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PRODUCTION AND PARTIAL CHARACTERIZATION OF EXTRACELLULAR ENZYMES BY ACTINOMYCETES ISOLATED FROM WATER SAMPLE OF LONAR LAKE.

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ABSTRACT
The alkaline Lonar Lake, located in Buldhana District of Maharashtra is the only hypervelocity meteoritic impact crater in basalt rock, ranking third in the world. The present study deals with the isolation, identification and enzymatic study of actinomycetes from Lonar Lake. Forty five strains were isolated by enrichment technique in Glycerol Asparagine Broth, these isolates were studied for their Morphological, Physiological and Biochemical Characterization and predominantly for their enzymatic potential. Actinomycetes isolates were tested for growth at different pH tolerance (4, 5, 6, 7, 8, 9, 10, 11), Temperature ranges (10, 15, 20, 25, 30, 35, 40, 45, 50) and salt ranges (4, 7, 10, 13% NaCl). Among all the isolates of actinomycetes, 13 (28%) were able to produces amylase, 7 (15%) isolates were produces lipase, 19 (42%) could produces caseinase, 24 (53%) produces pectinase and 11 (24%) strains were produces gelatinase enzymes. Two isolates out of forty five which showed maximum Caseinase and Pectinase enzyme producing ability were proceeded for identification and enzyme production. After production the enzymes were purified by ammonium precipitation, dialysis and column chromatography methods. Enzyme activity was also optimized by studying the parameters like Temperature, pH and Substrate concentration. The maximum production of enzymes was observed at 50°C. and at pH 9. From the Morphological and Biochemical Characters, the isolates may be *Streptoverticillium baldaccii and Streptomyces griseoruber*. The results obtained from the present work revealed that the isolated actinomycetes have potential to produce industrially and pharmaceutically important enzymes.

KEY WORDS: Actinomycetes, Enzymes, Lonar Lake, Purification, Salt tolerance.

INTRODUCTION

Actinomycetes are excellent source of biotechnological applications and that still makes them one of the most important place after microbes to research on it, despite decades of research dedicate to unravel their bioprospective potential (Rizvi and Kamble, 2014). Actinomycetes are an important group of filamentous, gram positive microbes which are most economical and biotechnologically valuable class of prokaryotes producing bioactive secondary metabolites specifically antibiotics and number of industrially important enzymes (Blunt and Prinsep, 2006), these are differ from fungi in their cellular composition. They do not possess chitin and cellulose that is found in the cell wall of fungi (Zahabi and Issazadeh, 2014). Actinomycetes represent a group of relatively abundant and metabolically diverse microbes in environment. Microbes reside in extreme environments like alkaline conditions tend to express proteins with different characteristics than those inhabiting in the normal environment.

Lonar Lake located in Buldhana district of Maharashtra is like one of the wonders of state and is the world's third largest crater believed to be formed due to a meteor impact. It is a saline soda lake having the pH in the range of 10-12. This provides an extreme environment and serves as an excellent habitat for isolation of halophilic and alkaliphilic organisms (Chande and Bhat, 2014). Lonar lake ecosystem has reported to contain rich bacterial diversity. The microorganisms, alkaliphilic bacteria in this environment would therefore be unique. Lonar lake crater is a classic beautiful bowl shaped depression in the basaltic flows of the Deccan traps in Southern India (Gopalkrishna, 2000). As the water of Lonar lake is alkaline most of the strains were alkaliphilic in nature, the high alkalinity is due to the high concentration of sodium carbonate (Kanekar and Joshi, 2008). These bacteria were produce industrially important enzymes at alkaline pH. Alkaliphilic microorganisms offer a multitude of potential applications in various fields of industry. Not only of them produce compounds of industrial interest, but also they possess useful physiological properties, which can facilitate their exploitation for commercial purposes (Ulkani and Digrak, 2002). Microbial population in natural alkaline environment has attracted attention because of possible biotechnological applications of various enzymes produced by actinomycetes of alkaline water habitat.

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The present study deals with the isolation and identification of actinomycetes from Lonar lake for screening of their industrially important enzymes producing ability like amylase, lipase, caseinase, pectinase, gelatinase etc. and access the production and characterization of enzymes. However production and characterization of these industrially important enzymes have not been reported so far.

MATERIALS AND METHODS

Sample collection

Five water samples from different sites of Lonar Lake were collected in sterile bottles and brought to laboratory. The pH of all water samples were recorded by using digital pH meter (Chande and Bhat, 2014).

Isolation of actinomycetes

1ml of each water sample were inoculated in sterile Glycerol Asparagine Broth and kept for two days on rotary shaker (120 rpm) at 37^0C for enrichment. After enrichment, the actinomycetes were isolated on Glycerol Asparagine Agar and Bennett's Agar plates containing antibacterial (Streptomycin) and antifungal (Greseofulvin) agents to avoid the growth of other bacteria and fungus. After 14 days well isolated colonies were transferred on respective media slants and given them number 1 to 45.

Testing for enzymatic potential of isolates

All the 45 isolates were tested for production of different enzymes like lipase, caseinase, pectinase and gelatinase by inoculating them on respective 1% substrate and 1.5% agar containing plates. The strains which showed maximum zone of substrate utilization were selected for biochemical characterization and enzyme production.

Identification of actinomycetes

After preliminary screening for different enzyme production, the characterization of isolated colonies were done according to Kharat and Kharat, 2009, by using morphological, cover slip technique and biochemical characterization including NaCl resistance, urea, starch, tween 20 hydrolysis, temperature and pH tolerance. Gram staining were observed under oil immersion (100X) microscope, all Strains were checked for colonial appearance, spore colour and diffusible pigments formation were compared with Bergey's Manual of Systematic Bacteriology, Volume 4 (1984).

Slide and cover slip method

The simple inclined cover slip technique was used for a wide range of species. Glass cover slips were sterilized by autoclaving and placed at an angle of 45^0C in to solidified medium in a Petri dish, half the cover slip was in the medium. An inoculum was then spread along a line, where the upper surface of cover slip meets the agar. During incubation, organisms grow both on the medium and in a line across the upper surface of the cover slip. This line of growth remains attached to the cover slip, they are carefully withdrawn from the medium and examined directly under the microscope.

Production and extraction of enzymes

The growth medium containing casein and pectin as substrates were used for production of caseinase and pectinase enzymes by strain number 6 and 7 which showed maximum zone of utilization during primary screening. The substrate containing sterile production medium were inoculated with strain number 6 and 7, kept on rotary shaker for 3 days at 37^oC. The broths were centrifuged at 4500rpm for 10 minutes, the supernatant was collected and used for enzyme assay on respective substrate containing plates by using disk diffusion method. Ammonium salt precipitation, dialysis and column chromatography techniques were performed for partial purification of enzymes, the zone of utilization of crude and partially purified caseinase and pectinase enzymes were measured by disk diffusion assay.

Effect of pH, temperature and substrate concentration on enzyme

The effect of pH (4, 5, 6, 7, 8, 9, 10, 11), temperature (10, 15, 20, 25, 30, 35, 40, 45, 50^{o}C) and substrate concentration ranges between 0.4 to 3.4% on enzyme activity were optimized.

RESULTS AND DISCUSSION

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The pH of all samples were recorded between 9 to 11 which revealed that the water samples were alkaline in nature the pH range was similar to the study of Kanekar and Joshi, 2008. The 45 isolates were showed different morphological characters with hard colony appearance and different colour of diffusible pigments showed in figure 1(a), but only 10 isolates were taken for further characterization. The Gram staining of those 10 isolates showed Gram positive rods under oil immersion microscope. The cover slip method after 15 days incubation revealed the mycelium growth on cover slip which was observed under 40X microscope. The different colours of diffusible pigments were showed on Bennett's agar plates which confirm that the isolated actinomycetes strains have ability to produce coloured pigments such as melanine. Morphological and biochemical characterization, optimum pH, temperature and salt tolerance were studied of selected strains. Our results showed different morphological characters of isolated actinomycetes.
Morphological characters:

Table 1: Morphological characteristics of isolated strains.

Actinomycetes strain	Sporemass colour	Melanin Production	Consistency	Gram nature
1	White	Negative	Hard	Gram Positive
2	Black	Positive	Hard	Gram Positive
3	Grey	Negative	Hard	Gram Positive
4	Black	Positive	Hard	Gram Positive
5	Black	Negative	Hard	Gram Positive
6	Grey	Positive	Hard	Gram Positive
7	Black	Positive	Hard	Gram Positive
8	Black	Positive	Hard	Gram Positive
9	Grey	Positive	Hard	Gram Positive
10	Grey	Positive	Hard	Gram Positive

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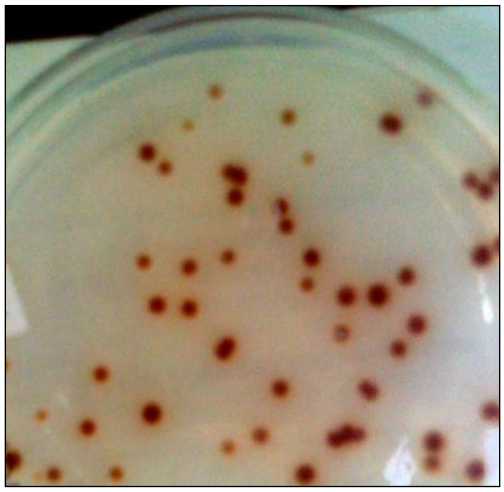
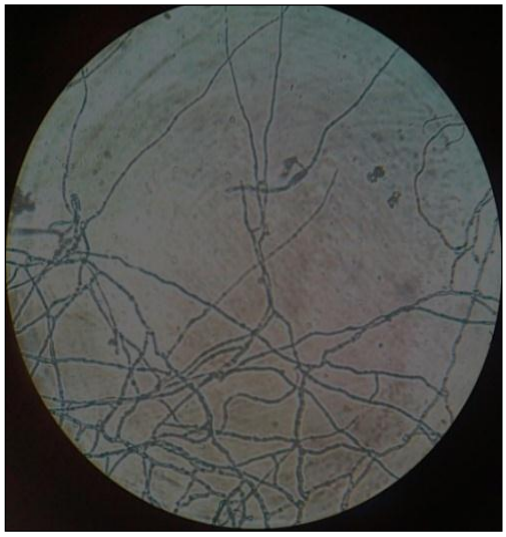


(a)

(b)



(c)

(d)

Figure 1. (a) Melanine pigments produced by actinomycetes on GAA
(b) Growth of Actinomycetes on Bennett's agar
(c) Actinomycetes 6 (Cover slip technique) showed aerial mycelium and spore chain under light microscope at 40X
(d) Actinomycetes isolate 7 (Cover slip technique) showed aerial mycelium under light microscope at 40X.

The two selected and purified isolates were identified according to description of species reported by Shirling and Gottlieb, 1966 and key of Bergey's Manual of Williams *et al.* 1984, depending on the colonial, morphological, physiological characters, microscopic examination and biochemical characterization.

Detailed analysis for identification

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Table 2: Different parameters for identification of actinomycetes.

Test	Specifications/ Set Points	Actinomycetes 6	Actinomycetes 7
Temperature	10^0c	-	+
	20^0c	-	+
	45^0c	+	+
NaCl	4%	+	+
	7%	+	+
	10%	-	+
	13%	-	+
pH	4.3	+	+
Tolerance to Phenol	0.1%	-	-
Sodium azide	1%	-	-
Potassium tellurite	1%	+	+
Tween20 hydrolysis	1%	+	+

(+) =growth (-) = no growth

Table 3: **Biochemical tests for identification.**

Actino isolates / Biochemical test	1	2	3	4	5	6	7	8	9	10
Urea hydrolysis	-	+	+	+	+	+	+	-	-	+
Starch hydrolysis	+	+	+	+	+	-	-	-	+	+
Casein hydrolysis	+	+	+	+	+	++	++	+	+	+
Pectin hydrolysis	-	+	-	-	-	++	++	-	-	-
Gelatin hydrolysis	+	+	+	-	-	+	+	-	+	+
Lipase	+	+	-	+	-	++	++	-	+	+
NaCl 7%	+	+	+	+	+	+	+	+	+	+
Lysine	+	-	+	+	+	-	+	-	+	+
Arginine	+	-	+	+	-	-	-	-	-	-
Asparagine	+	+	+	+	+	+	+	+	+	+
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-
Citrate utilization	+	-	+	+	-	+	+	-	+	+
Tween 80	-	-	-	-	-	+	-	-	-	-
Lysozyme sensitivity	+	+	+	-	-	-	-	+	+	-
Nitrate reduction	-	+	-	+	+	+	+	+	+	+
Tetracycline	-	-	-	-	-	+	-	-	-	-

(+) = growth, (-) = no growth

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Table 4: Sugar fermentation tests.

Sugar	Actinomycetes 6		Actinomycetes 7	
	Gas	Acid	Gas	Acid
Maltose	Negative	Negative	Positive	Positive
Mannitol	Positive	Positive	Positive	Positive
Fructose	Negative	Positive	Positive	positive
Sucrose	Positive	Positive	Positive	Positive
Dextrose	Negative	Negative	Positive	Positive
Glucose	Negative	Positive	Positive	Positive
Inositol	Positive	Positive	Positive	Positive
Galactose	Negative	Negative	Negative	Negative
Ribose	Negative	Negative	Negative	Negative
Dextrin	Negative	Negative	Positive	Positive
Mannose	Negative	Negative	Negative	Negative
Xylose	Negative	Negative	Negative	Negative

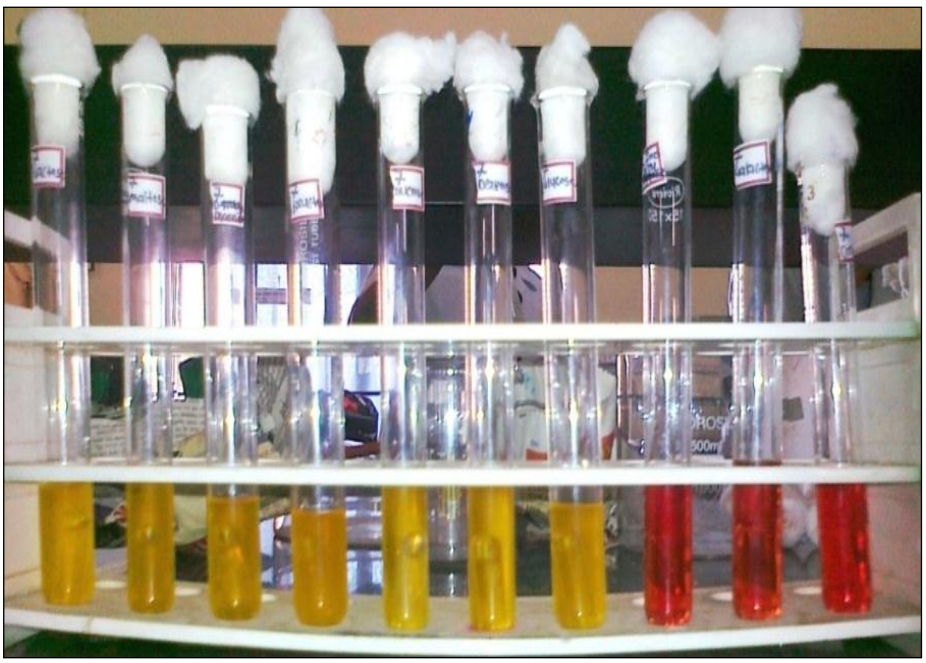

Figure 2: Sugar fermentation test.

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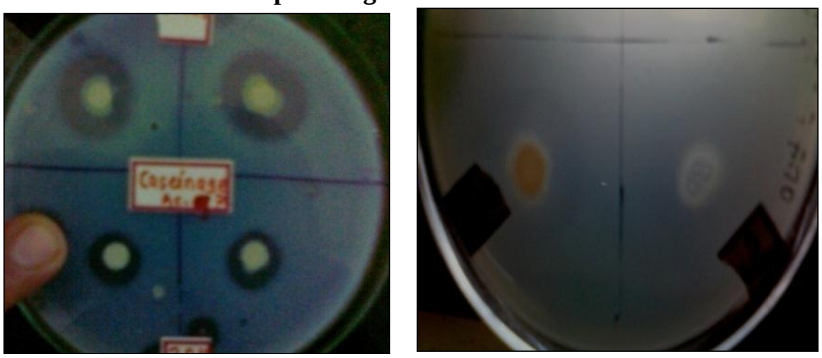
According to Bergey's manual of systematic bacteriology, volume 4, the resembling species were,

Table 5: Keys to related species.

Strain number	Species
Actinomycetes 6	*Streptoverticillium baldaccii*
Actinomycetes 7	*Streptomyces griseoruber*

Zone of substrate utilization on casein and pectin agar:



(a) **(b)**

Figure 3: (a) Casein utilization (b) Pectin utilization

Enzyme optimization: The effect of pH, temperature and substrate concentration on both enzymes revealed maximum stability at pH 9, temperature at 50^0C and substrate concentration at 1.6% were shown in table 5.

Table 5: Enzyme optimization.

Concentration of casein in %	Zone of utilization (mm)	Concentration of pectin in %	Zone of utilization (mm)
0.4	15	0.4	6
0.8	17	0.8	6
1.2	25	1.2	6
1.6	26	1.6	7
2.0	23	2.0	7
2.4	20	2.4	6
2.8	13	2.8	6
3.2	12	3.2	6

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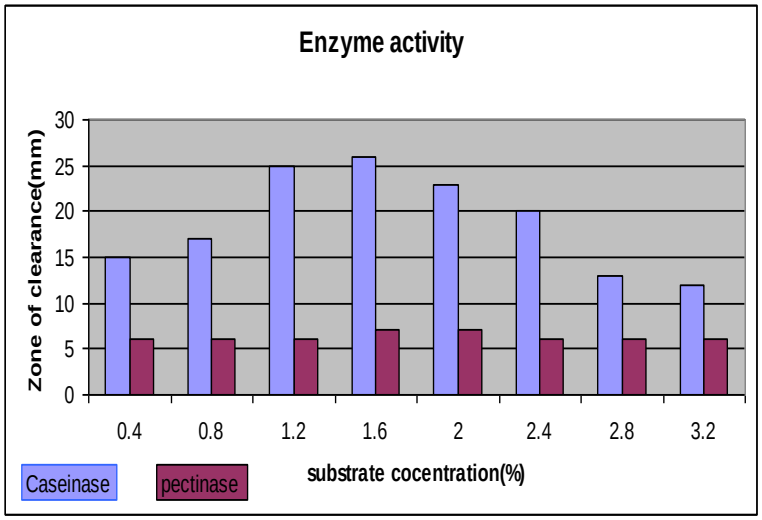

Figure 4: Effect of substrate concentration on enzyme activity.

CONCLUSION

The present work revealed that the water sample collected from Lonar lake have the habitats of actinomycetes which have great potential to produce industrially important enzymes at high alkaline condition. Out of 45 strains isolated from water sample, strain number 6 and 7 were proceeded for caseinase and pectinase production respectively and also for biochemical characterization. From the morphological, physiological and biochemical characters the strain 6 and 7 were the resembling species of *Streptoverticillium baldaccii* and *Streptomyces griseoruber*. The caseinase and pectinase enzymes revealed maximum stability at pH 9, temperature at 50^0C and substrate concentration at 1.6%. Among all the 45 isolates of actinomycetes, 13 (28%) were able to produces amylase, 7 (15%) isolates were produces lipase, 19 (42%) could produces caseinase, 24 (53%) produces pectinase and 11 (24%) strains were produces gelatinase enzyme. From our results it was concluded that the actinomycetes isolated from water sample of Lonar lake have abilities to produce different biotechnologically important enzymes. Identification of the isolated actinomycetes by 16S rRNA sequencing is the further milestones of this study.

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REFERENCES

Blunt J. and Prinsep M. (2006). Marine natural products. *Nat. Prod. Rep.* 23(4): 26-78.

Chande A. and Bhat M. (2014). Isolation and characterization of L-asparginase producing isolates from Lonar lake, Buldhana District, MS., India. *Res. J. Recent Sci*. 3(1): 38-41.

Goodfellow M. (1971). Numerical taxonomy of some *Nocardia* form bacteria. *J. Gen. Microbiol.* 69(1): 33-80.

Gopalkrishnan C. (2000). The geological horizon. The Hindu, dated: 2000/ 24/ 4.

Kanekar P. and Joshi A. (2008). Alkaline Lonar Lake: A treasure of alkaliphilic and halophilic bacteria. Proceedings of Taal The 12th World Lake Conference: 1765-1774.

Kharat K. and Kharat A. (2009). Antimicrobial and cytotoxic activity of *Streptomyces* sp. from Lonar Lake. *Afr. J. Biotechnol*. 8(23): 6645-6648.

Rizvi R. and Kamble L. (2014). A report on extracellular enzyme production potential of actinomycetes isolated from sediments of river Godavari, India. *Biosci. Discov.* 5(1): 121-122.

Shirling E. and Gottlib D. (1966). Method for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16(2): 313-340.

Ulkani Z. and Digrak M. (2002). Alkaliphilic microorganisms and habitats. *Turk. J. Biol.* 26(2): 181-191.

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Williams S. and Sharpe E. (1984). Bergey's Manual of Systematic Bacteriology: Volume 4, Williams and Wilkins Baltimore, Hong Kong, London, Sydney.

Zahabi I. and Issazadeh K. (2014). Antimicrobial activity of *Streptomyces* spp. isolated from marine sidements of iran against tested microorganisms. *Indian J. Fundam. Appl. Life Sci.* 4(4): 213-217.

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