Chitinolytic bacterial isolation, extracellular chitinase production and its optimization

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ABSTRACT

Chitinases are enzymes which perceive and hydrolyze chitin, a linear homopolymer of N-acetyl-D-Glucosamine. The bacteria produce a few chitinases, most likely to hydrolyze the decent variety of chitins found in nature. Chitinases have demonstrated various applications in squander treatment and the executives of shellfish processing ventures. Thus, in the current investigation, an endeavour was made to produce chitinase by the shrimp's gut bacteria.Chitinase production was found to be more (76.4 ± 0.01 U/ml) when the test strain P. alcaligenes was maintained at pH 7.5, followed by 72.0 ± 0.04 U/ml of enzyme produced by B. polymyxa at pH 6.5. The chitinase production decreased absolutely (39.3 ± 0.10 U/ml) when B. circulans was grown in the production medium was maintained at pH 8.5.The influence of carbon sources on chitinase production by the test organisms indicated that P. alcaligenes was found to be more (77.5 ± 0.24 U/ml) chitinase producer at CMC supplemented medium, whereas, the production was very much reduced (36.25 ± 1.00 U/ml) in sucrose added medium by B. polymyxa.

Key words: Bacteria, chitinase production, optimization.

INTRODUCTION

Microorganisms specifically have been viewed as fortune wellsprings of helpful enzymes (Shimizu et al., 1997). Naturally dynamic enzymes might be removed from living life forms. Enzymes are proteins which require a particular substrate on which to work (Okonko et al., 2006). Extracellular enzymes are liked, in light of the fact that troublesome and exorbitant strategies for cell disturbance are a bit much for their extraction. Microbial enzymes present a wide range of qualities that make them utilizable for very explicit applications (Hossain et al., 2005). Enzymes are steady and along these lines will stay dynamic over a wide scope of pH (PiaDesantis, 1983). They are typically equipped for processing insoluble supplement materials, for example, cellulose, protein and starch and the processed items are moved into the phone where they are utilized as supplements for development. A significant number of the *Bacillus* species produces an assortment of enzymes (Ajaya and Fagade, 2003). Various microbial sources may deliver enzymes,

however just a couple of chosen strains of bacteria and fungi may meet the criteria for business production (Reddy et al., 2003).The modern production of enzymes from microorganisms includes refined them in colossal tanks where enzymes are emitted into the fermentation medium as metabolites of microbial action (Okonko et al., 2006).Pandey et al. (2004) expressed that enzymes are among the most significant bio products and are being used in countless procedures in the zones of mechanical, natural and nourishment biotechnology. Also, current improvements in biotechnology are yielding new applications of enzymes.The enzymes are currently being applied to plant insurance from parasitic pathogens and creepy crawly pests (Fukamizo, 2000). Enzymes are basic in the digestion of every single living organism and are broadly applied as preparing helps in the nourishment and the refreshment industry (FAO, 2000, 2004; BREI, 2006).The interest for mechanical enzymes, especially of microbial inception is consistently expanding attributable to their applications in a wide assortment of procedures. Enzyme interceded responses are alluring options, in contrast to dull and costly synthetic strategies.Enzymes discover the incredible use in countless fields, for example, nourishment, dairy, pharmaceutical, cleanser, material and restorative enterprises and in biotechnology. (Saxena et al., 1993).

Chitin, a homopolymer of-1, 4-N-acetyl-D-Glucosamine (GlcNAc), is one of the most copious common polymers. This polymer is available as an auxiliary part in the exoskeletons of bugs, in the shells of crustaceans, in the cell dividers of numerous fungi, algae and furthermore in nematodes.Reusing of chitin from arranged materials and dead organisms result for the most part of the movement of chitinolytic microorganisms (Brurberg et al., 2000; Jindra et al., 2001; Wang et al., 2001; Wen et al., 2002).Chitin is potentially thesecond richest polysaccharide in nature, after cellulose (Nelson and Cox, 2000; Brurberg et al., 2000; Sheng et al., 2002). Chitin is light, white or yellowish shaded, fine/flaky (Subasinghe, 1999). The fish business goes about as a significant wellspring of chitinous squanders, the reusing of which is critical to hold the carbon-nitrogen balance in the biological system (Tsujibo et al., 1998; Reguera and Lesehine, 2003). The solvency of chitin and its inertness to most chemical operators requires the utilization of most possible biological procedures. The huge amounts of fish mechanical squanders can be bioconverted utilizing the enzyme chitinase, which break down the chitin (Nawani et al., 2002).The debasement of chitin is intervened principally by bacterial chitinases (Gary Lecleir et al., 2004). For endurance of amphibian biological systems, chitin is quickly catabolized by marine bacteria (Li and Roseman, 2004). Chitinases are enzymes which perceive and hydolyzed chitin, a linear homopolymer of N-acetyl-D-Glucosamine. Chitinases are omnipresent in nature, being found in eukaryotes, prokaryotes, archaea and viruses (Suzanne et al., 2001). They are created by bacteria, fungi, plants, insects and even mammals.As indicated by the attributes of hydrolyzing chitin, the chitinases are ordered into two sorts, exochitinase and endochitinase (Nielsen and Sorensen, 1999). The bacteria produce a few chitinases, likely to hydrolyze the assorted variety of chitins found in nature (Svitil et al., 1997).

Enzyme chitinases has a place with glycosyl hydrolases which hydrolyze the chitin to its monomer N-acetyl Glucosamine by breaking the glycosidic bonds. Chitin is a linear polymer which exists broadly in contagious cell dividers, arthropod exoskeletons, insect

cuticles, shell fishes, mammals and plants.Chitinases are put into two general classifications as endo-chitinases and exo-chitinases. Chitinases are delivered in three unique methods of fermentations - batch, fed batch and nonstop fermentations. Strong state fermentations and submerged fermentations are utilized. Entire cell immobilization was likewise accomplished for chitinase production. It assumes a wide job in parasitism, nutrition, morphogenesis, basic jobs, and protection and a vitality source of bacteria, fungi and plants. A portion of the regularly utilized hotspots for chitinase production are insects, plants, mammals, bacteria and fungi. Chitinases are created in a wide scope of pH from 4 to 8. Optimum temperature, extend for chitinase fluctuates from 30ºC to 50ºC.

Chitinase enzymes has an expansive of uses as biocontrol specialist, morphogenesis, bioconversion of waste containing chitin, pollution corruption, mosquito control, fungal biomass estimation, protoplast confinement and bio pesticides, antifungal operators and furthermore in skin moisturizers and creams for fungal infections.

In spite of the fact that significant data is accessible concerning the intestinal microflora of homeotherms and the job of these intestinal microfloras in processing, next to no data is accessible identified with the bacterial populace in the gastrointestinal tract of poikilotherms and their effectiveness in integrating explicit enzyme. The crustaceans, particularly the shrimps are not satisfactorily concentrated right now. Along these lines, the current examination was embraced with the accompanying goals: to confine and distinguish out all the oxygen consuming heterotrophic bacterial populace in the gastrointestinal tract of shrimp *Penaeus monodon* and to screen the chitinolytic qualities of the recognized bacteria.

MATERIALS AND METHODS

Collection of experimental animal

The tiger shrimps, *Penaeus monodon* were gathered from a nearby shrimp ranch at Rajakkamangalam, Kanyakumari District, Tamilnadu. The gathered shrimps were aseptically moved to the laboratory for additional investigation.

Isolation, identification and screening of gut bacterial flora

In the laboratory, the weight of the entire shrimp was noted and the gut samples of shrimps were aseptically dissected out. At that point the length and weight of the gut were estimated separately. The gut tests were pooled and serially diluted up to 10-5 dilution. From every dilution, 0.1 ml of the test 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 count (TVC) of the colonies were at long last noted. The detached cultures were sanitized separately by streaking on nutrient agar plates and were sub cultured. At that point the bacterial cultures were identified by performing biochemical tests like,Gram's staining, motility, indole production, methyl red, Voges- Proskauer, citrate utilization, oxidase, urease, triple sugar iron, fermentation of carbohydrate, starch hydrolysis, casein hydrolysis, gelatin hydrolysis, catalase, nitrate reduction, etc.

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 ; CACO3 : 1.0 g ; : M9 medium: Na2HPO4 : 0.65 g ; KH2PO4 : 1.5 g ; NaCl : 0.25 g ; NH4Cl : 0.5 g ; MgSO4 : 0.12 g ; CaCl2 : 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℃ 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 (Plate 7).

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 lt of ice cold ethanol (95%) under vigorous stirring for 30 minutes. This suspension was stored at 20℃ 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.

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 102 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. The five strains *Bacillus cereus*, *B. polymyxa*, *B. stearothermophilus*, *B. circulans* and *B. mycoides* belong to Gram positive group and the two strains *Pseudomonas alcaligenes* and *P. anguilliseptica* belong to Gram negative group.

Enzymatic characterization of identified bacterial species

All the identified bacterial strains showed chitinolytic positive activities.

Chitinase production

The efficacy of chitinase production by the test organisms was determined and it is presented in. At normal condition, the chitinase production ability of *P.alcaligenes* was more (72.0 ± 0.41 U/ml), followed by 67.5 ± 0.20 U/ml of chitinase produced by *B. stearothermophilus*. At the same time, the lowest (57.0 U/ml) level of chitinase produced by two organisms viz. *B. polymyxa* and *B.circulans*, respectively. The chitinase production capacity of the tested strains as observed from one-way ANOVA was found significant ($F = 645.32$; $P < 0.0001$). The chitinase production by all the test organisms was also determined at various temperatures. It was found that 35℃ was optimum for maximum (76.6 ± 0.09 U/ml) chitinase production by *P. alcaligenes*. The minimum (39.8 ± 0.14 U/ml) level of chitinase was produced by *P. anguilliseptica* when it was grown at 45℃. Two-way ANOVA conducted for

the variation between the test organisms as well as for the variation between tested temperature on chitinase production was more significant ($F = 11.895$ & 132.191 ; $P < 0.0001$). Chitinase production was found to be more (76.4 ± 0.01 U/ml) when the test strain *P. alcaligenes* was maintained at pH 7.5, followed by 72.0 ± 0.04 U/ml of enzyme produced by *B. polymyxa* at pH 6.5. The chitinase production decreased absolutely (39.3 ± 0.10 U/ml) when *B. circulans* was grown in the production medium was maintained at pH 8.5. The results on chitinase production revealed that the differences between the test organisms and the differences between the tested media pH was statistically (two-way ANOVA) more significant ($F = 7.510$ to $P < 0.001$ to $P < 0.0001$). The influence of carbon sources on chitinase production by the test organisms indicated that *P. alcaligenes* was found to be more (77.5 ± 0.24 U/ml) chitinase producer at CMC supplemented medium, whereas, the production was very much reduced (36.25 ± 1.00 U/ml) in sucrose added medium by *B. polymyxa*. The variation among the tested strains as well as the variation among the tested carbon sources on chitinase production was statistically (two-way ANOVA) significant ($F = 2.729$ &9.438 ; $P < 0.05$ to $P < 0.001$). Among the tested nitrogen sources, *P. alcaligenes* produced more amount (72.5 ± 0.12 U/ml) of chitinase by utilizing $NaNO_2$, followed by 70.0 ± 1.14 U/ml of chitinase produced by the same organism in NH_4Cl supplemented medium. But the chitinase production by *B.mycoides* was poor (42.5 ± 0.13 U/ml) when grown at KNO_3 supplemented medium. The influence of the test organisms on chitinase production was statistically significant ($F= 2.724$; $P < 0.05$). At the same time, the influence of nitrogen sources on chitinase production was statistically not significant (F 0.1933 ; $P > 0.05$).

DISCUSSION

The digestive tract of aquatic organisms are colonized by a great number of heterotrophic bacteria and they are responsible for production of certain digestive enzymes (Preetha and Palavesam, 2002). Bairagi et al. (2002) reported that a distinct microbial source of digestive enzymes such as, amylase, cellulase, lipase and protease, chitinase were found in the gut of Tilapia and Carp. To know much about gut microflora and their enzyme production capabilities, the present study was undertaken. Moriarty (1999) stated that *Bacillus* sp. are generally present in the sediment, on which shrimp are feeding and they colonize in the gut of shrimp Similarly Hoshino et al. (1997) isolated a *Pseudomonas* sp. from the intestine of fish which can capable of producing protease enzyme. In the present study also, different Bacillus sp. (*B. cereus*, *B. polymyxa*, *B. stearothermophilus* and *B. mycoides*) and *Pseudomonas sp.* (*P.alcaligenes*and *P. anguilliseptica*) were isolated and identified from the gut of shrimp *P.monodon* and their ability on production of five different enzymes namely amylase, protease, lipase, cellulose and chitinase were studied. In accordance with these, a wide variety of gram negative and gram positive bacterial species were reported to produce different gut enzymes (Gilbert, 1993; Gupta et al., 2004; Vandana and Bera, 2005).

In the present study, the gut microflora of selected shrimp *Penaeusmonodon* was studied and the production of important enzyme chitinase by these microflora and their mutants were also investigated. Isolation and identification of bacterial population in the gut of shrimp *P.monodon* was performed. The isolated bacteria were identified through different physiological and biochemical characteristics. Based on the mentioned characteristics, five

probiotic strains of *Bacillus sp.* (*B. cereus*, *B. polymyxa*, *B. stearothermophilus*, *B. circulans* and *B. mycoides*) and two *Pseudomonas sp.* (*P. alcaligenes* and *P. anguilliseptica*) were identified.

There are various factors that influence the nature of microbial metabolic process and enzyme production. It was reported that the medium composition is one of the factors that affects amylase production as well as sporulation in some *Bacillus* species (Strivastava and Baruah, 1986). Research efforts have also been directed mainly towards evaluating the effect of various nutritional sources like carbon and nitrogen sources and their cost effectiveness on the yield of enzyme production through fermentation process and also to optimize the environmental parameters such as pH, temperature, aeration, agitation, etc. for enzyme production through fermentation process (Adinarayana and Ellaiah, 2002). Fermentation temperature usually has a profound effect on the level of enzyme produced in the medium (Riaz et al., 2009). Similarly, pH of the growth medium also plays an important role by inducing morphological changes in microbes and in enzyme secretion (Mrudula and Kokila, 2010). The pH change observed during growth of microorganisms also affects the product stability in the medium (Rani et al., 2003). Supplementation of carbon sources in the form of monosaccharides, disaccharides and polysaccharides to the production medium shows different impact on enzyme production with different compounds (Mrudula and Kokila, 2018). Chitinase was produced from colloidal chitin (Mohammed et al, 2019).

In this study it was resulted that colloidal chitin, flake chitin and powder chitin were the mostly good source of for the production of microbial enzyme as well as in chitinase. And the application of chitinase was also evaluated.

SUMMARY

The gastro intestinal tract of fish can be generally described as a hollow tube into which food enters, processed and absorbed by the animals. Within this tract, there is a very large stable population of bacteria that are able to survive. The different species of bacteria that live within the gut lumen of vertebrates and invertebrates are termed as gut microflora. These microbes produce different enzymes needed for the breakdown of numerous dietary constituents in the gut and they have been regarded as treasure sources of useful enzymes. A variety of microorganisms such as bacteria, fungi, yeasts and actinomycetes are responsible for the production of gut enzymes. Considerable information are available concerning the intestinal microflora of homeotherms and also the role of those intestinal microflora in digestion; but very little information is available concerning the bacterial population in the gastrointestinal tract of poikilotherms. As shrimps are not adequately studied in this respect, the present study was undertaken.

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