

Isolation and Characterization of Amylase Inhibitor from Alkalophilic Bacteria Isolated from Lonar Crater and its Insecticidal Protein Producing Ability

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Five different isolates of alkalophilic bacteria were obtained from water and sludge sample of Lonar lake, in Buldhana district of Maharashtra. These isolates were identified as Cholesterol oxidase, Protease inhibitor, Amylase inhibitor, Alkaline Protease, Chitinase by 16S rDNA sequencing. In this paper, the characters of protease inhibitor produced by the five most potent isolates were studied for its insecticidal potential. Among five isolates, three isolates (isolate A, B, E) amylase inhibitor producing ability. Out of that isolate A i.e. *Bacillus thuringiensis* serovar *finitimus* showed the highest (0.031 Unit mg protein⁻¹) amylase inhibitory activity. The activity of Amylase inhibitor was almost constant over the range of pH. The optimum pH for the amylase inhibitor obtained from all the isolates was found to be pH 9. Specific activity of amylase inhibitor decreases at extreme pH (pH 12). The proteins showed stability upto 1hr when incubated on pH 9 to pH 11. The stability of enzyme decreases at extreme pH 12. AI isolated for all the alkalophile isolates were optimally active at 40°C and specific activity decreases at extreme temperature range 60°C. The thermal stability revealed that the enzyme was stable on 30°C to 40°C for upto 150 min. However, the enzyme activity declined drastically at temperature higher than 50°C.

Key words: Amylase inhibitor, Alkalophilic bacteria, Lonar Crater.

Amylase are hydrolytic enzyme that are widespread in nature being found in animal, microorganism and plants. (Octavio *et al.*, 2008). α -Amylase inhibitor (AI) inhibits the α -amylase, an important digestive enzyme. Thereby, hampered the carbohydrate digestion and leading to the death of insect. Femiola *et al.*, 2011 showed that the natural defence system of crop plants

to avoid damage may be improved through the use of transgenic technology. Amylase inhibitor that can inhibit the protein preventing the growth and development of insect larvae and prevent the further damage of crop plants. For this reason the amylase inhibitor is used for pest control and it is potential alternative for biocontrol agent.

Kluh *et al.* (2004) studied inhibitory specificity of a AI-1 with a panel of the digestive α amylases from 30 species of insects, mites, nematode and fungal phytopathogens with a focus on agricultural pests and important model species.

Exogenous chemical means to counteract Lepidopteran attack have become less feasible, mainly due to the development of pesticides resistance in insect and inherited possible

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environmental hazard. Chemical insecticides are widely used in agricultural pest control, but they impose serious negative effect on environment and human health. As a consequences alternative method such as biological control using entomopathogenic bacteria and their enzymes/ proteins having insecticidal potential needs to be explore further as ecofriendly pest control measure. Protein with insecticidal action need to be ingested by the insect to be active, since the insect's cuticle is impermeable to hydrophilic macromolecules such as proteins. As a natural consequence of this, the mechanism of action of most insecticidal proteins involves a step in which the protein interacts with components of the digestive tract of the insect.

Lonar lake ecosystem has reported to contain rich bacterial diversity. The microorganism, alkalophilic bacteria, in this environment would therefore be unique. Lonar crater is a classic beautiful bowl shaped depression in the basaltic flows of the Deccan traps in Southern India believed to be formed as a result of high velocity impact of huge meteor of extra terrestrial origin. It is situated in Buldhana district of Maharashtra. Rightly rated as the third largest and oldest meteoritic crater is about 52000 year old crater size 1800-2000m in diameter, height is 20-30m, depth 150m and placid water spread areas 77.69 ha. The water of this lake are characterised by very high alkaline pH of 8 to 10.5. (Gopalkrishna, 2000)

As the nature of Lonar lake is alkaline most of the strains were alkali tolerant and only two strains were obligate alkalophilic bacteria. These bacteria were produce biotechnologically important enzymes at alkaline pH. However production and characterization of insecticidal enzymes/protein have not be reported so far.

In the present paper, we report that amylase inhibitor obtained from Alkalophiles of Lonar lake were studied for their potential to express different insecticidal enzymes/proteins. Insecticidal enzymes/proteins are those which impede the important physiological process of insect and thereby inhibit the growth of the insect. Alkalophiles can be the good source of the insecticidal enzymes/ proteins and will be more effective in alkaline gut condition of the insect.

These insecticidal enzymes/protein thought to act effectively in insect gut (alkaline condition) because of their alkaline stability.

Considering this, present investigation is planned with the objective to isolate alkalophilic bacteria and explore their insecticidal protein producing ability.

MATERIALS

Soluble, starch, Glucose, Peptone, NaCl, α -Amylase, Soluble starch 1% DNSA

Methods

Isolation of bacterial strains from alkalophiles of Lonar Lake

Five strains of alkalophilic bacteria producing amylase inhibitors were isolated from water and sediment sample collected from Lonar lake, India. Purified culture were obtained on leuria broth by the single colony plating technique and five different alkalophiles were obtained.

Production of amylase inhibitors from alkalophiles of Lonar Lake

Alkalophiles were subjected for production of Amylase inhibitor by using the media given by Murao *et al.*, 1976 and its inhibitory activity assay were performed according to the protocol given by Moreno *et al.*, 1989.

Optimum pH and pH stability studies

The optimum pH was examined only by changing the pH of the Tris-HCl buffer used during assay was changed. Different pH range viz. 8, 9, 10, 11, 12 was used during assay. To measure the pH stability of the insecticidal enzymes isolated from alkalophiles a solution of enzymes (50 μ l/ml) was diluted with an equal volume of buffer with different pH range (pH 8-12). After 1 hr incubation in each buffer at 37°C, the residual inhibitory activity of all enzymes was measured.

Optimum temperature and temperature stability studies

The optimum pH and optimum temperature were determined by incubating the insecticidal enzymes at different temperature range viz 30°C, 40 °C, 50°C, 60°C. during enzyme assay. The assay was carried out as described above only the incubation temperature was changed from 30°C to 60°C. To measure the heat stability of the insecticidal enzyme isolated from alkalophiles was determined by incubation of the insecticidal enzyme (50 μ l enzyme extract and 60 μ l 0.1M Tris-HCl pH 9.0) at various temperature (30°C-60°C) for 1h. After treatment the aliquots were cooled

on ice and inhibitory assay was carried out for determination of residual activity of insecticidal enzymes from alkalophiles.

Insect bioassay against *Plutella xylostella* to study the insecticidal potential

The larvae and pupae of *P. xylostella* were collected from cabbage and Cauliflower field from outskirts of Akola. They were reared in the laboratory on the mustard seedlings up to F₄ generations for establishing homologous laboratory population. The rearing procedure described by Lu and Sun (1984) was followed to maintain the test culture of *P. xylostella*.

Bioassay of selected native isolates of against *Plutella xylostella*

The bioassay was carried out by cabbage

leaf disc dip method in triplicate as described by Tabashnik *et al.* (1987). Mortality in *Plutella xylostella* larvae was recorded and cumulative mortality after 72 hrs. was calculated.

RESULTS AND DISCUSSION

Different alkalophiles obtained from Lonar lake were studied for its amylase inhibitor producing potential. Among five isolates (isolate A, B, and E) has found amylase inhibitor producing ability and results were shown in detail in table 1

Imada and Simidu (1988) obtained a marine actinomycete which produce an extracellular α -amylase inhibitor which was isolated by starch

Table 1. Amylase inhibitor activity of alkalophiles obtained from Lonar lake

S. No.	Isolate name	Protein concentration ($\mu\text{g ul of broth}^{-1}$)	Inhibitory activity of AI (Umg protein^{-1})
1	<i>A-Bacillus thuringiensis</i> serovar <i>finitimus</i>	0.3 ± 0.001	0.031 ± 0.001
2	<i>B-Bacillus licheniformis</i>	0.3 ± 0.001	0.019 ± 0.003
3	<i>C-Bacillus Cereus</i>	Not detected	Not detected
4	<i>D-Halomonas Campisalis</i>	Not detected	Not detected
5	<i>E-Bacillus pseudofirmus</i>	0.6 ± 0.002	0.022 ± 0.005

Table 2. Optimum pH for Protease Inhibitor obtained from Lonar alkalophiles

pH range	<i>A-Bacillus thuringiensis</i> serovar <i>finitimus</i> Inhibitory activity of AI \pm S.E (U mg protein^{-1})	<i>B-Bacillus licheniformis</i>	<i>E-Bacillus pseudofirmus</i>
8	0.22 ± 0.01	0.24 ± 0.02	0.27 ± 0.02
9	0.29 ± 0.02	0.28 ± 0.04	0.29 ± 0.04
10	0.25 ± 0.03	0.24 ± 0.05	0.26 ± 0.03
11	0.23 ± 0.02	0.22 ± 0.01	0.23 ± 0.01
12	0.17 ± 0.01	0.15 ± 0.01	0.11 ± 0.04

Table 3. Optimum pH for Protease Inhibitor obtained from Lonar alkalophiles

Temp $^{\circ}\text{C}$	<i>A-Bacillus thuringiensis</i> serovar <i>finitimus</i> Inhibitory activity of AI \pm S.E (U mg protein^{-1})	<i>B-Bacillus licheniformis</i>	<i>E-Bacillus pseudofirmus</i>
30	0.26 ± 0.001	0.25 ± 0.001	0.24 ± 0.002
40	0.28 ± 0.004	0.29 ± 0.003	0.30 ± 0.003
50	0.20 ± 0.001	0.21 ± 0.004	0.19 ± 0.004
60	0.18 ± 0.003	0.15 ± 0.001	0.14 ± 0.006

agar plate method. They isolate 5,158 isolates from various sea areas. Only one strain which was isolated from sediment of meritic sea area was found to produce an amylase inhibitor.

The activity of Amylase inhibitor was almost constant at high pH. The optimum pH for the amylase inhibitor obtained from all the isolates was found to be pH 9. and the data pertaining to this were detailed in table 2. Similarly the protein showed stability 1hr at at pH 9 to pH 11

when incubated for 1 hr. The stability of enzyme decreases at extreme pH 12 .

Amylase inhibitor obtained from the alkalophile were optimally active at 40°C. The specific activity of these enzyme decreases at extreme temperature range 60°C. as shown in Table 2. Similarly, the thermal stability revealed that the enzyme was stable on 30°C to 40°C for upto 150 min. the data to this were depicted in fig 1.

Shanmughapriya *et al.*, (2009) studied an

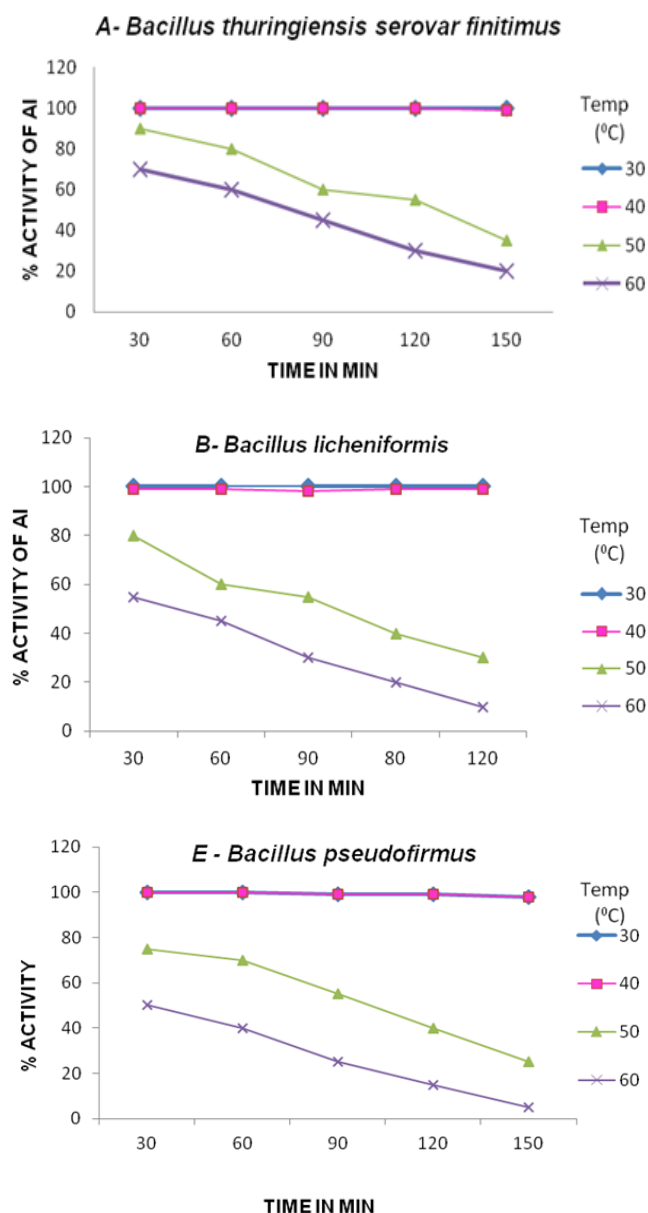


Fig. 1. Temperature stability for Amylase obtained from Lonar alkalophiles

endosymbiont *Halobacterium Salinarum* (strain MDD047), which produces high amylase inhibitor. In their study they showed that, AI possesses stability at 40°C, whereas, enzyme activity decreased at above 50°C. Based on this finding, they show that enzyme was characterized as relatively heat sensitive. But in our present finding, AI isolated from *Bacillus* spp (Lonar alkalophiles) showed the higher temperature stability than the AI reported by him which clearly indicates the heat resistance nature of AI.

Insecticidal enzymes isolated from alkalophiles obtained from Lonar lake were studied for their insecticidal potential by performing insect bioassay against third instar larvae of *Plutella xylostella* by exposing them to 1mg/ml protein concentration of each enzyme/protein. Effective insecticidal enzymes/protein can be identified on the basis of lower toxicity or higher mortality. Each bioassay was carried out in three replication containing 12 larvae. Cumulative mortality after 72 were recorded

However Highest mortality (66%) was obtained from isolate *E-Bacillus pseudofirmus* when larvae were exposed to PI obtained from isolate E, Lowest mortality was obtained from isolate B *Bacillus licheniformis* (33%) and The mortality figures are only indicative figures suggesting the insecticidal potential of these insecticidal enzymes/proteins.

Mortality obtained in the insect bioassay suggests that these enzymes/proteins obtained from the Lonar alkalophiles can act as good candidate biomolecule for developing the biopesticides or transgenic insect resistance plant. High pH temperature and stability makes these molecule more interesting to work upon in future.

REFERENCE

1. Femi-Ola, T.O. and B.M. Olowe, Characterization of Alpha amylase from *Bacillus subtilis* BS5. Isolated from Amitermen Evencifer Silvestri. *Res. J.Microbial*. 2011; **6**(2):140-146.
2. Chiaki Imada and Usio Simidu, Isolation and characterisation of an α -amylase inhibitor producing actinomycetes from marine environment, 1988; **54**: 10
3. Gopalkrishnan, C.V., The geological horizon. The Hindu, dt. 2000; 24
4. Ivan Klueh, Nartin Horn, Michael Mares, Inhibitory specificity and insecticidal selectivity of α -amylase inhibitor from *Phaseolus vulgaris*. *Physicochemistry*, 2004; 31-39. www.sciencedirect.com.
5. Jaquin Moreno, Teresa Altabeua and Marteen, Characterization of α -amylase inhibitor a Lectin like protein in seeds of *Phaseolus vulgaris* Plant physiology 1989; 703-709. 0032-0889 / 90 / 92 / 0703 /
6. Lu and Sun., Rearing diamond backmoth (Lepidoptera; Yponomeutidae) on rape seedling by modification of Koshihara and Yamada method. *J Econ Entomology*, 1984; **77**(6): 1608-1609
7. Sawao Murao, Kunio Ohyma and Eci Ogura, Isolation of Amylase inhibitor producing microorganism *Agric. Biol. Chem.* 1976; **41**(6): 919-924
8. Narimasa Satio., A thermophilic extracellular α -amylase from *Bacillus licheniformis*. *Archives of Biochem and Biophysics*. 1972; **155** (2): pp 290-298
9. Octavio, L.F., J.R. Daniel, R.M. Francislete, C. Bloch, J.R. Carlos and F.G. Maria, Activity of wheat α -amylase inhibitor towards bruchid α -amylase and structural explanation of observed specificities. *Eurp. J. Biochem.* 2008; **26**(1): 2166-2173
10. S. Shanmughapriya, G. Seghal Kiran, Joseph Selvin, R. Gandhimathi, T. Bastin Baskar, Aseer Manilal, and S. Sujith., Optimization, Production, and Partial Characterization of an Alkalophilic Amylase Produced by Sponge Associated Marine Bacterium *Halobacterium salinarum* MMD047 *Biotechnology and Bioprocess Engineering*, 2009; **14**: 67-75
11. Tabashnik, B.E., Cushing, N.L. and Johnson, M.W., Diamond black moth (Lepidoptera : Pyralidae) resistance to insecticide in Hawaii: intra-Island Variation and Cross Resistance. *J. Econ. Entomol.* 1987; **80**:1091-1099.