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Utilization of prawn waste for chitinase production by the marine fungus *Beauveria bassiana* by solid state fermentation

P.V. Suresh* and M. Chandrasekaran

Prawn waste, a chitinous solid waste of the shellfish processing industry, was used as a substrate for chitinase production by the marine fungus *Beauveria bassiana* BTMF S10, in a solid state fermentation (SSF) culture. The process parameters influencing SSF were optimized. A maximum chitinase yield of 248.0 units/g initial dry substrate (U/gIDS) was obtained in a medium containing a 5:1 ratio (w/v) of prawn waste/sea water, 1% (w/w) NaCl, 2.5% (w/w) KH_2PO_4, 425–600 μm substrate particle size at 27 °C, initial pH 9.5, and after 5 days of incubation. The presence of yeast extract reduced chitinase yield. The results indicate scope for the utilization of shellfish processing (prawn) waste for the industrial production of chitinase by using solid state fermentation.

Key words: *Beauveria bassiana*, chitinase, marine fungus, prawn waste, solid state fermentation.

Shellfish processing is one of the major agro-industries based on aquaculture, and has assumed great importance in recent years due to the ever-increasing demand for shrimps and crabs. The industries, however, are faced with severe problem in disposing of the formidable quantity of shellfish solid wastes (Nirmala 1991). About 14–27% of the dry weight of shrimp and 13–15% of crab processing waste, respectively in chitin (polymer of *N*-acetyl-D-glucosamine) depending upon the processing method (Ashford *et al*. 1977). The conventional method of seafood processing includes chitin disposal by ocean dumping, incineration and land filling. However, factors such as cost of transportation and environmental pollution have prompted the search for alternative disposal methods (Vyas & Deshpande 1991; Nirmala 1991). Bioconversion of waste is probably the most cost-effective and environment friendly procedure for waste utilization (Healy *et al*. 1994). A number of possibilities for bioconversion of shellfish waste have been reported (Hedges & Wolf 1974; Carroad & Tom 1978; Revah-Moiseev & Carroad 1981; Vyas & Deshpande 1991) but each of these have problems of practicability or economics.

Solid state (substrate) fermentation (SSF) is a low-cost technology fermentation process, particularly suitable for the needs of developing countries (Tengerdy 1992). The commonly used substrates in SSF include plant and animal products with high carbohydrate and/or protein content (Aidoo *et al*. 1982), but the widely exploited substrates for SSF are mostly of plant origin (Smith & Aidoo 1988). The prawn waste can also be used as substrate for SSF. Marine microorganisms, by virtue of their ability to adsorb onto solid particles, are ideal candidates for use in SSF (Chandrasekaran 1994). Exploitation of marine microorganisms employing SSF and utilizing fishery and prawn waste as solid substrate would revolutionize industrial biotechnology, and solve the problem of solid waste disposal (Chandrasekaran 1994, 1996; Balakrishnan & Pandey 1996).

The production of microbial chitinase (1, 4-β-poly-*N*-acetylglucosaminidase, EC 3.2.1.14) has received attention as one step in a bioconversion process to treat shellfish waste and the production of single cell protein for animal and aquaculture feed. The production of chitinase is thought to be one of the primary economic variables in the bioconversion of chitin, accounts for 12%

The authors are with the Microbial Technology Unit, Department of Biotechnology, Cochin University of Science and Technology, Kochi-682 022, India; fax: (+91) 484 532495. M. Chandrasekaran is now with the Department of Fermentation Technology, Hiroshima University, Hiroshima 739, Japan. *Corresponding author.

of the total production cost (Casio *et al*. 1982) and is presently uneconomic due to the high prices of the commercially available chitinase. Therefore, a more effective and viable process is essential for chitin utilization and the management of shellfish waste. In India 60,000–80,000 tonnes of chitinous wastes are produced annually (Madhavan *et al*. 1986). These may be converted into value-added products such as animal and aquaculture feed (Vyas & Deshpande 1991) or they can be used as the substrate for SSF production of enzymes (Chandrasekaran 1994, 1996; Balakrishnan & Pandey 1996). Bioconversion through SSF would be the right choice (Yang 1988) if economic utilization of prawn waste is desired. In the present study an attempt has been made to utilize the solid waste from the prawn-processing industry for chitinase production through SSF.

Materials and Methods

Substrate for SSF

Sun-dried prawn waste was kindly provided by the Central Institute of Fisheries Technology, Kochi, India. It contained 39.74% protein, 23.08% chitin, 5.054% fat and 21.13% ash on a dry weight basis (Madhavan & Nair 1975). This material was dried in a mechanical dryer at 50 ± 2 °C to a constant moisture content (10%), milled to < 425 μm particle size and stored in a dry place at room temperature. It was used as solid substrate for SSF without demineralization or deproteinization.

Microorganism and Inoculum Preparation

The fungus *Beauveria bassiana* BTMF S10 isolated from marine sediment of Kochi, India was used (Suresh 1996). It was grown on Bennet's agar prepared in 50% aged sea water and preserved at 4 °C. The culture was subcultured monthly. For inoculum preparation 10 ml of sterile distilled water containing 0.1% (w/w) Tween-80 was added to the newly raised agar slant containing conidia (2-weeks-old). The conidia were suspended by means of a sterile platinum loop. The concentration of conidia was adjusted to 23×10^6 c.f.u./ml suspension and used as inoculum.

Solid State Fermentation

Solid substrate (5 g) was taken in petri plates (86 mm diameter and 17 mm height), mixed with aged sea water to a solid/liquid ratio of 5:2 (w/v) and autoclaved at 121 °C for 1 h. After cooling to room temperature (28 ± 2 °C) it was inoculated with 2 ml of inoculum and incubated at 27 °C at 90% relative humidity.

Enzyme Extraction

After incubation, the fermented solid from the petri plates were transferred to 250 ml conical flasks, mixed with 25 ml (5 × volume, based on initial dry weight of the substrate) distilled water and mixed thoroughly on a rotary shaker (150 rev/min) for 30 min. The entire contents were squeezed through a cheese cloth (Nagendra & Chandrasekaran 1996). After extracting twice the extracts were pooled, centrifuged at 4 °C for 20 min at 10,000 rev/min and the clear supernatant was used as crude enzyme for various assays.

Analytical Procedures

Chitinase activity was measured by incubating 1 ml of enzyme solution with 5 mg of colloidal chitin in 1 ml of 0.1 M citrate–phosphate buffer (pH 6.0) at 37 °C for 2 h. The reaction was terminated by placing the tubes in a boiling water bath for 5 min, and the undigested material was removed by centrifugation at 5000 rev/min for 5 min. The reducing sugar produced was measured colorimetrically using the dinitrosalicylic acid (DNS) reagent (Miller 1959) with *N*-acetyl-D-glucosamine as standard. One unit of chitinase activity was defined as the amount of enzyme that releases 100 μg equivalent of reducing sugar under the reaction conditions (Pegg 1988). Enzyme yield was expressed as units/g initial dry substrate (U/gIDS).

Colloidal chitin was prepared by the method described by Gernot (1983). Reducing sugar of enzyme extract was measured using the DNS reagent (Miller 1959) with glucose as standard. The dry weight of the substrate and moisture contents were determined by weight after drying samples at 105 °C over night. The pH of the solid substrate was measured according to the procedure of Raimbault & Alezard (1980) as follows: 1 g of prawn waste was added to 50 ml of distilled water and mixed thoroughly using a blender; the mixture was allowed to settle and the pH was measured at room temperature using a standard digital pH meter (Systronic, India).

Optimization of Factors Influencing Chitinase Production

The protocol adopted for the standardization of fermentation parameters was to evaluate the effect of an individual parameter and to incorporate it at the standardized level before standardizing the next parameter (Sandhya & Lonsane 1994). The effect of initial moisture content was studied in the range of 5:0.5–5:4 ratio (w/v) prawn waste/sea water (initial dry weight basis). The effect of incubation temperature was evaluated in a range of 22–42 °C. The initial pH of the medium was varied between 6.0 and 10.5 by adding either 1 M HCl or NaOH to sea water before using it to moisten the medium. The effect of NaCl concentration was studied by adding different concentrations of NaCl (0–10% w/w, based on the initial dry weight of substrate) directly to the prawn waste before moistening with sea water. The effect of phosphate concentration was studied by using different concentration of either K_2HPO_4 or KH_2PO_4 (w/w based on dry weight of the substrate) alone and in various combinations (w/w). The influence of particle size of the substrate was evaluated by using prawn waste of varying size (< 425, 425–600, 600–1000, 1000–1400 and 425–1400 μm). Effect of additional nitrogen sources (yeast extract) 0–4% w/w (based on initial dry weight of the substrate) was also evaluated. Finally, the time-course of chitinase production was studied by using the standardized conditions for a period of 168 h. All experiments were conducted in triplicate and the average values are reported. Key results were repeated three times to establish their validity.

Results and Discussion

The *B. bassiana* strain BTMF S10 selected for this study was known to produce higher titres of chitinolytic enzyme in SSF using other solid substrates such as wheat bran (Suresh 1996). Visual observation showed that fungus grew uniformly throughout the moist prawn waste medium as a white cottony mass and tolerated

adverse conditions including the high protein, $CaCO_3$ and presence of other components. Naturally, the marine fungi are adapted to grow in nutritionally diluted environments under submerged conditions (Molitoris & Schaumann 1986). But in an arbitrarily selected prawn waste medium, *B. bassiana* produced 21.2 U/gIDS of chitinase after 5 days of incubation.

Factors Influencing Enzyme Production

Initial Moisture Content. Initial moisture content significantly affected the hydrolytic enzyme production in SSF (Nishio *et al*. 1979) and the moisture content of the medium is a critical factor that determines microbial growth and product formation in SSF (Ramesh & Lonsane 1990). Figure 1 shows effect of initial moisture content on the chitinase production by *B. bassiana*. The enzyme production was highest in prawn waste medium with a 5:1 ratio of prawn waste/sea water (w/v) and the peak in the maximal enzyme titres (22.1 U/gIDS) occurred at 96 h. The lower levels of moisture content for maximal yield were characteristic of fungal SSF (Ghidyal *et al*. 1985). The critical importance of moisture level in SSF media and its influence in the biosynthesis and secretion of enzyme can be attributed to the interference of moisture in the physiological properties of solid particles (Ramesh & Lonsane 1990). The data also showed that at lower and higher initial moisture levels, metabolic activities of the culture and consequently product synthesis were found to be variously affected. Similar results were reported in other fungal SSF processes (Hung *et al*. 1985). An increase in enzyme titres with increase in initial moisture content (above 5:3 ratio) was observed in all cases after 96 h of fermentation. It may be due to slow growth of the culture in media containing higher initial moisture content and the consequent late entry to stationary phase as well as late initiation of the enzyme production phase.

Incubation Temperature and Initial pH. Incubation temperature and initial pH of the medium strongly influenced the chitinase synthesis in SSF with prawn waste medium using *B. bassiana*. The optimum temperature that promoted maximal chitinase yield was 27 °C (22.8 U/gIDS). Nevertheless, significant chitinase yield was also recorded over a range from 22 °C (20.1 U/gIDS) to 32 °C (20.5 U/gIDS). However, above 32 °C the enzyme yield declined sharply. The data presented in Figure 2 indicated that the optimum pH was 9.5 for the maximal chitinase yield (31.4 U/gIDS). The double peak in the pH shows the characteristic nature of marine fungi (Jones & Irvine 1972). The effect of temperature and pH on growth of higher marine fungi is well known (Jones & Byrne 1976). Comparable results were obtained with the same culture using wheat bran in SSF and in submerged fermentation (Suresh 1996). These factors are largely characteristic of the organisms and were similar irrespective of the type of solid supports used (Chandrasekaran *et al*. 1991; Nagendra & Chandrasekaran 1996).

Enrichment of NaCl. Addition of NaCl to the prawn waste medium affected the chitinase yield. Maximal enzyme yield (56.0 U/gIDS) was observed in a medium supplemented with 1% (w/w) NaCl compared with the control (31.4 U/gIDS). But chitinase yields were observed at all concentrations of NaCl tested. The chitinase yield at

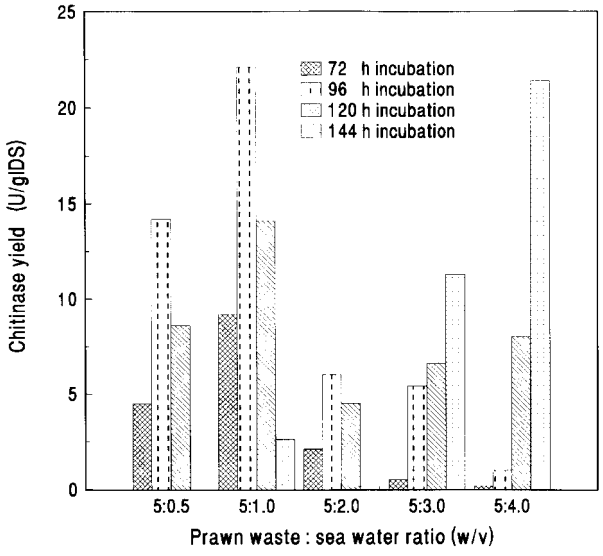

Figure 1. Effect of prawn waste:sea water ratio on chitinase production by *B. bassiana* at different incubation periods in SSF.

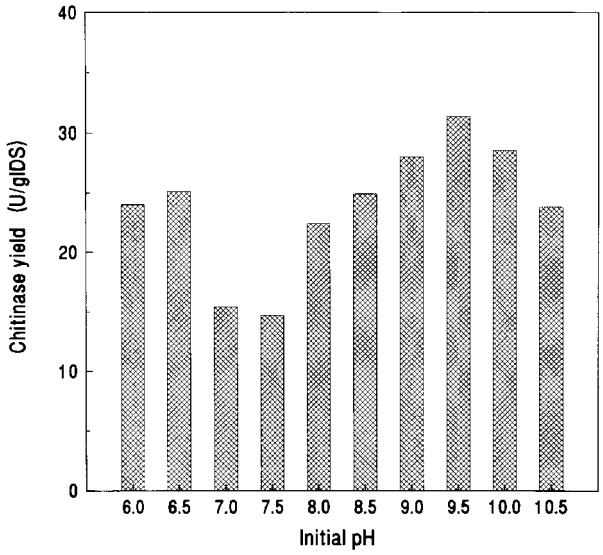

Figure 2. Effect of initial pH on chitinase production by *B. bassiana* in SSF using prawn waste.

higher concentrations of NaCl indicate the tolerance of high salt concentration by this organism. These results are in agreement with the reports of Moriguchi *et al*. (1994) and Chandrasekaran (1994, 1996).

Effect of Phosphate. Results presented in Figure 3 suggest that incorporation of phosphate affected chitinase yield significantly. Maximal enzyme yield (117.0 U/gIDS) was recorded in the medium supplemented with 2.5% (w/w) KH_2PO_4 as compared with the control (55.3 U/gIDS). However significant levels of chitinase yield were recorded with all concentrations of K_2HPO_4 and KH_2PO_4 tested. These results emphasize the critical role of phosphate in the enhanced secretion of chitinase.

Effect of Substrate Particle Size. As shown in Figure 4, the effect of particle size with respect to maximal chitinase production varied with incubation time. Maximal chitinase yield (248.3 U/gIDS) was recorded in a medium with particle size of 425–600 μm after 5 days of incubation. However, with smaller particles (< 425 μm) maximal chitinase yield (160.4 U/gIDS) was recorded on day 4 of incubation, whereas with all other larger particles tested, maximal chitinase yield was recorded on day 5. Particle size, and therefore the specific area of the substrate, is of great importance in SSF (Muniswaran & Charyulu 1994). The variation in optimum incubation time required for maximal enzyme yield with the substrate particle size could be attributed to the difference in their size and physical nature, which consequently influence the aeration rate and fungal growth during the SSF. With smaller particles, the available surface area for microbial growth is larger but the inter-particle space, and hence the porosity become less; with larger substrate particles, the situation is the opposite. These two opposing factors probably interact together and thus determine the growth and activity of microorganisms (Lonsane *et al*. 1985; Muniswaran & Charyulu 1994).

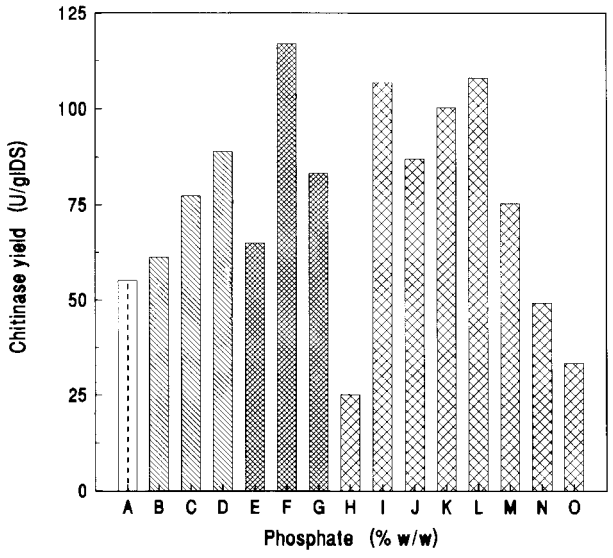

Figure 3. Effect of phosphate on chitinase production by *B. bassiana* in SSF using prawn waste. (A) Control; (B, C, D) 1, 2.5, 5% K_2HPO_4; (E, F, G) 1, 2.5, 5%, KH_2PO_4; (H, I, J, K, L, M, N, O) 1:1, 1:2.5, 1:5, 2:1, 2:2.5, 3:1, 3:2.5, 3:5% K_2HPO_4/KH_2PO_4 respectively.

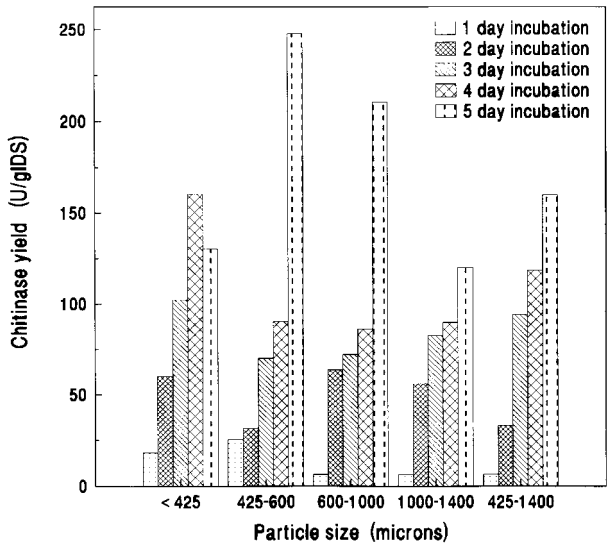

Figure 4. Effect of substrate particle size on chitinase production by *B. bassiana* at different incubation periods in SSF using prawn waste.

Effect of Nitrogen Enrichment. Incorporation of yeast extract as an additional nitrogen source significantly reduced chitinase synthesis. The enzyme yield was 143.6 U/gIDS with 1% (w/w) yeast extract as compared with the medium without yeast extract (163.2 U/gIDS). The yield was reduced to 98.8 U/gIDS when the yeast extract concentration was increased to 3% (w/w). This could be due to the preferential utilization of this nutrient in place of the chitin waste. In the case of other enzymes, the addition of yeast extract to the fermentation medium in SSF has promoted synthesis (Soni *et al*. 1996).

Time Course of Chitinase Production

Chitinase production in optimized medium was about 6.2 U/gIDS upto 24 h and it increased to about 90.3 U/gIDS at 96 h. In the subsequent period, the rate of enzyme formation was very high with a peak of 248.0 U/gIDS at 120 h. The enzyme yield was almost same up to 144 h (246 U/gIDS), and it was denatured beyond this period. However, the decrease was comparatively slower. Visual observation showed that at 168 h of incubation the prawn waste medium was completely covered with sporulated mycelium. Sporulation becomes an ultimate limiting factor in fungal biomass production and indicates the low substrate conversion in SSF (Raimbault &

Alezard 1980). There was a gradual increase in the concentration of sugar, due to liberation by the fungal culture, and this indirectly slowed the growth of the culture (data not shown). However, after 144 h the sugar concentration declined sharply and the subsequent sporulation agrees with the report of Raimbault & Alezard (1980).

Chandrasekaran (1994, 1996) pointed out that the solid waste from shellfish-processing industries could be explored as probable solid substrate for SSF-based production of enzymes and other desirable products using marine fungi and bacteria. The data obtained in this study indicate that prawn waste can indeed be used as a substrate for the production of chitinolytic enzymes. The marine fungus *B. bassiana* BTMF S10, a potent producer of chitinase (Suresh 1996) is an ideal organism for chitinase production using prawn waste. The maximal chitinase activity obtained was 248.0 U/gIDS without the addition of any other nutrients; this is an appreciable amount when compared with other reports.

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