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Impact of process parameters on chitinase production by an alkalophilic marine *Beauveria bassiana* in solid state fermentation

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Abstract

A chitinolytic fungus, *Beauveria bassiana* was isolated from marine sediment and significant process parameters influencing chitinase production in solid state fermentation using wheat bran were optimised. The organism was strongly alkalophilic and produced maximum chitinase at pH 9·20. The NaCl and colloidal chitin requirements varied with the type of moistening medium used. Vegetative (mycelial) inoculum was more suitable than conidial inoculum for obtaining maximal enzyme yield. The addition of phosphate and yeast extract resulted in enhancement of chitinase yield. After optimisation, the maximum enzyme yield was 246·6 units g^{-1} initial dry substrate (U $gIDS^{-1}$). This is the first report of the production of chitinase from a marine fungus.

Keywords: *Beauveria bassiana*; Chitinase; Marine fungus; Process parameters; Solid state fermentation; Wheat bran

1. Introduction

Chitin is the second most abundant polysaccharide (after cellulose) on earth [1] and is a long chain polymer of *N*-acetyl-glucosamine (2-acetamido-2-deoxy-D-glucose; Glc.NAc) linked by β-1,4 bonds [2]. Chitinases are a heterogeneous group of enzymes that catalyse the hydrolytic depolymerisation of chitin. The chitinase system consists of two fractions, which are known to be synergistic and consecutive. Endochitinase (E.C. 3.2.1.14) randomly cleaves chitin to produce chitobiose, whereas chitobiase (E.C. 3.2.1.30) completes the hydrolysis by converting chitobiose molecule to Glc.NAc [3]. Micro-organisms which degrade chitin occur widely in nature and prevent the polysaccharide deposited from dead animals and fungi from accumulating in land and marine sediments [4]. Chitinolytic enzymes have been produced from many micro-organisms including various groups of fungi and their enzymic properties have been investigated [5]. *Beauveria bassiana* is recognised as a potent producer of chitinase and was found to secrete it extracellularly in the presence of colloidal chitin in submerged fermentation (SmF) [6]. At present, chitinase is produced exclusively by the SmF processes.

The production of microbial chitinase has received attention as one step in a bioconversion process to treat shellfish waste chitin and the production of single cell protein (SCP) for animal and aquaculture feed. The production of chitinase enzyme is thought to be one of the primary economic variables in the bioconversion of chitin, estimated to account for 12% of the total production cost [7] and is presently uneconomic due to the high prices of the commercially available chitinase. A more efficient and economically viable process is essential for chitin utilisation and the management of shellfish wastes. In addition to these, chitinolytic enzymes have been used for the isolation of fungal protoplasts [8], for the preparation of bioactive chito-oligosaccharides [9] and used for the control of phytopathogenic fungi and pests [10,11]. In spite of such industrial significance, few of these applications

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have been commercially exploited, mainly due to the constraints imposed by the high cost of the enzyme.

Solid state (substrate) fermentation (SSF) has been known for centuries and used successfully for the preparation of oriental foods. More recently it has gained importance in the production of microbial enzymes due to several economic advantages over conventional SmF [12,13]. Among the various groups of micro-organisms used in SSF, the filamentous fungi are the most widely exploited because of their ability to produce a wide range of extracellular enzymes and grow on complex solid substrates [14,15]. Several fungal enzymes including amylase, cellulase, pectinase, protease and glucoamylase have been produced with this system [13]. Reports on chitinase production in SSF are not common except those on the properties of chitinase produced in the black koji process [16,17]. A thorough study of the literature showed that almost all the organisms used in solid state fermentation are of terrestrial nature except for a few reports on the use of marine bacteria [18–22] and there was no attempt to utilise the untapped sources of marine fungi. Marine micro-organisms, which are salt tolerant and have the potential to produce novel metabolites, are highly suitable for use in SSF by virtue of their ability to adsorb onto solid particles [23,24]. Their great potential to produce novel metabolites employing SSF remains untapped [20].

The purpose of the present work was to evaluate the possibility of production of chitinase by *Beauveria bassiana* isolated from marine sediment using solid state fermentation.

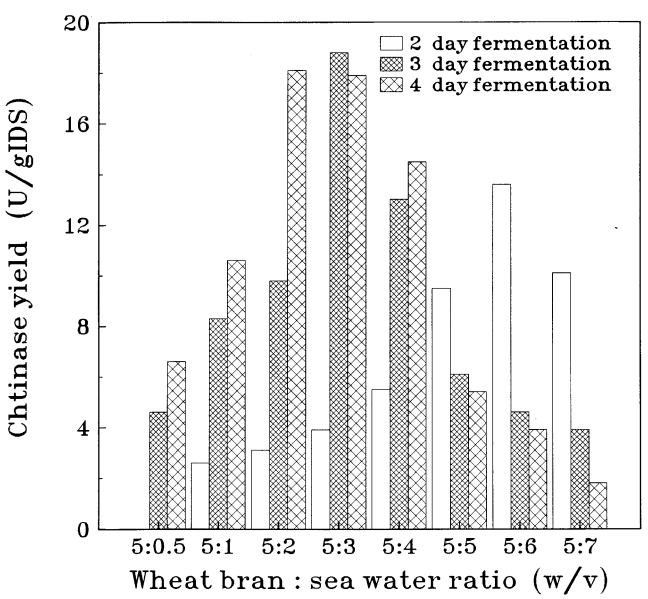

Fig. 1. Effect of wheat bran: sea water ratio on chitinase production by marine *B. bassiana* at different incubation periods in SSF.

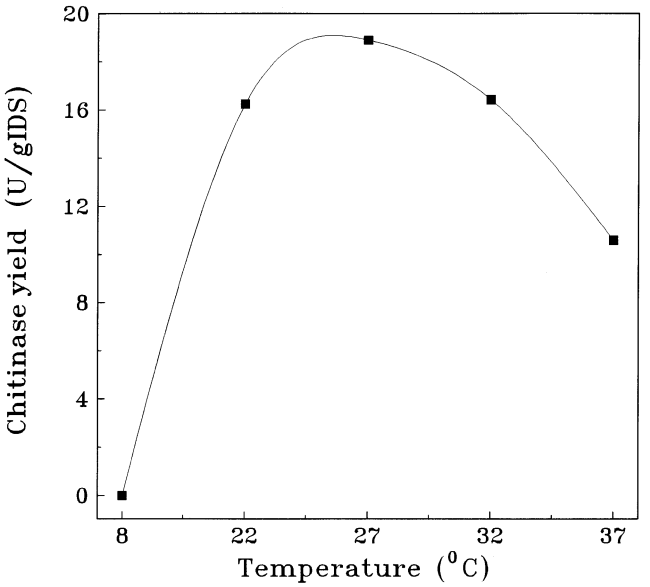

Fig. 2. Effect of incubation temperature on chitinase production by marine *B. bassiana* in SSF.

2. Materials and methods

2.1. Micro-organism

The fungus was isolated from marine sediments from Kochi, south coast of India, on colloidal chitin agar medium prepared in aged sea water and screened as described elsewhere [25]. The culture was identified using standard methods [26], maintained on Bennet's agar slants prepared in 50% aged sea water, subcultured monthly and stored at 4°C. A stock culture of the fungus was maintained at room temperature under sterile mineral oil.

2.2. Inoculum preparation

The conidial inocula was prepared by growing the culture on Bennet's agar slants in 50% aged sea water at 27°C for 12 days. Thc conidia from a fully sporulated culture were dispersed in 20 ml of 0·1% solution of Tween 80 by scraping with a sterile inoculum needle under strictly aseptic conditions. The concentration was adjusted to 16×10^6 colony forming units (cfu) ml^{-1} suspension. Vegetative (mycelial) inocula was prepared in a medium composed of (w/v) glucose 5%, peptone 5%, yeast extract 5%, in 50% aged sea water. Two hundred and fifty millilitres of medium in 1 l Erlenmeyer flasks was prepared, sterilised at 121°C for 15 min, cooled to room temperature (28 ± 2°C), inoculated with 10 ml conidial suspension (prepared as described above) and incubated at room temperature (28 ± 2°C) on a rotary shaker at 150 rpm. The mycelium was collected aseptically after 48 h by centrifugation and washed repeatedly with sterile physiological saline

Table 1
Effect of initial pH on chitinase production by marine *B. bassiana* in solid state fermentation using wheat bran moistened with aged sea water

pH of the moistening medium	pH of substrate		Chitinase yield (U gIDS^{-1})	pH of the enzyme extract
	before autoclaving	after autoclaving		
7·00	6·08	5·98	17·6	6·16
8·00	6·09	6·00	18·8	6·15
9·00	6·10	6·10	12·4	6·40
10·00	6·20	6·10	5·9	6·41
11·00	6·00	6·10	32·0	6·73
12·00	6·73	6·17	41·2	6·86
12·50	6·75	6·19	41·2	6·96
12·75	9·28	7·70	41·8	6·98
12·90	10·73	9·20	61·8	7·20
13·00	11·37	10·10	52·1	7·90

U gIDS^{-1} = Units g^{-1} initial dry substrate.

(0·85% w/v NaCl). Since the fungus grew in the liquid medium in the form of pellets, the biomass was homogenised in a blender for 1 min prior to its utilisation for inoculation. The concentration of the prepared suspension was about 2·5 mg dry weight equivalent of mycelia 10 ml^{-1} (mg dry wt equiv mycelia 10 ml^{-1}).

2.3. Preparation of colloidal chitin

The colloidal chitin was prepared according to Gernot [27] with some modification as described below. 5 g of purified chitin obtained from the Central Institute of Fisheries Technology, Kochi, India was taken in a beaker and 140 ml of 50% (v/v) H_2SO_4 added with continuous stirring in an ice bath for 60 min. The insoluble fraction was filtered off by passing through glass wool and the clear solution was poured into 2 l deionised distilled water in order to precipitate the chitin. The suspension was kept overnight at 4°C, the supernatant decanted out and the remaining mixture centrifuged at 5000 rpm for 10 min. The sedimented residue was resuspended in deionised distilled water, allowed to settle and decanted. This process was repeated until the pH of the liquid became neutral. The colloidal chitin was collected by centrifugation and stored at 4°C, after heating for 15 min in boiling water bath. It was used as a substrate for chitinase production and in the estimation of enzyme activity.

2.4. Solid state fermentation

Commercially available wheat bran (WB) was used as the solid substrate. Solid WB media for chitinase production was prepared by mixing 5 g WB of particle size >425 μm with 50 mg (1% w/w) colloidal chitin in Petri plates (86 mm diameter and 17 mm height). The WB supplemented with colloidal chitin was moistened with aged sea water to a ratio of 5:5 (w/v) substrate: sea water and mixed thoroughly to achieve uniformity. Media in Petri dishes were autoclaved at 121°C for 1 h, cooled to room temperature (28 ± 2°C) and inoculated with 2 ml of the prepared conidial suspension (unless otherwise mentioned). The suspension was mixed carefully under strictly aseptic conditions to achieve uniform distribution of conidia throughout the medium. The dishes were then incubated at room temperature (28 ± 2°C, unless otherwise mentioned) in a chamber with 90% relative humidity for desired period.

2.5. Enzyme extraction

After completion of fermentation, the fermented wheat bran was mixed, transferred to 250 ml Erlenmeyer flasks, with 5 volumes of distilled water added (based on initial dry weight of the substrate) and the mixture mixed at room temperature (28 ± 2°C) on a rotary shaker (150 rpm) for 30 min. The slurry was then squeezed through cheese cloth [20]. After extracting twice, the extracts were combined, clarified by centrifugation at 10 000 rpm at 4°C for 15 min and the clear supernatant used as crude enzyme for assays.

2.6. Optimisation of process parameters

The protocol adopted for the optimisation of process parameters influencing chitinase production was to evaluate the effect of an individual parameter and to incorporate it at the standardised level before standardising the next parameter [28]. The parameters optimised were: (1) impact of initial moisture content [substrate: sea water ratio (5:1 to 5:7 w/v)]; (2) incubation temperature (8–37°C); (3) the initial pH of the medium was varied between 5 to 11 by adding either 1 N HCl or NaOH to sea water before using it to moisten the medium. NaOH solutions of varying nor-

Table 2
Effect of initial pH on chitinase production by marine *B. bassiana* in solid state fermentation using wheat bran moistened with NaOH solution

Normality of NaOH solution	pH of substrate		Chitinase yield (U $gIDS^{-1}$)	pH of the enzyme extract
	before autoclaving	after autoclaving		
0·1	6·93	6·12	50·5	6·96
0·5	8·26	6·98	45·6	7·58
1·0	9·40	8·07	23·3	7·30
2·0	11·04	9·60	74·3	8·23

U $gIDS^{-1}$ = Units g^{-1} initial dry substrate.

mality (0·1–2) was also used; (4) effect of sodium chloride was evaluated by incorporating at various concentrations (0–20% w/w); (5) optimum concentration of colloidal chitin was determined by incorporating at various concentrations (0–15% w/w); (6) impact of phosphate was evaluated by incorporating either K_2HPO_4 or KH_2PO_4 singly (0–5% w/w), or in combination at various ratios (1:1–3:5 w:w/w); (7) ideal type and optimum concentration of inoculum, either conidial (32×10^6, 16×10^6, 8×10^6 and 4×10^6 cfu 5 g^{-1} WB) or vegetative (175, 250, 450 and 1000 mg dry wt equiv 100 g^{-1} WB) was evaluated; (8) optimum particle size of the substrate was determined by using < 425 μm (fine); 425–600 μm (medium); 600–1000 μm (coarse); and 1000–1425 μm (large) particles; (9) impact of incubation time was evaluated for both conidial and vegetative inoculum for a total period of 96 h; (10) effect of yeast extract (organic) and ammonium chloride (inorganic) as additional nitrogen sources were studied (0–4% w/w); (11) effect of type of moistening medium was evaluated by using aged sea water of various dilutions, distilled water and tap water.

2.7. Analytical methods

Chitinase activity was assayed colorimetrically using colloidal chitin as substrate. The reaction mixture consisting of 0·5 ml of 0·5% (w/v) colloidal chitin, 0·5 ml of 0·1 M MacIlvain's citrate phosphate buffer (pH 6·0) and 1 ml of appropriately diluted enzyme solution was incubated at 37°C in a water bath for 2 h. The reaction was terminated by boiling the reaction mixture for 5 min. The undigested materials were removed by centrifugation at 5000 rpm for 5 min and the amount of reducing sugar liberated estimated using a dinitrosalicylic acid (DNS) reagent [29]. One unit of chitinase activity was defined as the amount of enzyme which released 100 μg of reducing sugar as GlcNAc under the reaction conditions [30]. Enzyme production in SSF was expressed in terms of units g^{-1} of initial dry substrate (U $gIDS^{-1}$). The reducing sugar in the enzyme filtrate was analysed by the methods of Miller [29]. The pH of the solid substrate was measured by the method of Raimboult and Alazard [31]. pH measurements of the enzyme extract were made by a standard pH meter (Systronics,

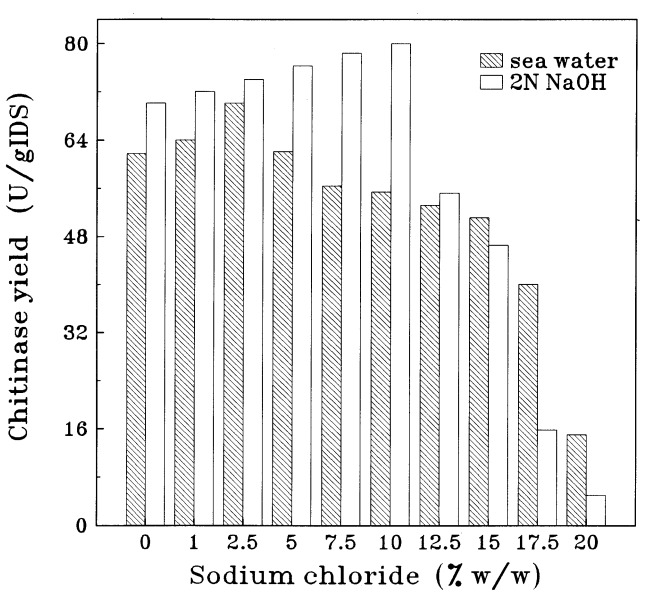

Fig. 3. Effect of NaCl concentration on chitinase production by marine *B. bassiana* in SSF using wheat bran moistened with aged sea water and 2 N NaOH solution.

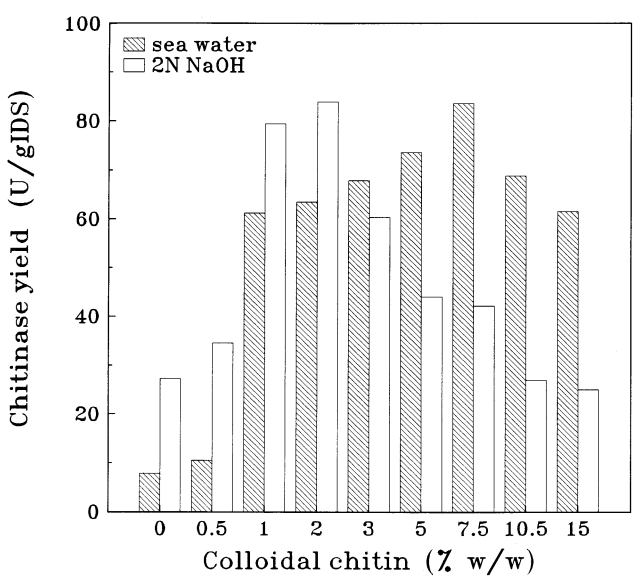

Fig. 4. Effect of colloidal chitin on chitinase production by marine *B. bassiana* in SSF using wheat bran moistened with aged sea water and 2 N NaOH solution.

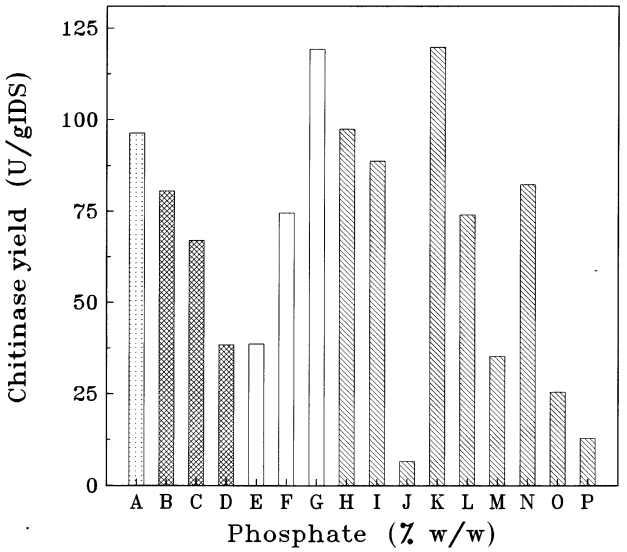

Fig. 5. Effect of phosphate on chitinase production by marine *B. bassiana* in SSF using wheat bran moistened with aged sea water. (A) control; (B, C, D) 1, 2·5, 5% K_2HPO_4, (E, F, G) 1, 2·5, 5% KH_2PO_4; (H, I, J, K, L, M, N, O, P) 1:1, 1:2·5, 1:5, 2:1, 2:2·5,3:1, 3:2·5,3:5 K_2HPO_4:KH_2PO_4, respectively.

India). All experiments were conducted in triplicate and the mean values are reported. Key results were repeated three times to establish their validity.

3. Results and discussion

A total of 21 chitinolytic fungal cultures was isolated from marine sediments and screened for a hyperproducing culture. *Beauveria bassiana* BTMF S10 was selected for its ability to produce chitinase at higher levels under SSF (data not shown). Naturally, marine fungi are adapted to grow in nutritionally diluted environment under submerged conditions [32] but this fungus grew in the SSF process and tolerated adverse conditions including the high carbohydrate concentration and the presence of other waste components. Wheat bran was used in the present study since it is a well recognised, widely used and most popular substrate in SSF enzyme production [33]. The fungus grew profusely and uniformly throughout the wheat bran medium supplemented with colloidal chitin and moistened with aged sea water. In arbitrarily selected medium, chitinase production was 9·5 U $gIDS^{-1}$ at 48 h of fermentation. This fungus is a spore former, which helped the uniform distribution of the culture during inoculation.

3.1. Factors affecting chitinase production in SSF

3.1.1. Initial moisture level

The data presented in Fig. 1 show that chitinase production was high in WB medium at a wheat bran to sea water ratio of 5:3 (w/v) and the peak in the enzyme titre occurred on the third day of fermentation (18·6 U $gIDS^{-1}$). The initial moisture content significantly affected hydrolytic enzyme production in SSF since the moisture content of the medium is a critical factor that determines microbial growth and product yield in SSF [34,35]. Moisture is reported to cause swelling and thereby facilitates better utilisation of the substrate by the micro-organisms [36]. Higher titres of enzyme with a lower ratio of moisture levels are characteristic of fungal solid state fermentation [37,38]. An increase in enzyme titres with increase in initial moisture content of the medium was observed in all cases after 24 h of fermentation. This may be due to faster growth of the culture in media containing higher initial moisture and the consequent early entry to the stationary phase as well as early initiation of enzyme production. However, the increase was observed only up to a 5:6 ratio of initial moisture levels. Comparable results were also reported in other SSF processes with fungal cultures [39,40]. Enzyme titres at 72 and 96 h were lower than those at 48 h in all the media containing an initial moisture content above 5:5 ratio. The slow increase in enzyme titres and the continuation of the enzyme production phase up to the fourth day with lower production of the enzyme may be due to a slower growth rate at lower moisture levels. At lower and higher initial moisture levels the metabolic activities of the culture and consequently product synthesis were variously affected [35]. In fungal and bacterial SSF, lower moisture was stated to lead to reduced solubility of the nutrients present in the solid substrates a lower degree of substrate swelling and higher water tension [41]. Similarly, higher moisture contents were reported to cause decreased porosity, loss of particle structure, development of stickiness, reduction in gas volume, decreased gas exchange and enhanced formation of aerial mycelium [13,34]. Moreover, the optimum moisture content is different for strains of the same species of bacteria and mould [35]. However, no information is available about the optimum moisture content of marine fungi under SSF.

3.2. Incubation temperature

The data presented in Fig. 2 clearly indicate the strong influence of incubation temperature on chitinase yield by *B. bassiana*. Enzyme production and yield were similar at 21 and 37°C and the optimal temperature for maximal yield was 27°C. This factor is largely characteristic of the organism and was similar irrespective of the type of solid support involved in SSF [20,42]. The incubation temperature has a profound effect on the enzyme yield and the duration of enzyme synthesis phase [43]. Most of the marine fungi

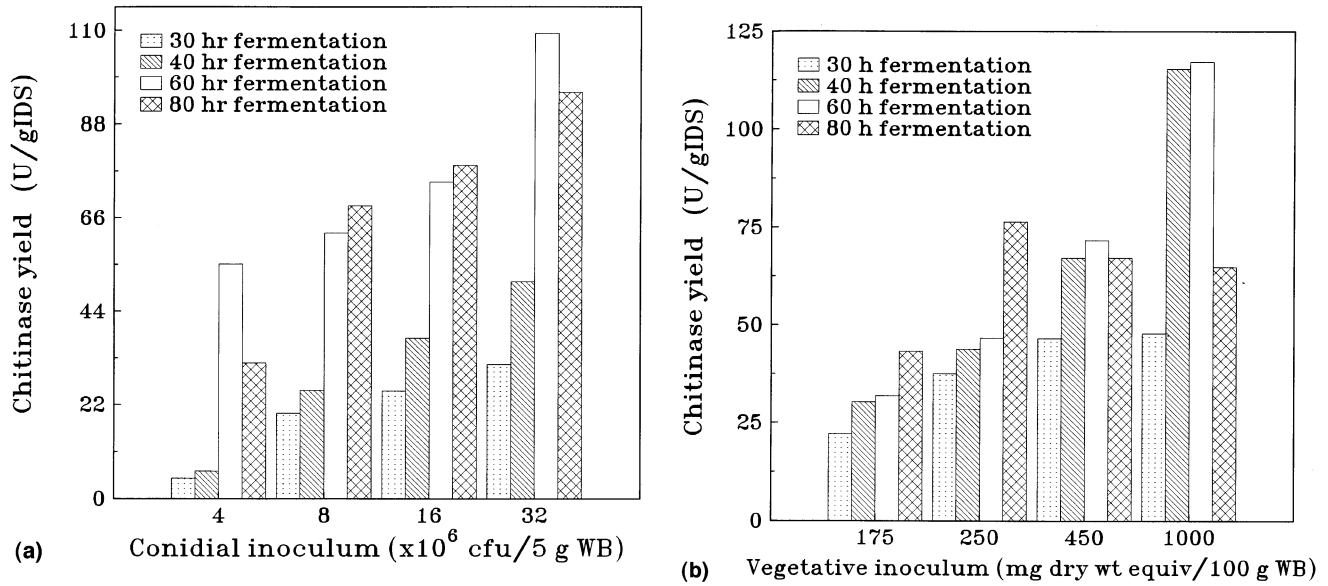

Fig. 6. (A) Effect of conidial inoculum on chitnase production by marine *B. bassiana* at different incubation periods in SSF. (B) Effect of vegetative (mycelial) inoculum on chitnase production by marine *B. bassiana* at different incubation periods in SSF.

investigated showed optimum growth in the range 10–20°C and none appeared to require a temperature above 30°C [44].

3.3. Effect of initial pH

The results obtained for WB medium moistened with aged sea water are shown in Table 1. The data indicate a strong influence of the initial pH of the medium on the rate of chitinase production. Maximal production (61·8 U $gIDS^{-1}$) was noted with the substrate having pHs of 10·73 and 9·2 before and after autoclaving the substrate, respectively. There was a 14% decrease in pH of the substrate after sterilisation at higher pH levels. However, the chitinase yield was also significant at all other pH values in the range with two peaks of optima, one at high alkaline pH (9·2) and the other at acidic pH (6·0) before autoclaving the medium. The double pH peak optima for growth is characteristic of most marine fungi [44,45]. The results for the NaOH solution also showed the same result (Table 2). and maximal chitinase yield (74·3 U $gIDS^{-1}$) was recorded with medium moistened with 2 N NaOH solution (medium pH 9·6 after autoclaving). Therefore *B. bassiana* appears to be unusual in its capability to produce chitinase at high pH. The effect of pH on the growth of micro-organisms is well known. Moreover, most microbial extracellular enzymes are produced in greatest yield at a growth pH near the maximal for enzyme activity [46]. It is known that the synthesis of extracellular enzymes by several micro-organisms is regulated by the pH value of the culture, particularly in the case of fungal strains [47].

3.4. Effect of NaCl

Marine fungi typically exhibit a broad tolerance to salinity, while terrestrial fungi are inhibited by higher salinity, especially their reproduction and spore germination [44]. One of the characteristics of marine microorganisms is salt tolerance [23,24,48]. Salt-tolerant micro-organisms and their enzymes may play significant roles in the industrial processes that require high salt environments [48]. The NaCl requirement of *B. bassiana* for maximal chitinase yield is shown in Fig. 3. It varied with the medium used for humidifying the wheat bran and the maximal chitinase yield is recorded in the presence of 25% w/w (67·5 U $gIDS^{-1}$) and 10% w/w (81·2 U $gIDS^{-1}$) of NaCl with aged sea water and 2 N NaOH solution, respectively.

3.5. Effect of colloidal chitin

It is known that an ideal substrate concentration in any fermentation process results in higher conversion efficiencies and optimum substrate utilisation [49]. The results of these studies presented in Fig. 4 indicated that the optimum concentration of colloidal chitin for maximal chitinase titres varied with the solvent used for moistening the WB. Maximal yields were recorded with 7·5% w/w (81·6 U $gIDS^{-1}$) and 2% w/w (83·9 U $gIDS^{-1}$) for aged sea water and 2 N NaOH solution, respectively. However, the yield was almost equal in both cases. Chitinase production was found even in the absence of colloidal chitin in both cases, while the rate was very high in the case of medium treated with NaOH solution. Chitinases are produced constitutively [50], but it is also reported

that the addition of chitin to culture medium greatly enhances enzyme production [3]. In *Beauveria bassiana* chitinase production was inducible [6]. However, all the previous work was carried out in SmF.

3.6. Impact of phosphate enrichment

The data presented in Fig. 5 show that chitinase production by *B. bassiana* was significantly affected by the enrichment of WB medium with phosphate when compared with medium without phosphate. An increase in chitinase titres with increase in the KH_2PO_4 content of the medium was observed at 5% (w/w), while concentration below 5% reduced chitinase titres. The addition of phosphate as K_2HPO_4 reduced enzyme yield at all concentrations tested (1–5%). When both K_2HPO_4 and KH_2PO_4 were combined at various ratios the enzyme yield declined compared with the control except at a 1:1 ratio (97·4 U $gIDS^{-1}$) and 2·5:1 (119·8 U $gIDS^{-1}$). These results suggest that addition of KH_2PO_4 at 5% and a combination of K_2HPO_4 and KH_2PO_4 at a ratio of 2·5:1 could alone promote en-

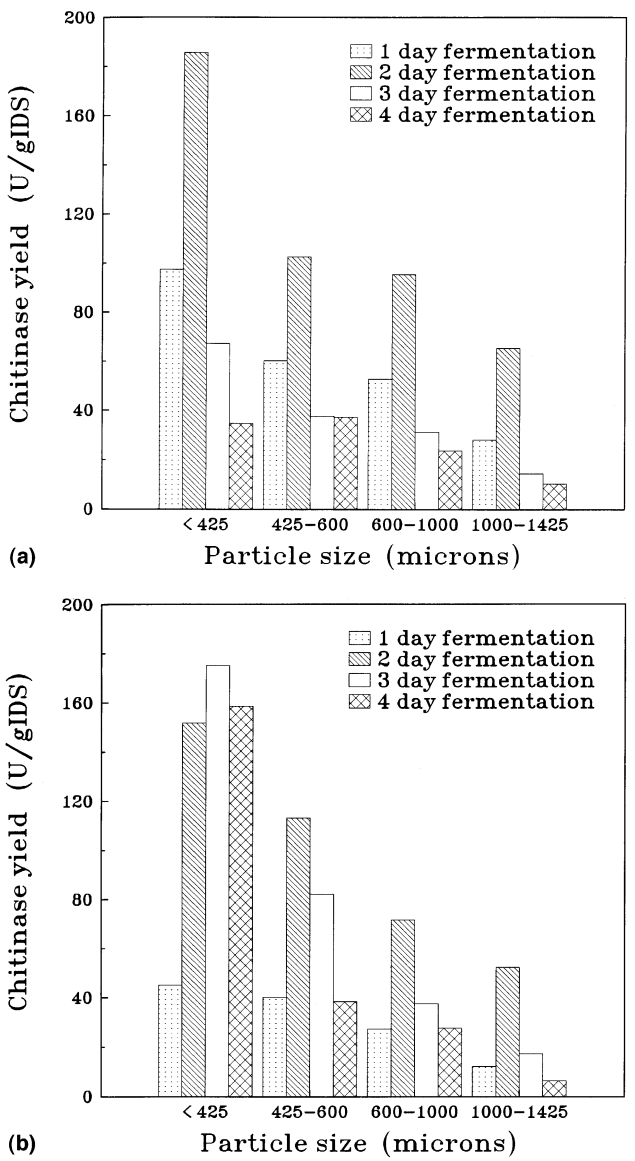

Fig. 7. (A)Effect of substrate particle size on chitinase production by marine *B. bassiana* at different incubation periods and vegetative inoculum in SSF. (B) Effect of substrate particle size on chitinase production by marine *B. bassiana* at different incubation periods and conidial inoculum in SSF.

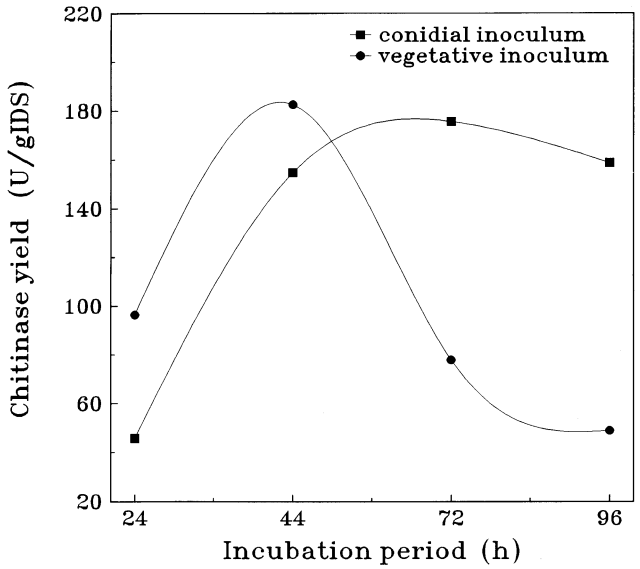

Fig. 8. Effect of incubation period on chitinase production by marine *B. bassiana* in SSF using wheat bran inoculated with conidial and vegetative inoculum.

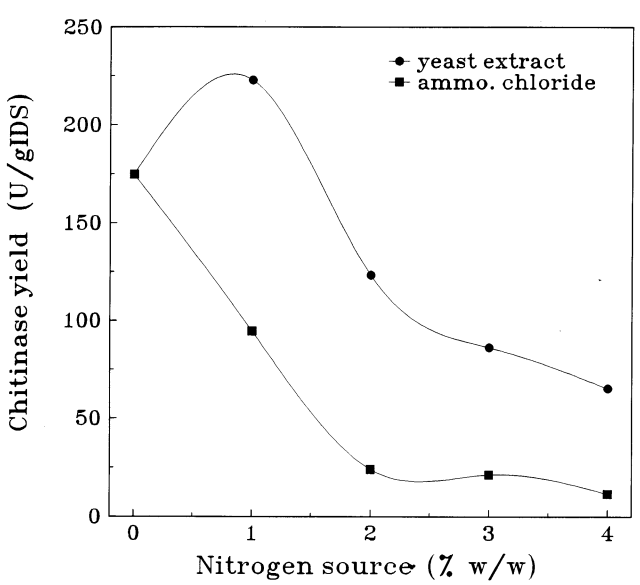

Fig. 9. Effect of additional nitrogen source on chitinase production by marine *B. bassiana* in SSF using wheat bran inoculated with conidial inoculum.

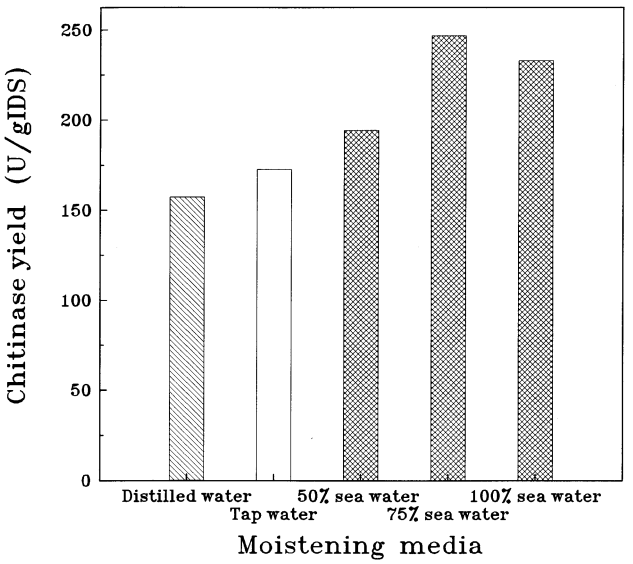

Fig. 10. Effect of type of moistening medium on chitinase production by *B. bassiana* in SSF using wheat bran inoculated with conidial inoculum.

zyme yield by 23·7% and 24·3%, respectively, as compared with the control. The phosphate present in the media plays an important role in buffering and thereby controls the pH of the media [51].

3.7. Impact of inoculum size and type

The affect of inoculum in SSF is well known [52,53]. The results presented in Fig. 6A and B suggest that there is a significant increase in chitinase yield with an increase in inoculum concentration. The results also showed that the inoculum type influenced the rate of enzyme production. In both types of inoculum, maximal yields of chitinase were observed after 60 h of incubation. However, with a conidial inoculum there was only a 55% yield (50·9 U $gIDS^{-1}$) at 40 h compared with maximal production (109·2 U $gIDS^{-1}$) after 60 h fermentation, which remained at approx. 95% of the yield (95·4 U $gIDS^{-1}$) after 80 h of fermentation (Fig. 6A). In the case of a vegetative inoculum the rate of chitinase production was very rapid and with 99·5% (115·4 U $gIDS^{-1}$) of maximal activity (117·2 U $gIDS^{-1}$) apparent at 40 h, but with only 60% (64·8 U $gIDS^{-1}$) of the maximum remaining after 80h (Fig. 6B). The faster rate of enzyme yield with a vegetative inoculum may be due to the rapid growth initiation of the cultures and early entry of the organisms into the production phase. At lower inoculum levels the yield was very low with both types of inoculum. The marginal decrease seen with later phases of incubation with larger inocula could be due to the shortage of nutrients available for the larger biomass and faster growth of the culture. However, for small scale fermentation, suspensions prepared from agar slants remain the most convenient [52]. Spores are preferable because of the convenience of preparation, their stability during storage and tolerance of mistreatment during harvesting [53].

3.8. Effect of particle size

The particle size and therefor the specific area of the substrate is of importance in SSF [54], and usually smaller particles stimulate greater growth [39]. Fig. 7A and 7B show the results obtained when fermentation was carried out with different sizes of WB. Maximum chitinase production was obtained with substrate particles of average size < 425 μm, irrespective of the inoculum type. With smaller particle, the surface area for growth was greater but the inter-particle porosity was less. With the larger size, the porosity was greater but the saturated surface area was less. These two opposing factors probably interacted to give the value corresponding to optimum growth and product formation [54]. In the case of a vegetative inoculum the rate of production was high and maximal yield (185·6 U $gIDS^{-1}$) was obtained on the second day of fermentation (Fig. 7A). Similarly with all other particle sizes maximal yield was observed on the second day of incubation. However, when using a conidial inoculum the maximal yield (175·4 U $gIDS^{-1}$) was recorded on the third day of fermentation (Fig. 7B) and approx. 80% of the activity was retained on the fourth day. However, all other particle sizes showed maximal yield on the second day of fermentation. The influence of substrate particle size, which determines the accessible surface area to the micro-organisms on product formation has been emphasised earlier [12,55].

3.9. Impact of incubation time

The time course of enzyme production under optimised conditions was approx. 45·0 U $gIDS^{-1}$ up to 24 h and it increased to approx. 154·8 U $gIDS^{-1}$ by 44 h (Fig. 8). In subsequent periods the rate of enzyme formation was very high with a peak at 72 h (175·7 U $gIDS^{-1}$) in the case of conidial inoculum. However, in the case of a vegetative inoculum the rate of enzyme yield was very rapid with about 96·4 U $gIDS^{-1}$ recorded at 24 h and a maximum (214 U $gIDS^{-1}$) recorded at 44 h of fermentation. In the case of a vegetative inoculum, incubation beyond the optimum time showed a rapid decline in the enzyme titres and was only 60% at 72 h, as compared with the maximum at 44 h. In the case of a conidial inoculum the decrease in yield was slow and was approx. 60% of the maximum at 92 h. The reduction in chitinase yield after an

optimum period (Fig. 8) is probably due to enzyme denaturation by protease secreted by the fungus. Similar results were obtained during glutaminase [22], and α-amylase [43] production in SSF. A sharp drop of chitinolytic activity after a maximal peak in *B. bassiana* was reported in liquid culture [6]. The variation in the incubation time required for maximal enzyme production with the type of inoculum could be attributed to differences in the physiological status/condition of the inoculum which consequently influenced the rate of growth and product formation of the fungus during SSF.

3.10. Effect of nitrogen enrichment

An external nitrogen source is essential for increased utilisation of soluble carbohydrates and reduction in fermentation time [28]. Commercial wheat bran contains 8·5 and 9·5% starch and protein, respectively, in addition to various minerals [56]. The uptake of nutrients from the WB by the culture, which is due to their ability to penetrate deeply in to the WB particles is well established in SSF processes [13]. Some of the vital nutrients necessary for optimum growth and product formation may also be present in WB at an optimal level. Hence, the supplementation of WB with other solid and/or water-soluble nutrients has been found to lead to enhanced product formation in SSF processes [38]. The supplementation of WB with yeast extract significantly increased the production of amylase and glucoamylase [56] in SSF processes. The result of the present study showed that addition of yeast extract enhanced chitinase yield to 127·5% in the presence of 1% yeast extract (Fig. 9). However, addition above 1% greatly reduced enzyme titres. Nitrogen enrichment with an inorganic nitrogen source such as ammonium chloride greatly affected adversely the enzyme yield. This could be due to preferential utilisation of these nutrients in place of chitin and the subsequent variation in the growth of the fungus.

3.11. Effect of type of moistening media

Enzyme production was maximal (246·6 U $gIDS^{-1}$) in WB medium moistened with 75% aged sea water (Fig. 10) and dilutions above and below the optimum resulted in enzyme yields of 94% and 77·8% of the maximum. In the case of wheat bran moistened with tap water and distilled water, enzyme yield was less than that observed with various concentrations of aged sea water. The results clearly indicate that the nature of media used to moisten the substrate had a clear impact on chitinase yield.

4. Conclusions

Marine micro-organisms, with their unique nature differ very much in many aspects from their terrestrial counterparts and are known to produce diverse spectra of novel useful substances. Currently, there is a lot of interest in the scientific community around the world in exploiting novel micro-organisms [20–24]. Although the oceans cover more than two-thirds of the world's surface, our knowledge of marine organisms, in particular marine fungi, remains very superficial. Research on marine fungi has been mainly concerned with their isolation, cultivation, morphology, taxonomy and systematics [32]. The results reported in this paper indicate the scope for utilisation of marine fungi for extracellular enzyme production through solid state fermentation. The fungus *B. bassiana* isolated from a marine sediment sample can be used as a novel strain for the production of chitinolytic enzymes by SSF using wheat bran as solid substrate. This enzyme system could be efficiently used as biopesticide against pests because *B. bassiana is* an entomopathogenic fungus and its enzyme systems are commercially used for the control of many pests.

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