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Isolation and Characterization of Cholesterol Oxidase 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 Cholesterol oxidase produced by five most potent isolate were studied for its insecticidal potential. , Among the five isolates, four isolates (i.e A, B, C and D) were found to produce Cholesterol oxidase. Out of that, *Bacillus thuringiensis* serovar *finitimus* showed the highest (0.835 U mg protein^{-1}) COX inhibitory activity. The activity of COX was almost constant at wide range of pH (8-11).The optimum pH for the COX obtained from all the isolate was found to be at pH 10.The protein showed stability upto 1hr when incubated at pH 9 to 10 and stability of enzymes decrease at extreme pH-12. COX obtained from all the alkalophile isolates were optimally active at 50^{o}C.The thermal stability revealed that enzyme was stable at 30^{0}C to 50 ^{o}C for upto 90 min. However ,the enzyme activity declined drastically at temperature higher than 50^{o}C.

Key words: Cholesterol oxidase (COX), Lonar crater, alkaophilic bacteria, *Bacillus thuringiensis*.

Cholesterol oxidase has been reported from variety of microorganism mostly from actinomycetes.It was found that COX was produced by soil mycobacterium (Stadtman *et al* 1954).Cholesterol Oxidase shows insecticidal activity that is vital part of pest control strategies.

The main role of Cholesterol Oxidase is the degradation of dietary cholesterol in insect gut. The enzyme is involved in the lysis of the midgut epithelial cells of the larvae.Cholesterol or the related sterol at the membrane of the boll weevill midgut epithelial seemed to be accessible to the enzyme and is oxidized by cholesterol oxidase, causing lysis of the midgut epithelial cells resulting in larval death Cholesterol oxidase shows insecticidal activity that in a vital part of pest control strategies employing transgenic crops (Doukyu *et al*., 2009).

Noriyuki Doukyu (2009) studied the enzyme cholesterol oxidase has potential application as a biocatalyst which can be used as an insecticide. Cox has been reported from variety of microorganism mostly from actinomycetes.

Exogeneous chemical means to counteract lepidopteran attack have become less feasible, mainly due to the development of pesticides

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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 as a eco friendly pest control methods.

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 volecity 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 cholesterol oxidase 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 acteria and explore their insecticidal protein producing ability.

MATERIALS

Media composition(g / L^{-1}) NH_4NO_3, (17), K_2HPO_4(0.2), $MgSO_4H_2O$ (0.25), $FeSO_4$ (0.001), NaCl (0.005), Cholesterol (2), Tween 20 (0.5 ml). Cholesterol substrate solution 500mM Sodium Acetate buffer Ethyl Alcohol

METHODS

Isolation of bacterial strains from alkalophiles of Lonar

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 cholesterol oxidase from alkalophiles of Lonar lake

Alkalophiles were subjected for production of Cholesterol Oxidase by using media given by Yazdi et.al 2001. To isolate cholesterol oxidase 1 g or 1 ml of bacterial isolates was suspended in 100 ml of distilled water by shaking the suspension for 30 min. 0.1 ml of the supernatant was inoculated on a plate containing 15 ml of medium with a cholesterol as sole carbon source.The media was incubated at 30°C for 7-12 days. and enzyme assay was carried out by protocol developed by Richmond,in 1973 to calculate the Cholesterol oxidase activity.

Optimum pH and pH stability studies

The optimum pH of COX was studied 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 COX was measured.

Optimum temperature and temperature stability studies

The optimum temperature and temperature stability was studied by incubating the COX 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. and residual activity of COX was determine.

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

Rearing of Plutella xylostella (DBM)

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 COX 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 48 hrs. was calculated.

RESULT AND DISCUSSION

Different alkalophhiles obtained from Lonar lake was studied for its Cholesterol oxidase producing potential. Different alkalophiles were grown in specific Cholesterol oxidase producing media of and its activity was determined as per the protocol given in materials and methods. Among the five isolates, four isolates (i.e A,B,C and D) was found to produce Cholesterol oxidase. Out of that, *Bacillus thuringiensis* serovar *finitimus* showed the highest (0.835 Unit mg protein^{-1}) COX inhibitory activity.as showm in table 1.

Purcell.*et al.* (1993) discovered a highly efficacious protein that killed boll weevil (*Anthonomus grandis* Botieman). Larvae was discovered in Streptomyces culture. The protein was identified as cholesterol oxidase EC (1.1.3.6). Purified cholesterol oxidase was found to be active against boll weevil larva and the results were Comparable with bioactivity of *Bacillus thurengeinsis* protein against other insects pest. Although this protein has shown toxicity to some

Table 1. Cholesterol oxidase (COX) activity of alkalophiles obtained from Lonar lake

S. No.	Isolate name	Protein concentration (µg ul of broth^{-1})	Specific activity of COX (U mg protein^{-1})
1	A- *Bacillus thuringiensis* serovar *finitimus*	0.163± 0.001	0.835± 0.001
2.	B - *Bacillus licheniformis*	0.176± 0.003	0.434± 0.003
3.	C -*Bacillus cereus*	0.177± 0.002	0.295± 0.005
4.	D-*Halomonas campisalis*	0.165± 0.004	0.209± 0.004
5	E-*Bacillus pseudofirmus*	Not detected	Not detected

Table 2. Optimum pH for Cholesterol Oxidase obtained from Lonar alkalophiles

pH	A *Bacillus thuringiensis* serovar *finitimus*	B *Bacillus licheniformis*	C *Bacillus cereus*	D *Halomonas campisalis*
	Specific activity of COX ±S.E. (U mg protein^{-1})			
8	0.163 ± 0.001	0.170 ± 0.003	0.169 ± 0.001	0.175 ± 0.002
9	0.162 ± 0.004	0.172 ± 0.002	0.174 ± 0.003	0.177 ± 0.001
10	0.163 ± 0.001	0.174 ± 0.001	0.176 ± 0.004	0.178 ± 0.004
11	0.161± 0.004	0.171 ± 0.003	0.173 ± 0.003	0.169 ± 0.002
12	0.142 ± 0.003	0.153 ± 0.002	0.155 ± 0.002	0.146 ± 0.003

Specific activity U mg protein^{-1} = COX activity retained after incubation at various range of pH

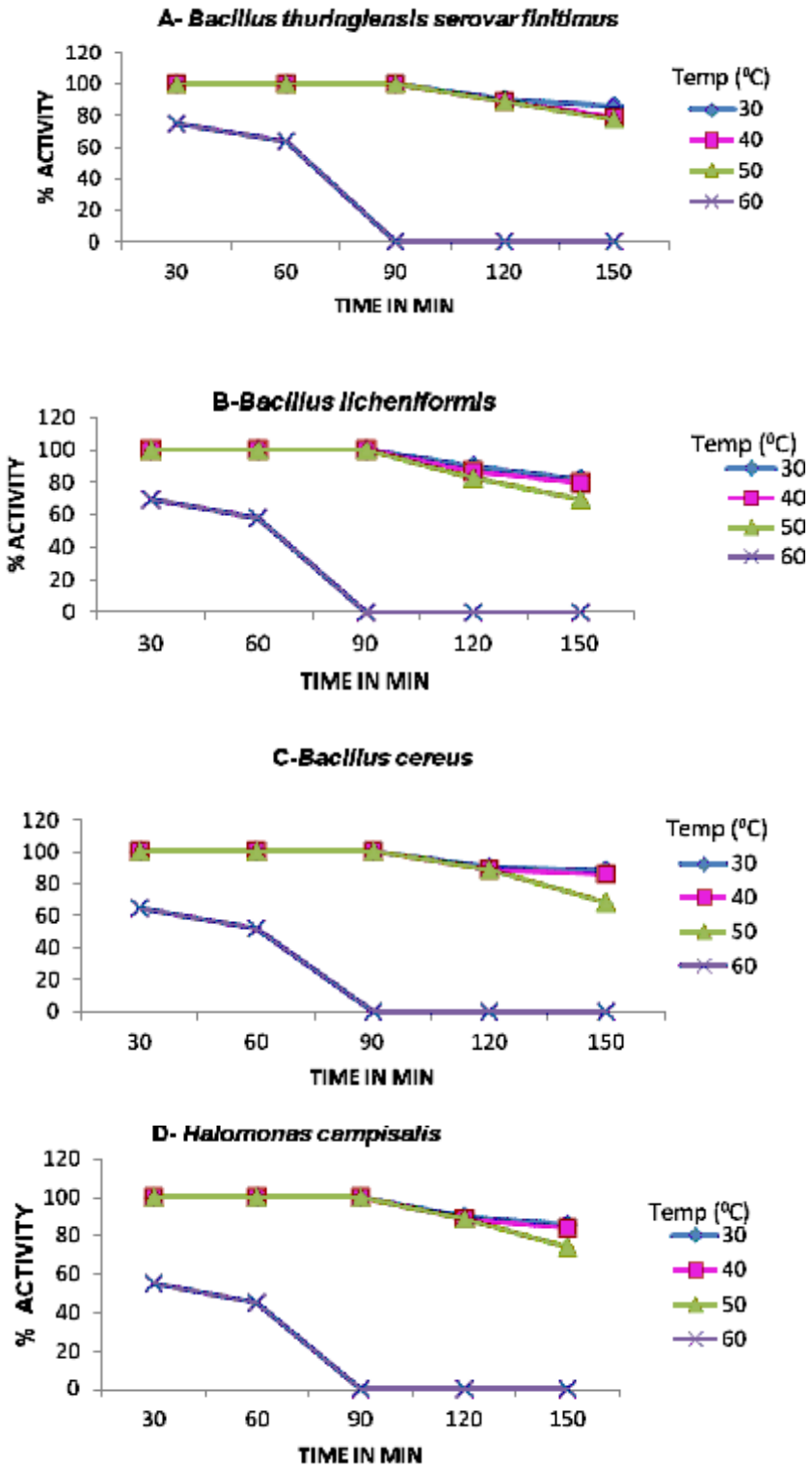

Fig. 1. Temperature stability of Cholesterol oxidase obtaine from Lonar alkalophiles

insects,its potential use to control pests in crops has not been demonstrated

The COX isolated from Lonar alkalopiles isolate A, B, C and D of was found to be highly active over the wide range of pH (pH 8-11). It was furthur found that COX activity decreased at extreme pH (pH12) as shown in Table 2 and Figure1.COX showed pH stability at pH 9-10.However stability of the enzyme was found to be decreased at pH 12

Cholesterol Oxidase obtained from Lonar alkalophiles showed high activity at 30^0C to 60^0C.COX activity was found to be decreased at extreme temperature as shown in Table 3 . COX showed thermal stability at temperature range 30^0C to 50^0C when incubated upto 90 min. However , enzyme was totally inactivated at 60^0C after 60 min incubation as shown in Figure 1

Similar results were showed by Tabatabai *et al.* (2001) showed that cholesterol of Rhodococcus sp PTCC (1633) has broad range of pH stability (pH 5.0 to 9.5), its range is narrower that of *Streptomyces fradie* which has pH stability in the range of 4-10. This enzyme showed complete stability at 4^0C and relatively good thermal stability at 40 and 50^0C.

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 identifiedon the basis of lower toxicity or higher mortality. Each bioassay was carried out in three replication containing 12 larvaes. Cumulative mortality after 72 hrs were recorded

However Highest mortality (33 %) was obtained when larvae was exposed to COX obtained from isolate A i.e Bacillus thuringienesis serovar finitimus the mortality figures are only indicative figures suggesting the insecticidal potential of these insecticidal enzymes/proteins. A detail insecticidal bioassays for depicting the LC_{50} values will give the clear picture, which was not possible in this study.

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 biopesticide or transgenic insect resistance plant. High temperature and pH stability makes these molecule more interesting to work upon in future.

Table 3. Optimum temperature for Cholesterol Oxidaseobtained from Lonar alkalophiles

pH	A *Bacillus thuringiensis* serovar *finitimus*	B *Bacillus licheniformis*	C *Bacillus cereus*	D *Halomonas campisalis*
	Specific activity of COX ±S.E. (U mg protein^{-1})			
30	0.163 ±0.002	0.174± 0.002	0.175 ± 0.002	0.178± 0.001
40	0.165 ±0.001	0.176± 0.004	0.177 ± 0.004	0.179 ± 0.003
50	0.166 ±0.001	0.177 ±0.001	0.178 ± 0.001	0.180 ± 0.001
60	0.052± 0.003	0.087± 0.002	0.140 ± 0.002	0.130 ± 0.004

Specific activity U mg protein^{-1} = COX activity retained after incubation at various range of pH

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