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Effective production of chitinase by mutant strains of *Pseudomonas* sp. and its optimization studies

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Abstract

The study was attempted to investigate the production of chitinase from wild and mutant strains of *Pseudomonas sp.* In the present investigation, the organism was isolated from gut of *Penaeus indicus* exhibited optimum chitinase activity at 72 h of batch fermentation. Chitinolytic activity of *Pseudomonas sp* was preliminarily confirmed by zone of clearance on Chitinase detection agar medium, chitinase production was carried out using chitinase production medium. Inducible effect of different carbon and nitrogen sources, pH and temperature were studied on chitinase enzyme production. Addition of lactose and Ammonium suphate in the production medium further improved chitinase production. The optimum temperature and pH on enzyme production was 40^{0}C and 6.0 respectively. The strain was mutated using Ampicillin and mutants exhibited a improved chitinase production when compared to wild type. Maximum chitinase production of 20.7 U/ml was observed in lactose and ammonium sulphate supplemented medium at 40^{0}C (pH 6.0) by the ampicillin mutants of *Pseudomonas sp*.

Keywords

chitinase, mutant strains, *Penaeus indicus*, *Pseudomonas* sp

Introduction

Chitin is one of the most abundant natural non-curable polysaccharides consisting of â-(1-4)-linked N-acetyl glucosamine units, which play an important role for the degradation of chitin. It is the major structural component of molluscs, insects, fungi, algae and marine invertebrates (Skaikh and Despande, 1993; Roberts and Selitrennikoff, 1988). It is widely distributed in bacteria, actinomycetes and plants (Vegauwen and Van Laere, 1997; Watanabe *et al.,* 1990; Yabuki *et al.,* 1986; Sakai *et al.,* 1998). The annual global yield of chitin is assumed to be 1 to 100 billion metric tonnes making chitin the second most abundant polysaccharide on the earth after cellulose (Brurberg *et al.,* 1996) and it largely exists in wastes from the processing of marine food products (crab, shrimp and krill shells) and about 10^{11} tonnes of chitin is produced annually in the aquatic biosphere alone. The waste generated from the worldwide production and processing of shell fish is a serious problem of growing magnitude.

Many bacteria and fungi produce extracellular chitinolytic enzymes known as chitinases, able to convert chitin into compounds (Metcalfe *et al.,* 2002) that can be of industrial interest, mainly N-acetyl-D-glucosamine. They have contributed to the recycling of vital carbon and nitrogen resources (Revah and Carroud, 1981). Several species of bacteria such as *Bacillus pabuli* (Frandberg and Schnurer, 1994), *B. licheniformis* (Takayanagi *et al.,* 1991) and mainly *Serratia marcescens* (Monreal and Reese, 1969) have shown chitinase producing ability. Chitinase promise to be safer pesticide and microbial biocontrol agents and its degradation products have a wide variety of medical and biological applications, chitin and chitinases are receiving more attention from biologists.

Materials and methods

Isolation and Identification of chitinolytic bacteria

The bacterial strain was isolated from the gut of *Penaeus indicus*, and was identified as *Pseudomonas* sp according to Bergey's manual of determinative bacteriology.

Preliminary screening of chitinolytic activity

Chitinolytic activity of *Pseudomonas sp* was initially screened on Chitinase detection agar medium. *Pseudomonas sp* was inoculated on agar medium

consisting (g/l) of Na_2HPO_4 - 0.65, KH_2PO_4 - 1.5, NaCl - 0.25, NH_4Cl - 0.5, $MgSO_4$ - 0.12, $CaCl_2$ -0.005, pH maintained at 6.5 and incubated at temperature of 37°C±2 for 6 days. After incubation the tributyrin agar plates were observed for zone of clearance (chitinolytic activity) around the colony.

Chitinase production

Chitinase production was carried out using Chitinase production medium (pH 5.0) containing Soybean powder – 20, Starch – 4, Peptone – 3, Yeast extract – 2, KH_2PO_4 – 0.3, $MgSO_4$ – 0.3, $CaCO_3$ – 1, Colloidal chitin – 10 (g/l). Inoculated production media was incubated under shaking condition at 100rpm at 37±2ºC. Extracellular enzyme production was checked after 72h. The culture filtrate was collected by centrifugation and the supernatent was assayed for chitinase productivity using Dinitrosalicylic acid (DNS) method.

Effect of different substrate on Chitinase production

To determine the suitable substrate for the production of Chitinase by *Pseudomonas sp*, ten different carbon (Glucose, Sucrose, lactose, Fructose, Maltose, Starch, Galactose, Xylose, Mannitol and Cellulose) and nitrogen sources (Ammonium chloride, Ammonium sulphate, Ammonium nitrate, Sodium nitrate, Potassium nitrate, Potassium sulphate, Peptone, Casein, Beef extract and Skim Milk) were used as substrates They were individually tested by replacing the substrate present in the above said medium at the concentration of 1%.

Effect of pH and temperature on chitinase productivity

The effect of pH and temperature on chitinase production was also determined by incubating the culture flasks with different pH (5, 6, 7, 8, and 9) and temperature (25, 30, 35, 40, 45, and 50 °C).

Enzyme production by mutant *Pseudomonas* sp

Generation of mutants was carried out using Ampicillin. 2 ml of overnight bacterial cultures were pelleted out, washed twice with saline and was resuspended in 2 ml of the saline buffer. Antibiotic was added to give a final concentration of 50ìg/ml and incubated at 37^{0}C in waterbath for 30 mins. The cultures were inoculated on Luria Bertani agar plates for production of mutant generation. The effect of pH, temperature, carbon and nitrogen sources on chitinase enzyme production by mutant strain was also performed according to standard procedures.

Results

Preliminary screening of chitinolytic activity

Pseudomonas sp hydrolysed colloidal chitin after 72 h of growth on chitinase detection agar medium supplemented with colloidal chitin as the sole carbon source. Large zones of clearing around the growing bacteria were observed and it is evident that, the *Pseudomonas* sp was able to produce chitinase.

Chitinase production

Preliminary experiments with colloidal chitin were carried out to detect exochitinase activity directly in supernatants of Pseudomonas wild-type and mutant culture. On comparison the mutant strain exhibited more chitinase production than the wild type strain. Chitinase production was determined by assaying chitinase activity in crude culture filtrate at standard assay conditions. Production parameters like effect of pH, temperature, different substrates (carbon and Nitrogen sources), were studied to maximize the chitinase production by *Pseudomonas sp.*

Effect of different substrate on Chitinase production

Among the ten different carbon tested for chitinase production, wild type and mutant strains produced maximum chitinase when supplemented with lactose and sucrose as carbon source. Morever, in mutant strain the production was again increased to 20.3% and 12.07% in lactose and sucrose supplemented medium respectively. Nitrogen sources also showed a considerable chitinase production by mutant strain than wild type strain. Wild strain produced maximum chitinase when supplemented with Ammonium sulphate as nitrogen source. Further, the production was enhanced to 28.86% by mutant at the same nitrogen source. The results were shown in Fig 1 and 2.

Effect of pH and temperature on chitinase productivity

For optimization of initial pH of the production medium for chitinase production, fermentation was carried out at different pH from 5.0 to 9.0. The effect of the initial pH of the medium on chitinase production by *Pseudomonas sp* indicated a linear increase from pH 5.0 to 6.0 and the maximum chitinase production of 5.4 units/ml was seen in the pH of 6.0 (Fig. 2). When the pH increases further, the chitinase production gradually decreased. However the enzyme production by mutant strain was 9.2 units/ml at pH 6.0 and there was observed an 41.3% increase in chitinase production when comparing with wild type. One unit of the enzyme activity was defined as the enzyme amount releasing 1 µmol of N-acetylglucosamine per minute.

Incubation temperature plays an important role in the metabolic activities of microorganism. Optimization was carried out by incubating the fermentation flask at 25°C, 30°C, 35°C, 40°C, 45°C, 50°C and maximum chitinase production by *Pseudomonas sp* was observed at 40°C (6.3 units/ml), the temperature increased the chitinase production until 40^{0}C and gradually reduced thereafter. The mutant strain produced 20.3% (11.7 units/ml) more increased chitinase than the wild strain. The results were depicted in Fig 3 and 4.

Fig 1: Chitinase enzyme production by wild and mutant *Pseudomonas* sp supplemented by ten different carbon sources

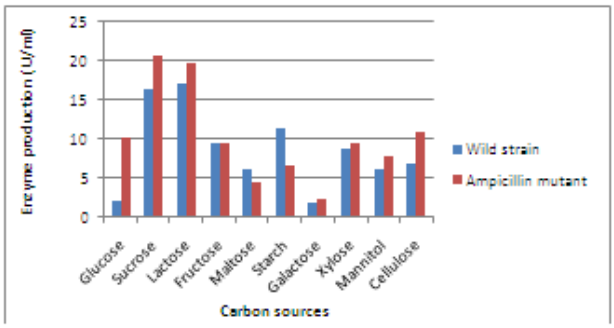


Fig: 2: Chitinase enzyme production by wild and mutant *Pseudomonas* sp supplemented by ten different nitrogen sources

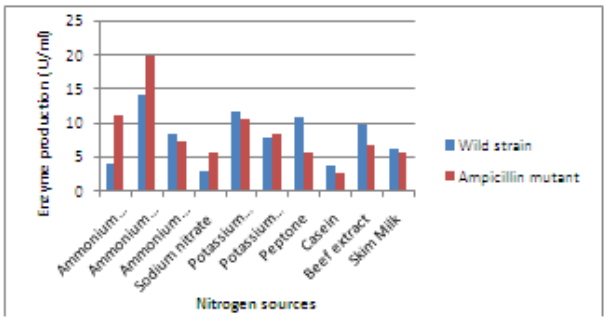


Fig 3: Chitinase enzyme production by wild and mutant *Pseudomonas* sp at the different medium pH

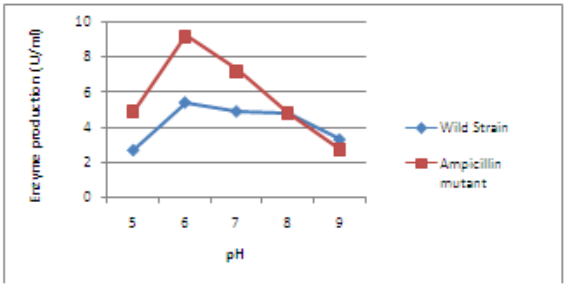
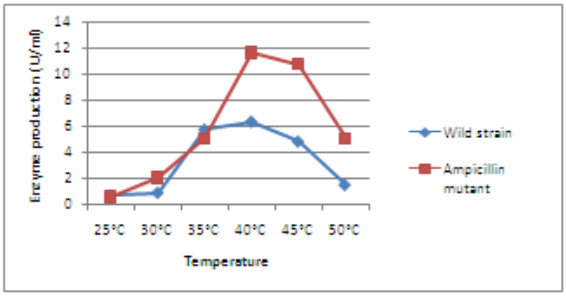


Fig 4: Chitinase enzyme production by wild and mutant *Pseudomonas* sp incubated at different temperatures



Discussion

In the study, an extracellular chitinase secreted by *Pseudomonas* sp under various environmental parameters were analysed. The chitinase production was increased manifold through strain improvement using ampicillin. Chitinase from *Pseudomonas* sp was produced optimally at 40^{0}C (pH 6.0). The pH optima reported for other chitinases were pH 4.0 for *Aeromonas* sp (Veda *et al.,* 1995), pH 5.0 for *Alcaligens xylosoxydans* (Vaidya e al., 2001), pH 5.5 for *Bacillus* sp. (Woo and Park, 2003), pH 6.0 for *Enterobacter* sp (Park *et al.,* 1997), pH 6.3 for *Bacillus* sp (Wen *et al.,* 2002), pH 6.5 for *Vibrio alginolyticus* (Ohishi-Kazoo *et al.,* 1996). *Pseudomonas sp* chitinase revealed that the enzyme was optimally active at 40^{0}C. The temperature optimum of *Pseudomonas* sp was in accordance with other reports in literature such as *Vibrio alginolyticus* (Ohishi-Kazoo *et al.,* 1996), *Arthrobacter* sp. NHBN-10 (Okazaki *et al.,* 1999) and *Vibrio* sp (Takahashi *et al.,* 1993). The optimum pH and temperature for chitinase production is thus near neutral since neutral chitinase is very useful for industrial applications performed at neutral conditions such as production of chitin oligosaccharides for medical purposes.

Results on the effect of carbon and nitrogen sources on the basal medium for the production of chitinase showed that it is an inducible enzyme. Chitinase production was increased by inducing substrate such as lactose and ammonium sulphate other than poly- and di-saccharide substituted medium. The amount of enzyme produced by inducible substrate is very high. The finding were consistent with that of El-Katatny *et al.* (2000), who found that specific activites of enzyme were secreted in the presence of cellobiose, lactose or xylose.

Here the ampicillin mutant strains produced many fold increase in chitinase production. The increase in enzyme production by the mutant strains may be due to genetic changes occurred by the action of ampicillin. Ampicillin was used by various researchers to increase the product formation. Dwight *et al.* (1988) used ampicillin and kanamycin for his mutagenesis study. Lampe *et al.* (1982) used ampicillin and reported an increase in enzyme production. It was evidenced from the above study that the ampicillin inducibility of the chitinase enzyme system of *Pseudomonas sp* had a positive effect.

Conclusion

The present study revealed that extracellular chitinase production by *Pseudomonas* sp was found to be accelerated at optimized culture conditions such as medium pH, temperature and various substrates (carbon and nitrogen sources). From the results, it could

be concluded that the medium pH of 6.0 and temperature of 40 °C were optimum for maximizing chitinase production by *Pseudomonas sp.* The assessment of various substrates for optimizing the production of chitinpase by *Pseudomonas sp* inferred that lactose and ammonium sulphate were the best substrate with the optimum concentration of 1 %. Moreover, the chitinase production was considerably increased when mutated by ampicillin. Further research is necessary in this aspect to determine the effect of ampicillin on chitinase enzyme production.

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