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Effect of different growth parameters on chitinase enzyme activity of acridine orange and ethidium bromide mutant bacteria of the gut environment

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The degradation of chitin is mediated primarily by bacterial chitinases. 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. Therefore, in the present study, an attempt was made to optimize chitinase production by one of the shrimp's gut mutant bacterial flora, *Pseudomonas alcaligenes*. Optimization of culture conditions revealed that the enzyme production was maximum in pH 7.5 (107.4 ± 0.50 U/ml), temperature 35°C (103.15 ± 1.74 U/ml) when the carbon and the nitrogen sources used were CMC (106.0 ± 1.89 U/ml) and KNO_3 (91.2 ± 1.51 U/ml), respectively. The total chitinase production for all optimum conditions is 105.07 ± 1.33 U/ml.

Key words: Chitinase, shrimp gut, mutant, *Pseudomonas alcaligenes*, optimization condition.

INTRODUCTION

Chitin, a homopolymer of β-1, 4-N-acetyl-D-glucosamine (Glc NAc), is one of the most abundant natural polymers. Recycling of chitin from disposed materials and dead organisms result mainly from the activity of chitinolytic microorganisms (Brurberg et al., 2000; Jindra et al., 2001). Species of the genera *Bacillus, Serratia* and *Vibrio* have been reported to secrete several chitinolytic enzymes and chitin binding proteins, which are thought to degrade chitin synergistically into the extracellular environment (Amit Kumar et al., 2007). 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). Several strains of microorganisms have been selected or genetically modified to increase the efficiency with which they produce enzymes (Okonko et al., 2006).

Chemical agents known to be effective in increasing the rate of mutations in higher organisms have similar

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effects on bacteria. The mutations induced by these agents in bacteria, as in higher organisms, seem to be random and non specific (Witkin, 1946). 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 to identify them and to screen their chitinolytic activity and to examine their enzyme producing ability with varying nutritional sources, pH and temperature.

MATERIALS AND METHODS

Penaeus monodon which inhabits in marine forms and is endemic in Peninsular India and other countries is an important marine crustacean. 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

The weight of 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 on nutrient agar. The plates were incubated at 37°C for 24 to 48 h and the total viable counts (TVC) of colonies were finally noted. The isolated cultures were purified individually by streaking on nutrient agar and were subcultured. Then, the bacterial cultures were identified by performing biochemical tests.

Chitinase activity

The chitinase detection agar (CHDA) (components (g/l) colloidal chitin, 10.0 g; agar, 20.0 g; soya bean powder, 20.0 g; starch, 3.0 g; peptone, 3.0 g; yeast extract, 2.0 g; $CaCO_3$, 1.0 g; M9 medium, Na_2HPO_4, 0.65 g; KH_2PO_4, 1.5 g; NaCl, 0.25 g; NH_4Cl, 0.5 g; $MgSO_4$, 0.12 g; $CaCl_2$: 0.005 g; 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 observed for zone formation. The colonies which formed a zone around them were chitinase positive strains, which were sub cultured regularly for further study.

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

Twenty gram of chitin powder was added into 180 ml of 37% HCl under vigorous stirring for 2 h. It was poured into 1 L of ice cold ethanol (95%) under vigorous stirring for 30 min. 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.

Chemical mutation

The test organisms were inoculated into the nutrient broth and incubated at 37°C for 24 h. After incubation, the organisms were incubated at 37°C for 24 h. After incubation, the organisms were inoculated into Luria Bertani broth and incubated at 37°C for 24 h. The above cultures were serially diluted on normal saline solution and the 10^{-2} dilution was selected and plated into LB agar plates with different chemical mutagens such as ethidium bromide (200 µg/ml) and acridine orange (150 µg/ml) by spread plating technique. In the above concentration of chemical mutagens, growth of *Bacillus cereus* was not formed. Therefore, the concentrations of chemical mutagens were reduced till observations of the growth. Hence, the concentration of ethidium bromide and acridine orange were fixed as 25 and 100 µg/ml, respectively. The plates were then incubated at 37°C for 24 h. After incubation, the number of colonies was noted and the isolated colony from each plate was streaked and subcultured.

Chitinase assay using mutant cultures

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

Mutagenic characterization of microbes

Bacterial culture which showed a maximum chitinolytic activitiy (*Pseudomonas alcaligenes*) was mutated by chemical means. The chemical mutation was done by chemical agents like Ethidium bromide and Acridine orange.

Chitinase assay (Tweddell et al., 1994)

Chitinolytic bacteria were inoculated individually into chitinase liquid medium ((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 culture filtrate 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 incubated 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 chitinase production 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°C) and pH (5.5, 6.5, 7.5 and 8.5).

Statistical analysis

The experiments were conducted following completely randomized design (CRD) with three replications. The significant difference, if any, among the means were compared by Duncan's multiple range test (DMRT). Whenever necessary, the data were transformed before statistical analysis following appropriate methods.

RESULTS

Isolation and identification of gut microflora

The total viable count of bacterial colonies recorded in

Table 1. Total colonies of *Pseudomonas alcaligenes* after chemical mutation with different mutagens**.**

Chemical mutagens	Number of colonies (CFU/ml)
Control (wild strain)	115 ± 0.66^{c}
Ethidium bromide (200)	96 ± 1.20^{b}
Acridine orange (150)	40 ± 0.40^{a}

Values in parenthesis indicate concentration (µg/ml) of chemical mutagens.

the gut samples of shrimps was 115 ± 0.66. Based on the morphological, physiological and biochemical characteristics, seven bacterial strains were identified (*B. cereus, Bacillus polymyxa, Bacillus stearothermophilus, Bacillus circulans* and *Bacillus mycoides*, *Pseudomonas alcaligenes* and *Pseudomonas anguilliseptica*).

Enzymatic characterization of identified bacterial species

All the identified bacterial strains showed a positive chitinolytic activity.

Enzyme production by mutated strains

The maximum level of chitinase producing strain *P. alcaligenes* as per the results obtained in the previous experiments was mutated using ethidium bromide and acridine orange as chemical mutagens.

Chitinase production by mutated *P. alcaligenes*

Chemical mutation

When *P. alcaligenes* mutated chemically with various mutagens, the load of the colonies decreased much when compared with that of the control plate (115 ± 0.66 CFU/ml) (Table 1). Among the tested chemical mutagens, *P. alcaligenes* treated with ethidium bromide recorded a maximum of 96 ± 1.20 CFU/ml, whereas, in the acridine orange treated plate, the number of colonies observed was 40 ± 0.40 CFU/ml, respectively.

Chitinase production at different media temperature

Among the tested chemical mutagens, ethidium bromide treated *P. alcaligenes* produced maximum level of chitinase production at 35°C (118.65 ± 2.77 U/ml) (Table 2). The differences between chitinase production between the changes in chemical mutagens as well as between the changes of media temperature were found statistically significant (F = 11.365 and 26.407).

Chitinase production at different media pH

Within the chemically mutated *P. alcaligenes*, the organisms treated with acridine orange produced a maximum of 107.4 ± 0.50 U/ml at pH 7.5. The wild strain produced a maximum of 74.1 ± 1.26 U/ml at pH 7.5 (Table 3). The influence of different chemical mutagens and also the influence of different media pH on chitinase production were observed statistically more significant (F = 26.192 and 8.888; P < 0.001 to P < 0.0001).

Chitinase production at different carbon sources supplied media

Among the tested chemical mutagens, the test organisms treated with acridine orange exhibited maximum of 106.0 ± 1.89 U/ml when utilizing CMC as the carbon source. At the same time, the wild strain produced a maximum of 79.2 ± 1.14 U/ml by utilizing CMC as the sole carbon source (Table 4). The variation between chemical mutagens and the variation between tested different carbon sources on chitinase production were statistically (two-way ANOVA) significant (F = 14.872 and 11.264; P < 0.001 to P < 0.0001).

Chitinase production at different nitrogen sources supplied media

Among the tested chemical mutagens, *P. alcaligenes* treated with ethidium bromide produced a maximum of 91.2 ± 1.51 U/ml at KNO_3 supplied medium. Invariably, the enzyme production by the control strain was within the range between 63.70 ± 2.35 and 73.2 ± 2.29 U/ml at different nitrogen sources supplied media (Table 5). Chitinase production on the influence of different chemical mutagens as well as different tested nitrogen sources were statistically more significant (F = 16.088 and 9.630; P < 0.001 to P < 0.0001).

DISCUSSION

A variety of methods have been employed to modify enzymes for their industrial usage including strain improvement (Chand et al., 2004). The mutation and screening of industrially useful microorganisms are important for the successful development of the various strains required in the fermentation industry. Acridine dyes are aromatic compound that intercalate within pairs of bases in the DNA molecule favouring insertions and deletions of nucleotide bases upon replication (Kapuscinski and Darzynkiewicz, 1984). In the present study, maximum (118.65 ± 2.77 U/ml) chitinase production was registered by the ethidium bromide treated mutant strain of *P. alcaligenes* grown at the incubation temperature of 35°C. Beyond this

Table 2. Chitinase production (U/ml) by the mutated strain *Pseudomonas alcaligenes* at different temperature.

Chemical mutagens	Chitinase production (U/ml) at different temperature (ºC)			
	30	35	40	45
Wild strain	62.1 ± 2.43[b]	78.4 ± 2.27[a]	59.20 ± 1.54[c]	47.9 ± 0.34[d]
Chemical mutation (CM)				
Ethidium bromide mutant	66.8 ± 1.58[d]	118.65 ± 2.77[a]	93.25 ± 2.95[b]	80.65 ± 3.47[c]
Acridine orange mutant	65.55 ± 0.99[d]	103.15 ± 1.74[a]	96.2 ± 1.85[b]	74.45 ± 2.17[c]

Table 3. Chitinase production by the mutated *Pseudomonas alcaligenes* at different pH.

Chemical mutagens	Chitinase production (U/ml) at different media pH			
	5.5	6.5	7.5	8.5
Wild strain	54.8 ± 1.19[c]	68.2 ± 1.24[b]	74.1 ± 1.26[a]	53.9 ± 2.34[d]
Chemical mutation (CM)				
Ethidium bromide mutant	84.60 ± 0.77[c]	87.30 ± 0.80[b]	88.30 ± 0.82[a]	75.3 ± 1.52[d]
Acridine orange mutant	86.5 ± 0.48[c]	98.95 ± 0.51[b]	107.4 ± 0.50[a]	82.25 ± 0.95[d]

Table 4. Chitinase production (U/ml) by the mutated strain of *Pseudomonas alcaligenes* at different carbon sources media.

Chemical mutagen	Chitinase production (U/ml) at different carbon sources			
	Glucose	Sucrose	Lactose	Carboxymethyl cellulose (CMC)
Wild strain	64.1 ± 1.30[b]	59.4 ± 1.20[c]	48.9 ± 0.86[d]	79.2 ± 1.14[a]
Chemical mutation (CM)				
Ethidium bromide mutant	87.95 ± 2.69[b]	71.5 ± 1.58[d]	83.40 ± 2.47[c]	93.0 ± 3.01[a]
Acridine orange mutant	90.00 ± 7.80[b]	72.85 ± 0.99[d]	75.85 ± 1.55[c]	106.0 ± 1.89[a]

Table 5. Chitinase production (U/ml) by the mutated strain of *Pseudomonas alcaligenes* at different nitrogen sources media.

Chemical mutagens	Chitinase production (U/ml) at different nitrogen sources			
	NH_4Cl	$NaNO_2$	KNO_3	NH_4SO_4
Wild strain	68.5 ± 2.29[b]	73.2 ± 2.29[a]	66.8 ± 2.33[c]	63.70 ± 2.35[d]
Chemical mutation (CM)				
Ethidium bromide mutant	86.35 ± 1.49[c]	88.55 ± 1.49[b]	91.2 ± 1.51[a]	75.20 ± 1.53[d]
Acridine orange mutant	78.05 ± 0.93[c]	90.5 ± 0.93[a]	82.10 ± 0.95[b]	70.9 ± 0.95[d]

temperature, chitinase production reduced. It may be due to the reduction of moisture content in the production medium, subsequently the growth of the organisms was reduced and it leads to reduction of enzyme production (Haq et al., 2010). Lactose- yeast extract in the production medium acted as the best source for chitinase production (25.03% higher than wild strain) by an EMS treated mutant of *Aspergillus terreus* (Narayanan et al., 2013). In contrast, in the present study, the production medium with CMC as the carbon source supported

maximum (106.0 ± 1.89 U/ml) chitinase production by the acridine orange treated mutant of *P. alcaligenes*. It is evident from the present study that the wild strain of *P. alcaligenes* produced only 74.1 ± 1.26 U/ml of chitinase, at the same time, the strain treated with acridine orange produced 107.4 ± 0.50 U/ml enzyme. It is important in epidemiology and ecology to be able to identify bacterial species and strains accurately. Thus, an attempt was made in the present study 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 have the potential for industrial application.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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