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ISOLATION AND IDENTIFICATION OF L-ASPARAGINASE PRODUCING *SALINICOCCUS* SP. M KJ997975 FROM SOIL MICROBIAL FLORA

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ABSTRACT: L-asparaginase is responsible for the hydrolysis of L-asparagine into L-aspartic acid and ammonia. L-asparaginase is used as an effective therapeutic agent against lymphocytic leukemia. It also finds applications in food industry. Though much has been unraveled about LA, it may appears today that it is at the tip of iceberg and there is tremendous scope in screening of novel LA sources and studying their properties and applications. In the current study L-asparaginase producing bacteria were screened from forest soil of Vasai, MS, India. Modified M9 medium was used for screening the L-asparaginase producers. Out of the total 885 organisms screened for L-asparaginase production, one isolate was found to produce substantial L-asparaginase (15.94 IU/ml). This isolate was further identified by 16S ribosomal RNA gene sequencing as a novel strain *Salinicoccus* sp. M KJ997975. This orange pigment producing isolate was found to be moderately halophilic and alkaliphilic. Not much literature is available on L-asparaginase producing gram positive alkalophilic and halophilc bacteria and this is the first kind of report on L-asparaginase producing *Salinicoccus* sp.

INTRODUCTION: The population of bacteria in the soil is maximum as compared to all other groups of microorganisms in both number and variety. Bacteria bring about a number of changes and biochemical transformations in the soil and thereby directly or indirectly help in the nutrition of higher plants growing in the soil. Soil bacteria play crucial role in decomposition of complex organic matter, ammonification, nitrification, denitrification, biological fixation of atmospheric nitrogen, oxidation and reduction of sulphur and iron compounds. Soil bacteria also produces many important enzymes.

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Which have been used in industries on large scale. Increased usage of enzymes is due to their excellent prospects. L-asparaginase, a relatively widespread enzyme, found in many microorganisms has attracted much attention in the past decade because of its antineoplastic activity.

L-asparaginase hydrolyses L-asparagine to L-aspartic acid and ammonia in leukemic cells, resulting in the depletion of L-asparagine, inhibition of protein synthesis, cell cycle arrest in G1 phase and apoptosis of susceptible leukemic cell populations[1].

Normal cells can synthesize L-asparaginase with the help of enzyme asparagine synthase, whereas malignant cells are deprived of this enzyme and thus needs exogenous source of L-asparagine for growth. Addition of L-asparaginase depletes exogenous L-asparagine and causes death of

malignant cells [2]. Due to this attribute, L asparaginase has become essential drug in the treatment of acute lymphoblastic leukemia (ALL) [3]. It is also used in food processing for reducing acrylamide, a carcinogenic agent from baked food[3]. L-asparaginase has been isolated from different microorganisms like bacteria, fungi, actinomycetes and algae. Out of these there are three main commercial sources of this enzyme. *E.coli* asparaginase, *Erwinia* asparaginase and pegaspargase asparaginase.

E. coli has two distinct forms of this enzyme. Asparaginase II is most active form against lymphoma. *Erwinia* Asparaginase is used in combination with other agents in the treatment of leukemia. Pegaspargase the modified form has been found to be a good alternative for the treatment [4]. Isolation of L- asparaginase from novel and better sources is the need of the hour due to allergic reactions and immunological side effects of existing sources of L-asparaginase[5]

Ventosa *et al.* described the genus of Gram positive cooci called *Salinicoccus* belonging to the family *Staphylococcaceae*[7]. *Salinicoccus* is a halophilic bacterium isolated from different habitats such as salt lakes, saline soils and salted food [6]. The genus *Salinicoccus* is characterized chemotaxonomically by having menaquinone-6 as the predominant isoprenoid quinone, a cell-wall peptidoglycan type based on L-Lys–Gly5 and a DNA G+C content of 46–51 mol% [7]. There are total 11 species of genus *Salinicoccus.* They are *S. roseus, S. albus, S. alkaliphilus, S. halodurans, S. hispanicus, S. iranensis, S. jeotgali, S. kumingensis, S. luteus, S. salsiraiae, S. siamensis*[7].

The present study is an attempt to isolate and identify a novel soil bacterium that produces L-asparaginase in quantities that can prove to be a good source for commercial productions.

MATERIALS AND METHODS:

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

Primary Screening: Soil samples were collected from Garden and Mangroove soil of CBD Belapur, MS, India and forest soil of Vasai, 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 100 ml of sterile modified M9 broth (g/l) (3.0 KH_2PO_4, 6.0 Na_2HPO_4, 0.5 NaCl, 0.12 $MgSO_4.7H_2O$, 0.001 $CaCl_2.2H_2O$) containing 1% L-asparagine, and incubated at 30^0C for 48-72 hours.

The diluted samples were spread on sterile modified M9 agar plate containing 1% L-asparagine and 0.005% phenol red. Enzyme production was accompanied by an increase in pH of the medium, which resulted in the formation of pink zone that help in identification [8, 9]. The selected colonies were maintained on nutrient agar slant at 4^oC.

Secondary screening of potential isolates:

Agar cup method was carried out to quantify the enzyme. Sterile nutrient agar was modified by adding 1% L-asparagine and 0.005% phenol red. Sterile nutrient agar without L-asparagine and phenol red served as control. The isolates were grown in 10 ml sterile nutrient broth for 18 hrs and 20 microliters each were added in the respective wells. Plates were incubated for 24 hrs at 30^0 C. Diameter of pink zones around the well were measured to quantify the amount of enzyme produced.

Identification of bacterial isolate:

Morphological, cultural and biochemical characteristics of the isolate were studied and compared with standard description of Bergey's Manual of Determinative Bacteriology [10]. pH and salt tolerance study was carried out in sterile nutrient broth and agar with different pH and salt concentrations. The potential isolate was identified by 16S rRNA sequencing method (Chromus Biotech pvt ltd, Bangalaore). The sequence was aligned with representative sequence. The sequence was then submitted to NCBI for accession number.

Assay of L-asparaginase:

The enzyme was assayed by direct nesselerization method [11]. 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 [11, 12].

Enzyme Preparation:

The isolate was cultivated for 16 hrs at 30^0 C in shaking condition and the harvested culture was transferred to 250 ml Erlenmeyer flasks with 100 ml sterile modified M9 broth medium incubated in a shaker incubator (120 rpm, 30^0 C) for 72 h. After incubation, the cells were removed by centrifugation at 10000 rpm for 10 min. The supernatant was used to assay extracellular L-asparaginase activity [11, 12]. The enzyme was assayed by nesslors method[12].

Statistical analysis:

The results were expressed as mean + standard deviation (S.D.). Chi square test was applied to the primary and secondary screening data. Statistical significance indicated with p value.

RESULTS & DISCUSSION:

Microbes in the soil are important for soil fertility, biogeochemical cycles and various industrial products such as enzymes, antibiotics, vitamins, hormones and organic acid. Agricultural practices, research on plant growth and bioremediation are the major thrust areas for exploiting the potential of soil microbial flora. Commonly enzymes have been isolated and purified from microorganisms because of their broad biochemical diversity, feasibility of mass culture and easy genetic manipulation. LA plays important role of N mineralization in soil. LA has been isolated from various soil bacteria. Soils may possess novel microbial strains with novel properties of biotechnological interest.

Mangrove soil plays important role in nutrient recycling. Several marine bacteria especially halophilic estuarine bacteria are the potential sources of LA, which can be exploited on large scale for industrial productions [13]. Various soil microbes have been reported to produce industrially important enzymes.

Novel LA producing bacteria with antileukemic potential have been reported from these ecosystems. Hence in the current study mangrove and forest soil had been chosen for screening of LA producing bacteria. There are reports that cultivated soil also has potential microbial flora. Hence garden soil used in study served the control of cultivated soil.

Primary screening of L-asparaginase producing isolates from soil:

Out of total 885 screened isolates only 15 isolates were found to be positive for L-asparaginase production by rapid plate assay method [9]. Pink zone around the colonies indicated production of L-asparaginase (**Fig 1** and **3).** The isolates were further tested for LA production. Selected 15 isolates through primary screening were positive in that they showed intense pink zone around the colonies on modified M9 medium. It is noteworthy that in primary screening mangrove soil exhibited maximum number of LA positive isolates, indicating that this niche may prove to be a valuable source of microbial flora for biotechnology (***p <0.0001).

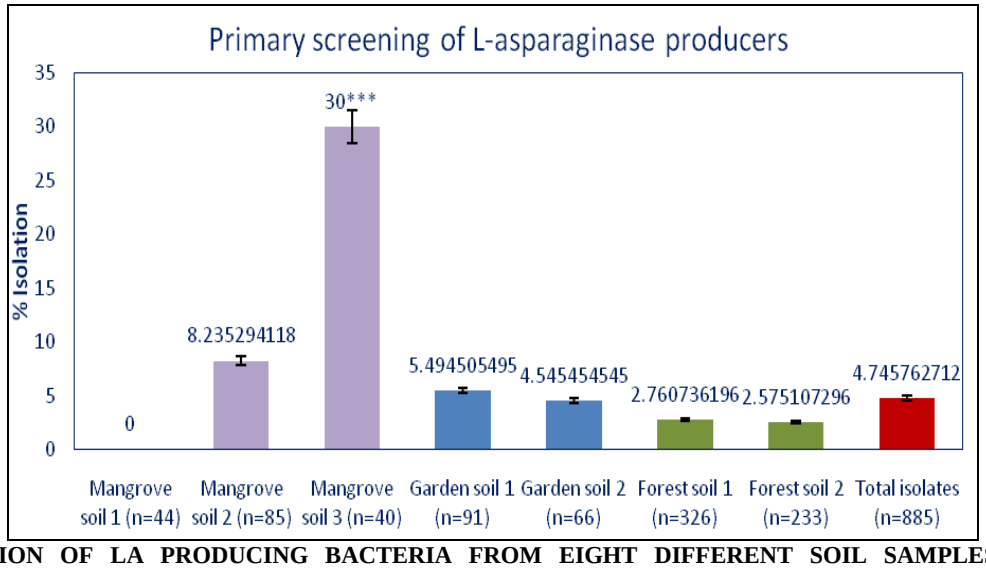

FIG.1: ISOLATION OF LA PRODUCING BACTERIA FROM EIGHT DIFFERENT SOIL SAMPLES BY PRIMARY SCREENING METHOD. VALUES ARE EXPRESSED IN PERCENTAGE AS MEAN + S.D. IN FIGURE SYMBOLS REPRESENT STATISTICAL SIGNIFICANCE *** P<0.0001.

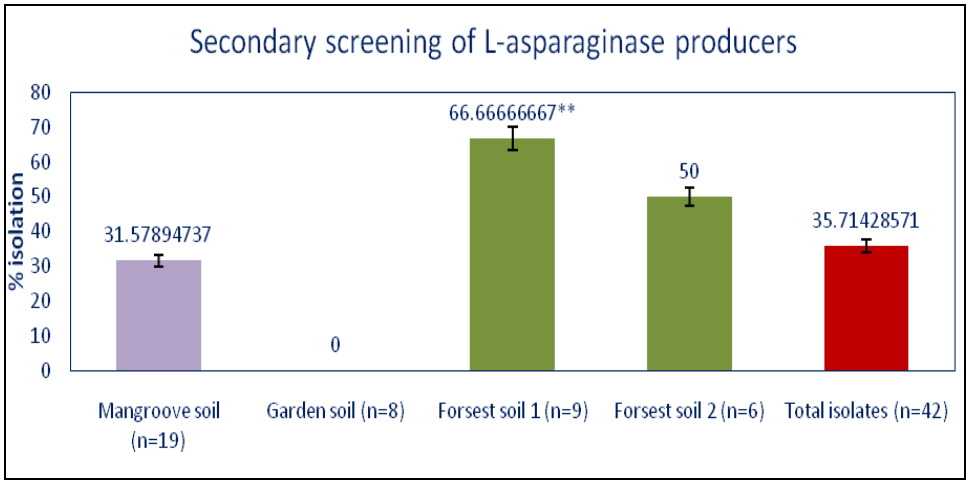

FIG. 2: ISOLATION OF LA PRODUCING BACTERIA FROM FIVE DIFFERENT SOIL SAMPLES BY SECONDARY SCREENING METHOD. VALUES ARE EXPRESSED IN PERCENTAGEAS MEAN + S.D. IN FIGURE SYMBOLS REPRESENT STATISTICAL SIGNIFICANCE ** P<0.001.

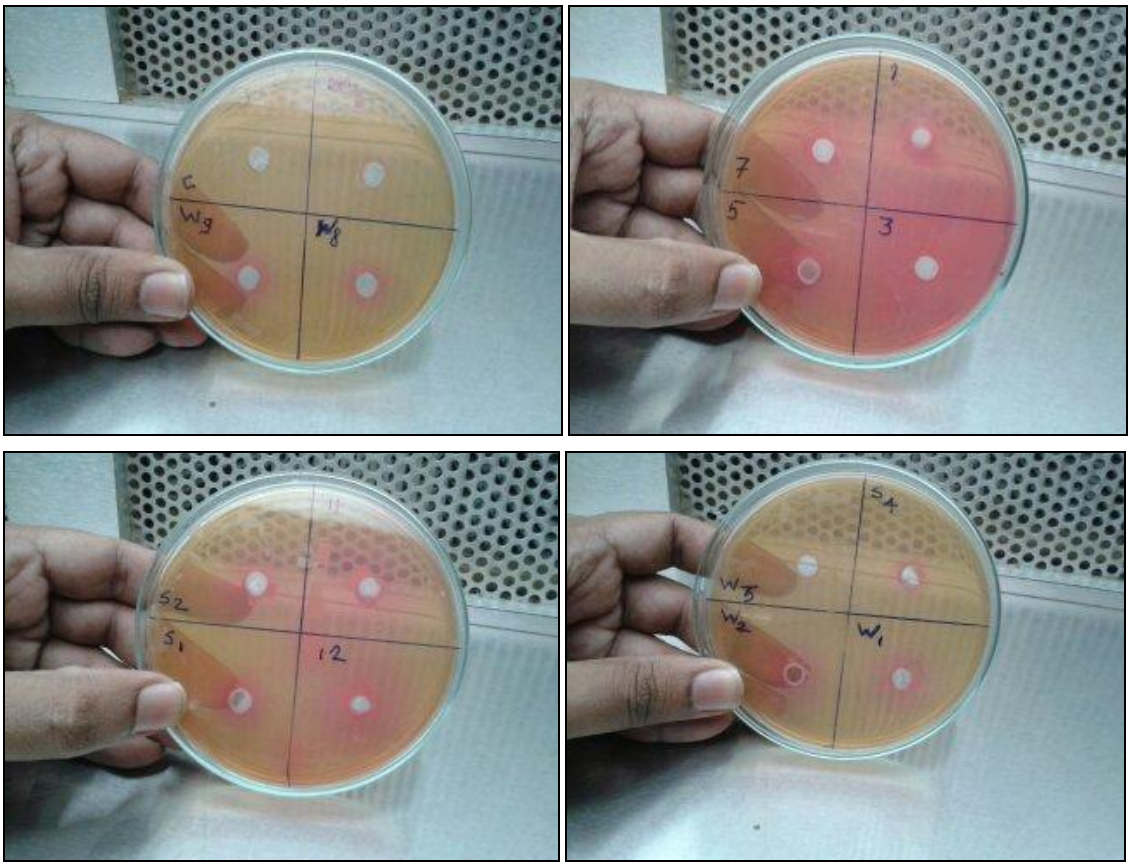

FIG. 3: SHOWING THE DIAMETER OF PINK ZONE OBTAINED BY VARIOUS PRIMARY SCREENED LA PRODUCING COLONIES ON MODIFIED M9 MEDIUM BY AGAR CUP METHOD. PINK COLORATION AROUND WELLS INDICATES LA PRODUCTION DUE TO BREAKDOWN OF PRESENT L-ASPARAGINE IN THE MEDIUM BY LA PRODUCED BY ISOLATES.

Secondary screening:

Secondary screening was carried out by agar cup method followed by enzyme assay. Among 15 selected bacterial isolates tested by rapid plate assay, only four isolates gave prominant pink zone with diameter ranging from 2 to 2.5 cm after 24 h incubation. Out of these, isolate M from forest soil was found to be one of the good LA producer as it exhibited 2.5 cm pink zone (**Table 2**). Isolate number W2/M gave maximum pink color zone (**Table 1**) with enzyme activity of 15.94 IU /ml (**Table 2**). All other isolates were found to produce less extracellular enzyme than isolate M. Hence Isolate M was selected for further study. There are no reports of use of agar cup method for screening of LA producers; however there are many reports

that justify the selection of potential isolate depending on the diameter of pink zone around LA producing colonies [9, 17, 18].

TABLE 1: THE DIAMETER OF PINK ZONE OBTAINED BY VARIOUS PRIMARY SCREENED LA PRODUCING COLONIES ON MODIFIED M9 MEDIUM BY AGAR CUP METHOD.

Isolate designation	Diameter of Zone in cm (Plate with L-asparagine)
1	2
3	2
5	2.5
7	2.5
11	2.5
12	2
S1	1.5
S2	1.4
S4	1
W1	1.5
W2/M	2.5
W5	-
W7	-
W8	1.3
W9	1.2
Control	-

TABLE 2: ENZYME ACTIVITY OF LA IS EXPRESSED AS MEAN ± S.D. LA ACTIVITY OF PRIMARY SCREENED ISOLATES IN IU/ML OBTAINED BY L-ASPARAGINASE QUANTITATIVE ASSAY

Sr. No.	Isolates	LA activity IU/ml
1	1	9.14 ± 0.84
2	3	5.14 ± 0.54
3	5	12.17 ± 0.30
4	7	8.97 ± 0.30
5	11	11.52 ± 0.27
6	12	9.13 ± 0.77
7	S1	12.88 ± 0.48
8	S2	0.49 ± 0.06
9	S4	11.58 ± 0.60
10	W1	12.69 ± 0.28
11	W2/M	15.94 ± 0.46
12	W5	1.79 ± 0.28
13	W7	1.04 ± 0.16
14	W8	12.34 ± 0.50
15	W9	12.72 ± 0.65

Primary screening exhibited 30% of total mangrove bacterial population produced LA. However, forest soil bacterial population was found to produce more amount of extracellular LA quantitatively in comparison to mangrove soil bacteria. Hence isolate M (isolated from forest soil) was selected for further study (**Fig.2**).

There are reports that soil samples can have potential LA producers. Production of L-asparaginase by a new isolate *Bacillus aryabhattai* Strain ITBHU02 had been reported[14]. *Fusarium equiseti* isolated from rhizosphere soil of *Ipomoea muricata* has the ability to produce significant amount of L-asparaginase enzyme [15]. L-asparaginase producing marine *Actinomycetes* have been isolated and studied for their anticancer potential[16]. L-asparaginase production by moderate halophilic bacteria have been studied from Maharloo salt lake[17]. Screening and characterization of L-asparaginase producing *Bacillus subtilis* strain hswx from Taptapani hot spring has been reported recently[18]. L-asparaginase from *Helicobacter pyroli* was found to be a promising chemotherapeutic agent[19].

Identification and characterization:

The morphological and cultural characteristics of the isolate M are appended herewith. The colonies of isolate were opaque, with entire margin, flat elevation, dry granular surface with orange pigmentation. The isolate was found to be gram positive cocci (**Table 3**). It was found to be non motile sporulating bacteria. The isolate M was exposed to different pH range (3-12) and concentrations of sodium chloride (2%-14%), exhibited growth at pH range of 6-12 and tolerated sodium chloride concentrations of 2%-12% respectively.

TABLE 3: COLONY CHARACTERISTICS OF ISOLATE M, GROWN ON STERILE MODIFIED M 9 MEDIUM AFTER 24 H OF INCUBATION.

Characteristics	Observation
Size	2-4 mm
Shape	Cicular
Colour	Orange
Margine	Regular
Opacity	Opaque
Consistency	Soft
Elevation	Convex
Gram Nature	Gram positive cocci

The isolate showed no growth at pH 3-5 and beyond 12 % sodium chloride (**Table 4**). The isolate M was subjected to various biochemical tests. The isolate showed negative results for IMVic tests. It was nitrate reductase positive, produced catalase and oxidase enzymes. It was noteworthy that the isolate M did not produced acid by utilization of any sugar. It utilised only glucose, manitol, sucrose, lactose, maltose and rhamnose sugars (**Table 5**).

TABLE 4: PH AND SALT TOLERANCE EXHIBITED BY ISOLATE M IN STERILE NUTRIENT BROTH AFTER 24 H OF INCUBATION. KEY + INDICATES GROWTH AND – INDICATES NO GROWTH.

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

TABLE 5: RESULTS OF BIOCHEMICAL TESTS OF ISOLATE M AFTER 24 H OF INCUBATION IN BROTH.

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	No acid
Rhamnose	No acid
Sucrose	No acid
Lysinedecarboxylase	Negative
Ornithine	Negative
Phenylalanine	Negative
H_2S	Negative

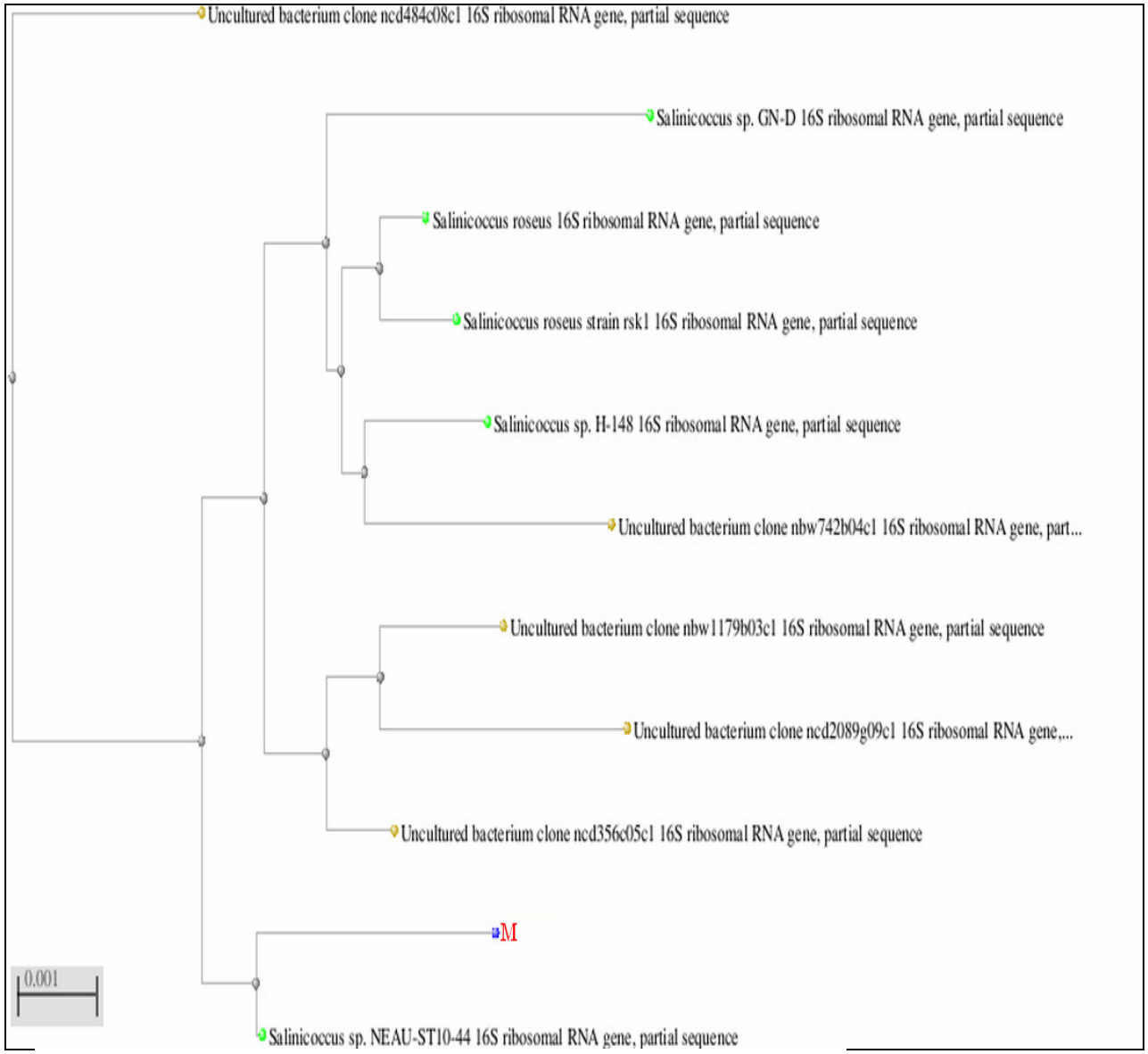

FIG.4: PHYLOGENETIC TREE OF ISOLATE M, OBTAINED WITH THE HELP OF SOFTWARE SEQ SCAPE_ V 5.2. NEIGHBOUR JOINING TREE BASED ON 16S RRNA GENE SEQUENCE WITH APPLICATION OF JUKES-CANTOR MODEL AND BOOTSTRAPPING A STATISTICAL TOOL. INDICATING THAT ISOLATE M AS A NOVEL UNEXPLORED ISOLATE

The results of biochemical tests are in accordance with *Salinicoccus sp.* Based on the morphological, physiological and biochemical characterization, the isolate M was identified as *Salinicoccus* sp. In order to confirm the same and to identify the sub-species level 16S rRNA sequencing was carried out. It revealed that the isolate belongs to genus *Salinicoccus.* Blast analysis revealed that the isolate under study is a novel *Salinicoccus* sp. M with Gene Bank Accession Number as KJ99797[20]. Phylogenetic tree was constructed by taking the sequences obtained in the blast search (**Fig. 4**). Enzymes of alkalophilies and halophilies have made great impact in industrial applications and tremendous use in human life. Sparse literature is available on LA producing *Salinicoccus* sp. and thus this warrants extensive study to identify its commercial prospects. This is the first kind of report of LA production from any *Salinicoccus* sp. from soil sample.

CONCLUSION: Our findings suggest that the isolate under study is a novel, extracellular L-asparaginase producing gram positive, moderate alkalophilic and halophilc bacteria. The isolate tolerated 12% of salt concentration and pH 12 and was 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 this is very first report of L-asparaginase production from any *Salinicoccus sp*

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