Isolation, Screening and Characterization of Novel Halotolerant L-Glutaminase Producing Bacteria from Lonar Lake

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Abstract*— L-glutaminase is an amidohydrolase that catalyzes the hydrolytic deamination of L-glutamine, resulting in the production of L-glutamic acid and ammonia. The L-glutaminase has received a significant attention due to its potential as a flavor enhancer in food industry. In the present study, water and soil sediments were collected from the Lonar Lake. The isolated bacterial strains were screened for L-glutaminase production, and the potent strain was characterized and identified as Halomonas salifodinae MM4 (Accession no: MZ674396.1).

Index Terms- L-glutaminase, screening, Lonar lake, Halomonas salifodinae.

I. INTRODUCTION

L-Glutaminase (EC 3.5.1.2) is an amidohydrolase enzyme that catalyses the deamidation of L-glutamine, in the presence of water, to L–glutamic acid and ammonia (Balagurunathan et. al., 2010). It plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes (Kashyap et. al., 2002; Katikala, et. al., 2009). This enzyme supplies the required nitrogen and carbon source of the biosynthesis of several intermediates in metabolic pathways such as DNA structural units and amino acids, therefore plays a crucial role in cellular metabolism (Mosallatpour et. al., 2019).

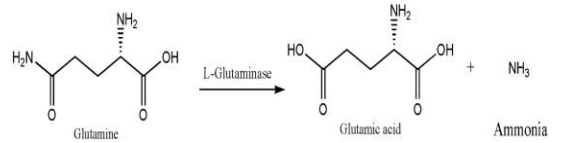


Fig. 1: Schematic representation of the mechanism of action of L-Glutaminase.

Bacterial glutaminases are generally produced extracellularly (Pandian et. al., 2014; Satish et. al., 2016) although intracellular production has also been reported (Klein et. al., 2002). Extracellular enzymes have greater advantages than intracellular enzymes; they may be produced abundantly under normal conditions and the process of extraction and purification would be simpler than for intracellular enzymes.

L-Glutaminase is used as flavor enhancing agent in the food industry, due to its involvement in the synthesis of L-glutamic acid, the main compound which is responsible for the delicious taste or flavor and aroma of many fermented products like soy sauce, miso, sufu etc (Ayodeji et al., 2019; Unissa et al., 2014).

Lonar Lake in India is a unique ecosystem formed by the meteorite impact on basaltic rock. The Lonar Lake appears green for most of the year due to the presence of dense blooms of cyanobacteria such as *Arthrospira spp*. (Surakasi et. al., 2010) bacteria and archaea belonging to diverse functional (Joshi et. al., 2008; Kumar et. al., 2012; Paul et. al., 2016) have been reported. The objective of this study was to isolate halotolerant L-glutaminase producers from Lonar Lake and further their characterization and identification.

II. MATERIALS AND METHODS

Sample collection

The water and sediment samples were collected from the Lonar Lake, district: Buldhana (Latitude: 19°58′30″N, Longitude: 76°30′27″E) in March 2016 Maharashtra, India.

The samples were brought to the laboratory in sterile containers and stored at 4°C till further use.

Enrichment and isolation of microorganisms

One ml of Lonar Lake water sample and one gram of Lonar Lake sediment sample was mixed with 100 ml of sterile distilled water separately and shaken well for one hour at room temperature. From that, 1 ml sample was taken and inoculated in 50 ml broth containing (g/L) glutamine, 10.0; K_2HPO_4, 1.0; KH_2PO_4, 0.1; $MgSO_4$, 1.0; NaCl, 0.5 and yeast extract, 0.5; adjusted to pH 7.2 (Pandian, 2015). About 50 µg /ml of nystatin was added into the medium, after sterilization, in order to retard the growth of fungi. The culture medium was incubated in an orbital shaker at 150 rpm at 37±2ºC for 72-96 hours. To obtain pure cultures producing L–glutaminase the strains were serially diluted with sterile distilled water and 100 µl of that sample was spread on glutamine containing agar plates and incubated at 37±2ºC. After incubation, morphologically different bacterial colonies were selected, purified and subcultured on the same media agar slants. All the isolates were preserved at refrigerated conditions as slant culture.

Screening of isolates for L–glutaminase production by rapid plate assay method

The microorganisms isolated from Lonar Lake water and sediment samples were tested for L–glutaminase activity by rapid plate assay method as used by Gulati et. al., (1997); Padma and Singhal, (2010). Activity of extracellular enzyme was checked by well diffusion assay.

Identification of the isolate

Identification of the isolate was done on the basis of Morphological, Physiological, biochemical characterization and 16S rRNA gene sequencing.

Morphological Characterization of isolates

Bacterial strains were studied for their colony, cell morphology, Gram's staining and motility. The morphological and cultural characteristics of all isolates were studied. Morphological characters like shape, margin, elevation, colour and opacity of colonies were recorded.

Physiological characterization

i) Effect of NaCl (Halotolerance) on the growth of isolates

The halotolerance of these isolates was determined, as in the food industry halotolerant L-glutaminases are of significant importance. The halotolerance of Lonar Lake MM4 isolate was investigated by supplementing the media with NaCl in the range of of 0 to 20%.

ii) Effect of pH and temperature on growth

Effect of pH and temperature on growth of isolate was estimated by inoculating culture in medium of pH in the range of 5 to 12 with an increment of 1 and 20 to 60ºC temperature with an increment of 5ºC respectively. The sterile uninoculated broth medium was used as a blank. (Upasani and Desai, 1990; Deshmukh et. al., 2011; Sahay et. al., 2012).

Biochemical Characterization of isolates

Various biochemical tests were performed as per the methods described by Aneja, (2007) and (Kannan), 2002.

Molecular characterization and phylogenetic analysis:

The molecular characterization was carried out by 16S ribosomal RNA gene sequencing at the National Collection of Industrial Microorganisms (NCIM), Council of Scientific & Industrial Research- National Chemical Laboratory (CSIRNCL), Pune, India.

The 16S rRNA gene sequence of the isolates were used as a query to search for the homologous sequence in the nucleotide sequence databases by running the BLASTN program (Altschul et. al., 1997). The high scoring similar to 16S rRNA gene sequences were identified from the results and retrieved from the GenBank database. The identified sequences were aligned using CLUSTAL-W algorithm in MEGA X software. Phylogenetic trees were inferred by using the neighbor-joining (Saitou & Nei 1987) bootstrap analysis with the help of MEGA X software.

III. RESULT AND DISCUSSION

In the present investigation from Lonar Lake water sample total 3 isolates and from sediment sample 01 isolate were obtained and these were initially designated as LW-1, LW-2, LW-3 and LS-4.

Table 1. Isolates obtained from Lonar lake.

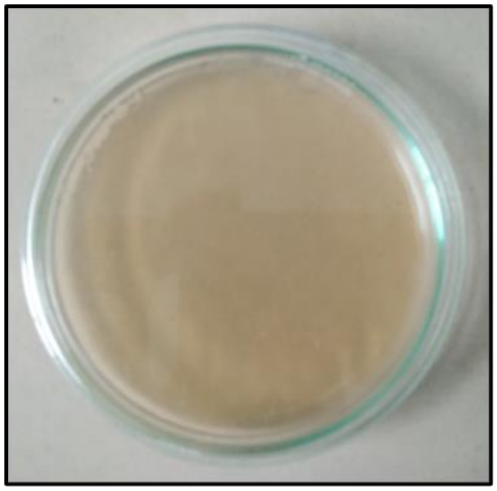
Sample source	Isolates
Lonar lake water	LW-1
	LW-2

	LW-3
Lonar lake sediment	LS-4

Screening of isolates for L–glutaminase production by rapid plate assay method

All the 04 isolates viz LW-1, LW-2, LW-3 and LS-4 showed positive results in rapid plate assay method. The media was supplemented with a dye indicator viz, phenol red. The indicator is pH sensitive normally it gives yellow colour to the media in acidic conditions, and it gives pink or red colour to the media when the pH changes from acidic to alkaline. The formation of pink colour around the bacterial colony indicates the pH alteration which originated from ammonia accumulation in the medium.

Among four isolates from Lonar Lake sample only one isolate viz LW-1 (Fig. 2) showed considerable enzyme production. The isolate LW-1 was later designated as MM4.



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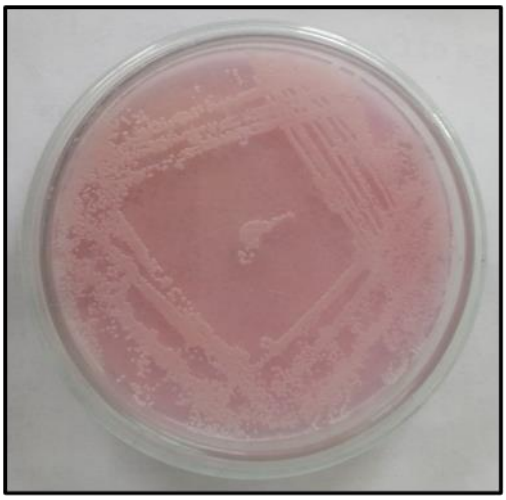

LW-1

Fig. 2: Screening of L–glutaminase producing bacteria by rapid plate assay method

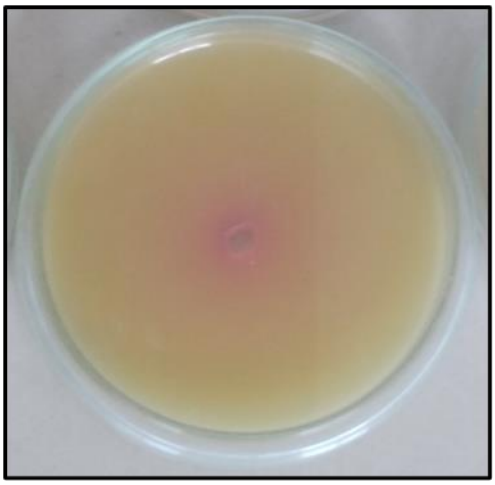

Fig. 3: Activity of extracellular enzyme. The formation of pink colour around the well confirms that, extracellular L-glutaminase produced by the isolate.

Identification of isolates:

Morphological characteristics of isolate

The colonies of isolate MM4 were circular in shape. The margin of colonies of isolate MM4 was entire. Isolate MM4 showed creamish colour colonies (Table 2).

Microscopic observations revealed that isolate MM4 was Gram negative and Rod shaped.

The isolate MM4 was motile.

Table 2: Morphological characteristics of isolate

Characteristics	Isolate MM4

Configuration	Circular
Margin	Entire
Elevation	Convex
Surface	Smooth
Colony pigmentation	Cream
Opacity	Opaque
Gram nature	-
Cell Shape	Rod
Motility	Motile

Table 3: pH and Temperature Profile of isolate MM4

pH	Isolate MM4	Temperature (°C)	Isolate MM4
5	++	20	+
6	++	25	++
7	+++	30	++
8	++	35	+++
9	++	40	++
10	++	45	++
11	+	50	+
12	+	55	+

The lonar lake isolate MM4 showed growth in the range of pH 5 to 12, the optimum pH for its growth was 7. Whereas, it showed growth in the temperature range of 20 to 55°C and the optimum temperature for its growth was 35 °C.

Table 4: Salt (NaCl) tolerance profile of the isolate.

NaCl Conc. (%)	Isolate MM4
0	+
2	+
4	++
6	+++
8	++
10	++
12	+
14	+
16	+
18	-
20	-

The lonar lake isolate MM4 showed growth in the range of 0 to 16% of NaCl concentration, the optimum salt concentration for its growth was 6%.

The isolate MM4 was subjected towards biochemical characterization as shown in Table 5.

The isolate was Positive for Oxidase, Catalase, Urease, Citrate and Voges Proskauer tests. It carried out the hydrolysis of casein, gelatin, starch, tributyrin and cellulose.

Table 5: Biochemical Characteristics of isolate

Test	Isolate MM4	Utilization of	Isolate MM4
Oxidase	+	Arabinose	-
Catalase	+	Fructose	+
Urease	+	D-Glucose	+
Nitrate reduction	-	D-Galactose	-
H2S production	-	Mannose	-
Citrate	+	Ribose	-
Methyl Red	-	Lactose	+
Voges Proskauer	+	Xylose	+
Indole Production	-	Maltose	-
Hydrolysis of:		Hydrolysis of:	
Casein	+	Tributyrin	+
Gelatin	+	Cellulose	+
Starch	+		

+ Positive Test and – Negative Test.

Molecular characterization and phylogenetic analysis: The identification of bacterial isolates based upon conventional techniques, most commonly involving the standard biochemical tests has been observed to be not as accurate as the identification based upon genotypic methods. Therefore, in the present study, after a preliminary phenotypic identification, a final confirmatory identification of the isolate MM4 was

carried out by using molecular techniques involving 16S rRNA gene sequencing.

The sequence obtained after 16S rRNA sequencing of the isolate MM4 is as follows

16S rRNA gene partial sequence of Isolate MM4:
>MM4
CGAGCGGCGGACGGGTGAGTAATGCATAGG
AATCTGCCCGAGAGTGGGGGATAACGTGGGG
AAACTCACGCTAATACCGCATACGTCCTACG
GGAGAAAGCAGGGGATCTTCGGACCTTGCGC
TATCGGAGGAGCCTATGTCGGATTAGCTAGT
TGGTGAGGTAACGGCTCACCAAGGCGACGAT
CCGTAGCTGGTCTGAGAGGATGATCACCCAC
ACTGGGACTGAGACACGGCCCACACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGGACAAT
GGGGGAAACCCTGATCCAGCCATGCCGCGTG
TGTGAAGAAGGCCTTCGGGTTGTAAAGCACT
TTCAGCGAGGAAGAAGGCCTGAGGGCTAATA
CCCTTCAGGAAGGACATCACTCGCAGAAGAA
GCACCGGCTAACTCCGTGCCAGCAGCCGCGG
TAATACGGAGGGTGCGAGCGTTAATCGGAAT
TACTGGGCGTAAAGCGCGCGTAGGTGGCTTG
ATAAGCCGGTTGTGAAAGCCCCGGGCTCAAC
CTGGGAACGGCATCCGGAACTGTCAGGCTAG
AGTGCAGGAGAGGAAGGTAGAATTCCCGGT
GTAGCGGTGAAATGCGTAGAGATCGGGAGG
AATACCAGTGGCGAAGGCGGCCTTCTGGACT
GACACTGACACTGAGGTGCGAAAGCGTGGGT
AGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCGACTAGCCGTTGGGT
TCCTTGAGAACTTTGTGGCGCAGTTAACGCG
ATAAGTCGACCGCCTGGGGAGTACGGCCGCA
AGGTTAAAACTCAAATGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTC
GATGCAACGCGAAGAACCTTACCTACCCTTG
ACATCGTGCGAACTTGGTAGAGATACCTTGG
TGCCTTCGGGAACGCACAGACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTTGTGAAATGTTG
GGTTAAGTCCCGTAACGAGCGCAACCCTTGT
CCCTATTTGCCAGCGATTCGGTTGGGAACTCT
AGGGAGACTGCCGGTGACAAACCGGAGGAA
GGTGGGGACGACGTCAAGTCATCATGGCCCT
TACGGGTAGGGCTACACACGTGCTACAATGG
TCGGTACAAAGGGTTGCAATACCGCGAGGTG
GAGCTAATCCCATAAAGCCGGTCTCAGTTCG
GATCGGAGTCTGCAACTCGACTCCGTGAAGT
CGGAATCGCTAGTAATCGTGAATCAGAATGT
CACGGTGAATACGTTCCCGGGCCTTGTACAC
ACCGCCCGTCACACCATGGGAGTGGACTGCA
CCAGAAG

The BLAST analysis of the obtained sequence was performed to know the sequence similarities with available sequences of NCBI for confirmation and identification of isolate.

The halotolerant L-glutaminase producer MM4 was identified as *Halomonas salifodinae*.

The nucleotide sequence data derived in this study has been deposited in the NCBI Gene Bank database, the strain designation and the accession number assigned for the submitted sequence is *Halomonas salifodinae* MM4 (Accession no: MZ674396.1). The isolate belonged to phylum Gamma proteobacteria.

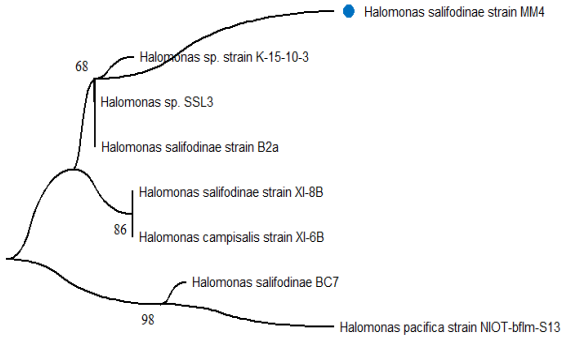

Fig 4: Phylogenetic relationship of *Halomonas salifodinae* MM4.

Isolate MM4 showed 99.03% similarity with the *Halomonas salifodinae* BC7 (NR_044263.1).

CONCLUSION

In present study it was found that the samples collected from Lonar Lake were good source of halotolerant L-glutaminase producing bacteria. The present study shows that *Halomonas salifodinae* MM4 has the ability to produce extracellular L-glutaminase. The further study for this isolate is required for maximum production of L-glutaminase by optimization of various physicochemical parameters. The *Halomonas salifodinae* MM4 possesses a promising industrial application potential for L-glutaminase production.

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