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The Occurrence of potential and novel isolates of *Oceanobacillus* sp. JAS12 and *Salinicoccus* sp. JS20 recovered from West Coast of Arabian Sea, India

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Abstract

Many halophiles were considered to be extremophiles due to their inborn industrial potentials and tolerance to hostile environmental conditions. The isolated halophilic bacteria described in the present study are not only grown at environmentally adverse conditions, also they can be able to produce bioactive molecules. Among the isolated strains, Oceanobacillus iheyensis strain JAS12 and Salinicoccus roseus strain JS20 are known for the unique biotechnological applications. The isolate Oceanobacillus sp. grows well at 35–55^{o}C (optimum 45^{o}C) and pH 6 to 12 (maximum growth at pH 8), interestingly the strain could hydrolyze casein, starch and gelatin. The G+C content was 40.2 mol % and the major fatty acids are iso-15:0: 30.52%, primary-C15: 0 (29.29 %), iso-14:0 (16.15%) anteiso-C17: 0 (4.03%). Another isolate was Salinicoccus sp. JS20 The DNA G+C content was 50.4 mol % and the major fatty acids are anteiso-C15: 0 (26.23%), iso-15:0, (17.62%), 16:0 (11.5%), anteiso-C17: 0 (7.7 %), iso- C16: 0 (10.20 %), iso-17:0: (5.43%) and iso-C14: 0 (3.97 %).

These isolates are also producers of many extracellular enzymes such as protease, amylase, inulinases, gelatinase and β-fructofurinosidase above the optimal conditions. Oceanobacillus sp. JAS12 16S rRNA gene sequence similarity is 99% similar to the reported genera. Salinicoccus sp. JS20 indicated 96% 16S rRNA sequence similarity with near species Salinicoccus genus, thus, they were found to be novel concerning to their genetic makeup and biochemical features.

Keywords: *Oceanobacillus*, *Salinicoccus*, protease, amylase, halophiles.

Introduction

Past few decades, plants and animals have explored higher than the microorganisms.[10] Since microbes occur ubiquitously and their nutritional requirement is very diverse, only 8-10% of the microbial population has been explored. Undoubtedly, these microorganisms habitat is in aquatic, marine, terrestrial, deserts, volcanic erupted land, glaciers, stratosphere etc. Among these organisms, halophilic bacteria are the microbes that can grow under higher salt concentrations. Halophiles inherently possess different industrially important metabolites producing abilities. Industrial processes are usually carried out at relatively extreme physiological conditions such as higher temperatures, pH, or dissolved oxygen concentrations.

Therefore, it is critical to have potential microbes that can sustain such harsh physiological conditions provided with producing definite value-added product at an industrial scale. Sánchez-Porro et al[23] in 2003 reported extracellular enzymes such as protease, lipases, amylase, pectinase, gelatinase and inulinase enzymes have enormous value in the food, feed, chemical and biomedical industries.

Many researchers have revealed the occurrence of *Oceanobacillus* genera in coastal regions, the first being Lu et al.[14] They have isolated the *Oceanobacillus* from the mud sample of deep-sea and type species identified as *Oceanobacillus iheyensis* DSM 14371T. Concurrently rigorous work was conducted by the different researchers around the globe and corrected the genus upon the isolation of *Oceanobacillus oncorhynchi subsp. oncorynchi* JCM 12661T.[37] The reclassification of *Virgibacillus picturae*[7] as *Oceanobacillus picturae* DSM 14867T was also done.[13] Until 2014 twelve *Oceanobacillus* species along with two subspecies were recognized.

The *Salinicoccus* genus was first described by Ventosa et al in 1990[29] and the type species was much similar to *Salinicoccus roseus* DSM 5351T. The *Salinicoccus* sp. generally are aerobic and moderately halophilic. The previously identified species under the *Salinicoccus* genus are as follows: *Salinicoccus roseus* and *Saliniccus hispanicus* as described by Ventosa et al,[29,30] *Salinicoccus salsiraiae* described by Franca and coworkers,[6] *Salinicoccus alkaliphilus* described by Zhang and colleagues,[38] *Salinicoccus jeotgali* identified by Aslam and others.[1] Numerous industrial important moderate and extreme halophilic bacteria and archaeons were isolated from unique habitats such as ancient salt deposits and that tend to the isolation of novel taxon.[4]

In Algerian Sahara of hypersaline soils, archaeal strains were identified and enumerated for antimicrobial activities,[5,21] Similarly, Taha et al[27] enumerated the existence of extremely halophilic archaea bacteria from Algerian arid and

semi-arid wetland ecosystems. According to the editorial report of Erika, the enzymes obtained from the marine bacteria have a pretty good source of biotechnological applications and their fatty acid esters were further explored for biomarkers development.[9]

The West Coast of the Arabian Sea near Karwar and Mangalore, situated in south India, is the unique habitat for the new species of moderately halophilic bacteria. Thus, the microbes living in hostile environments inherently produce extracellular enzymes at relatively higher salt concentrations and higher temperatures. In the present work, we describe the detailed occurrence, physiology, molecular identification, fatty acid profiles and extracellular enzymes produced by the novel isolates such as *Oceanobacillus* strain JAS12 and *Salinicoccus* Strain JS22. In concerning the *Salinicoccus* sp., 96% of sequence similarity strongly indicated its potential novelty in its occurrence.

Material and Methods

Isolation media: Bacterial strains were isolated from the west coast of India by following the protocol of Spring et al.[24] The medium contains tryptone 5 g, sodium citrate 3g, casamino acids 5g, potassium chloride 2g and magnesium sulfate 20g/L; pH 7.5[17] which was ideal for the growth and maintenance of the bacterial strains. All selected tests were performed using the medium containing 10% sodium chloride (W/V), at pH 7.5 and allowed to grow at 35°C for 48 hours.

Morphological characterization: To analyze the morphological feature possessed by the isolated strains, they were allowed to grow on the PYA medium contained peptone 1g, yeast extract 0.5g, K_2HPO_4 0.1g, $MgSO_4$ 0.02g, NaCl 3g and Bacto agar 1.5g to 100ml of distilled water and pH 7.5. After 24-36 hrs of incubation at temperature, 35^0 C gram staining was carried out. The morphology of the cells of each strain was observed under Phase-Contrast Microscope.

16S rRNA sequencing: The genomic DNA extraction was carried out according to Ausubel et al[2] followed by PCR amplification and the 16S rRNA analysis was performed according to the Sulochana et al. Novel strains of *Oceanobacillus* and *Salinicoccus* species gene sequences were deposited in the National Center for Biotechnological Information (NCBI) on accession numbers JX104218 and HQ834854 respectively.

Cellular G+C content analysis: The percentage of guanine and cytosine content of DNA was critical in the description of strain.[16] During the process, 2g of wet biomass (centrifugal pellet) of each isolate was used. Initially, isolates were grown in halo bacterial medium (ATCC 213) containing 10% of NaCl for 18 hr. After incubation, centrifugation of culture suspension was carried out at 10000 rpm for 10 min. Bacterial cells lysed using the French press method and then desired DNA was purified using hydroxyapatite as per Cashion et al.[3] The DNA hydrolyzed with P1 nuclease and the nucleotides dephosphorylated after the treatment with alkaline phosphatase.[24] The subsequent deoxynucleosides were analyzed using HPLC.[25,31] Lamda DNA and 3 DNAs with already published genome sequences representing 43-72% were used as standards. G+C values were calculated as the ratio of guanosine and thymidine as defined by Mesbah et al.[16]

Fatty Acid Analysis: Analysis of cells containing fatty acids in halophilic bacteria was carried out by growing them on saline medium. These plates were incubated for 48 hours and further the saponification process, methylation and extraction of cellular fatty acids were carried out as per the protocol developed by the Sherlock Microbial Identification System (MIDI).[1] Screening of newly isolated strains of *Oceanobacillus* and *Salinicoccus roseus* strain JS20 for extracellular enzyme production was carried out as follows.

Extracellular protease production: To determine the protease producing ability in 10% w/v of skim milk, 2% agarose with 20% of salt medium, the halophilic strains were inoculated.[22,32] After seven days of incubation, zone of hydrolysis indicated a positive test.

Amylase production: *Oceanobacillus* and *Salinicoccus* sp. were screened for the production of amylase enzyme. The qualitative analysis was carried according to Amoozergar et al where starch agar medium consisted of 20% of salt. The plates were incubated at 34-37^0C temperature for about seven days. After incubation, 0.3% I_2 -0.6% KI solution was spread on Petri dishes. The zone of hydrolysis around the inoculated strains indicated the amylase activity.[31]

Gelatinase activity: 15% of gelatin was added to the saline medium and 2 ml transferred to a small test tube that was inoculated with the testable strains and incubated at 30^0C. Along with the negative control, after incubation, the cultures were again incubated for about 10 minutes at 4^0C. The liquefaction of gelatin confirms the production of gelatinase and was recorded.

Inulinase activity: To determine inulinase activity the strains were allowed to grow on medium (g/liter) containing inulin 2, ammonium sulfate 0.5, magnesium hydroxide 0.2, potassium dihydrogen phosphate 3, agar 20 and 20% w/v NaCl salts. The substrate inulin was a sole carbon source in the medium; consequently, bacterial growth after two days of incubation at 37^0 C was designated as the presence of inulinase activity.

β-galactosidase production: The halophilic bacterial isolates were initially inoculated into the skimmed milk agar plates and incubated at 40-45^0 C and cultured on lactose broth for about 16 hours at 43°C. After the incubation, centrifugation was carried out at 8000 rpm for 10 minutes and to the collected supernatant, 2-3ml of acetone were added to obtain the β-galactosidase enzyme.[20]

Results and Discussion

Novelty of *Oceanobacillus iheyensis* strain JAS12: *Oceanobacillus iheyensis* strain JAS12 (Nucleotide data obtained and deposited under accession number: JX104218) was found to be gram-positive, flagellated, rod-like structure (Fig. 1). And interestingly, it is non-spore forming which contributes significant difference among commonly occurring *Oceanobacillus* group and *Oceanobacillus oncorynche* subsp. *incaldanensis* are the lone non-spore former groups accumulated in a similar type of characteristic feature. *Oceanobacillus iheyensis* sp. JAS12 forms circular and cream-white colored obligatory under aerobe or facultative alkaliphilic.

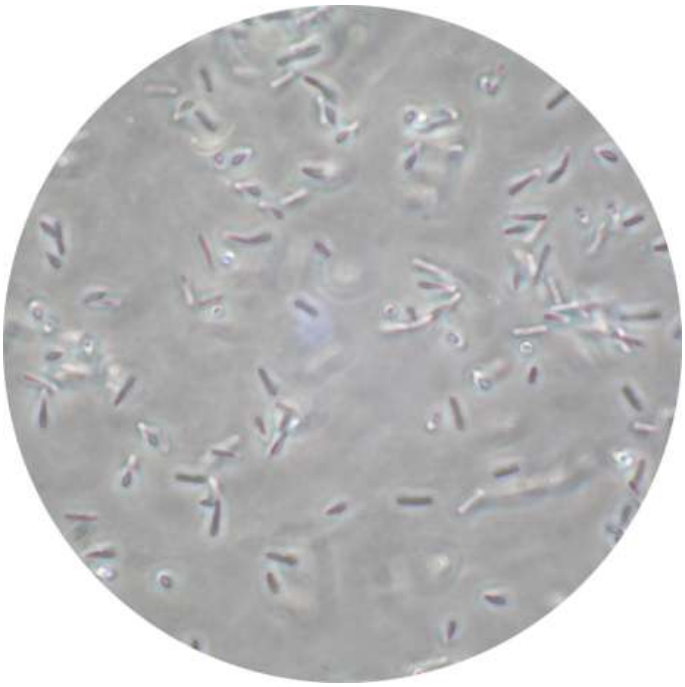

Figure 1: Phase Contrast image of *Oceanobacillus* sp.

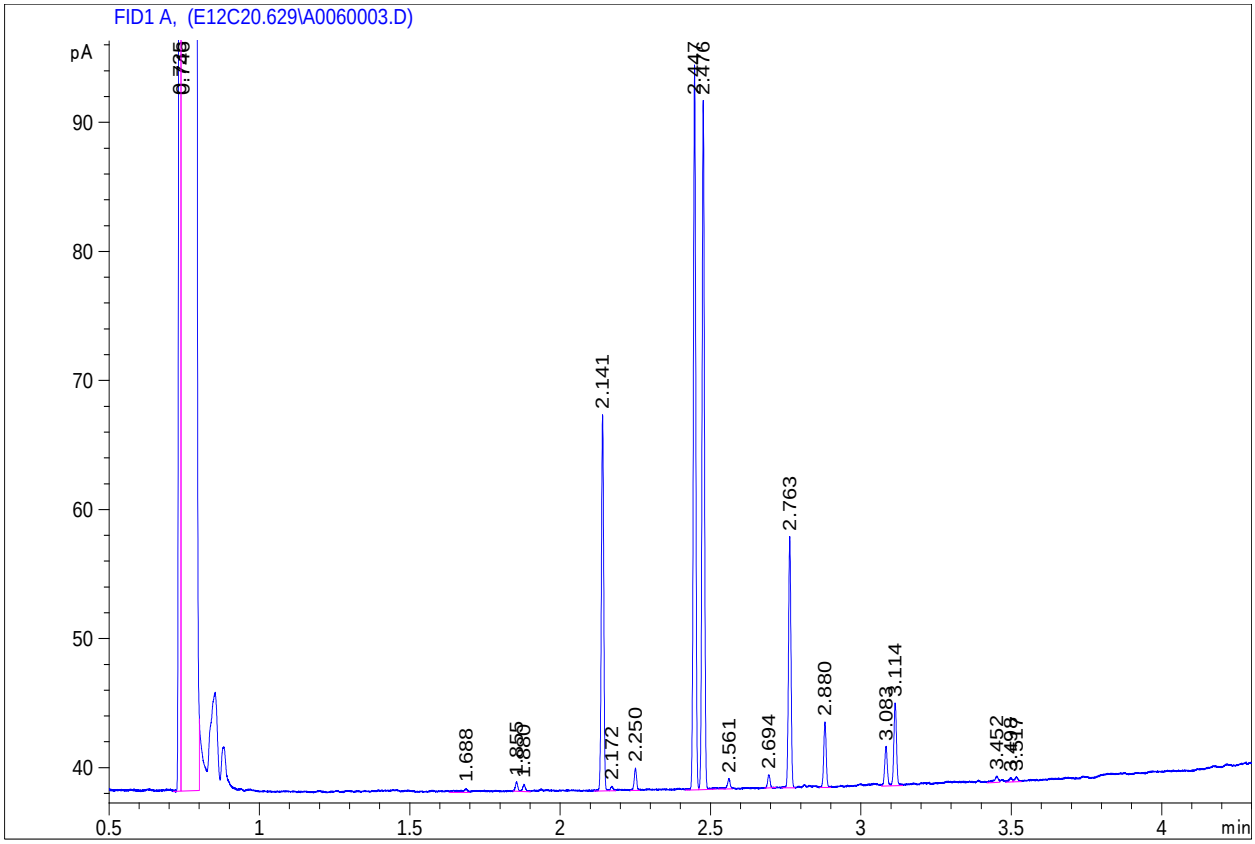

Figure 2: Fatty acid methyl ester analysis of proposed novel species Oceanobacillus strain JAS12.

In HiCarbo^{Tm} Kit 20 strips, a positive result was obtained on fermentation of dextrose, xylose, maltose, fructose, trehalose, sucrose, inulin, sorbitol, glycerol and citrate utilization. However, the assimilation of lactose, α-methyl D-glucoside, rhamnose, ribose, melezitose, α-methyl D-mannose, cellobiose, xylitol, D-arabinose, sorbose and malonate utilization was negative. The major fatty acids were iso-15:0: 30.52%, anteiso-C15: 0 (29.29 %), iso-14:0 (16.15%) anteiso-C17: 0 (4.03%) (Fig. 2).

The morphological and biochemical characterization was carried out for the following strains 1. JAS12 (*Oceanobacillus* sp.); 2. *O. iheyensis* KCTC 3954^{T,14} 3. *O. oncorhynchi* R-2^{T,37} 4. *O. picturae* KCTC 3821^{T,7}. All strains were motile, gram-positive, oxidase and catalase-positive and produced elliptical spores and test for indole production was negative. Not a single isolate produced acid from D-arabinose, L-rhamnose, Myo-inositol, L-fucose, or 5-keto-D-gluconate.

On the other hand, *Salinicoccus roseus* strain JS20 cells were observed to be gram-positive, motile, non-spore former. Oxidase and catalase-positive, obligately aerobic cocci (0.8–1.2 mm) appear to be singly or in pairs, tetrads, or clumps (Fig. 3). Colonies are round, convex, pinkish-pigmented and non-translucent with glistening surfaces and form entire margin, 2–3 mm in diameter after three days on halobacteria medium ATCC 213 containing 10% (w/v) salts at pH 7.2 and temperature about 32ºC.

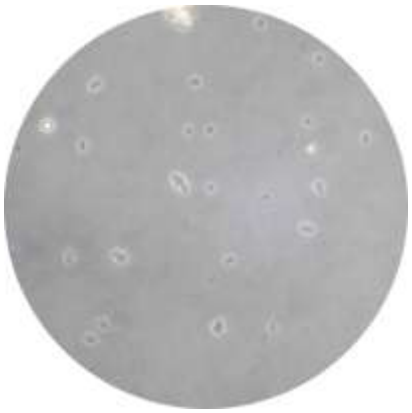

Figure 3: Phase Contrast image of *Salinicoccus* sp. JS20.

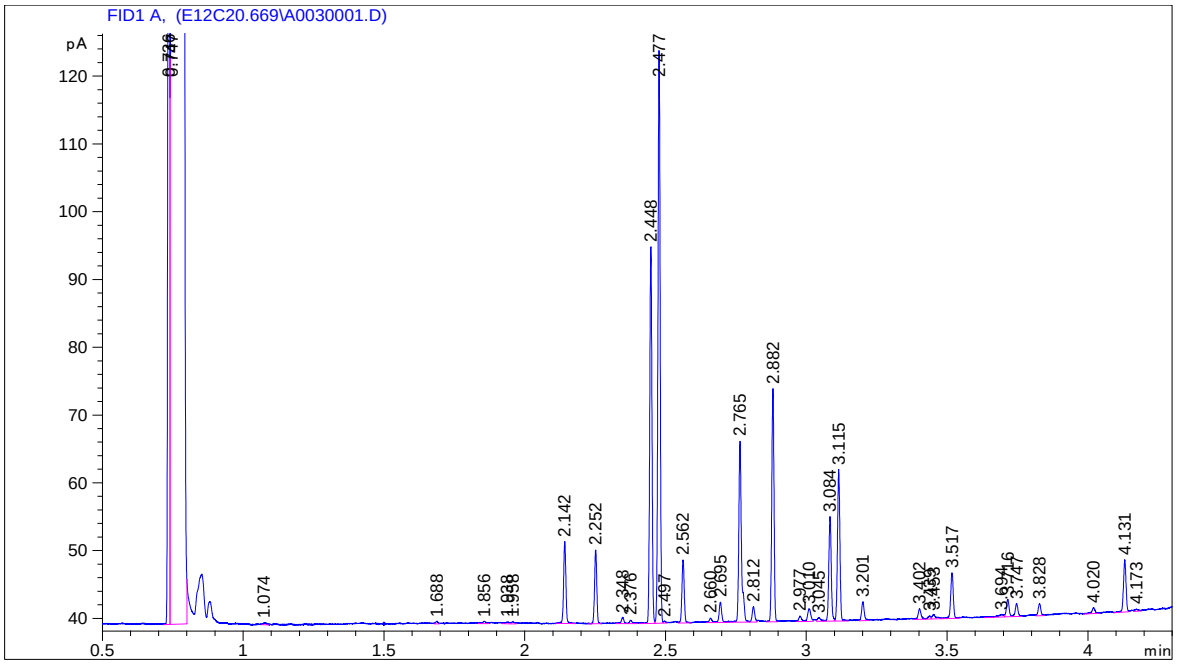

Figure 4: Fatty acid methyl ester analysis of proposed novel species *Salinicoccus* strain JS20.

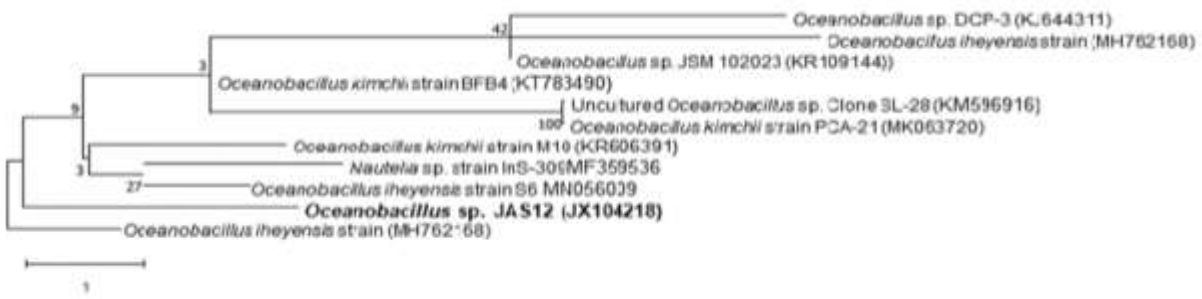

Fig. 5: Phylogenetic tree constructed using Mega 7 (Evolutionary analysis by Maximum Likelihood method) software where *Oceanobacillus* sp. JAS12 compared with relevant species.

Table 1
Extracellular enzyme production from *Salinicoccus* sp. *strain JS20* and *Oceanobacillus* sp. JAS12.

S.N.	Extracellular enzymes	*Salinicoccus* sp. *strain JS20*	*Oceanobacillus* sp. JAS12
1	Protease	+	+
2	Amylase	+	-
3	Gelatinase	-	+
4	Inulinase	+	+
5	β-galactosidase	+	+

Note: "+"is positive for respective hydrolytic enzymes production, "-" is no enzyme production.

Temperature range of growth is 22–45ºC (optimum 32ºC). The strain could utilize casein, inulin, starch and β-galactosidase, but unable to hydrolyze gelatin and aesculin. Nitrate reduction test was positive and methyl red and Voges–Proskauer, indole and H_2S were negative. The strain can utilize fructose, dextrose, trehalose, sucrose, inulin, sodium gluconate, sorbitol, mannitol, adonitol, ONPG, citrate utilization, malonate utilization and was unable to utilize lactose, xylose, maltose, galactose, raffinose, melibiose, L-arabinose, mannose, glycerol, salicin, glucosamine, dulcitol, Inositol, α-methyl D-glucoside, Ribose, Rhamnose, Cellobiose, Melezitose, α-methyl D-mannoside, xylitol, aesculin hydrolysis, D-arabinose and sorbose.

Phenotypic, biochemical and genotypic Cellular G+C content comparison were deliberated for Strains: 1. SS5 (*Salinicoccus roseus*); 2. *S. alkaliphilus* JCM 11311$^{T, 38}$ 3. *S. roseus* DSM 5351$^{T, 29}$; 4. *S. hispanicus* DSM 5352^{T} Marquez et al, 5. *S. jeotgali* KCTC 13030^{T,1} 6. *S. salsiraiae* LMG 22840^{T,6} and were compared.

The DNA G+C content was 50.4 mol % and the major fatty acids are anteiso-C15: 0 (26.23%), iso-15:0, (17.62%), 16:0 (11.5%), anteiso-C17: 0 (7.7 %), iso- C16: 0 (10.20 %), iso-17:0: (5.43%), iso-C14: 0 (3.97 %). Fatty acid composition of members of the genus *Salinicoccus* strains was deliberated (Table 1): 1. SS5 (*Salinicoccus* sp. from the study); 2. *S. alkaliphilus* JCM 11311$^{T, 38}$; 3. *S. roseus* DSM 5351^{T};[36] 4. *S. hispanicus* DSM 5352$^{T. 28}$ 5. *S. jeotgali* KCTC 13030$^{T\ 1}$; 6. *S. salsiraiae* LMG 22840$^{T, 6}$.

With respect to the potential enzyme production and occurrence of halobacteria, contrast results were obtained when compared to previous reports of Ventosa[31] where he found maximum potential isolates to produce hydrolytic enzymes belonging to gram-negative genera *Salinivibrio* or *Halomonas*. However, in the present study, potential isolates in moderately saline habitats of Karwar and Mangalore were namely *Salinicoccus* and *Oceanobacillus* sp. Respectively. Both strains are gram-positive.

These strains *Oceanobacillus* and *Salinicoccus* prefer to grow under minimum salt concentration. The mangroves ecosystems of Karwar, coastal belt of Mangalore were provided with untapped habitat diverse halo bacterial communities. Since they possess the abundant sources of a wide variety of halophilic bacteria, Yaradoddi et al[33] identified and classified the different communities of halophilic bacteria belonging to *Virgibacillus, Halobacillus, Pontibacillus, Oceanobacillus, Salinicoccus, Marinobacter, Nesrenkonia* and *Staphylococcus*. They have thus obtained halobacteria classified under the moderately halophiles based on morphological, physiological, biochemical adaptations. Interestingly, species among these communities can be able to produce at least four extracellular enzymes such as protease, amylases, inulinases, β-galactosidases and gelatinase enzymes. These enzymes have precise biotechnological and industrial applications such as food, beverages, textiles, detergents, leather industries.

When compared to the reports of Lee et al,[13] the significant difference among existing *Oceanobacillus* sp. was discussed as follows: the JAS 12 could be able to tolerate higher temperature (up to 55ºC) and optimum temperature was observed at 45ºC, whereas most of the reported *Oceanobacillus* sp. can tolerate up to 42ºC temperature, the maximum pH required for the growth is 13 whereas relevant *Oceanobacillus* sp. can tolerate and grows well up to pH 10. The major parameter in defining the classification of halobacterium is the ability of salinity tolerance; JAS12 was sustained even at salinity of 30% which is significantly higher than the earlier reported for *Oceanobacillus* sp. (Maximum 22% salinity). Interestingly acid production from galactose was observed for JAS12 as weakly positive (few species) and acid production from galactose is negative for most of the reported *Oceanobacillus* sp., aesculin hydrolysis with respect to *Oceanobacillus* sp. was reported in the literature where the JAS12 strain used in the present study was not able to hydrolyze aesculin. The percent G+C content of the JAS12 strain was found to be little higher 40.2 when compared to the existing one (maximum 40% G+C content).

The morphological, biochemical and the molecular characteristic features of the isolated *Salinicoccus* sp. JS20 were compared as per the reports described by the previous researchers.[1,6,29] JS 20 strain used in the present work requires at least 3% of NaCl for their growth whereas most of the reported species among the *Salinicoccus* genera require 0.5%. It looks almost similar in pigment production (pinkish in color) compared to previous studies; the maximum pH required for the growth of the *Salinicoccus* sp. JS20 is 10 which is little bit higher than the previously reported. Acid production from the sucrose was observed for the present strain whereas it was negative for the relevant strains.

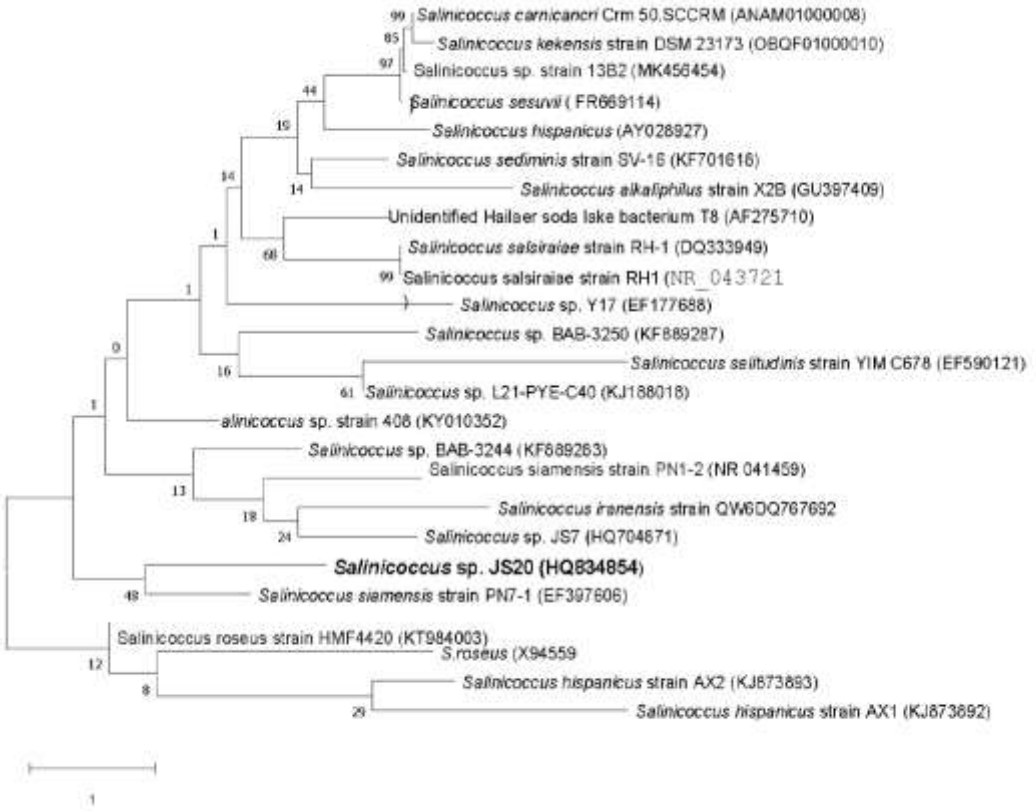

Fig. 6: Phylogenetic tree constructed using Mega 7 (Evolutionary analysis by Maximum Likelihood method) software where *Salinicoccus* sp. JS20 was compared with relevant species.

The significant differences were observed with respect to molecular systematics of the present strain; it has shown 96% of the similarity when we carried out the BLASTn analysis which is most promising result to claim as potential novel isolate and the molecular G+C content of the strain *Salinicoccus* sp. JS20 is 50.4% which is considerably more than the existing *Salinicoccus* species (Fig. 4). According to the evolutionary analysis by Mega 7 maximum likelihood method,[8, 12] these two isolated strains could be placed under the subgroups among *Oceanobacillus iheyensis* and *Salinicoccus siamensis* respectively.

Conclusion

Much work was carried out on cellulases, amylases, DNases, lipases, proteases and pullulanase[11,34]. However, most of the studies restricted to the screening, production and purification of the extracellular enzymes from these halophiles. Our research was not only limited to the selection of extracellular enzymes but also exposed physiological and biochemical features possessed by *Oceanobacillus* strain JAS12 and *Salinicoccus* strain JS20. Recent reports have shown the enzymes acting on carbohydrates have received significant interest.[35] The unique metabolites produced by these two strains are unrivaled, since, these isolates not only tolerate higher salt concentrations but they also possess inherent industrial properties like they can duplicate and produce extracellular enzymes in adverse temperature and pH conditions where most of the organisms have failed to grow and proven the enzymatic activities.

In support of the report, the 16S gene sequence analysis alone always cannot be a standard analyzing tool to identify the microbes at the strain level, certainly the heterogeneity of genes and the massive sequences in the nucleotide databases leading to the ambiguity in classification.[26] The present work provides crucial information about the morphological, biochemical, fatty acid profiles, 16S rRNA and cellular GC content of the industrially desired extracellular enzymes producing strains such as *Salinicoccus* and *Oceanobacillus.* However, their activities in microbial interaction and plant protection will be prospective.

Morphological and biochemical multiplicity characteristics possessed by these strains have a definite influence on understanding the molecular dynamics of the halophiles. The tremendous biotechnological importance and potentiality of producing bioactive molecules by a novel isolate is the immediate requirement of the industries which implies the versatile biotechnological applications for future opportunities.

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