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# Isolation and Characterisation of Protease Inhibitors From Alkalophilic Bacteria Isolated From Lonar Crater and Its Insecticidal Protein Producing Ability

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**ABSTRACT**

Proteinase inhibitors (PIs) are anti-metabolic proteins which interfere with digestive process of insects. It is one of the important defence strategies existing in plants against predators. 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 the five isolates, four isolates (i.e A, B, D and E) showed the activity of protease inhibitor. Out of that, isolate E i.e *Bacillus pseudofirmus* showed highest (12.2 U mg protein$^{-1}$) protease inhibitory activity. All the enzyme exhibited maximum activity in the neutral to alkaline range of pH from (8 to 10) with wide stability range from pH 9 to 10.the temperature optima for all the enzymes were slightly highest between 30 to 50$^0$C ,with a stability range from 30 to 40$^0$C. The enzyme PI retained above 95% thermal stability after incubation for 90 min.

**Keywords ;-** Protease inhibitor PI , alkalophilic bacteria , Lonar crater

## I. INTRODUCTION

Protease inhibitors are anti-metabolic proteins which interfere with the defensive strategies existing in plants against predators. The use of plants derived PI genes for developing insect resistant transgenic plants expressing PIs have been created and these plants have shown enhanced resistance against insect pests. Protease inhibitor (PI) was obtained from variety of sources (virus, bacteria, fungi, plants and insect) have toxicity towards insects. Some of these insecticidal protease inhibitor evolved as herbivore resistance factors and play roles in antibiosis mechanism of plant against insect development or digestion. The sites of protease toxic inhibitor activity range from insect midgut to the homocoel (body cavity) to the cuticle **(Harrison and Bonning 2010).** The defensive capacities of plants protease inhibitors rely on inhibition of protease present in insect guts or secreted by microorganism causing reduction in the availability of amino acids necessary for their growth and development. **(De Leo et al 2002).**

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

Importance protease as inhibitor in insect control is well known and probably this is the most studied insecticidal gene apart from insecticidal *Bt* genes. Schuler et al., 1998 showed that, PI as potent toxic protein against predators and pathogens. PIs are protein that occurs naturally in wide range of plants as a part their defense system and provides immunity to plant against insect and pathogen

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 meteror of extra terrestrial origin. It is situated in Buldhana district of Maharashtra. Rightly rated as the third largest and oldest meterotic 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 protease 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.

**II MATERIAL ;-** Soyameal Leuria broth Trypsin ,1Mm HCl ,0.1M Tris-Hcl buffer (Ph 8.0) ,0.1M $Cacl_2$ , Azocasein 2% Acetic Acid (5%),1 N NaOH

**III METHOD**

**Isolation of bacterial strains from alkalophiles of Lonar lake**

Five strains of alkalophilic bacteria producing protease 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 protease inhibitors from alkalophiles of Lonar lake**

Alkalophiles were subjected for production of Protease inhibitors by using media given by Dash and Rao 2001.The protease inhibitors production was estimated by the protease inhibitors assay by the protocol given by Ceelia et al. 2002.

**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 µl/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 decribed 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 µl enzyme extract and 60µl 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 $F_4$ 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.

**IV RESULT AND DISCUSSION**

Different alkalophhiles obtained from Lonar lake were studied for its protease inhibitor producing potential. Different alkalophiles were grown in specific fermentation media of protease inhibitor and its activity was determined as per the protocol given in materials and methods. Among the five isolates, four isolates (i.e A, B, D and E) showed the activity of protease inhibitor. out of that *Bacillus pseudofirmus* showed the highest (0.213 Unit mg $protein^{-1}$) Proetease inhibitory activity as shown in **Table 1**

Apart from use of PI solely as an (**Macintosh et al. 1990)** showed that several serine protease inhibitor enhanced the insecticidal activity of the insect control proteins from *Bacillus thuriengensis* against their target insect tobacco budworm and other Lepidopteran respectively. Antimetabolic properties of soybean proteinase inhibitor (SPI) on sugarcane borer. SPI retarded the growth rate development of larvae,

reduction in population in this study. SPI was to be potent for protecting sugarcane

The PI isolated from Lonar alkalophiles, isolates was almost constant over the range of pH. The data pertaining to optimum pH of protease inhibitor, obtained from Lonar alkalophiles, are detailed in **Table 2** and The PI obtained from Lonar alkalophiles showed remarkable stabiltiy at pH 9 and 10. The PI showed decrease specific activity at pH12.

The PI obtained from Lonar alkalophiles showed highest activity at wide range of temperature (30 to 50$^0$C). The PI activity was decreased at 60$^0$C. The details regarding the optimum temperature are given in **Table 3** The temperature stability of PI indicates that, the inhibitor exhibited varying degree Among the four inhibitors, three inhibitor (*Bacillus thuringiensis* serovar *finitimus*,*Bacillus licheniformis*,*Bacillus pseudofirmus*), was most stable at 30$^0$C to 50$^0$C. They showed above 95% thermal stability when incubated for 90 min. However, PI obtained from *Halomonas Campisalis* showed stability upto 40$^0$C, after that activity was found to reduce. **Fig 1**

**(Siddalingeshwara et al. 2010)** studied the Screening and characterization of PI from *Bacillus* spp. isolated from different regions of Bangalore. and observed that Optimum pH for the PI obtained from *Bacillus* was found to be 10, activity was found to be decreased at neutral pH 4. The optimum temperature was observed to be at 37$^0$C to 65$^0$C and activity reduced at 65$^0$C. The PI showed good stability at temperature 37$^0$C.

**(Pandhare et al 2002)** studied the differential stabilities of protease inhibitor obtained from actinomycetes. They isolated three strains producing protease inhibitors i.e API -1,API -2,API -3.In their study they showed that API -1 was stable at 60$^0$C,but API -2 showed stability at 45$^0$C,whereas API-3 exhibited least thermal stability with complete loss of activity at 37$^0$C.

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 larvaes. Cumulative mortality after 72 were recorded.

However Highest mortality (58%) was obtained when larvae were exposed to PI obtained from isolate D, Lowest mortality was obtained from isolate E (42%) 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

## V CONCLUSION

Lonar Crater is only alkaline lake present in India. Its microbial ecology and diversity has already been reported .But the study on insecticidal enzymes/protein harbour by the Lonar alkalophiles have not been studied so far. So considering the promising niche of important microorganism and thereby insecticidal enzymes/proteins, present investigation was carried out. *Bacillus thuriengiensis* serovar finitimus ,*Bacillus licheniformis*,*Bacillus cereus* , *Halomonas campisalis, Bacillus pseudofirmus* have been isolated from the Lonar lake and it was confirmed by 16S rRNA sequencing..

Protease Inhibitor insecticidal enzymes/proteins have been found to produced by these Lonar alkalophiles.Insecticidal enzymes/proteins isolated from Lonar alkalophiles are found to be highly stable at alkaline pH and higher temperature. Insecticidal enzymes/protein obtained from Lonar alkalophiles showed the significant toxicity against Lepidopteran pest.

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**Table 1.** Protease inhibitor activity of alkalophiles obtained from Lonar lake

| Sr. No. | Isolate Name | Protein concentration (µg ul of broth$^{-1}$) | Inhibitor activity PI (U mg protein$^{1}$) |
|---|---|---|---|
| 1 | A-.*Bacillus thuringiensis* serovar *finitimus* | 0.181 ± 0.001 | 10.4 ± 0. 2 |
| 2 | B-*Bacillus licheniformis* | 0.187± 0.003 | 10.8 ± 0. 2 |
| 3 | C- *Bacillus Cereus* | Not Detected | Not Detected |
| 4 | D-*Halomonas campisalis* | 0.189 ± 0.003 | 10.9 ± 0.4 |
| 5 | E-*Bacillus pseudofirmus* | 0.213 ± 0.005 | 12.2 ± 0.1 |

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

| pH | A- *Bacillus thuringiensis* serovar *finitimus* | B - *Bacillus licheniformis* | D- *Halomonas campisalis* | E –*Bacillus pseudofirmus* |
|---|---|---|---|---|
| | Inhibitory activity of PI ± standard error (U mg protein$^{-1}$) | | | |
| 8 | 0.161 ±0.001 | 0.158 ±0.004 | 0.162 ±0.004 | 0.208 ±0.003 |
| 9 | 0.179 ± 0.003 | 0.181 ± 0.004 | 0.186± 0.003 | 0.209 ± 0.002 |
| 10 | 0.181 ± 0.004 | 0.187 ± 0.001 | 0.191± 0.004 | 0.212 ± 0.004 |
| 11 | 0.165 + 0.005 | 0.164 + 0.002 | 0.168+ 0.001 | 0.200 + 0.003 |
| 12 | 0.159 ± 0.002 | 0.154 ± 0.002 | 0.159± 0.003 | 0.198 ± 0.001 |

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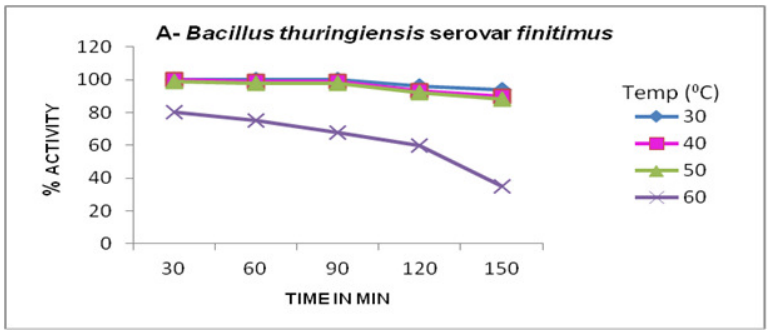
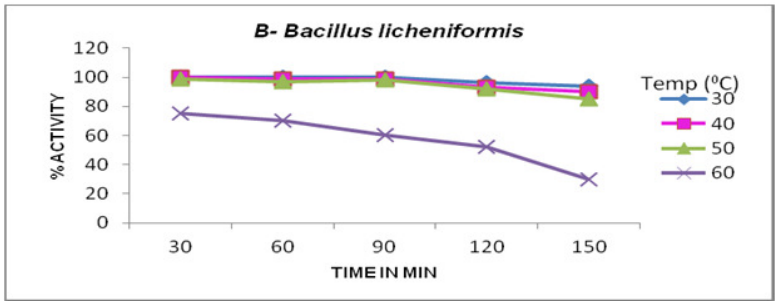
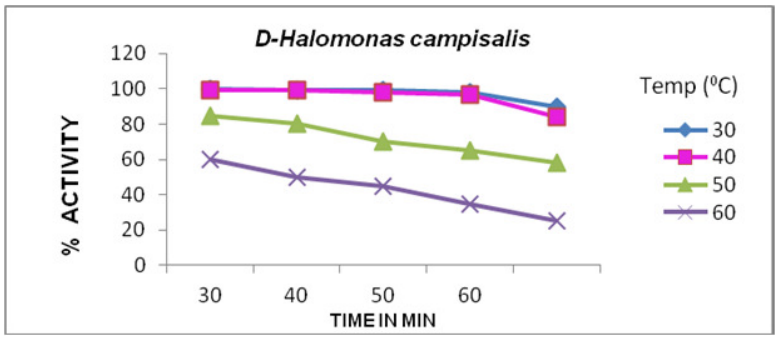
**Table 3** Optimum Temperature for Protease Inhibitor obtained from Lonar alkalophiles

| Temperature (ºC) | A-*Bacillus thuringiensis* serovar *finitimus* | B-*Bacillus licheniformis* | D-*Halomonas campisalis* | E-*Bacillus pseudofirmus* |
|---|---|---|---|---|
| | Inhibitory activity of PI± standard error (U mg protein$^{-1}$) | | | |
| 30 | 0.180 ±0.001 | 0.186 ±0.001 | 0.188 ±0.001 | 0.212 ±0.001 |
| 40 | 0.179 ±0.002 | 0.185 ±0.002 | 0.187 ±0.002 | 0.212 ±0.001 |
| 50 | 0.180 ±0.001 | 0.184 ±0.003 | 0.178 ±0.011 | 0.210 ±0.003 |
| 60 | 0.142 ±0.039 | 0.156 ±0.031 | 0.160 ±0.029 | 0.18 ±0.033 |



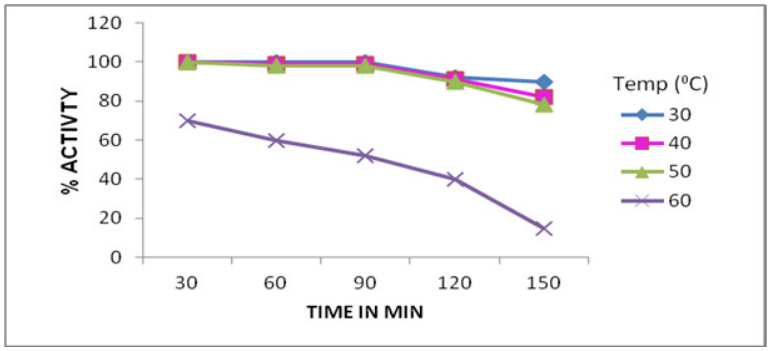



**Figure 1 ;** Temperature stability for protease inhibitor obtained from Lonar alkalophiles.

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