MUTATIONAL EFFECTS ON THE PROTEASE PRODUCING MARINE ACTINOMYCETES ISOLATED FROM *SCYLLA SERRATA*.

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Summary

Studies on proteolytic Marine actinobacteria especially in the Southeast coast of Tamilnadu have not been carried out adequately. Actinomycetes were isolated from the flesh content of the Mud crab, *Scylla serrata.* This is the first report on isolation of protease producing actinobacteria from crab. These crabs have a high tolerance to both nitrate and ammonia. The seven morphological different marine actinobacteria was isolated. The potential protease producing strains were mutated using UV radiation (physical) and sodium azide (chemical). This study suggests that mutation is one of the good methods for strain development to increase the efficiency of the Actinomycetes for protease production.

Keywords: Crab, Marine actinobacteria, protease, Sodium azide

Introduction

Microorganisms represent as a good source of enzymes due to a number of characteristics. For instance, their broad biochemical diversity, the rapid growth of microorganisms, the limited space required for cell cultivation, as well as the ease at which the enzymes can be genetically manipulated to generate new enzymes for various applications. [1]

The ability to produce a variety of proteolytic enzyme is a well Known phenomenon in mesophilic actinobacteria. Actinobacteria proteases, as well as other proteases of microbial origin, are of considerable commercial value as they provide a source of free and / or immobilized enzyme for use in the food, pharmaceuticals and tanning industries.

Actinobacteria occur in wide range of environments producing a variety of scientifically interesting and commercially useful high value metabolites. Marine actinobacteria have recently emerged as rich source for the isolation of industrial enzymes. Enzymes, after antibiotics, are the most important product of actinobacteria. For many years, actinobacteria were best known as the source of large numbers of antibiotics.

More recently, they have been found to be a promising source of a wide range of important enzymes. Examples includes, *Streptomyces* protease preparations that are commercially useful include PRONASE 7M (*S. griseus)* and FRADIASE 7M (*S. fradiae*). While alkaline proteases from bacteria are extensively characterized, similar attention has not been paid to actinobacteria. To date antibiotics are the major bioactive compounds from the Actinobacteria. [2, 3] However, the ability to produce a variety of enzymes may be an attractive phenomenon in these prokaryotes. This is the first report on isolation of protease producing actinobacteria from marine crab. [4-6]

Therefore, the present study was undertaken to isolate the proteolytic actinomycetes from the crab and to check their protease production efficiency by mutation methods.

Materials and methods

Isolation of actinobacteria

Mud crab, *Scylla serrata* was collected using cast net from the south east coast of India. Samples were kept in sterile polyethylene bags and transported to the laboratory under ice for microbiological analysis, before the flesh of the crab was removed; the body surface was wiped with 70% ethanol by using sterile cotton. The flesh content was removed aseptically and then one gram of the squeezed out flesh contents were taken and homogenized separately in a sterile mortar and pestle. The sample was serially diluted with filtered and sterilized 50% seawater. One ml of the serially diluted sample was plated in petriplates containing Kuster's agar medium and incubated at 35°C for seven days. The leathery colonies of actinobacteria that appeared on the petriplates were counted from the 5th day onwards up to 28th day.

Screening of proteolytoic actinobacteria

Protease activity of the strains was screened qualitatively in Gelatin agar medium. After inoculation, the plates were incubated at 36±1°C for five days. The cultures were noted and the plates were flooded with 20% aqueous solution of salphosalycylic acid. The colonies, which produced the clear zone, were indicated as the protease activity and the zone was measured. [7]

Effect of mutation:

The strains which showed efficient protease activity were further selected to study the effect of mutation on their protease production. Enzyme production can be increased by strain improvement. This is usually done by mutating the micro organism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens (X-rays, γ-rays, UV-rays etc.) and chemical mutagens (NTG, sodium azide etc.).

Physical mutation

The selected strains were cultured in test tubes containing 9 ml starch casein broth. The tubes were inoculated with one loop full growth the strain and incubated in a rotatory shaker at 250 rpm at 30°C for 72-96 hours. After incubation, the tubes were removed form the shaker and 3ml of each culture was exposed to UV-radiation at a distance of 30 cm for 3 min. Then, 1ml of the exposed cultures was transferred to 9 ml of Glycerol-starch broth medium and the tubes were incubated for 72-96 hrs on a rotatory shaker at 250 rpm for 30°C. The strain was used to examine the post-mutation effect on the strain for proteolytic activity. [8]

Chemical mutation

The selected strains were streaked on the KUA plates containing varying concentration (10-100 ppm) of sodium azide and incubated at 28°C for 5-6 days. After incubation, the plates were examined for the post mutation effect on the strain on its proteolytic activity.

Taxonomic Investigation

Thin Layer Chromatography (TLC): Spotting of the whole cell hydrolysates was made carefully on TLC plate using a micropipette. Spots were of 5-10 mm in diameter. This was done by multiple applications on the same spot of very small portions of the sample, which were dried by a hand drier.

Amino acids*:* Each sample (3 µl) was applied on the baselines of TLC plate (20 cm x 20 cm). Adjacent to this, 1µl of DL-diaminopimelic acid (an authentic material mixture of DAP isomers) and 1µl of amino acetic acid (glycine) were spotted as standards. TLC plate was developed with the solvent system containing methanol: pyridine: glacial acetic acid: H_2O (5: 0.5: 0.125: 2.5 v/v). It took approximately more than 4 h for development. The spots were visualized by spraying with 0.4% ninhydrin solution in water-saturated n-butanol, followed by heating at 100^0 C for 5 min. Spots of amino acetic acid ran faster than DAP. The sample spots were immediately compared with the spots of the standards since spots gradually disappeared in few hours. [9-11]

Whole-Cell sugars*:* On a cellulose TLC plate (20 cm x 20 cm), 5µl of samples was spotted, along with 3 µl of sugar solutions as standards on the same plates. Galactose, arabinose, xylose and madurose were the sugars, which were used as standards. TLC plate was developed with the solvent mixture containing ethyl acetate: pyridine: acetic acid: distilled water (8: 5: 1: 1.5 v/v). The developing time was more than 4 h. Spots were visualized by spraying with aniline phthalate reagent (3.25 g of phthalic acid dissolved in 2 ml of aniline and made upto 100 ml with water saturated n-butanol). The sprayed plate was heated at 100^0 C for 4 min. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots. [12]

Statistical Analysis

Data are expressed as means ±SD statistical analysis was performed with SPSS (8th version) Least standard deviation test were used for analysis of variance (ANOVA) and Post hoc test respectively. Difference on statistical analysis of data were considered significant at $P<0.05$.

Results and Discussion

Protease activity

A total of 9 strains were isolated from flesh content of the crab *S.serrata* and isolated strains were checked for their protease activity. Out of 9 strains, only 7 strains show protease activity in that LK-2, LK-3, LK-4 AND LK-5 show high protease activity. This is first report on isolation of actinobacteria from crab, compare to other marine animal it show less density of actinobacteria. The reports on actinobacteria population from other marine animals like fish and shellfish show 62% and shrimp 17%. [13]

Effect of mutation on protease activity

Strain improvement

The isolated strains were mutated using physical (UV radiation) and chemical (sodium azide) mutation methods. Strains exposed to UV radiation showed variation in clear zone from gelatin agar. As compared the control, the UV-mutatad strain showed an increase clear zone like (LK-1 (+4mm), LK-5 (6mm), LK-6 (2mm) and LK-7(+8mm).It also decrease protease activity like LK-3(-2mm), and LK-4(-19mm).No changes in LK-2strains (Table 1). All strains were exposed to UV radiation and showed variation in proteolytic activity. Adsual et al (2007) reported the strain of *Penicillium janthinellum* NCIM 1171 was subjected to mutation involving treatment by UV-irradiation for 3 min. Successive mutants showed enhanced cellulase production. [14]

Table 1.Variation in the clear zone of UV (physical) mutated strains and non-mutated strains

Strain no.	Mutated strains (mm)	Non mutated strains (mm)	variation
LK-1	5	1	+4
LK-2	20	20	0
LK-3	26	28	-2
LK-4	4	23	-19
LK-5	25	19	+6
LK-6	8	6	+2
LK-7	9	1	+8

The Proteolytic strains were then grown on plates containing 10 to 100 ppm sodium azide. The growth was observed only on plate containing 10 ppm sodium azide. In the plate, LK-3, LK-4 and LK-5 strains grown slowly and LK-1, LK-2, LK-6 and LK-7 strains grown fastly compare to other strains. The chemically mutated strains showed variation in the clear zone. Mutated strains LK-1(+7mm), LK-2(+3mm), LK-3(+10mm), LK-5(+6mm), LK-6(2mm) and LK-7(+8mm) showed increase activity.LK-4(-19mm) Strain only show decrease protease activity (Table 2).

Table 2.Variation in the clear zone of sodium azide (chemical) mutated strains and non-mutated strains

Strain no.	Mutated strains (mm)	Non mutated strains (mm)	Variation (mm)
LK-1	8	1	+7
LK-2	23	20	+3
LK-3	38	28	+10
LK-4	4	23	-19
LK-5	25	19	+6
LK-6	8	6	+2
LK-7	9	1	+8

Sodium azide mediates point mutations (mostly AT-GC transitions) and thus the modified biochemistry of the treated Streptomyces strain was most likely due to the mutagenic effect of the chemical. Kiran et al., (2007) observed mutagenic effects of sodium azide in Streptomyces sp. identified both loss of function (LOF) and gain of function (GOF) biochemical activities mediated by sodium azide treatment. Here we also observed both LOF and GOF. [15]

In the present study, after the mutation proteolytic activity of the strain viz. LK-4 (UV-treated) and LK-3 (sodium azide-treated) was increased. Due to the mutation, partial active gene of the strains which are responsible for the production of protease could have been activated.

Taxonomic investigation

All the seven proteolytic actinobacteria strains were found to possess LL-DAP and all of them contained glycine in their cellwall. Presence of LL-DAP indicates the cellwall chemotype-1 i.e. the wall property of the genus *Streptomyces.* (Table 3) Data are expressed as means ±SEM statistical analysis was performed with SPSS (8th version). Difference on statistical analysis of data were considered significant at $P<0.05$ (Table 4 & 5).

Table3. Cell wall amino acids and whole cell sugars

Strain No.	LL-DAP	Meso-DAP	Glysine	Whole cell sugars	Wall type
LK-1	+	-	+	-	I
LK-2	+	-	+	-	I
LK-3	+	-	+	-	I
LK-4	+	-	+	-	I
LK-5	+	-	+	-	I
LK-6	+	-	+	-	I
LK-7	+	-	+	-	I

+ denotes presence; - denotes absence

Table 4.Variation in the clear zone of UV (physical) mutated strains and non-mutated strains

Strain no.	Mutated strains(mm)	Non mutated strains (mm)	variation
LK-1	5.10±0.20	1.16±0.15	4.20±0.10
LK-2	20.16±0.14	20.20±0.09	0
LK-3	26.10±0.19	28.16±0.05	-2.10±0.10
LK-4	4.30±0.20	23.23±0.14	-19.3±0.3
LK-5	24.60±1.12	19.26±0.14	6.03±0.14
LK-6	8.33±0.15	6.26±0.20	2.03±0.15
LK-7	9.13±0.14	1.33±0.5	8.10±0.09

Table 5.Variation in the clear zone of sodium azide (chemical) mutated strains and non-mutated strains

Strain no.	Mutated strains(mm)	Nonmutated strains (mm)	Variation (mm)
LK-1	8.10±0.21	1.20±0.1	7.13±0.43
LK-2	23.30±0.19	19.93±0.14	3.23±0.14
LK-3	38.13±0.14	28.13±0.15	10.06±0.11
LK-4	4.33±0.15	23.10±0.19	-19.20±0.11
LK-5	25.13±0.09	19.13±0.41	6.13±0.14
LK-6	8.10±0.20	6.10±0.11	2.33±0.13
LK-7	9.20±0.12	1.10±0.13	8.16±0.81

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References

1. Adsul MG, Bastawde KB, Varma AJ, Gokhale DV. Strain improvement of *Penicillium janthinellum NCIM 1171* for increased cellulase production. Mutation Research. 2007; 98: 1467-1473

2. Chaphalkar SR, Deys. Thermostable alkaline mettalloprotease from newly isolated alkaliphilic *Streptomyces diastaticus Strain SSI*. Indian J. Bichem. Biophys 1998; 35: 34–40

3. Cummins CS, Harris HA. Comparison of cell wall composition in *Nocardia, Actinomyces, Mycobacterium and Propionbacterium*. J. Gen. Microbiol 1956; 15: 9.

4. Cruickshank R, Duguid JP, Marmian BP, Swain RHA. Medical Microbiology, 12th ed. Churchill livingstone, London. 1975: 74

5. Dieter A, Hamm A, Fiedler HP, Goodfellow M, Muller WE, Brun R, Bringmann G. Pyrocoll. An antibiotic, antiparasitic and antitumor compound produced by a novel alkaliphilic *Streptomyces* strain. J. Antibiot 2003; 56:639–646.

6. Ellaiah P, Raju KV, Adinarayana K, Adinarayana G, Prabhakar T, Premkumar J. Bioactive rare actinomycetes from indigenous natural substrates of Andhra Pradesh. Hindustan Antibiot Bull 2002; 44: 17–24.

7. Kiran BT, Keshav B, Vishwanath PA. Enhanced Antibacterial activity of sodium azide treated mutant *Streptomyces* strain. Journal of Nepal Association for Medical Laboratory Sciences 2007; 8: 67-8.

8. Maloy KS, SivaKumar K, Kannan L. Alkaline protease production by an Actinomycete isolated from tiger shrimp *Penaeus monodon*. Nat. Acad. Sci. Letter 2008; 30:1.

9. Muthurayar T, Maloy KS, Siva KK, Kannan L. Mutational Effects on the Antibacteial activity of some marine actinomycetes isolated from *Chanos chanos*. Environment and Ecology 2006; 24:46-50

10. Nonomura H. Key for classification and identification of 458 species of the *Streptomycetes* included in ISP. J. Ferment. Technol 1974; 52: 78 - 92.

11. Pridham TG, Anderson P, Foley C, Lindenfelser LA, Hesseltine CW, Bendict RG. A selection of media for maintenance and taxonomic study of *Streptomycetes*. Antibiotics Annual 1957; 1: 947–953.

12. Pridham TG. Colour and Streptomycetes. Report of an international workshop on determination of color of *Streptomycetes*. Appl. Microbiol 1965; 13: 43 - 64.

13. Rao BM, Tanksale MA, Cihatge SM , Deshpande VV. Molecular and Biotechnological aspects of microbial protease. Microbial. Mol. Boil 1998; 62: 597-635.

14. Schippers A, Bosecker K, Willscher S, Sproer C, Schumann P, Kroppenstedt RM. *Nocardiopsis metallicus sp. Nov*., a metal-leaching actinomycetes isolated from an alkaline slag dump. Int. J. Syst. Evol. Microbiol 2002; 52: 2291–2295.

15. Tsuchiya. Cloning and expression of an intercellular alkaline protease gene from alkalophilic *Thermoactinomyces sp. H5682*. Biosci. Biotechnol. Biochem 1997; 61: 298–303.