MINOR RESEARCH PROJECT

Summery

1. **Name & Post of the Project Investigator**- Dr. A. G. Jadhav
   Assistant Professor in Microbiology
2. **Name of the Research Project Sanctioned by UGC with date**
   "Isolation and characterization of salt tolerant (halotolerant and halophilic) bacteria from Lonar crater, MS India and their screening for extracellular halostable enzymes"
   (File No: 47-1635/10 WRO date 1st May 2011)
3. **Research Design and Methodology**:-
   - Collection of water and soil sediments from Lonar lake.
   - Screening for halotolerant and halophilic bacteria.
   - Determination of salt tolerance of obtained isolates.
   - Plate Assay for various selected enzymes.
   - Production of enzymes from selected isolates.
   - Halo stability of enzymes.
   - Optimization of pH and temperature.
   - Morphological and biochemical characterization of efficient cultures.
   - Phylogenetic study of efficient bacterial isolate.
4. **Report of work done/Major Findings**

Lonar lake ($19^0$58' N and $76^0$31' E) is in the formerly volcanic Deccan trap geological region. water and Sediment samples were collected from various sites of lake. Water samples were treated and analyzed for chemical and physical properties.

1. **Physico-chemical analysis of Lonar water**

Water temperature is found 26^{o}C which may vary according to season, highest in summer, pH of the water sample was 9.0. The electrical conductivity is found to be 11710µS/cm and was found related to high concentration of ionisable substances in water. Dissolved oxygen shows maximum level of 1.9mg/lit. The total solids are found to be 15.77gm/lit and total dissolved solids in 5.7gm/lit this is due to the accumulation of many dissolved substances like minerals, organic, and inorganic pollutant. The value of alkalinity is found to be 2.85gm/lit it might due to dissolved salt, dilution of surface water in rain. The hardness of water is found 0.09gm/lit. The concentration sodium in water is 3.260gm/lit which is higher than the natural fresh water so it can be related to cause adverse effect. This high concentration of sodium also affects the permeability of soil.

Table 1: Physico-chemical characterization of Lonar water

PARAMETERS	OBSERVATION
Colour	Greenish (less in rainy season)
Temperature	26
Electric Conductivity	11710µS/cm
pH	9.0
Total Solids	15.7gm/lit

Total Dissolve Solids	5.7 gm/lit
Total Hardness	0.09gm/lit
Total Alkalinity	2.85gm/lit
Biological Oxygen Demand	0.304gm/lit
Chemical Oxygen Demand	148 gm/lit
Sodium	3.260 gm/lit
Calcium	0.1 gm/lit

2. Screening of salt tolerant bacteria from collected soil samples:

Salt tolerance for isolates were studied at varying NaCl concentration in the same medium. For unknown reasons many isolates fail to grow, total 20 isolates were grown, obtained.

3. Screening for extracellular enzymes:

All isolates were screened for extracellular CMCase, amylase, protease, DNAase and lipase. So many studies were focused on such approaches to find potential of the obtained isolates from unusual, extreme habitat. Akhtar *et al.*, 2008, have studied culturable bacterial biodiversity and industrial importance of the isolates indigenous to Khewra salt mine, Pakistan. Also analysed isolates for production of industrial enzymes (amylase, carboxymethyl cellulase, xylanase, cellulase and protease) against starch, carboxymethyl cellulose (CMC), xylane, cellulose, and casein degradation in plate assays.

Screening was performed on media containing excess amount of salt, mostly 5% of the NaCl. Results were as follows.

Sr. No.	Extracellular DNAase activity +Ve Isolates	Extracellular Lipase activity +Ve Isolates	Extracellular Cellulase + Ve Isolate No.	Extracellular amylase activity +Ve Isolates	Extracellular protease activity +Ve Isolates
1	S-2	S-3	S- 9	S-14	S-3
2	S-3	S-4	S- 11	S-4	S-4
3	S-4	S-5	S- 13	S-12	S-8
4	S-7	S-10	S- 14	S-3	S-12
5	S-8	S-12	S- 16	S-8	S-14
6	S-10	S-16	S- 17	SO9	-
7	S-12	S-101	S- 91	S-7	-
8	S-102	-	-	-	-
9	S-104	-	-	-	-
10	S-105	-	-	-	-

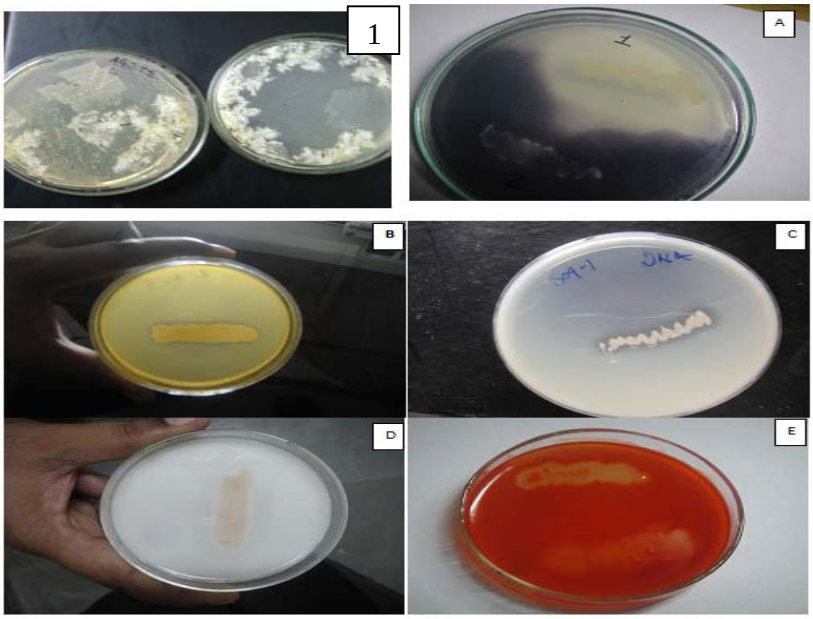
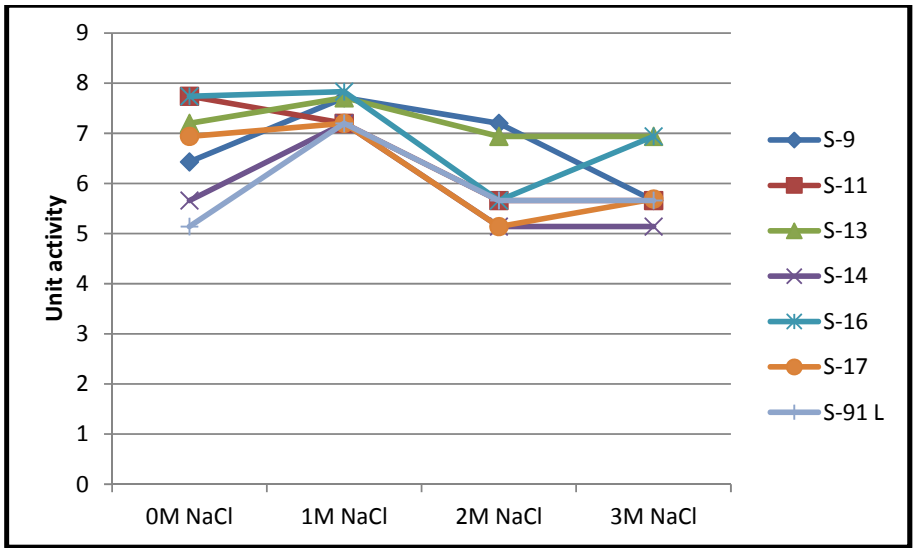


Photo: 1. Growth on salt containing medium, A. Amylase, B. Lipase, C. Dnase, D. Protease and E. Cellulase producers.

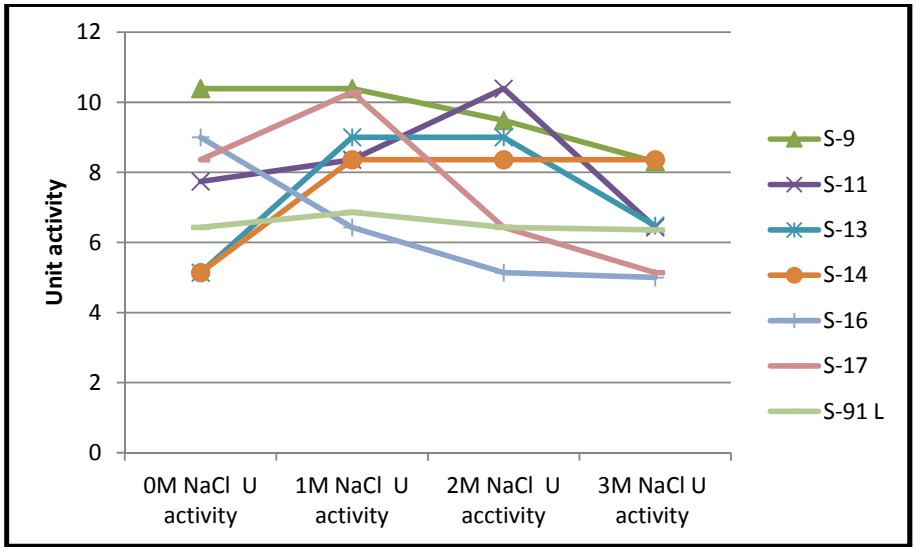
All the isolates which found producers of extracellular enzymes are Gm+Ve rods.

4. Determination of cellulase enzyme activity:

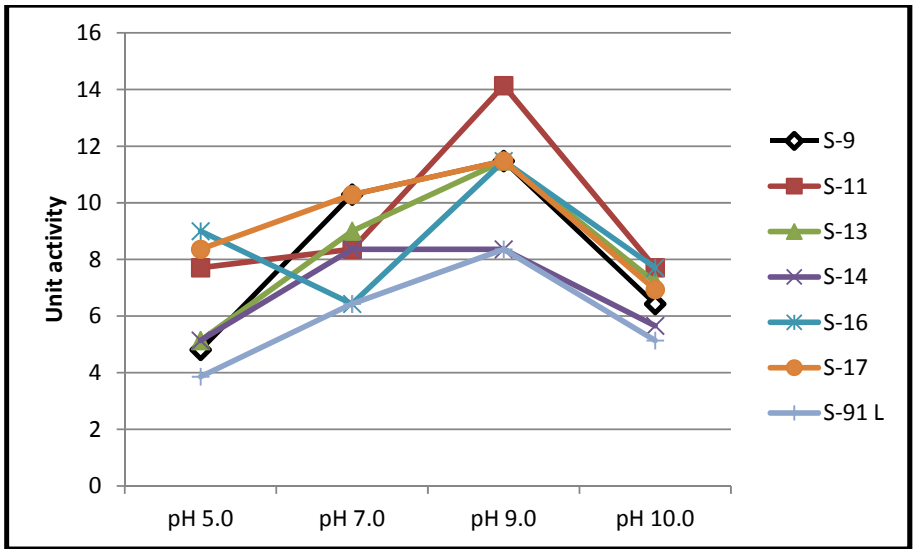
Activity of enzyme was determined by DNSA and measured by taking A_{540}. Activity of crud enzymes were determined by using standard graph of glucose. This is then converted to enzyme unit, one unit of enzyme is amount of enzyme required to produce 1µmol of product i.e. glucose from CMC. Influence of pH, temperature and salt on the activity of cellulase was studied. Results obtained are as shown in Graph No. 5.1 to 5.4.



Graph No.4.1: Activity of cellulase of selected isolates at pH 9.0 and different NaCl concentrations.



Graph No. 4.3: Activity of cellulase of selected isolates at pH 7.0 and different NaCl concentrations.



Graph No. 4.3.: Influence of pH on the activity of cellulase enzyme from selected isolates in terms of enzyme unit.

From the obtained readings it was found that the enzyme for all isolates is having optimum pH 9.0 and temperature 60^0C. The results obtained in this study elucidate production halo-stable cellulase from all above isolates. Our results are similar in many respects to already reported halo-stable cellulase enzymes. Cellulase obtained from an alkaliphilic strain, *Bacillus sphaericus* also has specific activity of 38.4 U/mg (Kaur *et al.,* 2007). For *Bacillus* sp. AC-1 and *Melanocarpus* sp., the specific activities of endoglucanase were 35.0 and 35.3 U/mg, respectively (Li *et al.,* 2006 and Singh *et al.,* 2004).

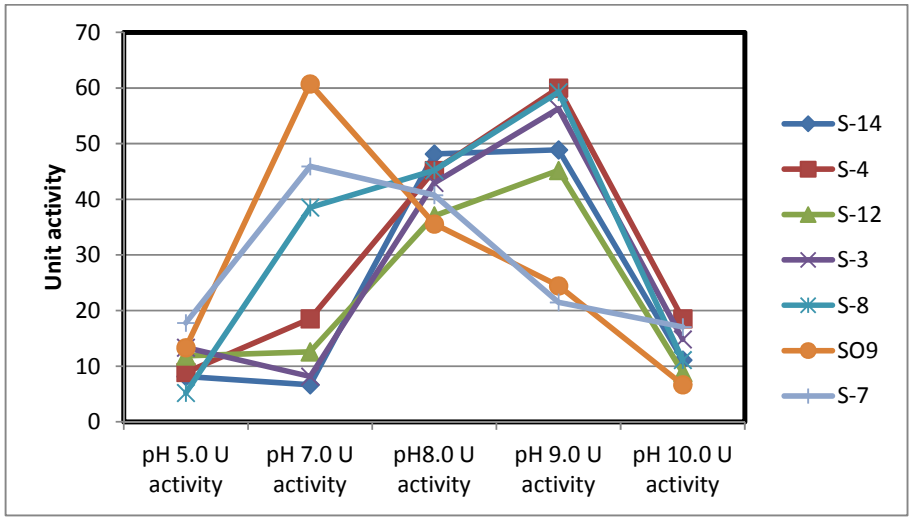
Though in our study enzyme unit activity seems less, we need to consider an important fact that, in this study we have not done any kind of media formulation for enzyme production.

5. Determination of Amylase enzyme activity:

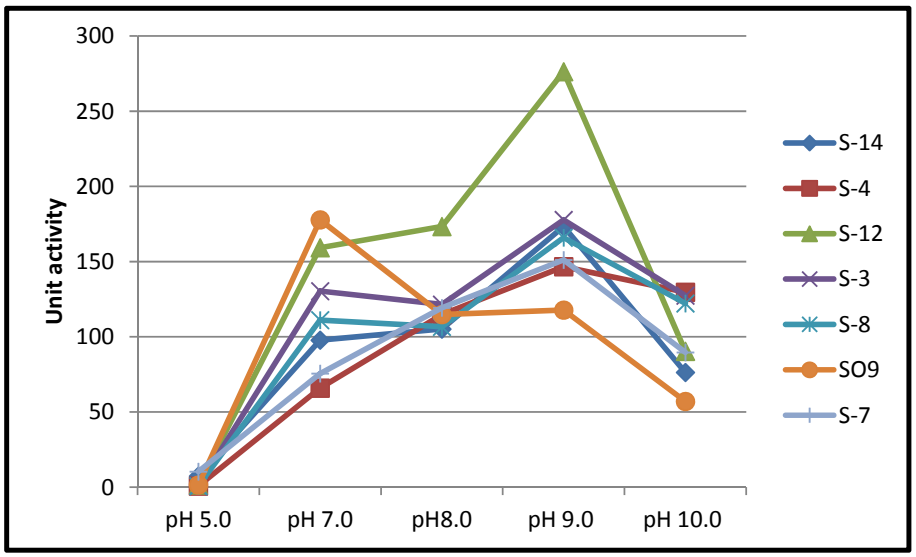
After incubation the content of broth were centrifuged at 15000g for 20 min at 4^0C. Supernatant is collected in separate sterile bottles for every culture and used as crude

enzyme. Total protein content present in the crude enzyme was determined by Folin and Lowry method. A standard graph of BSA was used to find out the respective protein concentrations/ amount in the samples. Protein content of crude enzyme from starch broth for amylase positive cultures is as shown in Graph No.6.1. Activity of enzyme was determined by DNSA method and measured by taking A_{540}. Readings obtained further used to calculate amount of glucose formed due to activity of used crude enzyme by using standard graph of glucose as mentioned in 7.2. This further converted to calculate total units of amylase enzyme and unit activity. One unit of amylase is amount of enzyme required to produce 1µmol of glucose molecule per minute under assay conditions.

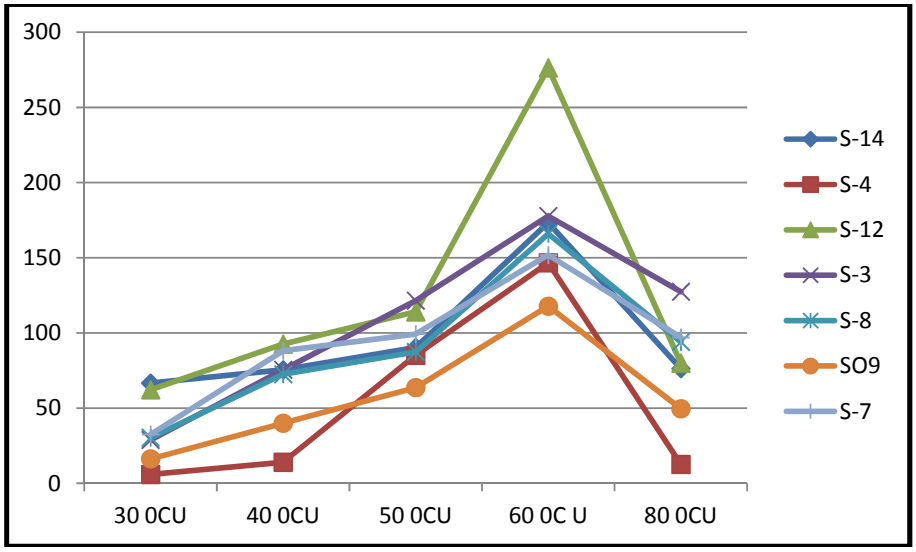
The activity of crude enzyme for selected isolates and influence of various pH and temperature conditions were determined.



Graph No.5.1: Activity of amylase enzyme from different isolate at 5% of NaCl.

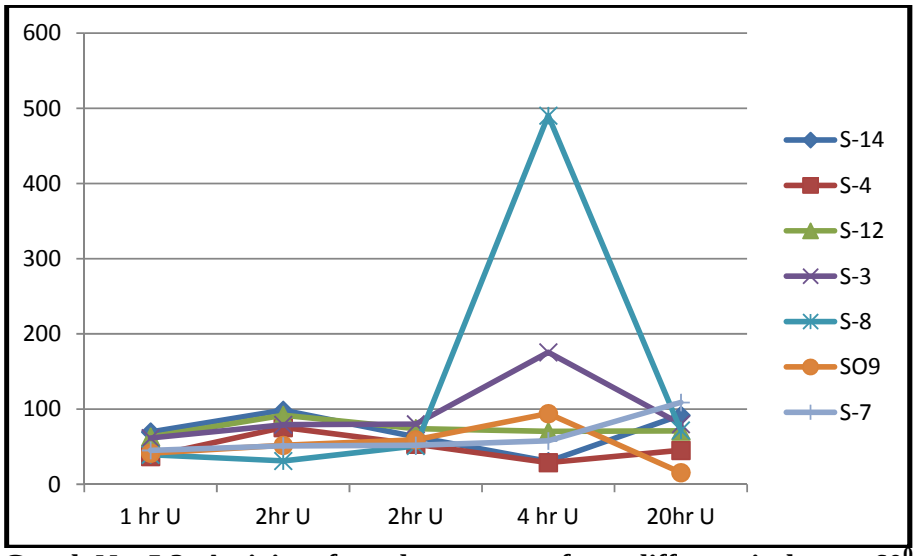


Graph No. 5.2: Activity of amylase enzyme from different isolate at various pH conditions.



Graph No. 5.3: Activity of amylase enzyme from different isolate at various temperatures.

From the obtained readings it was found that the enzyme for all isolates is having optimum pH 9.0 and temperature 60^0C. In order to find out the stability of enzyme, crude enzyme was enriched with 5% of NaCl (w/v) and activity of enzyme determined after interval of 1 hour. Obtained values are as follows



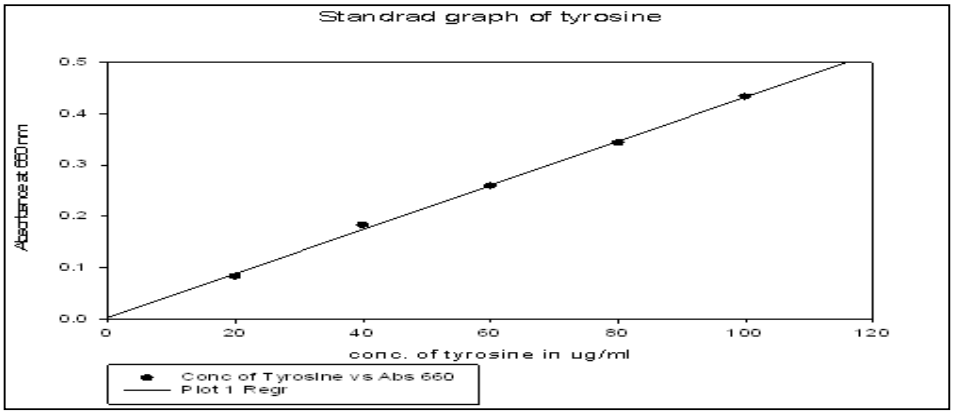
Graph No. 5.3: Activity of amylase enzyme from different isolate at 60^0C and 10% NaCl.

The amylases from isolates shown activity at 5% NaCl, further studies on activity over a broad range of salt concentrations (0–25%) is essential to find its halo-tolerance.

The stability of amylase with respect to salt concentrations indicates that amylase produced is highly stable in presence and the absence of salt.

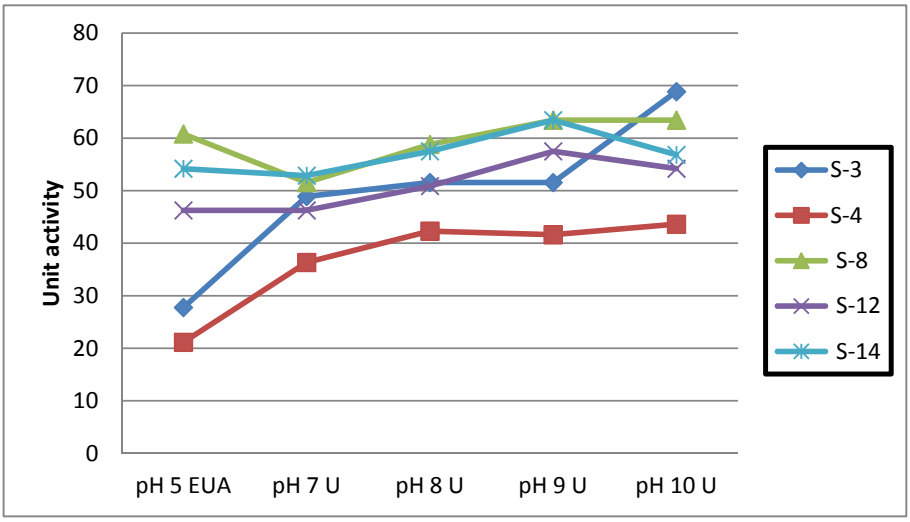
6. Determination of Protease enzyme activity:

For determination of protease activity firstly a standard graph of tyrosine was prepared. Absorbance obtained for various concentrations of glucose was plotted against concentration. Details of these were as mentioned in graph 7.1.

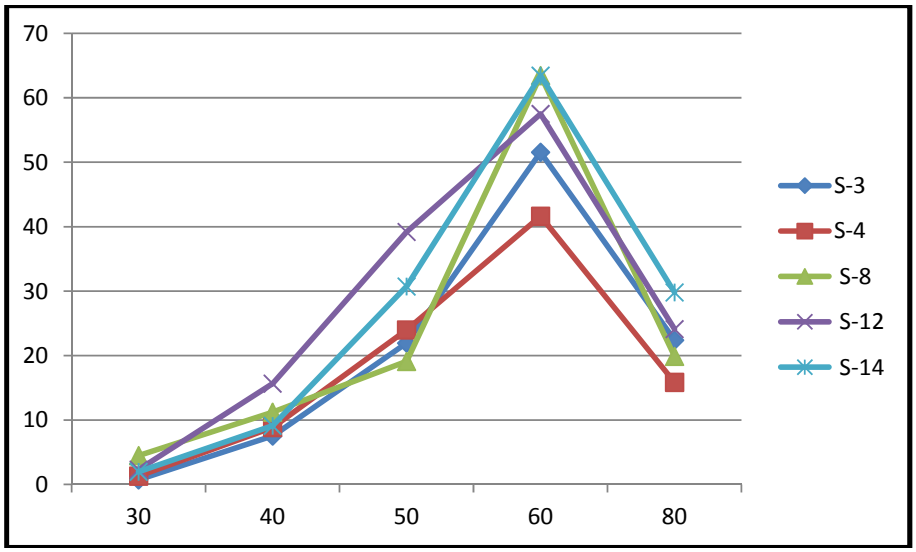


Graph No. 6.1: Standard graph of tyrosine.

After incubation the broth was centrifuged at 15000 g for 10 min at 4^0C. Supernatant were collected in separate sterile tubes and used as crude enzyme.



Graph No. 6.2: Influence of pH on the activity of protease enzyme from selected isolates in terms of enzyme unit activity.



Graph No. 6.3: Influence of temperature on activity of protease enzyme from selected isolates in terms of enzyme unit activity.

This result clearly indicated the potential of isolates in producing protease enzyme at 5% of salt, also stability of the enzyme. Studies to determine optimum temperature revealed that the protease showed maximum activity at 60^0C and can thus be classified as moderately thermo-active protease. On the other hand, the enzyme was active over a broad pH range (5.0–10.0) and had the optimal activity at pH 9-10, which is a typical characteristic of alkaline proteases. Moreover, the protease of all isolates was active even without NaCl and at 5% salt concentration.

7. Biochemical identification:

This reveals that the isolates obtained in this study belong to following types. Morphological and biochemical studies carried out to place these isolates in various genus. The details of the isolates obtained in this study were as given in the table 10.1.

Table no. 7.1. Biochemical properties of the obtained bacterial groups.

Character	*B. subtilis*	*B. licheniformis*	*B. megaterium*
Morphology	Rod	Rod	Rod
Gram property	+	+	+
Oxidase	+	+	+
Catalase	+	+	+
Spore formation	+	+	+
Growth pH range	7 .0 – 10.0	7 .0 – 10.0	7 .0 – 10.0
Growth in Salt range	0-5	0-8	0-5
Temperature	20-50	20-50	20-50
H2S production	ND	ND	ND
Lipase	+	+	-
Protease	+	+	+
Cellulase	+	+	-
Glucose	+	+	+
Lactose	+	+	+
Hydrolysis of:			
Casein	+	+	-
Gelatin	+	+	-
Starch	+	+	+

Resuls obtained in biochemical tests indicates presence of above mentioned bacteria producing tested extracellular enzyme belong to Bacillus.

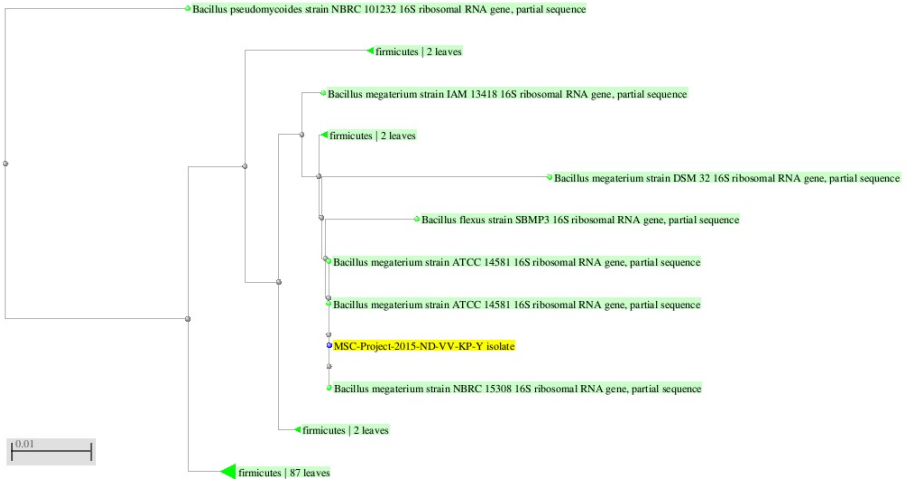
8. Identification of the isolates by 16S rRNA sequencing:

Though we did biochemical identification of the obtained isolates, but to acquire accuracy in the assignment of genus and species isolates showing response towards higher NaCl concentrations were subjected to 16S rDNA sequencing. Total 02 isolates were sequenced by using universal primers. Sequencing facility is availed from Paul Herbert center for DNA barcoding, Dr. BAMU, Aurangabad

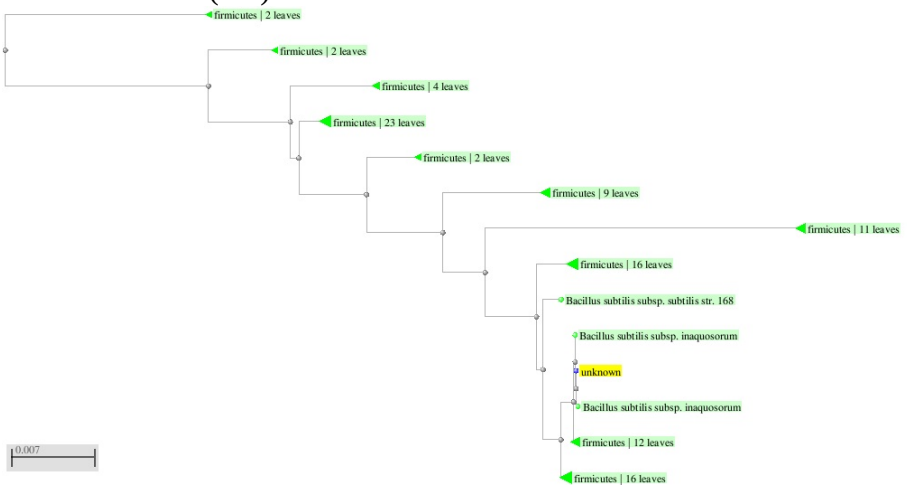
Both the isolates showed exact match with the 16 S rDNA database sequence. The alignment of both sequences was with positive strand comparison with good E value, identity.

Phylogenetic tree

Total 100 hits, sequences obtained in Blast comparison all these were used for plotting the tree of particular isolate. The tree diagram of Neighbour- Joining type was obtained for each isolate by exploring distance tree option available at Blast output.



Tree 1. Isolate Y (S-8)



Tree 2. Isolate w (S-14)

Phylogenetic tree of NJ type based on homologues sequences obtained by comparison with 16 S rDNA database, the isolate Y occupies position near to *Bacillus megaterium* and W isolate *Bacillus subtilis.* Both the sequences showed 100% query coverage and 100% sequence identity in partial sequence (Y-591 and W- 602 Bases). This indicate that obtained isolates are *B. megaterium and B. subtilis.*

CONCLUSION

The results obtained in this investigation put insights on the salt tolerant bacteria present in the saline water of Lonar lake. Bacterial isolates of this study majorly belong to salt tolerant/ halotolerant, only few are of moderately halophilic. Bacterial isolates found from this region during investigation showed good tolerance i.e. above 5%. Bacterial isolates from this study are of different genera revealed by biochemical investigation but among these only bacillus found useful in production of extracellular enzymes viz, amylase, protease and cellulase. All this genera have been reported from saline and hypersaline habitats and are already reported from the Lonar lake water. Thus isolate of this study identified as *Bacillus species* are catabolically diverse in nature i.e capable to produce extracellular enzymes.

As compared to this other isolates probably belongs to Pseudomonas, Alcaligens, Micrococus and staphylococcus are not found so much useful in utilizing selected mediums. A probable reason for this might be the presence of excess amount of salt in the medium. Screening of all the enzymes was made with higher salinity; characterization of these enzymes will put focus on their suitability in so many processes where excess salinity exits.

Finally we conclude that Lonar lake possess huge diversity of salt tolerant bacteria. These diverse group have shown great potential for checked few enzymes and thus can also be useful in remediation studies of this type or other toxic pollutants. Most importantly all these potentials have found and carried out at excess amount of salt (Salinity). This knowledge can be used for variety of purposes wherever higher salinity exit.