminase plays a significant role in the food industry[6-9]. This enzyme is also used as biosensing agent and an analytical agent[10] to determine the free glutamine in the cell culture broths. The γ-glutamyl transfer reactions of L-glutaminase is also useful in the production of the high marketed value specialty chemicals like threonine[11]. Based on all above applications the scientific community encouraged to explore the different resources for effective production of this enzyme.

Among different sources of L-glutaminase enzyme producers, microbial strains possess an elevated edge over other enzyme producers due to their growth requirements, easy processing and handling etc[3]. The literature reports suggested that the enzyme produced by different microbial strains differed in some physiological, biochemical, catalytic and immunological properties. This led to the continuous screening program for isolation of novel microbial strains that could produce an effective enzyme with few limitations at usage sectors.

In the previous publications the authors explained the various optimization procedures for the improvement of the L-glutaminase production in both submerged[4] and solid state fermentation[3]. The present study deals with the isolation, identification and selection of the high yielding L-glutaminase producing microorganism from the various soil samples.

MATERIALS AND METHODS

Screening of L-Glutaminase Producing Microorganism by Rapid-Plate Assay Method

L-glutaminase producing micro-organisms were isolated from the soil samples by using the method of[12]. The L-glutamine media[9] was supplemented with 0.135 µl of 2.5 % of phenol red as an indicator. Two control plates were also prepared using L-glutamine media - one was without dye while the other was without L-glutamine (use $NaNO_3$ as a nitrogen source). L-glutaminase activity was identified by formation of a pink zone around colonies. Zone diameters were measured after 24 hours. Based on the zone diameter the colonies were picked and broth studies were also carried out in order to compare the results obtained with the plate assay.

Biochemical and Phenotypic Characterization

Morphological studies were conducted under scanning electron microscope (model: JOEL-JSM 5600). Conventional physiological and biochemical characterization tests were carried out at Institute of Microbial Technology (IMTECH) Chandigarh, India, according to Bergey's Manual.

Measurement of L-Glutaminase Activity

L-glutaminase activity was determined using L-glutamine as substrate and the product ammonia, released during the catalysis was measured by using Nesseler's reagent according to method of[13]. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µMol of ammonia under optimal assay conditions.

Molecular Characterization of Isolate

DNA Extraction

The isolate RSP-GLU cell pellet was suspended in 5mL of lysis buffer (pH 8.0) consisting of 10 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 20 mM EDTA. The bacterial cells were then lysed using 0.5 mL of 10mg/mL lysozyme and incubated at 37 ^{0}C for 30 to 60min. Then 2 µL of RNase (500µg/mL) added and incubated for another 15 min. To this, 18µL of 10mg/mL proteinase-k (final conc.50µg/mL) and 180µL of 10% SDS (final concentration 0.5%) added and incubated at 37^{0}C for 60min to remove the protein contamination. Then the lysate was subjected to phenol : chloroform (25:24) extraction followed by ethanol precipitation. The obtained DNA pellet then re-suspended in 1mL of DNase and RNase free water.

Amplification and Sequencing of 16S rRNA Gene

The 16S rRNA gene was PCR amplified according to the Embley (1991), Using a GeneAmp 2700 PCR system (Applied Biosystems, Foster City, Calif., USA). For amplification the following primers were used 16S-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-1500r (5'-AAAGGAGGTGATCCAGCC-3') which represented a 16S rRNA gene of *Escherichia coli*. Chromosomal DNA was used as a template. The amplified DNA fragments were analyzed by agarose gel electrophoresis under standard conditions. The Qiaquick PCR purification kit was used to recover amplicon from the agarose gel. Purified PCR product was sequenced directly by ABI DNA sequenetor model 377a (Applied Biosystems) using Big-Dye Terminator kit (Applied Biosystems) using primers r1L (5′-GTATTACCGCGGCTGCTGG-3′), r2L (5′-CATCGTTTACGGCGTGGAC-3′), r3L (5′-TTGCGCTCGTTGCGGGACT-3′), r4L (5′-ACGGGCGGTGTGTACAAG-3′), 926f (5′-AAACTCAAAGGAATTGACGG-3′) and f3L (5′-GTCCCGCAACGAGCGAAC-3′). The DNAsis program was used for connecting the sequence of fragments; and the BLASTN program was used for a gene homology search with standard defaults. The nuclear sequence of the 16S rRNA gene for the strain was deposited in the GenBank database.

Phylogenetic Analysis of the Strain

The 16S rRNA gene sequence of the isolated strain was used as a query to search for homologous sequence in the nucleotide sequence databases by running BLASTN program[14]. The high scoring similar to 16S rRNA gene sequences were identified from the results and retrieved from GenBank database. The identified sequences were aligned using CLUSTAL-W algorithm in MEGA 4.0 software. Phylogenetic trees were inferred by using the neighbor-joining[15] bootstrap analysis with the help of MEGA 4.0 software package[16] based on 1000 resamplings.

RESULTS AND DISCUSSION

Enrichment and Isolation of Microorganisms

Among the 10 soil samples tested only 2 samples collected from the Godavari river sediments showed growth in L-glutamine enrichment media. From these two samples 5 isolates were obtained and these isolates were sub-cultured and used for further studies. All the isolates produced opaque, rough surface and irregular colonies. These were initially designated as RSP-GLU, RSP-GLU1, RSP-GLU2, RSP-GLU3 and RSP-GLU4.

Screening of L-Glutaminase Positive Cultures by Rapid-Plate Assay Method

All isolated five bacterial cultures grow on the glutamine media supplemented with a dye indicator (Phenol red). The indicator is pH sensitive normally it gives yellow color to media (in acidic and neutral conditions), it gives the pink color to the media when the pH changes from acidic to alkaline condition. The pink zone around bacterial colony indicates the pH alteration which originated from ammonia accumulation in the medium. All five isolates gave positive results in agar plate assay however, showed variation in zone formation. The isolate RSP-GLU exhibited the large pink zone when compared with other isolates. Further, to evaluate the potential of these isolates for production of L-glutaminase enzyme, submerged fermentation broth studies were performed using L-glutamine medium. The quantitative estimation of produced L-glutaminase revealed that among five isolates RSP-GLU showed the highest enzyme production under submerged fermentation also. Based on above observations further studies were continued with the isolate designated as RSP-GLU. Figure 1 depicts the pink zone formed by the isolate RSP-GLU.

Biochemical Characterization of Isolate

The colonies of strain RSP-GLU on nutrient agar plate were round, wavy margins rough surface and opaque density. The cell growth is aerobic, gram positive in nature and spore forming rod shaped bacteria. Figure 2 shows the scanning electron microscopic pictures of the isolated bacteria RSP-GLU. The cells could survive and grow in the medium pH ranging from 5.0 to 11.0. The growth was studied in presence of different NaCl concentrations, it was observed that RSP-GLU would grow even 10% NaCl. All other biochemical characterization tests and their results are depicted in the Table 1 & 2. From the above results, it was concluded that this isolate RSP-GLU belongs to *Bacillus subtilis* and it was designated as *Bacillus subtilis* RSP-GLU.

Molecular Characterization of Isolate RSP-GLU (16S Ribotyping)

Though based on above morphological and biochemical characterization the strain RSP-GLU belongs to *Bacillus subtilis* family, further conformation studies were performed by molecular characterization. This study was performed because in earlier studies the strain identified by biochemical characterization differed with ribotyping identification[17]. In order to evaluate the same 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 1539 base pairs consisting of Adenine - 24.7%, Cytosine - 23.6%, Guanine - 31.3% and Thymine - 20.4%; with AT:CG ratio of 45:55. Blast analysis denoted 99% similarity to the *B. subtilis* family. Phylogenetic tree was constructed by taking the sequences obtained in the blast search, using *Microbacterium indicum* (AM158907) as an outer group. Figure 3 shows the phylogentic tree from this it was observed that the isolate RSP-GLU belongs to the *Bacillus subtilis* family. The partial sequence of 16s rRNA gene was

submitted to the GenBank database and can be accessed under Genbank accession number AM990996. The culture was deposited in MTCC which was designated as *Bacillus subtilis* RSP-GLU MTCC 9727.

CONCLUSION

L-glutaminase producing microbial strain was isolated from soil samples collected from Godavari River bank. The isolates potential of L-glutaminase enzyme production was evaluated by spread plate method. The strain was purified and SEM studies indicated that this strain is rod shaped. Morphological and biochemical characterization revealed that this strain belongs to *Bacillus subtilis*. Robotyping analysis performed and deposited in Genbank under accession number AM990996.

REFERENCES

1. Brosnan JT, Ewart HS, Squires SA: Hormonal control of hepatic glutaminase. Adv. Enzyme Regul 1995;35:131–46.
2. Iyer P, Singhal RS: Production of glutaminase (E.C.3.2.1.5) from *Zygosaccharomyces rouxii*: Statistical optimization using response surface methodology. Bioresource Technolo 2007;99: 4300-07.
3. Sathish T, Lakshmi GS, Rao ChS, Brahmaiah P, Prakasham RS: Mixture design as first step for improved glutaminase production in solid-state fermentation by isolated Bacillus sp. RSP-GLU. Lett Appl Microbiol 2008;47: 256–62.
4. Sathish T, Prakasham RS: Enrichment of glutaminase production by *Bacillus* sp. RSP-GLU in submerged cultivation based on Neural Network –Genetic Algorithm approach. J Chem Technol Biotechnol 2010; 85: 50–58.
5. Schmid FA, Roberts J: Antineoplastic and toxic effects of *Acinetobacter* and *Pseudomonas* glutaminase-asparaginases. Cancer Chemother Rep 1974;58: 829-40.
6. Nakadai T, Nasuno S: Use of glutaminase for soy sauce made by Koji or a preparation of proteases from Aspergillus oryzae. J Ferment Bioeng 1989;67:158-62.
7. Nandakumar R, Yoshimune K, Wakayama M, Moriguchi, M: Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. J Mol Catal B: Enz 2003;23: 87–100.
8. Chou CC, Hwan CH: Effect of ethanol on the hydrolysis of protein and lipid during the ageing of a Chinese fermented soya bean curd-sufu. J Sci Food Agric 1994;66: 393-98.
9. Wakayama M, Yamagata T, Kamemura A, Bootim N, Yano S, Tachiki T, Yoshimune K, Moriguchi M: Characterization of salt-tolerant glutaminase from *Stenotrophomonas maltophilia* NYW-81 and its application in Japanese soy sauce fermentation. J Ind Microbiol Biotechnol 2005;32: 383–90.
10. Mulchandani A, Bassi AS: Determination of glutamine and glutamic acid in mammalian cell cultures using tetrathiafulvalene modified enzyme electrodes. *Biosens Bioelectron* 1996;11: 271-80.
11. Tachiki T, Yamada T, Mizuno K, Ueda M, Shiode J, Fukami H: γ-Glutamyl transfer reactions by glutaminase from *Pseudomonas nitroreducens* IFO 12694 and their application for the syntheses of theanine and γ-glutamylmethylamide. Biosci Biotechnol Biochem 1998; 62: 1279-83.
12. Hymavathi M, Sathish T, Subba Rao Ch, Prakasham RS: Enhancement of L-Asparaginase Production by Isolated *Bacillus circulans* (MTCC 8574) Using Response Surface Methodology. Appl Biochem Biotechnol. 2009; 159:191-98.
13. Imada A, Igarasi S, Nakahama K, Isono M: Asparaginase and glutaminase activities of microorganisms. J Gen Microbiol 1973:76, 85-99.
14. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25: 3389–02.

15. Saitou N, Nei M: The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4: 406–25.
16. Tamura K, Dudley J, Nei M, Kumar S: MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 2007;24:1596-99.
17. Subba Rao Ch. Fermentation kinetics of protease production by microbial sp. Ph.D thesis 2008.

Table 1: Morphological and physiological characteristics of the RSP-GLU

Tests	Results	Tests	Results
Colony Morphology		**Growth at Temperatures**	
Configuration	Circular	4 °C	-
Margin	Wavy	10 °C	-
Elevations	Low Convex	15 °C	+
Surface	Dull and Rough	25 °C	+
Density	Opaque	30 °C	+
Pigments	-	37 °C	+
Gram's Reaction	+ve	42 °C	+
Cell Shape	Fat Rods	45 °C	+
Size	Long	55 °C	±
Arrangement	Singles or short chain	65 °C	-
Spore(s)		**Growth at pH**	
Endospore	+	pH 5.0	+
Position	Central	pH 5.7	+
Shape	Oval	pH 6.8	+
Sporangia Bulging	+	pH 8.0	+
Capsule		pH 9.0	+
Motility	+	pH 11.0	+
Fluorescence (UV)	-	**Growth on NaCl (%)**	
		2.5 %	+
		5.0 %	+
		7.0 %	+
		8.5%	+
		10.0	+

Table 2: Biochemical characteristics of the RSP-GLU

Tests	Results
Growth on Mac Conkey Agar	-
Indole Test	-
Methyl Red Test	+
Voges Proskauer Test	+
Gas Production From Glucose	-
Citrate Utilization	+
Casein Hydrolysis	+
Starch Hydrolysis	+
Gelatin Hydrolysis	+
Urea Hydrolysis	-
Nitrate Reduction	+
Nitrite Reduction	-
H_2S Production	-
Cytochrome Oxidase	+
Catalase Test	+
Oxidation/Fermentation/Negative (O/F/-)	–
Arginine dihydrolase	-
Lysine decarboxylase	-
Ornithine decarboxylase	-

Fig. 1: Agar plates showing the RSP-GLU colonies producing L-glutaminase and its detection pink zone

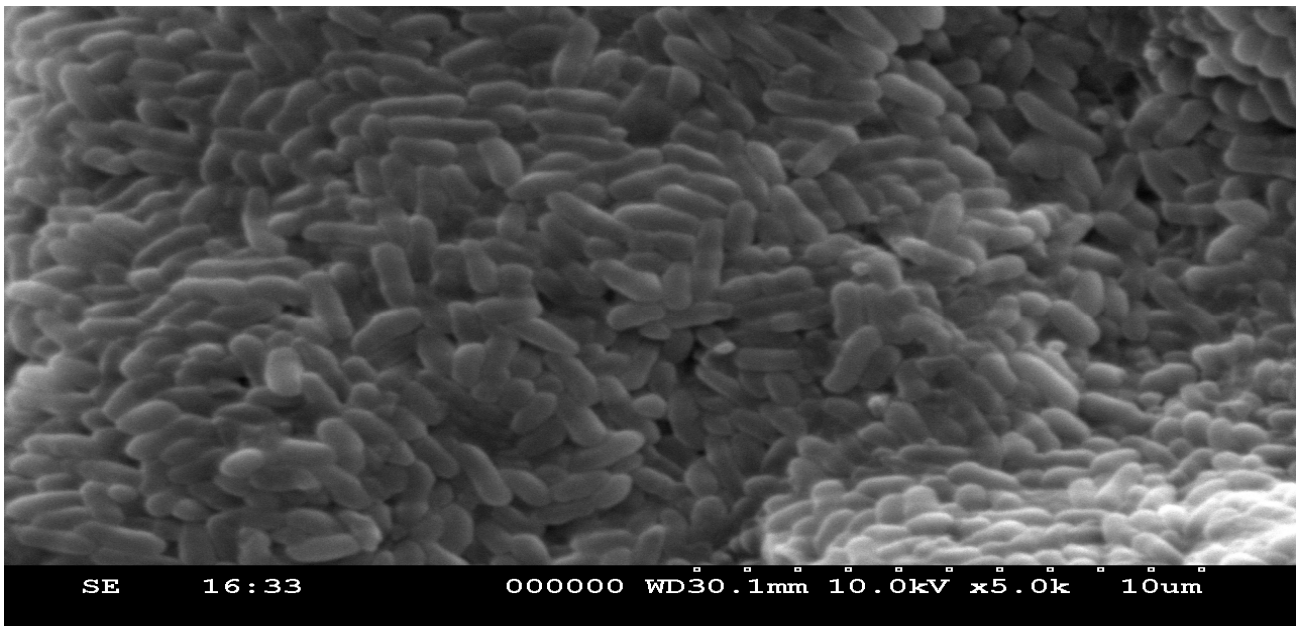

Fig. 2: Scanning electron microscopic (SEM) picture of isolated bacterial RSP-GLU colony

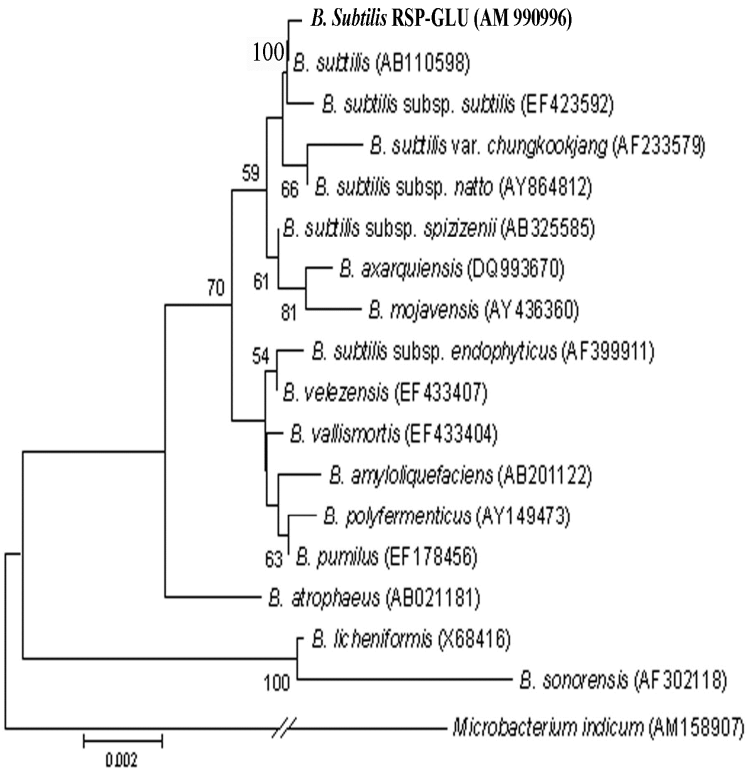

Fig. 3: Neighbour joining phylogenetic tree constructed according to Kimura two parameter model is showing phylogenetic relationship of *Bacillus subtilis* RSP-GLU with the members of the genus *Bacillus*. Bootstrap values (>50) calculated from 1000 replications are indicated in the branch nodes and the accession numbers for the reference sequences are given in the parenthesis. The bar represents 2 substitutions in 1000 nucleotides

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