INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

www.scienceandnature.org

EFFECT OF DIFFERENT GROWTH PARAMETERS ON CHITINASE ENZYME ACTIVITY BY MUTANT BACTERIA OF THE GUT ENVIRONMENT

[1]Ann Suji , H., [2]Palavesam, T. A., [2]Immanuel, G. & [3]Suthin Raj
[1]CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University
[2]Center for of Marine Science and Technology, MS University, Rajakkamangalam
[3]Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Chidambaram – 608 002

ABSTRACT
Chitinases are enzymes which recognize and hydolyze chitin, a linear homopolymer of N-acetyl-D-glucosamine. Bacteria produce several chitinases, probably to hydrolyze the diversity of chitins found in nature. Chitinases have shown numerous applications in waste treatment and management of shellfish processing industries. So, in the present study, an attempt was made to optimize chitinase production by one of the shrimp's gut mutant bacterial flora, *P. alcaligenes*. Optimization of culture conditions revealed that the enzyme production was maximum in pH7.5 (110.7 ± 1.76U/ml), temperature 35OC (99.9 ± 1.41U/ml) when the carbon and the nitrogen sources used were glucose (119.55 ± 4.29U/ml) and $NaNO_2$ (103.2 ± 1.72U/ml) respectively

KEY WORDS: Chitinase, mutant *P. alcaligenes*, Optimization.

INTRODUCTION

The seafood industry acts as a major source of chitinous wastes, the recycling of which is extremely important to retain the carbon-nitrogen balance in the ecosystem (Tsujibo *et al.,* 1998; Reguera and Lesehine, 2003). For survival of aquatic ecosystems, chitin is rapidly catabolized by marine bacteria (Li and Roseman, 2004). Bacteria produce several chitinases, probably to hydrolyze the diversity of chitins found in nature (Svitil *et al.,* 1997; Amit Kumar *et al.,* 2007). The enzymatic degradation of chitinous waste involves chitinases, playing a dual role in utilization of chitinous wastes and decreasing the production cost of the microbial chitinases (Gohel *et al.,* 2005). Quantitative enhancement of enzyme over production by bacteria requires strain improvement as the quantities produced by wild strains are usually too low (Bapiraju *et al.,* 2004; Pandey and Gupta, 2011).Several strains of microorganisms have been selected or genetically modified to increase the efficiency with which they produce enzymes (Okonko *et al.,* 2006). Physical and chemical agents known to be effective in increasing the rate of mutations in higher organisms have similar effects on bacteria. The mutations induced by these agents in bacteria, as in higher organisms, seem to be random and non specific (Evelyn Witkin, 1946). However, reports on ultra violet (UV) mutation of *Pseudomonas* sp. for production are very few (Dutta and Banerjee, 2006). Strain improvement by induced mutagenesis has been developed with rational selection procedures for an efficient screening of the mutants (Gromada and Fiedurek, 1997). Although naturally occurring organisms provide a major source of chitinolytic enzymes, genetic improvement plays an important role in their biotechnological applications (Vipul Gohel *et al.,* 2006). There are a number of different methods available for strain improvement for increasing the chitinase production (Vipul Gohel *et al.,* 2006). Molecular approaches for characterizing microbial species and assemblages have significantly influenced our understanding of microbial diversity and ecology (Vipul Gohel *et al.,* 2006; Mackie *et al.,* 1999). Detection and identification methods using the PCR to amplify DNA have been used for some organisms (Hartskeerl *et al.,* 1989). Considering the paucity of information available on enzyme production by wild and mutant microflora of shrimps gut environment, an attempt was made in the present study to isolate and identify them and to screen their chitinolytic activity and to examine their enzyme producing ability with varying nutritional sources, pH and temperature.

MATERIALS & METHODS

Penaeus monodon is an important marine crustacean which inhabits in marine forms and is endemic in Peninsular India and other countries. It was collected from the Rajakkamangalam estuary at Rajakkamangalam, Kanyakumari District, Tamilnadu. The collected shrimps were aseptically transferred to the laboratory for further study.

Isolation, identification and screening of gut bacterial flora

In the laboratory, the weight of the whole shrimp was noted and the gut was aseptically dissected out and serially diluted upto 10^{-5} dilution. From each dilution, 0.1 ml of sample was taken and spread plated on nutrient agar medium. The plates were then incubated at 37°C for 24 to 48 h. The total viable counts (TVC) of the colonies were finally noted. The isolated cultures were purified individually by streaking on nutrient agar plates and were sub cultured. Then the bacterial cultures were identified by performing biochemical tests.

Chitinase activity

The chitinase detection agar (CHDA) (Components (g/l) Colloidal chitin: 10.0g ; Agar: 20.0 g; Soya bean powder: 20.0g ; Starch : 3.0 g ; Peptone : 3.0 g ; Yeast extract : 2.0g ; $CACO_3$: 1.0g ; : M9 medium: Na_2HPO_4 : 0.65g ; KH_2PO_4 : 1.5g ; NaCl :0.25g ; NH_4Cl : 0.5 g ; $MgSO_4$: 0.12g; $CaCl_2$: 0.005g ; pH : 6.5) plates were prepared. The isolated gut microbes were single streaked individually into the CHDA plates and were incubated at 37°C for 72 h. They were then observed for zone formation. The colonies which formed a zone around them were the chitinase positive strains, which were then sub cultured regularly for further study.

Preparation of colloidal chitin (2%) (Roberts and Selitrennikoff, 1988)

20 g of chitin powder was added into 180 ml of 37% HCl under vigorous stirring for 2 h. It was then poured into 1 litre of ice cold ethanol (95%) under vigorous stirring for 30 minutes. This suspension was stored at 20°C until further use. When in need, 10 ml of the suspension was centrifuged at 5,000 rpm for 15 min. The precipitate was collected and washed with 50 ml of 50 mM sodium acetate buffer (pH 6.8). The above process was repeated 3 times and the precipitate derived was dissolved in 90 ml of 50 mM sodium acetate buffer (pH 6.8). This was the prepared 2% colloidal chitin.

Mutagenic characterization and PCR analysis of microbes

The bacterial culture which showed a maximum chitinolytic activitiy (*P. alcaligenes*) was mutated by physical means. Physical mutation was done by exposing sterile plates of Luria Bertani (LB) agar with cultures to UV light source.

Physical mutation (Miller, 1992)

The test organisms were inoculated into nutrient broth and incubated at 37°C for 24 h. After incubation, the organisms were individually inoculated into LB broth and incubated at 37°C for 24 hours and the above cultures were serially diluted on normal saline solution. The 10^{-2} dilution was selected for further use. The dilution was spread plated into sterile LB agar plates. The spreaded plates were exposed to short wavelength UV light (distance = 39 cm; 280 nm) for four different time intervals (5, 10, 15 and 20 min.). After exposure, the plates were incubated at 37°C for 24 h. simultaneously; control plates with test organisms were also maintained without any UV treatment. The colonies formed were then noted and the isolated colony from each plate was streaked and subcultured into nutrient agar slants for further assay.

Enzyme assay using mutant cultures

Chitinase assay was performed for the examination of enzyme production by the selected mutant strains.

Chitinase assay (Tweddell *et al.*, 1994)

The chitinolytic bacteria were inoculated individually into chitinase liquid medium (Components (g/l) : Soya bean powder : 20.0 g ; Starch : 4.0 g ; Peptone : 3.0 g ; Yeast extract : 2.0 g ; KH_2PO_4 : 0.3 g ; $MgSO_4$: 0.3 g ; $CaCO_3$: 1.0 g ; pH : 6.5) and were incubated at 30°C for 48 to 72 h in a shaker. After incubation, the enzyme source was taken by centrifuging the culture fluid at 5,000 rpm for 15 min.

To 1 ml of the enzyme source, 1 ml of 2% colloidal chitin was added and was incubated in a water bath at 50°C for 1 h. Then 3 ml of DNS reagent was added to it and was boiled for 10 min. Finally, it was centrifuged at 3,000 rpm for 20 min and the OD of chitinase enzyme production was measured by an UV-spectrophotometer at 530 nm.

Effect of various nutrient sources, pH and temperature on the production of chitinase by mutant strains

The assay procedure described earlier was performed for chitinase activity by mutated strains individually using different sources of carbon (Glucose, Sucrose, Lactose and CMC), nitrogen (NH_4Cl, NH_4SO_4, $NaNO_3$ and KNO_3), temperature (30, 35, 40 and 45^oC) and pH (5.5, 6.5, 7.5, and 8.5).

Determination of Genetic characteristics of normal and mutated strains

The DNA isolation, RAPD-PCR and plasmid isolation were performed in order to confirm mutation. *P. alcaligenes* was selected based on its maximum occurrence in the shrimp gut. The pellet of selected bacterial cell was ground in a glass homogenizer with 300 µl of CTAB (Cety Trimethyl Ammonium Bromide) DNA extraction buffer (1% w/v CTAB ; 1.4 M NaCl ; 10 mM EDTA ; pH 8.0 ; 100 m Tris – HCl ; pH 8.0 ; 0.2% v/v mercaptoethanol). The mixture was emulsified with equal volume of phenol: chloroform (1:1). It was then centrifuged at 10,000 rpm for 5 minutes at room temperature. The aqueous phase was collected and mixed with equal volume of chloroform: isoamyl alcohol (24: 1). The mixture was again centrifuged at 10,000 rpm for 5 min. and the ethanol was air-dried. The pellet was dissolved in 50 µl of TE buffer (Tris 10 mM, pH 8.0 and EDTA 1 mM, pH 8.0). The isolated DNA was quantified by a Spectrophotometer (260 nm) and the quality was tested by agarose gel electrophoresis.

RAPD-PCR analysis

20 mg of DNA was dissolved in 20 µl of PCR reaction buffer containing 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.2 mM dNTPs, 21 pM of primer and 0.5U of DNA polymerase. Primers (RAPD kit A1 to RAPD kit A10) obtained from IDT were used for RAPD-PCR studies. PCR was conducted according to the method of Williams *et al.* (1990); initial heating step (94°C for 5 min.), 40 cycles of denaturation (94°C for 1 min.), annealing (36°C for 1 min.) and extension (72°C for 2 min.) and a final extension step (72°C for 7 min.) were done. Amplification was performed using a programmable thermal cycler PTC-150 (MJ Research, USA). The products of PCR and DNA size markers [DNA digested with Eco RI and Hind III (Bangalore Genei)] were loaded into a 1.6% *tris*-borate-EDTA (Sambrook *et al.*, 1989) agarose gel and run for 4 h. at 50V. The gels were stained with ethidium bromide and photographed. Each lane of RAPD profiles was subjected to gel documentation system (Vilbert-Lourmat, France). The dendrogram analysis and similarity index was carried out using Bioprofile 1D software (Vilbert-Lourmat, France).

Statistical analysis

The results obtained in the present study were subjected to the following statistical analysis (Zar, 1974).

i. Mean ± SD
ii. One-way ANOVA
iii. Two-way ANOVA

RESULTS

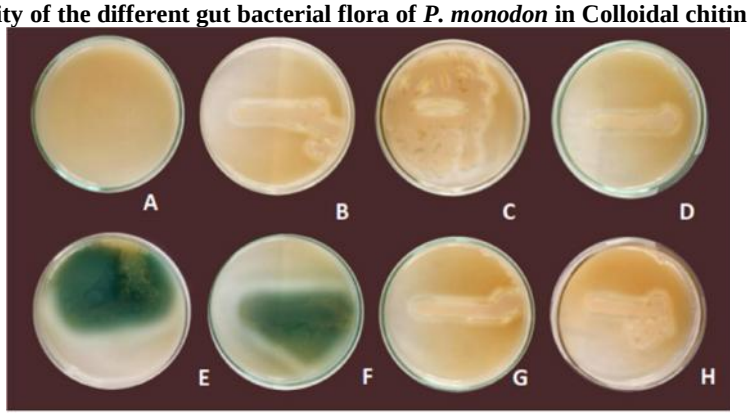
Isolation and identification of gut microflora

The total viable count of bacterial colonies recorded in the gut samples of shrimps was 43 ± 0.16 x 10^2 CFU/ml in 10^{-1} dilution and it was only 2 ± 1.13 CFU/ml in 10^{-5} dilution. Based on the morphological, physiological and biochemical characteristics, seven bacterial strains were identified(*Bacillus cereus, B. polymyxa, B. stearothermophilus, B. circulans* and *B. mycoides*, *Pseudomonas alcaligenes* and *P. anguilliseptica*).

Enzymatic characterization of identified bacterial species

All the identified bacterial strains showed a positive chitinolytic activitiy.

Chitinase activity of the different gut bacterial flora of *P. monodon* in Colloidal chitin agar medium



A : Control ; B : *B. cereus*, C : *B. polymyxa* ; D : *B. stearothermophilus* ;E : *B. circulans* ; F : *P. alcaligenes*; G **:** *B. mycoides*; H : *P. Anguilliseptica*

Enzyme production by mutated strains

The maximum level of chitinase producing strain namely, *P. alcaligenes* as per the results obtained in the previous experiments was mutated using UV as a physical mutagen.

Chitinase production by mutated *P. alcaligenes*

Physical mutation

The CFU of physically mutated *P. alcaligenes* was related with the exposure duration of UV treatment. For instance, at 5 min UV rays exposed plates, the number of colonies observed was 27 ± 1.36 CFU/ml, but it gradually decreased in the 10, 15 and 20 min UV treatment with the bacterial colonies of 23 ± 0.33, 20 ± 0.21 and 16 ± 0.01 CFU/ml, respectively. At the same time, in the control plate, the number of colonies observed was 110 ± 0.52 CFU/ml.

Observation of CFU of *P. alcaligenes* after physically mutated with UV rays at different time intervals

Sl. No.	Exposure to UV rays (time in minutes)	Number of colonies (CFU)
1.	0 (Control plate)	110 ± 0.52
2.	5	27 ± 1.36
3.	10	23 ± 0.33
4.	15	20 ± 0.21
5.	20	16 ± 0.10

Each value is the mean ± S.D of three estimates.

Chitinase production at different media temperature

The result on chitinase production revealed that a maximum enzyme production (99.9 ± 1.41 U/ml) was recorded by 15 min. UV treated *P. alcaligenes* at the temperature of 35°C. When the temperature level increased, the enzyme production gradually decreased.

Chitinase production (U/ml) by the mutated strain *P. alcaligenes* at different temperature

Sl. No.	Physical mutagens	Chitinase production (U/ml) at different temperature (°C)			
		30	35	40	45
1.	Wild strain	62.1 ± 2.43	78.4 ± 2.27	59.20 ± 1.54	47.9 ± 0.34
	Physical mutation (PM)				
2.	5 min UV mutant	83.15 ± 1.46	90.5 ± 2.56	98.40 ± 2.72	94.35 ± 3.20
3.	10 min UV mutant	41.90 ± 1.58	97.95 ± 2.77	84.15 ± 2.95	43.0 ± 3.47
4.	15 min UV mutant	51.95 ± 0.80	99.9 ± 1.41	79.5 ± 1.50	56.2 ± 1.76
5.	20 min UV mutant	55.10 ± 1.83	98.25 ± 3.22	83.4 ± 3.42	67.55 ± 0.02

Each value is the mean ± S.D of three estimates.

The chitinase production as a function of variation between different time intervals of UV mutation and also the variation between different tested media temperature were statistically significant (F = 3.718 & 9.437; P < 0.05 & P < 0.001)

Chitinase production at different media pH

Among the tested media pH, 20 min. UV mutated *P. alcaligenes* produced high amount (110.7 ± 1.76 U/ml) of chitinase at the pH 7.5. Whereas, only 40.7 ± 0.71 U/ml of chitinase was produced by 5 min. UV treated *P. alcaligenes* at 5.5 pH. The statistical two-way ANOVA test revealed that the variation between different time intervals of UV mutation and also the variation between different media pH were highly significant (F = 46.177 & 7.034 ; P < 0.01 to P < 0.0001)

Chitinase production (U/ml) by the mutated *P. alcaligenes* at different pH

Sl. No.	Physical mutagens	Chitinase production (U/ml) at different media pH			
		5.5	6.5	7.5	8.5
1.	Wild strain	54.8 ± 1.19	68.2 ± 1.24	74.1 ± 1.26	53.9 ± 2.34
	Physical mutation (PM)				
2.	5 min UV mutant	40.7 ± 0.71	49.2 ± 0.74	66.8 ± 0.75	70.95 ± 1.40
3.	10 min UV mutant	43.75 ± 0.77	49.95 ± 0.80	69.75 ± 0.82	63.00 ± 1.52
4.	15 min UV mutant	63.70 ± 0.39	66.45 ± 0.41	73.50 ± 0.41	72.65 ± 0.77
5.	20 min UV mutant	73.05 ± 0.89	80.45 ± 0.93	110.7 ± 1.76	82.9 ± 0.95

Each value is the mean ± S.D of three estimates.

Chitinase production at different carbon sources supplied media

The production of chitinase was tested in different carbon sources supplied media by various time intervals of UV mutated *P. alcaligenes*. Accordingly maximum (119.55 ± 4.29 U/ml) chitinase was produced by 10 min. UV treated *P. alcaligenes* which utilized glucose as the carbon source. At the same time, fewer amounts (56.5 ± 1.83 U/ml) of enzyme was produced by the 20 min. UV treated *P. alcaligenes* which utilized sucrose. The chitinase production as a function of the differences between the time intervals of UV mutation was statistically non-significant (F = 1.81075; P > 0.05) whereas the differences between tested carbon sources on enzyme production was statistically significant (F = 3.486; P < 0.05).

Chitinase production (U/ml) by the mutated strain of *P. alcaligenes* at different carbon sources supplemented media

Sl. No.	Physical mutagens	Chitinase production (U/ml) at different carbon sources			
		Glucose	Sucrose	Lactose	CMC
1.	Wild strain	64.1 ± 1.30	59.4 ± 1.20	48.9 ± 0.86	79.2 ± 1.14
	Physical mutation (PM)				
2.	5 min UV mutant	99.80 ± 3.95	66.35 ± 1.46	83.80 ± 2.28	79.2 ± 2.78
3.	10 min UV mutant	119.55 ± 4.29	58.0 ± 1.58	68.00 ± 2.47	84.62 ± 3.01
4.	15 min UV mutant	84.95 ± 2.18	76.35 ± 0.80	79.20 ± 1.25	75.90 ± 1.53
5.	20 min UV mutant	67.90 ± 4.97	56.5 ± 1.83	70.15 ± 2.86	85.10 ± 3.49

Each value is the mean ± S.D of three estimates.

Chitinase production at different nitrogen sources supplied media

When $NaNO_2$ was used as the nitrogen source, a maximum of 103.2 ± 1.72 U/ml of chitinase was produced by the 20 min. UV mutated *P. alcaligenes*, whereas a minimum of 57.95 ± 1.37 U/ml chitinase was produced by 5 min. UV treated *P. alcaligenes* by utilizing NH_4Cl as the nitrogen source. The statistical two-way ANOVA revealed that the differences between the time intervals of UV mutation on chitinase production was non-significant (F = 2.352 ; P > 0.05), but the differences between the tested nitrogen sources on chitinase production was found to be significant (F = 6.427 ; P < 0.01).

Chitinase production (U/ml) by the mutated strain of *P. alcaligenes* at different nitrogen sources supplemented media

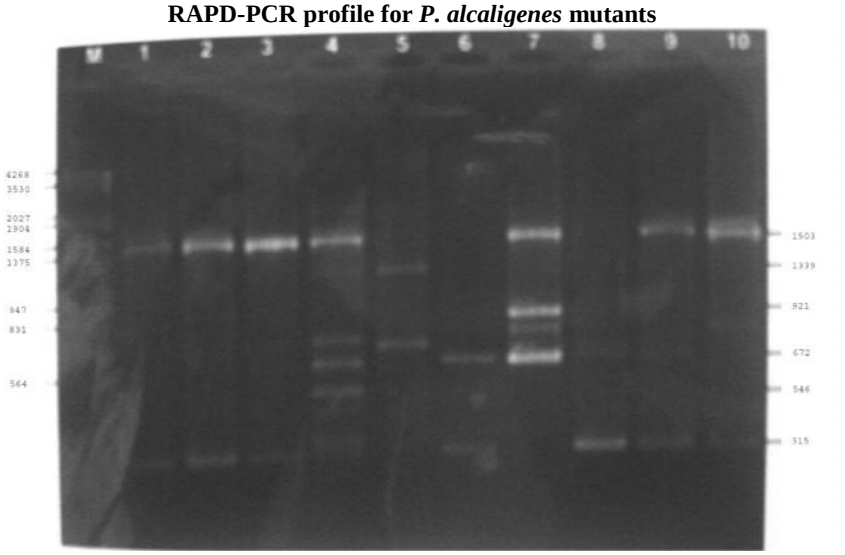
Sl. No.	Physical mutagens	Chitinase production (U/ml) at different nitrogen sources			
		NH_4Cl	$NaNO_2$	KNO_3	NH_4SO_4
1.	Wild strain	68.5 ± 2.29	73.2 ± 2.29	66.8 ± 2.33	63.70 ± 2.35
	Physical mutation (PM)				
2.	5 min UV mutant	57.95 ± 1.37	70.80 ± 1.37	63.25 ± 1.39	59.4 ± 1.41
3.	10 min UV mutant	59.90 ± 1.49	84.15 ± 1.49	67.15 ± 1.51	58.90 ± 1.53
4.	15 min UV mutant	63.60 ± 0.75	86.70 ± 0.75	66.55 ± 0.77	86.85 ± 0.77
5.	20 min UV mutant	70.35 ± 1.72	103.2 ± 1.72	70.3 ± 1.75	64.80 ± 1.77

Each value is the mean ± S.D of three estimates.

RAPD-PCR analysis

The primer RAPD kit A_{10} amplified a DNA fragment of 1584 base pairs (bp) with low intensity in wild *P. alcaligenes*, but two DNA fragments of 1584 bp and 315 bp were obtained in the 5 min UV treated *P. alcaligenes* (PM1). In 10 min. UV treated *P. alcaligenes* (PM2), the magnified DNA fragment size of 1503 bp was obtained. In the case of 15 min. UV treated *P. alcaligenes* (PM3), three DNA fragments with the size of 1503, 672 and 546 bp, respectively were found. In 20 min. UV treated strain (PM4), two DNA fragments (1339 & 672 bp) were obtained. The RAPD profiles of mutant *P. alcaligenes* were used for analyzing the similarity index.

RAPD-PCR profile for *P. alcaligenes* mutants



Lanes :

1 – Wild strain
2 – 5 min. UV mutant
3 – 10 min. UV mutant
4 – 15 min. UV mutant
5 – 20 min. UV mutant

Similarity index of *P. alcaligenes* mutants

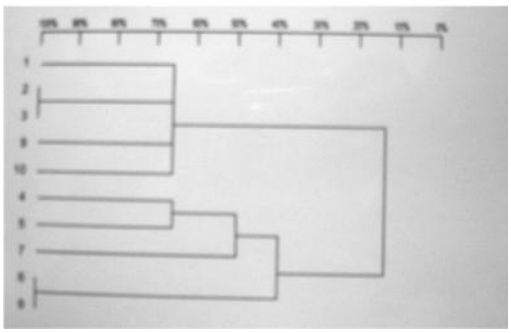
	1	2	3	4	5	6	7	8	9	10
1	1.00									
2	0.67	1.00								
3	0.67	1.00	1.00							
4	0.40	0.33	0.33	1.00						
5	0.12	0.12	0.12	0.67	1.00					
6	0.12	0.12	0.12	0.40	0.12	1.00				
7	0.40	0.67	0.67	0.50	0.12	0.40	1.00			
8	0.12	0.12	0.12	0.40	0.12	1.00	0.40	1.00		
9	0.67	0.50	0.50	0.67	0.12	0.67	0.67	0.67	1.00	
10	0.67	0.50	0.50	0.33	0.12	0.12	0.67	0.12	0.50	1.00

1 – Wild strain
2 – 5 min. UV mutant
3 – 10 min. UV mutant
4 – 15 min. UV mutant
5 – 20 min. UV mutant

The RAPD fragment of wild (control) *P. alcaligenes* had a similarity index of 0.67 with mutants 2and3; a similarity index of 0.40 with mutant strain 4 and similarity index of 0.12 with isolate 5. In *P. alcaligenes* strain (PM1 : 5 minutes UV exposed), the RAPD fragments showed a similarity index of 0.33 with strain 4 and a similarity index of 0.12 with strains 5. Likewise, the PM2 strain (10 minutes UV exposed) registered a similarity index of 0.33 with mutant 4 and a similarity index of 0.12 with mutant 5. The PM2 strain (15 minutes UV exposed) revealed a similarity index of 0.67 with the 5th strain. Dendrograms in the phylogram form depicting the phylogenetic relatedness

between various strains of *P. alcaligenes* were generated by the Unweighed Pair Group Method using Arithmetic averages (UPGMA).

Dendrogram for *P. alicaligenes* mutants



1 – Wild strain
2 – 5 min. UV mutant
3 – 10 min. UV mutant
4 – 15 min. UV mutant
5 – 20 min. UV mutant

Examination of dendrogram results indicated that the mutant strains were grouped into a single cluster.

DISCUSSION

The ability of breakdown of chitin into nutritive monomers may be the result of a relationship between fish and enteric bacteria which release extracellular chitinases (Gutowska, 2004). Among Gram negative bacteria, chitinolytic activity has been described for strains from the genera *Aeromonas, Alteromonas, Enterobacter, Pseudomonas, Serratia, Ewingella* and *Vibrio* (Chernin *et al.*, 1998). In a study by Cody (1989), seventeen of 52 strains representing ten species of *Bacillus* were chitinolytic positive. But in the present study, five of seven strains representing *Bacillus* sp. were chitinolytic positive.

A variety of methods have been employed to modify enzymes for their industrial usage including strain improvement (Chand *et al.*, 2004). In the present investigation, the isolated bacterial strain was subjected to strain improvement through physical mutation by UV rays.UV rays are important inducers of strain mutations (Prabakaran *et al.*, 2009; Nashima *et al.*, 2012). The mutation and screening of industrially useful microorganisms are important for the successful development of the various strains required in the fermentation industry. The evidences for such studies are put forth by several authors. For instance, the productivity of the parent strain of *Pseudomonas* sp. was improved by UV mutation by Dutta and Banerjee (2006). The mutation of bacterial cultures using UV rays by different time duration of 5 minutes interval upto 30 minutes was carried out by Saha and Bhattacharya (1990). In the present study, the experimental strains were exposed to UV rays upto 20 minutes with the time interval of 5 minutes, simultaneously, the cultures were kept in darkness for stabilization of thymine dimmers. Gohel *et al.* (2004) examined the production of chitinase enzyme by both the wild as well as UV treated mutant strain of *Pantoea dispersa* and found that the mutant strain produced 9.52 ± 0.21 IU/ml of chitinase while the wild strain produced only 7.74 ± 0.26 IU/ml. In the present study, chitinase production by wild as well as UV treated mutants of *P. alcaligenes* were examined, in which, 10 minutes UV treated strain produced maximum amount (119.55 ± 4.29 U/ml) of chitinase, while the wild strain produced only 79.2 ± 1.41 U/ml of chitinase. In consistence with this, Gohel *et al.* (2005) reported that, 10 minutes UV treated *Pantoea dispersa* exhibited the highest chitinolytic activity of 13.97 ± 0.25 IU/ml as compared to that of the wild type. Similarly, Lim *et al.* (1991) also reported the maximum chitinase production achieved by UV mutated *P. stutzeri* YPL-M26.

RAPD analysis

The randomly amplified polymorphic DNA (RAPD)-PCR technique is a modification of the polymerase chain reaction which can be used to produce genome fingerprints of the strains under examination (Shehata, 2008). It has been widely used for studying the genetic relationship of different bacteria including *B. sphaericus* and other bacilli (Stephan *et al.*, 1994).

In the present study, the diversity (% occurrence) of *P. alcaligenes* was greater than that of other strains. Therefore, this strain and its mutants were subjected to RAPD-PCR analysis. Further, genetic fingerprinting and phylogenetic diversity between different mutant *P. alcaligenes* isolates were determined by converting RAPD data into a similarity index and analyzed by UPGMA to produce a phylogenetic tree.

It could be observed that, the difference in gene expression of these mutants may be the reason for hyperactivity of enzyme in the respective strains. The banding patterns of these strains were analyzed by a dendrogram, which showed a similarity between mutant strains of *P. alcaligenes*. The possible relationship between host origin, mutation and genetic variation among the mutants of *P. alcaligenes* were also examined.

In conclusion, an attempt was made to mutate *P. alcaligenes* to increase chitinase production. The overall results indicated that mutant strains of *P. alcaligenes*, based on their characterization, could be useful sources of enzymes and has the potential for industrial application.

REFERENCES

Atienzar, F.A., Venier, P., Jha, A.N. and Depledge, M.H. (2002) Evaluation of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations. *Mutat. Res.*, 521: 151-163.

Amit kumar, S., Baruah, K., Debnath, D. and pal, A. (2007) Nutrizymes ideal nutraceuticals in aquafeed: potential and limitations, *Aquaculture Health International*, Issue 11(12), ISSN 1176-86330.pp.4-6.

Bapiraju, K.V.V.S.N., Sujatha, P., Ellaiah, P. and Ramana, T. (2004) Mutation induced enhanced biosynthesis of lipase. *African J. Biotechnol.*, 3(11): 618-621.

Chand, P., Aruna, A., Maqsood, A. and Rao, L. V. (2004) Novel mutation method for increased cellulase production. *J. Appl. Microbiol*., 98(2): 318 – 323.

Chernin, L., Michael, K. Winson, Jacquelyn, M. Thompson, Shoshan Haran, Barrie W. Bycroft, Ilan Chet, Paul Williams and Gordon, S.A.B. Stewart (1998) Chitinolytic activity in *Chromobacterium violaceum:* substrate analysis and regulation by quorum sensing. *J. Bacteriol.*, 180 : 4435-4441.

Cody, R.M., 1989. Distribution of chitinase and chitobiase in *Bacillus. Curr. Microbiol.*, 19(4): 201-205.

Dutta, J. R. and Banerjee, R. (2006) Isolation and characterization of a newly isolated *Pseudomonas mutant* for protease production. *Brazilian Arch. Biol. Technol.*, 49(1) : 37 – 47.

Evelyn, M. Witkin (1946) Genetics of resistance to radiation in *Escherichia coli.* Ph.D. Thesis, Columbia University.

Gary, R. Lecleir, Alison Buchan and James T. Hollibaugh (2004) Chitinase gene sequences retrieved from diverse aquatic habitats reveal environment – specific distributions. *Appl. Environ. Microbiol.,* 70(12): 6977-6983.

Gohel, V., Vyas, P. and Chhatpar, H.S. (2005) Activity staining method of chitinase on chitin agar plate through polyacrylamide gel electrophoresis. *Afr. J. Biotechnol.,* 4: 87-90.

Gohel. V., Trivedi, S., Vygas, P. and Chhatpar, H. S. (2004) Formulation of medium constituents by multiresponse analysis of central composite design to enhance chitinase production in *Pantoea dispersa*. *Indian J. Exp. Biol,* 42(11): 1123-1131.

Gromada, A. and Fiedurek, J. (1997) Selective isolation of *Aspergillus niger* mutants with enhanced glucose oxidase production. *J. Appl. Microbiol.,* 82: 648-652.

Gutowska, M., Darzen, J. and Robinson, B. (2004) Digestive chitinolytic activity in marine fishes of Monterey Bay, California, *Comp. Biochem. Physiol.*, A139 : 351 – 358.

Hartskeerl, R.A., De Wit, M.Y. and Waster, P.R. (1989) Polymerase chain reaction for the detection of *Mycobacterium leprae*. *J. Gen. Microbiol,* 135(9): 2357-2364.

Li, X. and Roseman, S. (2004) The chitinolytic cascade in *Vibrios* is regulated by chitin oligosaccharides and a two component chitin catabolic sensor/kinase. Proceedings of National Academy of Science, 101(2) : 627 - 631.

Lim, H. S., Kim, S. D. and Kim, S. D. (1991) *Pseudomonas stutzeri* YPL-1 genetic transformation and antifungal mechanism against *Fusarium solani*, an agent of plant root rot. *Appl. Environ. Microbiol*., 57 : 510 – 516.

Mackie, R.I., Aminov, R. I., Gaskins, H. R. and White, B. A. (1999) Molecular microbial ecology in gut ecosystems. In*:* Bell, C.R., Brylinsky, M. and Johnson-Green, P., (eds.), microbial biosystems: New frontiers, proceedings of the 8th international symposium on microbial ecology, Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Miller, J. H. (1992) A short course in bacterial genetics: A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold spring laboratory press. New York, 876.

Nashima, K., Santhiya, P. and Palanisamy, A. (2012) Production and optimization of lipase from wild and mutant strains of *Bacillus* sp. and *Pseudomonas* sp., *J. Acad. Indus. Res.*1(2): 97 – 100.

Okonko, I. O., Olabode, O. P. and Okeleji, O. S. (2006) The role of biotechnology in the socio-economic advancement and national development: An overview. *African J. Biotechnol.*, 5(19): 2354-2366.

Pandey. A and Gupta, L. (2011) Effect of UV Radiations on Enzyme Kinetics of Extracellular Amylases Isolated from Bacillus subtilis, *Advanced Biotech.*, 11(6) : 20-24.

Prabakaran, M., Thennarasu, V., Ayeswariya Mangala, R., Bharathidasan, R., Chandrakala, N. and Mohan, N. (2009) Comparative studies on the enzyme activities of wild and mutant fungal strain isolated from sugarcane field. *Indian J. Sci. Technol*., 2(11) : 46 – 49.

Reguera, G. and Lesehine, S.D. (2003) Biochemical and genetic characterization of chi-A, the major enzyme component for the solubilization of chitin by *Cellulomonas uda. Arch. Microbiol.,* 18: 434-443.

Roberts, W. K. and Selitrennikoff, C. P. (1988) Plant and bacterial chitinases differ in antifungal activity. *J. Gen. Microbiol*., 134: 169 – 176.

Saha, N. and Bhattacharyya, B. C. (1990) Strain improvement: mutagenesis and random screening procedure for *Rhizopus oryzae* IIT KG-1. Proceedings of International Symposium on Industrial Biotechnology, Department of Microbiology, Osmania Univ., Hyderabad.

Sandhya, C., Binod, P., Nampoothiri, K.M., Szakacs, G. and Pandey, A. (2005) Microbial synthesis of chitinase in solid cultures and its potential as a biocontrol agent against phytopathogenic fungus *Colletotrichum gloeosporioides. Appl. Biochem. Biotechnol.,* 127(1): 1-15.

Shehata, A. I. (2008) Phylogenetic diversity of Staphylococcus aureus by Random Amplification of Polymorphic DNA. *Australian J. Basic and Appl. Sci.,* 2(4): 858 – 863.

Stephen, R., Schraft, H. and Untermann, F. (1994) Characterization of *Bacillus licheniformis* with the RAPD technique (Randomly Amplified Polymorphic DNA). *Lett. Appl. Microbiol*., 18 : 260 – 263.

Svitil, A. L., Sinead, M. Nichadhain, Jessica, A. Moore and David L. Kirchman (1997) Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. *Appl. Environ. Microbiol.,* 63(2): 408 - 413.

Tsujibo, H., Orikoshi H. and Shiotani, K. (1998) Characterization of chitinasec from marine bacterium *Alteromonas* sp. strain 0-7 and its corresponding gene and domain structure. *Appl. Environ. Microbiol.,* 64: 472-478.

Tweddell, R. J., Jabaji-Hare, S. H. and Charest, P. M. (1994) Production of chitinases and 1,3-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhizoctonia solani*. *Appl. Environ. Microbiol.,* 60: 489 - 495.

Vipul, G., Singh, A., Vimal, M., Ashwini, P. and Chhatpar, H. S. (2006) Bioprospecting and antifungal potential of chitinolytic microorganisms. *African J. Biotechnol.,* 5(2): 54-72.

Zar, J. H. (1974) Biostatistical analysis, Prentice Hall, New Jersy, pp620.