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L-Asparaginase Producing *Gordonia terrae* from Lonar lake.

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ABSTRACT

L-asparaginase is a potential antileuckemic enzyme. It does not affect normal cells in the human body but target only cancerous cells. L- asparaginase had been isolated from many different microbial sources. Lonar Lake is a saline soda lake located at Lonar in Buldana district, Maharashtra, India. It is the hub of halophilic and alkalophilic microorganisms. In the current study the bacteria having the potential of L-asparaginase production have been isolated, characterized and identified. The potential isolate was identified as *Gordonia terrae strain 5-Sj-4-3-2-M* on the basis of biochemical, morphological characteristics and 16sDNA sequencing. This is the first kind of reports of L-asparginase producing *Gordonia terrae* from Lonar lake.

Keywords: L-asparaginase*, Gordonia terrae* , Lonar lake, halophile, alkalophile

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INTRODUCTION

Lonar Lake is a saline soda lake located at Lonar in Buldana district, Maharashtra, India, (lattidue 19 ^{0} 58', longitude. 76 ^{0} 34'). It was created by a meteor impact in Pleistocene period [7].The lake, has a basalt impact structure within and water is highly saline and alkaline in nature. Lonar lake is considered to be one of the wonder of Maharashtra. It ranks third as naturally formed crater in the world. It is belived to be 50,000 years old crater. Lonar lake served to be naturally occurring extreme environmental habitat for its flora and fauna. The lake has found to be ideal habitat for diverse microbial flora which are halophilic and alkaliphilic. Scentific focus so far with reference to the Lonar lake microbiota is isolation and characterization of comercially important microorganisms [3]. L-asparaginase acts on the substrate l-aspargine and convert it into L-aspartic acid along with the release of ammonia. L-asparaginase has been isolated from almost all types of microorganisms like bacteria, fungi , actinomycetes, yeast, algae as well as from various medicinal and non medicinal plants.L – asparaginase mainly acts on leuckemic cells hence serves the potential of antileuckemic agent in chemotherapy [4]. Currently L-asparaginase used in the chemotherapy is basically from E.coli and Erwinia species. It is the need of the hour to screen more and more sources of L-asparaginase as many forms of isolated enzyme had shown toxicity or allergic reactions [1, 9]
The organisms belonging to the genera Gordonia are aerobic, gram positive,non spore forming actinomycetes . They are non motile and found with rod or cocci shaped morphology. The organisms of this genus are widely distributed to aquatic, terrestrial , soil ,wasterwaters, marine sediments like habitat. Most interestingly some species are opportunistic pathogens to humans and animals. There are total more than 26 genera of gordonia reported. Some of them are *G.bronchialis, G.alkanivorans, G.aichiensis, G.amare, G.rhizosphera ,G.soli ,G. terrae* etc. *G. terrae* produces red to orange pigmented colonies on nutrient agar. GC content is approximately 64-69% [5]

MATERIALS AND METHODS

Chemicals

All chemicals used in this study investigation were of analytical grade and procured from Hi – media (India) and Merck (India).

Isolation of L asparaginase producing bacteria

Water and soil samples were collected from various sites of Lonar lake,Buldhana Maharashtra State, India, in sterile polythene bags, transported to the laboratory aseptically and stored at 4°C for further use. 10 gram of the soil sample was inoculated in 100ml of Sterile Modified M9 broth (g/l) (3.0 KH_2PO_4, 6.0 Na_2HPO_4, 0.5 NaCl, 0.12 MgSO4.7H_2O, 0.001 $CaCl_2$.2H_2O) containing 1% L-asparagine , and incubated at 30^{0}C for 48-72 hours. The samples were spread on Sterile Modified M9 agar plate containing 1% L-asparagine and few drops of phenol red. Enzyme production was accompanied by an increase in pH of the medium, which

results in the formation of pink zone [8]. The selected colonies were maintained on asparagine rich agar slant at 4°C.

Secondary screening of potential isolates

The special technique (Agar cup) was carried out to check the status of the enzyme [1]. Sterile Nutrient agar was modified by adding 1% L-asparagine and few drops of Phenol red. Sterile Nutrient agar without L-asparagine and Phenol Red served as control. The potential isolates were grown in 10 ml sterile nutrient broth for 18 hrs and 0.1 O.D. suspensions at 530 nm 20 micro liters each were added in the respective wells. Plates were incubated for 24 hrs at 30^0 C. Diameter of pink zones around the wells were measured.

Identification of bacterial isolate

All these isolates were further characterized by standard biochemical test according to Bergey's manual of systematic bacteriology.

Identification of bacteria on the basis of 16SrRNA sequencing

Maximum L-asparaginase producing isolate was identified by using 16SrRNA sequencing technique. Identification of the selected isolates was done by 16S rRNA gene sequencing. Genomic DNA was isolated using a DNA isolation kit (Himedia Laboratories Pvt. Ltd., Mumbai, India). 16S rRNA gene was amplified by PCR using 16s Forward Primer:

5'- CDGGHCTANCAVATGCWAGTS -3'

16s Reverse Primer:

5'- GMCGGRTGKGTACHAGGY -3'. were used to amplify the 1294 bp fragment The identification of phylogenetic neighbours was carried out by the BLASTn against the 16S rRNA database. Phylogenetic tree was constructed using Weighbor Tree.

Enzyme Assay

L-asparaginase is assayed with the help of nesselerization method [6]. One unit of L-asparaginase activity (IU) is defined as the amount of enzyme which liberates l µmol of ammonia per min at 30 ^{0}C and pH 7.4. [10]. The potential isolate was grown in Sterile modified M9 medium for 24 hrs and spinned at 5000 rpm for 10 minutes at 4^0 C . Supernatent was used for enzyme assay. 0.25ml of crude enzyme (supernatant) along with 1.25 ml of 0.2 M Borate buffer (pH 8) and 0.5 ml of 0.004 M. L-asparagine in borate buffer, then this reaction mixture was incubated at 37 °C for 15 min and the reaction was stopped by the addition of 0.5ml of 1.5M trichloroacetic acid (TCA). Precipitated proteins are removed by centrifugation and the clear supernatant is used for Nesseler's reaction. 0.5ml of the above clear supernatant along with 0.5ml of Nesseler's reagent and 3.0 ml of water are incubated for 10 min at 37^0C and the

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liberated NH_3 is determined by spectrophotometer at 480 nm. The concentration of ammonia in test solution was determined from standard curve of ammonium sulphate as a source of dissolved ammonia [10].

RESULTS

Table 1: Enzyme activity of potential isolates

No.	Isolate No.	Optical Density @ 480 nm	Enzyme activity IU/ml
1	S1	0.098	7.88
2	S2	0.107	9.18
3	S3	0.125	10.73
4	S4	0.138	11.86

Table 2: Colony characteristics

No.	Characteritic	Observation
1	Size	2-3 mm
2	Shape	Round
3	Color	Orange
4	Margin	Regular
5	Opacity	Tranlucent
6	Consistency	Semi soft
7	Elevation	Raised/convex
8	Gram Nature	Gram positive short rods
9	Motility	Non motile

Table 3: Biochemical characteristics of *Gordonia terrae strain DSM 43249*

Test	Result
Indol	Negative
M.R.	Negative
V.P.	Negative
Citrate	Negative
Catalase	Positive
Oxidase	Positive
Nitrate Reduction	Positive
Glucose	No acid
Arabinose	No acid
Lactose	No acid
Adonitol	No acid
Sorbitol	No acid
Manitol	Acid
Rhamnose	Acid
Sucrose	No acid
Sorbitol	Acid
Ornithine	Negative
Phenylalanine D'nas	Negative
H_2S	Negative

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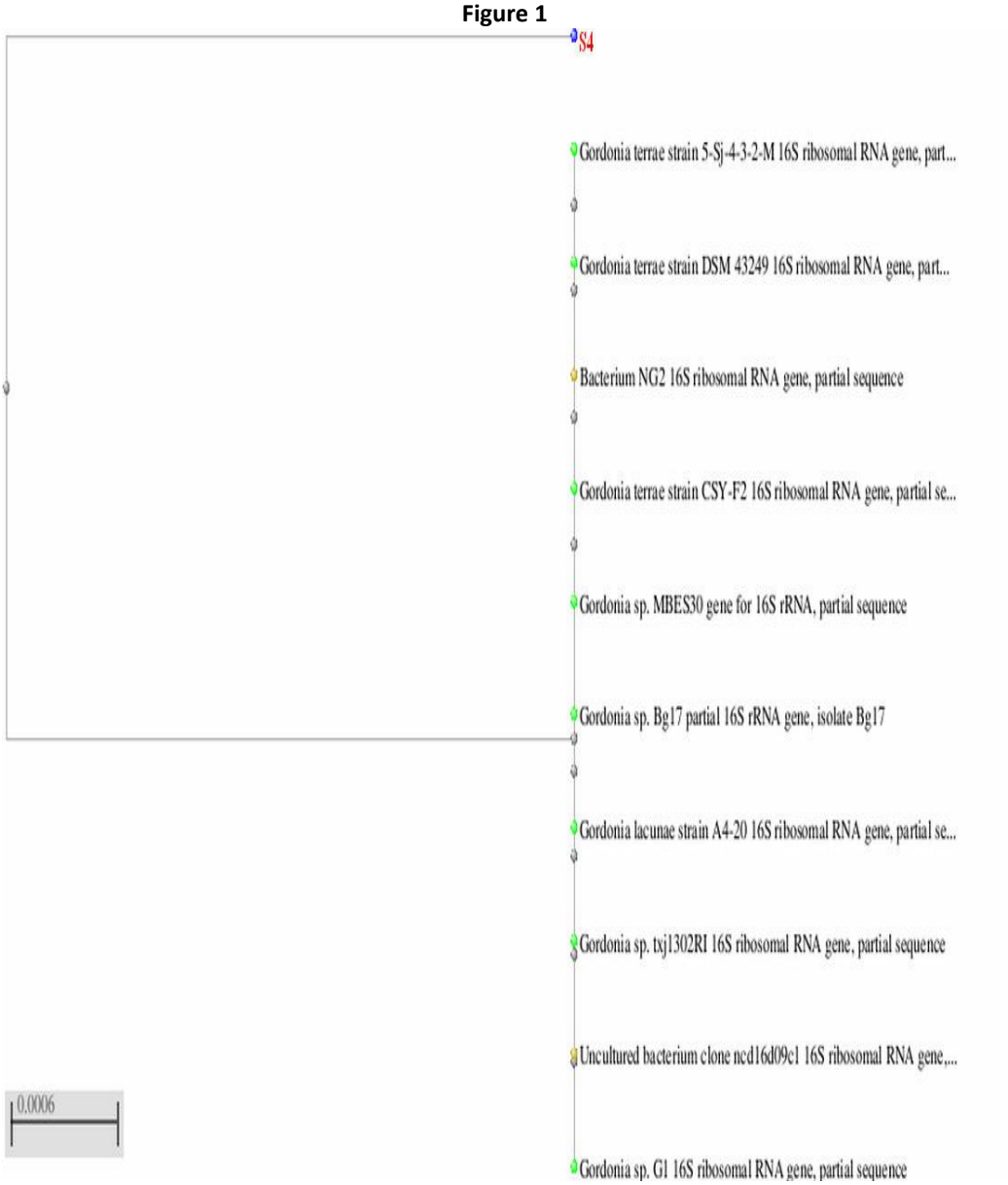
Table 4 pH and salt tolerance of *Gordonia terrae strain DSM 43249*

pH	Growth	Salt %	Growth
2	+	2	+
4	+	4	+
6	+	6	+
7	+	8	+
8	+	10	+
10	+	12	+
12	-	14	-

Figure 1



Phylogenetic tree of *Gordonia terrae strain DSM 43249*

From 3 samples total 144 colonies were obtained. Out of that only 4 isolates gave pink zone around them [Table 1]. Hence they were selected for secondary screening. Secondary screening was carried out by agar cup method followed by enzyme assay. S4 gave maximum pink color zone with enzyme activity of 11.86 IU /ml [Table 2]. The isolate S4 was characterized morphologically and biochemically. The potential isolate produced orange colored , raised, regular margined , translucent colonies [Table 2]. The isolate was found to be gram positive short rods, non-motile. The isolate gave MRVP test negative, catalase and oxidase and nitrate reductase tests positive [Table 3]. The isolate was found to be halophilic and alkalophilic [Table 4]. The 16S rRNA sequencing result showed that the isolate belong to genus *Gordonia* and species was found to be terrae [Figure1]

DISCUSSION

Total 4 isolates gave pink color zone around them. The pink color zone was obtained due to release of ammonia as L-asparaginase acts on L-asparagine and converts it into L-aspartic acid along with ammonia. The selected four isolates designated as S1, S2, S3, S4. S4 gave maximum L-asparaginase activity hence it was selected for further studies. S4 was found to be producing orange pigmented colonies on nutrient agar. The isolate gave MRVP test negative. It was found to be catalase, Oxidase, nitrate reductase positive. The isolate found fermenting mannitol, sorbitol and rhamnose with acid production. All these biochemical characteristics are in accordance with biochemical characteristics of *Gordonia terrae* [5]. As isolate tolerated ph up to 12 and 12% salt concentration its evident that its halophilic and alkalophilic organism. Further the isolate was sequenced by 16sRNA sequencing method. The result of PCR sequence blasted with other sequenced bacteria in NCBI showed similarity to the 16S small subunit rRNA of other bacteria. Edited sequences were used as queries in BLASTN searche to determine the nearest identifiable match present in the complete GenBank nucleotide database. Data analysis software used was Seq Scape_ v 5.2. Neighbour joining tree based on 16S rRNA gene sequence was constructed Weighbor Tree. The isolate was designated as *Gordonia terrae strain DSM 43249.*

CONCLUSION

The findings suggest that the potential isolate yields extracellular L-asparaginase. It is gram positive, akalophilic and halophilc bacteria. The isolate tolerated 12% of salt concentration and pH 12. The isolate was found to be an orange pigment producer. The characterization of pigment, purification of L asparaginase and study of its cyotoxic effect if any could lead to novel application of the isolate as there are no reports of L-asparaginase production from *Gordonia sp.*

This is the first kind of reports of L-asparginase producing *Gordonia terrae* from Lonar lake.

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