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ORIGINAL PAPER

Biodegradation of shrimp processing bio-waste and concomitant production of chitinase enzyme and *N*-acetyl-D-glucosamine by marine bacteria: production and process optimization

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Abstract A total of 250 chitinolytic bacteria from 68 different marine samples were screened employing enrichment method that utilized native chitin as the sole carbon source. After thorough screening, five bacteria were selected as potential cultures and identified as; *Stenotrophomonas* sp. (CFR221 M), *Vibrio* sp. (CFR173 M), *Phyllobacteriaceae* sp. (CFR16 M), *Bacillus badius* (CFR198 M) and *Bacillus* sp. (CFR188 M). All five strains produced extracellular chitinase and GlcNAc in SSF using shrimp bio-waste. Scanning electron microscopy confirmed the ability of these marine bacteria to adsorb onto solid shrimp bio-waste and to degrade chitin microfibers. HPLC analysis of the SSF extract also confirmed presence of 36–65 % GlcNAc as a product of the degradation. The concomitant production of chitinase and GlcNAc by all five strains under SSF using shrimp bio-waste as the solid substrate was optimized by 'one factor at a time' approach. Among the strains, *Vibrio* sp. CFR173 M produced significantly higher yields of chitinase (4.8 U/g initial dry substrate) and GlcNAc (4.7 μmol/g initial dry substrate) as compared to other cultures tested. A statistically designed experiment was applied to evaluate the interaction of variables in the biodegradation of shrimp bio-waste and concomitant production of chitinase and GlcNAc by *Vibrio* sp. CFR173 M. Statistical optimization resulted in a two-fold increase of chitinase, and a 9.1 fold increase of GlcNAc production. These results indicated the potential of chitinolytic marine bacteria for the reclamation of shrimp bio-waste, as well as the potential for economic production of chitinase and GlcNAc employing SSF using shrimp bio-waste as an ideal substrate.

Keywords Chitinase · *N*-acetyl-D-glucosamine · Marine bacteria · Solid state fermentation · Shrimp bio-waste · Biodegradation

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Introduction

Shrimp processing bio-wastes include the head, shell and tail portions (non-edible parts) which account for about 50–70 % of the total volume of raw materials. The global implications of this scenario are that enormous shrimp bio-wastes are being generated by the seafood industry due to the escalating demand for shrimp products. In addition, continuous disposal of these voluminous bio-wastes into coastal and near shore environments has contributed to intense environmental pollution and consequent deterioration in affected ecosystems. On the other hand, by disposing the valuable byproducts without recycling and proper utilization, the sea food industries are missing prime opportunities for deriving several value added products such as chitin and other bioactive substances (Synowiecki and Al-Khateeb 2003; Bhaskar et al. 2010; Suresh et al. 2011a). In India, a major producer of shrimp, more than 1,00,000 tons of shrimp bio-waste are generated annually and only an insignificant amount of that bio-waste is utilized for the extraction of chitin while the rest is discarded or underutilized (Mathew and Nair 2006; Suresh et al. 2011a, b; Suresh and Anil Kumar 2012). Therefore, effective utilization of shrimp bio-waste has an important

role not only in the maintenance of a clean environment but also in enhanced economic value through valorization of the same towards deriving a range of economically viable biomolecules (Suresh and Chandrasekaran 1998; Chang et al. 2007; Bhaskar et al. 2010). In this context, bioconversion of chitinous materials into value added products has been proposed as a waste treatment alternative for the disposal and efficient management of shrimp bio-wastes and it is well accepted as the most effective and eco-friendly strategy for their utilization (Suresh and Chandrasekaran 1998; Synowiecki and Al-Khateeb 2000; Wang and Hwang 2001; Wang et al. 2006, 2009, 2010; Mathew and Nair 2006; Annamalai et al. 2011; Suresh et al. 2011b).

N-acetyl-D-glucosamine (GlcNAc), the basic structural unit of chitin, is known to have antimicrobial, antitumor, antihypertensive, and hypocholesterolemic activities. Recently, it has attracted special attention for the treatment of osteoarthritis, inflammatory bowel diseases, gastritis, and as a food supplement (Sashiwa et al. 2003; Synowiecki and Al-Khateeb 2003; Andrew et al. 2005; Wang et al. 2006; Suresh et al. 2011a; Suresh and Anil Kumar 2012). GlcNAc is produced by complete hydrolysis of chitin with strong mineral acid. However, this acid hydrolysis has several disadvantages such as low yield, formation of acid wastes, high production cost, and deacetylation of products (Felse and Panda 2000; Sashiwa et al. 2003; Binod et al. 2007; Wang et al. 2006; Suresh et al. 2011a; Suresh and Anil Kumar 2012). Recently investigators have developed enzymatic methods using chitinases as an alternative for the production of GlcNAc (Sashiwa et al. 2003; Binod et al. 2007; Suresh et al. 2011a; Suresh and Anil Kumar 2012). However, commercial exploitation of chitinases for the production of GlcNAc is currently limited not only due to the high cost of the enzyme but also the intensive shrimp and crab shell pretreatment processes (Wang and Hwang 2001; Suresh et al. 2011a; Suresh and Anil Kumar 2012).

Chitinases (E.C.3.2.1.14) are glycosyl hydrolases which catalyze the hydrolysis of insoluble chitin to its soluble derivatives. They are produced by various groups of bacteria and fungi, and their physiological and ecological role vary with the source organism (Gooday 1990). Nevertheless, the production of microbial extracellular chitinases has received much attention recently due to their potential applications in the reclamation of seafood processing crustacean bio-waste, production of bio-active *N*-acetyl chitooligosaccharides, and production of GlcNAc (Suresh and Chandrasekaran 1998; Yoyi et al. 2004; Suresh et al. 2011a). In spite of such industrial, environmental, and biological significance, the high cost of the enzyme together with low activity and stability of available purified chitinases restrict its commercial exploitation (Suresh and Chandrasekaran 1998; Yoyi et al. 2004; Neetu et al. 2005; Suresh and Anil Kumar 2012). Hence, enormous efforts are being invested by investigators in harnessing new species of microorganisms, as well as different bioprocesses for economic enzyme production. In this context solid state fermentation (SSF) has been reported as a better alternative over conventional submerged fermentation (SmF) for cost effective production of microbial chitinases, since it can be carried out using cheap and readily available agro-industrial residues such as shrimp bio-waste (Suresh and Chandrasekaran 1998; Pandey et al. 1999; Nopakarn et al. 2002).

Marine microorganisms have recently emerged as a novel source for the isolation of industrial enzymes (Chandrasekaran 1997; Subramani and Narayanasamy 2009). Although more than 10^{11} tons of chitin is produced annually in the aquatic biosphere alone, there is no substantial accumulation of chitin in ocean sediments (Keyhani and Roseman 1999) due to the fact that they are biodegraded by the naturally occurring bioconversion process mediated by chitinolytic marine bacteria (Gooday 1990; Suginta et al. 2000; Claudiana et al. 2011). In fact, marine bacteria have been recognized as excellent sources of chitinase (Austin 1988) and they have been implicated in the degradation of chitin in the ocean ecosystem (Gooday 1990; Claudiana et al. 2011). Several investigators have also isolated, purified, and characterized chitinase from marine bacteria (Hiraga et al. 1997; Suginta et al. 2000; Shiro et al. 2007). However, information available on the processes parameters that influence chitinase production by marine bacteria during bioprocesses is rather scanty. In this context, the present investigation was undertaken towards effectively harnessing the marine bacteria for the bioconversion of shrimp bio-waste along with the concomitant production of enzyme and bio-active GlcNAc. This study is conceptualized as an integrated biotechnological solution for the valorization of the abundant seafood processing shrimp bio-waste into commercially valuable products.

Materials and methods

Materials

Zobell marine broth, Zobell marine agar, *N*-acetyl-D-glucosamine (GlcNAc) (Sigma Chemical Co., St. Louis, USA), HiCarbo™ Kit, HiPurA™ DNA-Xpress™ Kit, Polymerase Chain Reaction (PCR) purification kit (Himedia, Mumbai, India.), Taq DNA polymerase and other molecular biology reagents (Genei, Bangalore, India) were procured from commercial vendors. Universal PCR primers were obtained after synthesis by BioServe, Hydrabad, India. High Performance Liquid Chromatography (HPLC) standards for monomeric GlcNAc and oligomeric GlcNAc were procured from Associates of Cape Cod, Inc. East

Falmouth, MA, USA. All other reagents and chemicals were of analytical grade.

Shrimp processing bio-waste

Shrimp (*Penaeus* sp.) processing bio-wastes containing heads and carapaces were obtained from a seafood processing factory located at Kochi, Kerala, in the South coast of India, and were transported to the laboratory under frozen condition. After thawing under running water, the samples were dried in a drying oven (Kilburn, Mumbai, India) at 55 ± 2 °C for 12 h, and stored at room temperature (28 ± 2 °C) in air tight plastic containers until use.

Analysis of proximate composition of shrimp bio-waste

The moisture, lipid, protein and ash contents of the samples were determined according to the Standard methods of AOAC (AOAC 2000). Chitin was determined according to the procedure of Spinelli et al. (1974). The pH of shrimp bio-waste was determined according to Suresh and Chandrasekaran (1998).

Preparation of pure α-chitin, native chitin and colloidal chitin

Pure α-chitin and colloidal chitin from fresh shrimp shell were prepared as reported earlier (Suresh et al. 2011b). Native chitin (deproteinized shrimp shell) was prepared from fresh shrimp bio-waste by treating with 1 N NaOH, as reported previously, for pure chitin preparation, but omitting the acid treatment (demineralization) and decolorization steps (Suresh et al. 2011b). It was further dried in a drying oven (Kilburn, Mumbai, India) at 55 ± 2 °C for 12 h, powdered to <22 mesh size, and stored at room temperature in an air tight plastic container until use.

Culture media

Native chitin seawater broth (NCSB): Artificial seawater containing native chitin (~0.5 % w/v) was used as a selective medium to enrich fastidious chitin hydrolyzing microorganisms from marine samples. Modified artificial seawater (pH 7.6) was prepared according to APHA (1998) by constituting with 12 major compounds (mg/l): NaF, 3; $SrCl_2.6H_2O$, 20; H_3BO_3, 30; KBr, 100; KCl, 700; $CaCl_2.2H_2O$, 1,470; Na_2SO_4, 4,000; $MgCl_2.6H_2O$, 10,780; NaCl, 23,500; $Na_2SiO_3.9H_2O$, 20; Na_4EDTA, 1 and $NaHCO_3$, 200. *Colloidal chitin seawater agar (CCSA)*: Artificial seawater containing colloidal chitin (1 %; w/v; wet weight basis) and agar (2 %, w/v) was used as a selective medium for the isolation and cultivation of chitinolytic marine microorganisms. The pH of CCSA was initially adjusted to 9.0 ± 0.2 with 4 N NaOH to achieve a final pH of 7.6 ± 0.2 after autoclaving. Zobell marine broth and Zobell marine agar were used as general purpose media for the cultivation of marine bacteria.

Enrichment isolation and screening of chitin hydrolyzing marine microorganisms

Marine samples including seawater, sediments, decomposing crustaceans and seaweeds were collected from the South and East coasts of South India, transported to the laboratory under chilled condition (~4 °C) in an ice box, and were processed within 24 h. Prepared NCSB was inoculated with 0.5 ml of sediment/solid sample after 1:10 (w/v) dilution with sterile physiological saline (0.85 % NaCl, w/v) or 0.5 ml of undiluted water sample to 10 ml of NCSB taken in 100 ml Erlenmeyer conical flasks towards enrichment of chitinolytic microorganisms. Inoculated flasks were incubated at 32 ± 2 °C in the dark with occasional shaking for 2 weeks. From the enriched samples, full loop aliquots were streaked on CCSA plates and incubated at 32 ± 2 °C for one week in order to obtain pure cultures. Colonies that showed a zone of clearance against the white creamy background were regarded as chitin hydrolyzing microorganism. They were selected and re-streaked on CCSA plates until pure cultures were obtained. They were later maintained at 4 °C on Zobell agar slants containing 0.05 % (w/v) colloidal chitin and subcultured monthly.

All the isolates obtained from the enriched samples were initially screened for extracellular chitinase (qualitative) production by growing them on CCSA plates of 4 mm thickness at 32 ± 2 °C for 6 days. Diameter of microbial colonies and their clearing zones were measured and their chitinolytic activity ratios (ratio of diameter of chitinolytic clearing zone to diameter of microbial colony) were calculated. Those isolates which showed high chitinolytic activity ratio were further subjected to SSF for their concomitant production of extracellular chitinase (quantitative) and GlcNAc using solid shrimp bio-waste medium (described below). Five cultures (CFR221 M, CFR173 M, CFR16 M, CFR198 M and CFR188 M) which produced significant level of extracellular chitinase and GlcNAc were further characterized.

Identification of selected microorganism

All the five selected cultures (CFR221 M, CFR173 M, CFR16 M, CFR198 M and CFR188 M) were characterized for their morphological, physiological and biochemical features according to Bergey's Manual of Systematic Bacteriology (Bergey et al. 1984). Further, 16S DNA (16S

rRNA gene) sequence homology analyses for these strains were performed to confirm their identity as described below.

Amplification, sequencing and phylogenetic analysis of 16S DNA

The cells harvested after cultivation in Zobell marine broth for 24 h were washed thoroughly using Tris–HCl-EDTA buffer (pH 8.0) and their Genomic DNA was extracted using HiPurA™ DNA-Xpress™ Kit according to the vendors' instructions. Amplification of 1.4 kb long fragments of 16S DNA from genomic DNA of all five cultures was carried out in a Thermocycler (Primus-25, PeQLab, Germany) using specific forward (CTAGAGCGATTA CTAGCGATTCCGACTTCG) and reverse (GACGTCGG CTCAGGATGAACGTC GGCGGC) primers. The PCR reaction mixture (25 µl) contained template DNA ($\sim$10 ρg), primer sets (10 ρg), Taq polymerase (1 Unit), dNTPs (2 mM) and 10X assay buffer (Tris HCl, pH 8). The Thermo cycling procedure utilized was as follows: initial denaturation at 94 °C for 5 min, followed by denaturation at 94 °C for 1 min, annealing at 65 °C for 30 s, polymerization at 72 °C for 1 min for 35 cycles, and a final extension at 72 °C for 10 min. The PCR product was then analyzed by agarose (1 %) gel electrophoresis. Later the PCR product was purified using a PCR purification kit. The DNA sequences of purified PCR product were obtained using a DNA sequencing service of SciGenom Pvt. Ltd. (Kochi, India). Homology sequence alignment of the 16S DNA sequence with other origin from GenBank (National Centre for Biotechnology information, NCBI, USA) was subjected to BLAST (Basic and Local Alignment Search Tool) analysis (Altschul et al. 1990) and a phylogenic tree was constructed using MEGA 5 software (Kumar et al. 2004).

SSF of shrimp bio-waste by marine bacteria and production of chitinase and GlcNAc

Substrate preparation

The dried shrimp bio-waste was ground to obtain small particles of 1–2 cm particle size using a Stephen Mill (UM5 Universal, Hong Kong), washed with tap water (1:10, w/v) two times and dried in a drying oven (Kilburn, Mumbai, India) at 55 ± 2 °C for 12 h. The dried shrimp bio-waste chips were then milled with an electric blender and sieved through 22 mesh. The shrimp bio-waste powder (<22 mesh size) thus prepared was used as solid substrate for SSF without any further demineralization or deproteinization treatment.

Inoculum preparation

Potent bacterial cultures (CFR221 M, CFR173 M, CFR16 M, CFR198 M and CFR188 M) were grown in Zobell marine broth at 32 ± 2 °C on a rotary shaker (150 rev/min) for 24 h. Later the cells were harvested by centrifugation (10,000 rev/min, 10 min) at room temperature, washed and resuspended in sterile physiological saline. The concentration of each cell suspension was adjusted at A_{600} to 3.5 using a spectrophotometer and the adjusted suspension was then used as an inoculum.

Media preparation and fermentation

Shrimp bio-waste powder was used as a solid substrate in the media for SSF. Five grams of the substrate taken in a 100 ml Erlenmeyer conical flask was mixed with 4 ml of artificial sea water and was then autoclaved (15 lb pressure, 30 min). After cooling, the medium was inoculated with 3.5 ml of inoculum (arbitrarily selected) and incubated at 32 ± 2 °C in an incubator without air moisture control. The solid shrimp bio-waste medium had 55 % (w/w) initial moisture content after addition of inoculum and a pH of 8.7 ± 0.2. After incubation, the fermented substrate in each flask was combined with 50 ml of chilled citrate phosphate buffer (pH 6.8, 0.1 M) and mixed well on an environmental shaker (150 rev/min) for 20 min at 20 ± 2 °C. The slurry was then allowed to settle for about 5 min followed by centrifugation (12,000 rev/min) at 4 °C for 20 min in order to separate and collect the clear extract for various assays.

Determination of chitinase activity

Chitinase (E.C.3.2.1.14) activity was assayed using colloidal chitin as the substrate (Suresh and Chandrasekaran 1999). The reaction mixture which contained 1 ml of 1 % (w/v) colloidal chitin in citrate phosphate buffer (pH 6.8, 0.1 M) and 1 ml of the SSF extract was incubated at 32 ± 2 °C for 2 h. The reaction was terminated by heating the reaction mixture in a boiling water bath for 10 min. The undigested material was removed by centrifugation (8,000 rev/min, 10 min) and the amount of GlcNAc produced in the supernatant was estimated according to Ressing et al. (1955). Heat inactivated enzyme along with the substrate was used as a blank. One unit of chitinase activity was defined as the amount of enzyme which released 1 µmol of GlcNAc under the reaction conditions. Enzyme production in SSF was defined in terms of units/g of initial dry substrate (U/g IDS) (Suresh and Chandrasekaran 1999).

Determination of GlcNAc

GlcNAc present in the clear extracts of SSF was measured colorimetrically using ρ-dimethyl aminobenzaldehyde reagent according to Ressing et al. (1955).

Scanning electron microscopy

Scanning Electron Microscopy (SEM) was used to monitor the growth of all five marine bacteria (CFR221 M, CFR173 M, CFR16 M, CFR198 M and CFR188 M) on moist solid shrimp bio-waste and its degradation during SSF. Samples from fermented (48 h) shrimp bio-waste substrate were fixed by immersion in 2.5 % (v/v) gluteraldehyde for 12 h at 4 °C and washed with phosphate buffer (0.1 M, pH 7.0) for 1 h at room temperature. They were further dehydrated in a graded 20–100 % (v/v) ethyl alcohol series and dried under vacuum at room temperature. The dried samples were coated on a double sided conducting adhesive tape pasted on to a metallic stub and subjected to gold covering (~100°A). Finally the prepared samples were examined in a Scanning Electron Microscope (LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, UK) at 20 kV.

Analysis of SSF extract by high performance liquid chromatography (HPLC)

A HPLC system containing a LC-10AT pump and RID-10A RI detector (Shimadzu, Japan) and Spherisorb® 3 µm NH_2 (3.9 × 300 mm) column (Waters, Ireland) was used to analyze the products released during the biodegradation of shrimp bio-waste under SSF (Suresh et al. 2011b). The clear SSF extract obtained after centrifugation was filtered with a membrane filter (0.45 µm pore) (Whatman Inc, NJ, USA) and 20 µl of the sample was injected onto the column using a mixture of acetonitrile and water (70:30) as the mobile phase at a flow rate of 0.8 ml/min. A mixture of *N*-acetyl-D-glucosamine, Di-*N*-acetyl-Chitobiose, Tri-*N*-acetyl-Chitotriose, Tetra-*N*-acetyl-Chitotetrose, Penta-*N*-acetyl-Chitopentose and Hexa-*N*-acetyl-Chitohexaose was used as HPLC standard.

Optimization of SSF process parameters by 'one factor at a time' approach

The SSF process for biodegradation of shrimp bio-waste and concomitant production of chitinase and GlcNAc by five different marine bacteria was optimized by 'one factor at a time' approach. The effect of different levels of incubation temperature (27–42 °C), particle size of the solid substrate (<60 mesh [fine]; <22 mesh [medium]; 60–22 mesh [coarse]; and 22–18 mesh [large]), inoculum concentration (A_{600} at 1.5, 2.5 and 3.5), and initial moisture content (55, 60 and 65 %, w/w) were evaluated by growing the bacteria under SSF and estimating the enzyme and GlcNAc. Finally, the impact of incubation time was evaluated for a total period of 96 h under optimized conditions and estimating the rate of production of enzyme and GlcNAc at varying intervals of incubation time.

Optimization of SSF process parameters by experimental design

A fractional factorial design of response surface methodology (RSM) was developed to analyze the interactions of various process variables and to optimize their levels statistically for the maximal degradation of shrimp bio-waste and concomitant production of chitinase and GlcNAc by the bacterial strain CFR173 M. The matrix incorporated with 4 factors at three equidistant levels (−1, 0 and +1) in one block encompassing 27 runs. Four independent variables were used in the design experiment; viz. incubation temperature (°C, X1) initial moisture content of the substrate (%, w/w, X2); incubation time (h, X3) and inoculum concentration (ml, A_{600} at 3.5, X4). Tables 1 and 2 give the actual and coded levels of independent variables tested and the design matrix. Dependent (response) variables evaluated included chitinase activity (Y1) and GlcNAc yield (Y2). All analyses were conducted in triplicate and the results were analyzed using statistical software package Statistica Release 7 (Statsoft 1999). The data obtained were subjected to the Analysis of Variance (ANOVA). The responses obtained were subjected to multiple non-linear regression analysis in order to obtain the co-efficient and the second order model equation (Eq. 1).

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicated response; β_o is the intercept (regression coefficient); β_i is the linear coefficient; β_{ii} is the quadratic coefficient; β_{ij} is the interaction coefficient. X_i and X_j are the independent variables.

Table 1 Independent variables in coded and actual levels used in the biodegradation of shrimp shell by-product by marine *Vibrio* sp. CFR173 M in solid state fermentation

Symbol	Independent variables		Levels		
	Factors	Unit	−1	0	+1
X1	Incubation temperature	°C	20	30	40
X2	Initial moisture content of the substrate	%, w/w	43	53	63
X3	Incubation time	h	30	64	98
X4	Inoculum concentration	ml	0.5	2.2	3.9

Table 2 Fractional factorial experimental design matrixes with independent variables at coded and actual values and response variables of GlcNAc (μmol/g IDS) and chitinase (U/g IDS) at observed and predicted values by marine *Vibrio* sp. CFR173 M in solid state fermentation

Run	Independent variables								Response variables			
	X1		X2		X3		X4		GlcNAc		Chitinase	
	C[a]	A[b]	C	A	C	A	C	A	O[c]	P[d]	O	P
1	−1	20	−1	43	−1	30	−1	0.5	0.61	0.61	3.71	3.70
2	−1	20	−1	43	0	64	1	3.9	2.39	2.74	2.05	1.87
3	−1	20	−1	43	1	98	0	2.2	4.97	2.33	3.39	4.16
4	−1	20	0	53	−1	30	1	3.9	0.71	0.99	1.97	0.55
5	−1	20	0	53	0	64	0	2.2	1.90	4.77	3.88	3.23
6	−1	20	0	53	1	98	−1	0.5	2.66	3.73	8.77	7.03
7	−1	20	1	63	−1	30	0	2.2	0.59	−2.85	0.82	2.81
8	−1	20	1	63	0	64	−1	0.5	0.56	0.30	5.23	7.00
9	−1	20	1	63	1	98	1	3.9	0.85	2.62	2.50	1.96
10	0	30	−1	43	−1	30	1	3.9	7.87	7.47	5.62	5.96
11	0	30	−1	43	0	64	0	2.2	9.29	12.87	4.88	5.47
12	0	30	−1	43	1	98	−1	0.5	14.33	13.45	5.71	6.10
13	0	30	0	53	−1	30	0	2.2	8.46	9.30	6.57	6.53
14	0	30	0	53	0	64	−1	0.5	16.62	14.07	6.59	7.55
15	0	30	0	53	1	98	1	3.9	21.25	19.08	3.65	5.33
16	0	30	1	63	−1	30	−1	0.5	1.68	4.62	12.83	9.50
17	0	30	1	63	0	64	1	3.9	14.89	13.81	3.76	4.10
18	0	30	1	63	1	98	0	2.2	12.80	12.54	7.94	7.00
19	1	40	−1	43	−1	30	0	2.2	5.27	3.59	1.91	1.76
20	1	40	−1	43	0	64	−1	0.5	9.07	9.98	0.94	−0.40
21	1	40	−1	43	1	98	1	3.9	16.92	17.68	0.62	0.19
22	1	40	0	53	−1	30	−1	0.5	5.58	4.59	1.01	3.04
23	1	40	0	53	0	64	1	3.9	18.40	16.46	0.84	0.46
24	1	40	0	53	1	98	0	2.2	14.21	16.81	0.62	0.18
25	1	40	1	63	−1	30	1	3.9	2.73	5.18	0.64	1.23
26	1	40	1	63	0	64	0	2.2	11.58	9.71	2.48	1.34
27	1	40	1	63	1	98	−1	0.5	9.68	9.44	1.31	2.56

X1 incubation temperature (°C), *X2* initial moisture contentment of the substrate (%, w/w), *X3* incubation time (h), *X4* inoculum concentration (ml)

[a] *coded;* [b] *actual;* [c] *observed;* [d] *predicted*

Statistical analysis

Statistical analyses were carried out with the statistical software Statistica Release 7 (Statsoft 1999) using the Analysis of Variance (ANOVA) techniques. All experiments were conducted in triplicate and the mean values are reported.

Results

Proximate composition of shrimp bio-waste

The wet shrimp bio-waste used in the present study had a moisture content of 84.6 ± 1.8 % and its pH was 8.7 ± 0.20. The fat, protein, chitin and ash contents were 6.8 ± 0.40, 28.7 ± 0.43, 25.4 ± 1.1 and 40.5 ± 1.4 (%, dry weight basis, dwb). The washed shrimp bio-waste material contained (%, dwb) 0.6 ± 0.03 of fat, 22.3 ± 0.40 of protein, 26.6 ± 0.60 of chitin and 44.7 ± 1.3 of ash. The washed shrimp bio-waste had a pH of 8.8 ± 0.2. The native chitin prepared had 37 ± 0.8 % chitin and 42 ± 1.4 % ash content on dwb.

Enrichment, isolation, screening and identification of chitin hydrolyzing marine microorganisms

Sixty-eight marine samples were screened for chitin hydrolyzing microorganisms by an enrichment method using selective enrichment medium (NCSB) and native chitin as the sole source of carbon. A total of 250

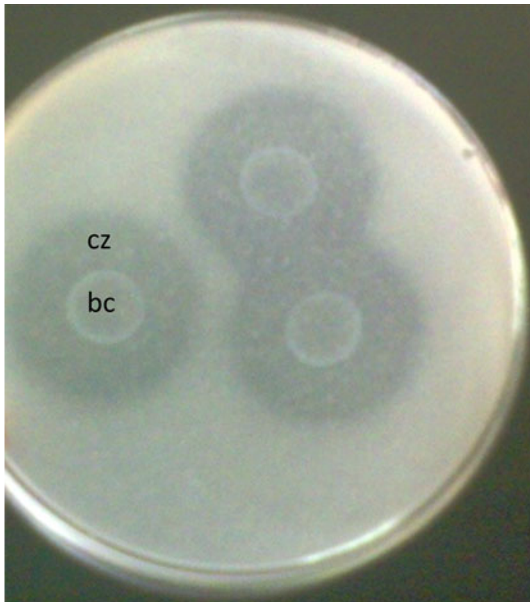


Fig. 1 Colloidal chitin artificial sea water medium (pH 7.6) showing the growth of a marine *Vibrio* sp CFR173 M) (*bc*) and characteristic chitinolytic clearing zone (*cz*) after 6 days of incubation at 32 ± 2 °C

chitnolytic bacteria were obtained from the enriched samples using the selective culture medium (CCSA). Initial qualitative screening of all the 250 isolates for chitinase production on CCSA plates (Fig. 1) resulted in the selection of 11 different isolates, which showed good chitinolytic activity ratio. Quantitative screening of these 11 isolates led to the final selection of 5 bacterial isolates (CFR221 M, CFR173 M, CFR16 M, CFR198 M, and CFR188 M) that showed significantly ($p \leq 0.05$) high levels of extracellular chitinase activity and GlcNAc using solid shrimp bio-waste as the solid substrate during SSF (data not shown). Based on the morphological, physiological and biochemical characteristics presented in Table 3 and 16S DNA homology analysis the five marine bacteria were identified as *Stenotrophomonas* sp. (CFR221 M), *Vibrio* sp. (CFR173 M), *Phyllobacteriaceae* sp. (CFR16 M), *Bacillus badius* (CFR198 M) and *Bacillus* sp. (CFR188 M). The phylogenic tree constructed with respect to all five cultures based on their 16S DNA homology studies is presented in Table 4. The 16S DNA sequences of these cultures have been deposited in the NCBI GenBank database with the accession numbers of HM440048 (CFR221 M); JN190925 (CFR173 M); HM440047 (CFR16 M); HM440045 (CFR198 M) and HM440046 (CFR188 M).

Optimization of SSF processes parameters by 'one factor at a time' approach

The results presented in Fig. (Online Resource 1) clearly indicate the strong influence of incubation temperature on concomitant production of extracellular chitinase activity and GlcNAc by the marine bacteria during SSF using shrimp bio-waste medium. The optimum incubation temperature for maximum GlcNAc production was found to be 27 ± 2 °C for all of the strains except *Bacillus* sp. CFR188 M which preferred 37 ± 2 °C for the same (Online Resource 1a). With respect chitinase activity, with the exception of *Vibrio* sp. CFR173 M, all the other cultures grew with 37 ± 2 °C as the optimum temperature for maximal enzyme production (Online Resource 1b). The particle size of the solid substrate was found to have differential influence on the rate of GlcNAc production with respect to the bacteria concerned (Fig. 2a). A particle size of <22 mesh size was observed to be the optimum for maximal chitinase activity by all the bacterial strains tested, except for *Bacillus* sp. CFR188 M (Fig. 2b).

The results presented in Fig. 3 indicate that there was a significant ($p \leq 0.05$) difference in GlcNAc yield and chitinase activity in response to an increase in inoculum concentration. The *Stenotrophomonas* sp. CFR221 M and *Vibrio* sp. CFR173 M were observed to produce maximum concentrations of GlcNAc with a high level of inoculum (3.5 OD) while *B. badius* CFR198 M produced maximum concentration of GlcNAc at moderate level of inoculum (2.5 OD). However, *Bacillus* sp. CFR188 M produced maximum GlcNAc production at very low levels of inoculum (1.5 OD) (Fig. 3a). In the case of chitinase activity, maximum enzyme production was found to be supported by high levels of inoculum for *Vibrio* sp. CFR173 M and *B. badius* CFR198 M, while low levels of inoculum (Fig. 3b) were preferred by *Bacillus* sp CFR188 M and *Stenotrophomonas* sp. CFR221 M.

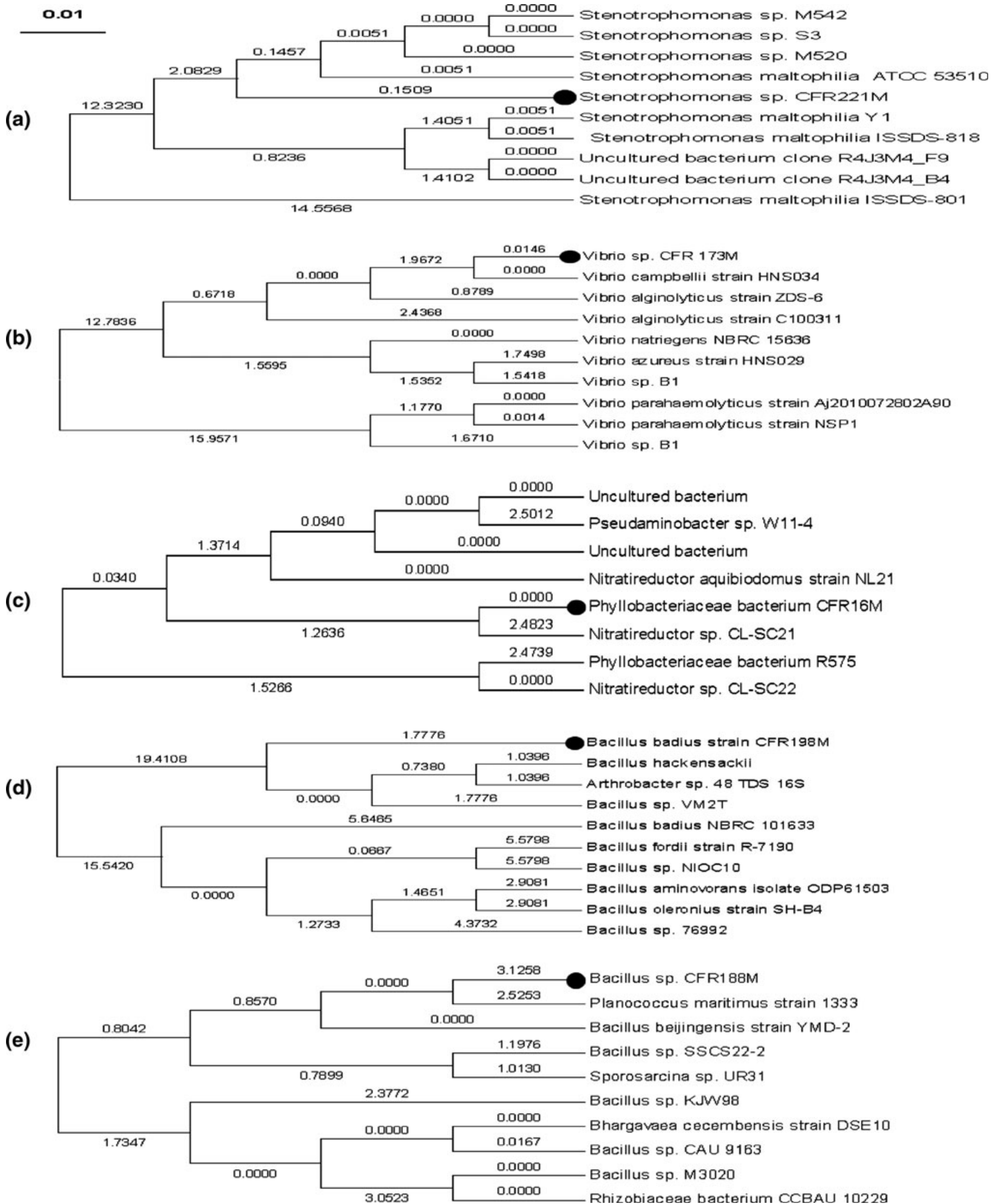
From the data presented in Fig (Online Resource 2) it was inferred that maximum levels of GlcNAc in the solid medium could be produced with 60 % (w/w) initial moisture content of the substrate by *Stenotrophomonas* sp. CFR221 M, *Vibrio* sp CFR173 M, *Phyllobacteriaceae* sp. CFR16 M and *B. badius* CFR198 M while 55 % (w/w) initial moisture content of the substrate was found to support maximal levels of GlcNAc by *B. badius* CFR198 M (Online Resource 2a). At the same time, maximum chitinase activity was generated at 60 % (w/w) initial moisture content of the substrate by *Stenotrophomonas* sp. CFR221 M, *Phyllobacteriaceae* sp. CFR16 M, *B. badius* CFR198 M and *Bacillus* sp. CFR188 M. However, *Vibrio* sp CFR173 M and *Bacillus* sp. CFR188 M generated maximum chitinase activity at 55 % (w/w) initial moisture content (Online Resource 2b).

Results presented in Fig. (Online Resource 2) indicated that the optimum incubation time for maximum chitinase activity and GlcNAc production varied for the marine bacteria. Thus after optimization by 'one factor at a time' approach the maximum amount of chitinase was recorded at 48 h for *Stenotrophomonas* sp. CFR221 M (3.3 U/g

Table 3 Morphological, physiological and biochemical characteristic of marine bacterial isolates CFR221 M, CFR173 M, CFR16 M, CFR198 M and CFR188 M

Parameter	CFR221 M	CFR173 M	CFR16 M	CFR198 M	CFR188 M
Cell morphology	Cocci	Cocci	Cocci	Rod	Rod
Gram reaction	−ve	−ve	−ve	+ve	+ve
Spore	−	−	−	+	+
Motility	+	+	+	+	−
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Indole	−	+	−	−	−
Methyl red	+	+	−	−	−
Voges Proskauer	−	+	−	−	+
Citrate	+	+	+	+	+
Nitrate	+	+	+	−	−
Urease	−	−	+	−	−
Gelatin	−	+	−	−	+
Starch	+	+	−	−	−
Casein	−	−	−	−	−
H_2S	−	−	−	−	−
O-Glucose	+	+	−	−	−
F-Glucose	+	+	+	+	−
Skim milk	−	+	−	−	+
Sugar utilization					
Lactose	−	−	−	−	−
Xylose	+	−	−	−	−
Maltose	+	+	−	−	−
Fructose	+	+	−	−	−
Dextrose	+	+	−	−	−
Galactose	+	−	−	−	−
Raffinose	−	−	−	−	−
Trehalose	−	+	−	−	−
Melibiose	−	−	−	−	−
Sucrose	+	+	−	−	−
L-Arabinose	+	−	−	−	−
Mannose	+	+	−	−	−
Inulin	−	−	−	−	−
Sod. gluconate	−	−	−	−	−
Glycerol	+	+	−	−	−
Salicin	−	−	−	−	−
Glucosamine	+	−	−	−	−
Dulcitol	−	−	−	−	−
Inositol	−	−	−	−	−
Sorbitol	−	−	−	−	−
Mannitol	+	−	−	−	−
Adonitol	−	−	−	−	−
α-Methyl-D-glucoside	−	−	−	−	−
Riboose	+	+	−	−	−
Rhamnose	−	−	−	−	−
Cellobiose	+	−	−	−	−
Melezitose	−	−	−	−	−
α-Methyl-D-mannoside	−	−	−	−	−
Xylitol	−	−	−	−	−
o-nitrophenyl-β-D-galactopyranoside	+	−	−	−	−
Esculin	−	−	−	−	+
D-arabinose	+	−	−	+	−
Malonate	+	+	+	+	+
Sorbose	−	−	−	−	−

Table 4 Phylogenetic tree obtained by neighbour-joining analysis of 16S rDNA sequences, showing the position of *Stenotrophomonas* sp. CFR221 M (a); *Vibrio* sp. CFR173 M (b); *Phyllobacteriaceae* CFR16 M (c); *Bacillus badius* CFR198 M (d) and *Bacillus* sp. CFR188 M (e) strains among the other related species



Numbers on the branch indicate the bootstrap confidence value (100 replicates). The *scale bar* represents 0.01 substitutions per nucleotide position

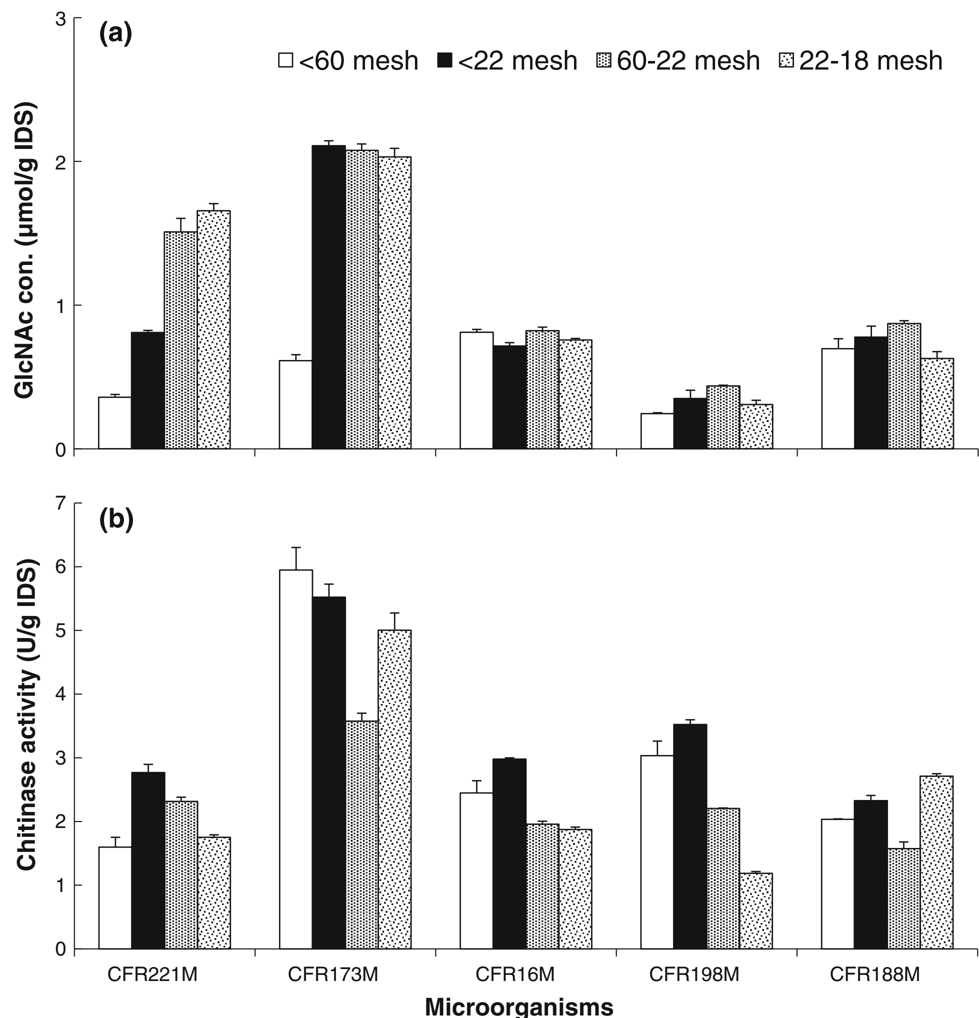


Fig. 2 The effect of particle size of the substrate on the biodegradation of shrimp bio-waste by different marine bacteria viz. *Stenotrophomonas* sp. CFR221 M; *Vibrio* sp. CFR173 M; *Phyllobacteriaceae* CFR16 M; *Bacillus badius* CFR198 M and *Bacillus* sp. CFR188 M in solid state fermentation and on production of *N*-acetyl-D-glucosamine (GlcNAc) (**a**) and chitinase (**b**)

IDS), *Vibrio* sp. CFR173 M (4.8 U/g IDS) and *B. badius* CFR198 M (3.2 U/g IDS). The maximum amount of chitinase was recorded at 72 h for *Phyllobacteriaceae* sp. CFR16 M (3.0 U/g IDS), and at 96 h for *Bacillus* sp. CFR188 M (2.7 U/g IDS). In addition, the maximum amount of GlcNAc (µmol/g IDS) was recorded at 96 h for all the bacteria (3.6, *Stenotrophomonas* sp. CFR221 M; 4.7, *Vibrio* sp. CFR173 M; 3.0, *Phyllobacteriaceae* sp. CFR16 M; 0.6, *Bacillus badius* CFR198 M and 2.9, *Bacillus* sp. CFR188 M).

Scanning electron microscopy

SEM micrographs of fermented shrimp bio-waste substrate (Fig. 4a) evidenced significant degradation of chitin microfibers, while no such degradation was observed in the SEM micrographs of non-fermented shrimp bio-waste substrate (Fig. 4b) indicating chitinolytic activity of bacteria during solid state fermentation.

HPLC analysis of products of biodegradation

HPLC chromatograms for the clear supernatant of SSF extract and GlcNAc yield presented as Fig. (Online Resource 3) testify that all the tested bacteria produced monomeric GlcNAc as the main chitin hydrolytic product along with small quantities of oligomeric GlcNAc. The maximum quantity of GlcNAc (% by HPLC) was found to be produced by *Stenotrophomonas* sp. CFR221 M (64.4) followed by *Vibrio* sp. CFR173 M (44.9), *Phyllobacteriaceae* sp. CFR16 M (47.8), *B. badius* CFR198 M (43.4) and *Bacillus* sp. CFR188 M (36.2).

Fractional factorial design

The mean responses obtained along with the predicted responses generated are presented in Table 2. Multiple regression analysis was used for the experimental data and

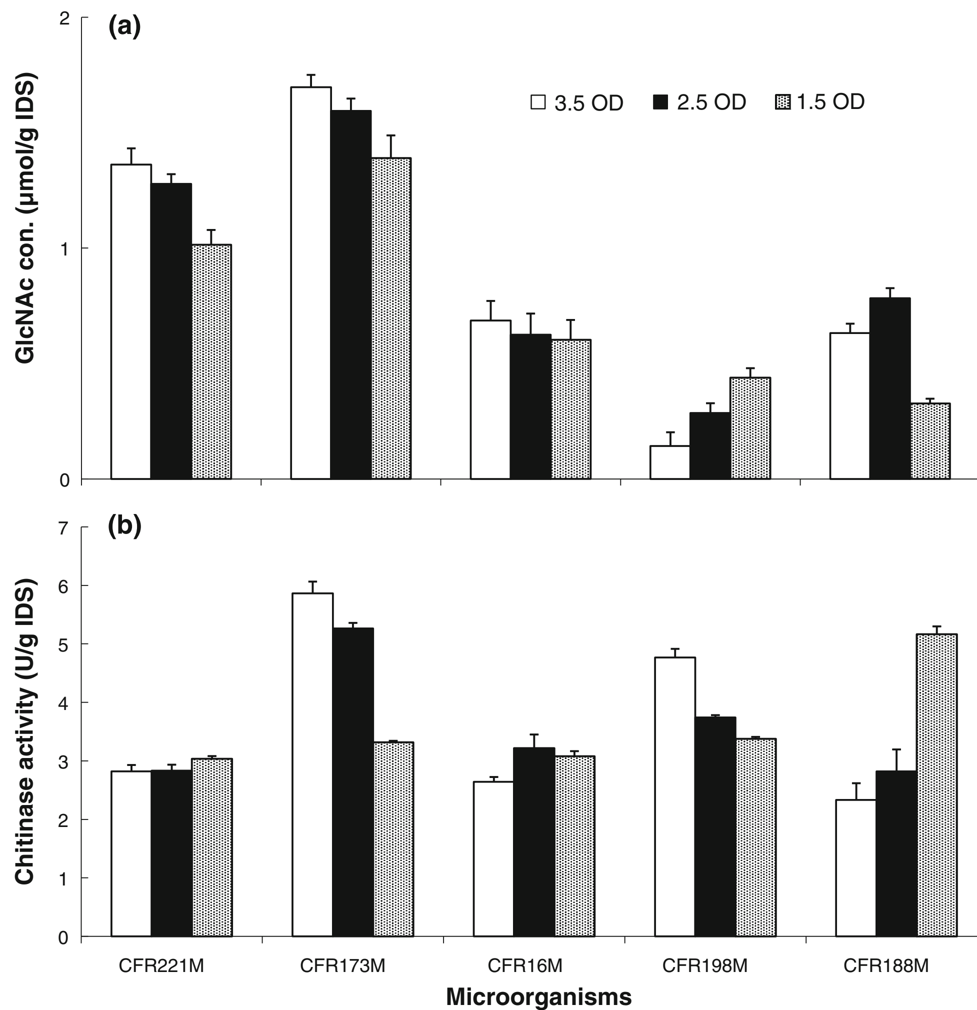


Fig. 3 The effect of inoculum concentration on the biodegradation of shrimp bio-waste by different marine bacteria viz. *Stenotrophomonas* sp. CFR221 M; *Vibrio* sp. CFR173 M; *Phyllobacteriaceae* CFR16 M; *Bacillus badius* CFR198 M and *Bacillus* sp. CFR188 M in solid state fermentation and on production of *N*-acetyl-D-glucosamine (GlcNAc) (**a**) and chitinase (**b**)

the following second order polynomial equations were derived for extracellular chitinase (Y1) and GlcNAc (Y2) production, which account for the natural logarithm of responses, as a function of four independent variables and their linear, quadratic and interactive functions.

$$\begin{aligned}\text{Chitinase(Y1)} = &(-34.22) + (2.3 * \text{X1}) + (-0.04 * \text{X1}^2)\\ &+ (0.22 * \text{X2}) + (-0.001 * \text{X2}^2)\\ &+ (0.18 * \text{X3}) + (0.0004 * \text{X3}^2)\\ &+ (0.88 * \text{X4}) + (0.05 * \text{X4}^2)\\ &+ (0.001 * \text{X1} * \text{X2}) + (-0.003 * \text{X1} * \text{X3})\\ &+ (0.051 * \text{X1} * \text{X4}) + (0.0004 * \text{X2} * \text{X3})\\ &+ (-0.066 * \text{X2} * \text{X4}) + (0.001 * \text{X3} * \text{X4})\end{aligned} \tag{2}$$

$$\begin{aligned}\text{GlcNAc (Y2)} = &(-122.3) + (3.4 * \text{X1}) + (-0.059 * \text{X1}^2)\\ &+ (2.96 * \text{X2}) + (-0.03 * \text{X2}^2)\\ &+ (0.153 * \text{X3}) + (-0.002 * \text{X3}^2)\\ &+ (-5.1 * \text{X4}) + (0.17 * \text{X4}^2)\\ &+ (0.001 * \text{X1} * \text{X2}) + (0.006 * \text{X1} * \text{X3})\\ &+ (0.07 * \text{X1} * \text{X4}) + (-0.0002 * \text{X2} * \text{X3})\\ &+ (0.045 * \text{X2} * \text{X4}) + (0.013 * \text{X3} * \text{X4})\end{aligned} \tag{3}$$

where X1, X2, X3, and X4 represent the incubation temperature, initial moisture content of the substrate, incubation time, and inoculum level, respectively.

ANOVA for the four variables indicated that the quadratic model derived from fractional factorial design could

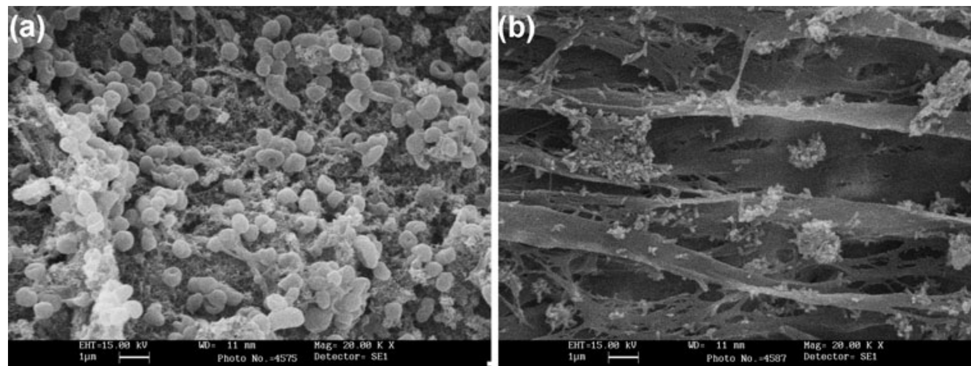


Fig. 4 Scanning electron micrograph of solid state fermented shrimp bio-waste substrate showing the growth of *Vibrio* sp. CFR173 M (**a**) and a control of non-fermented shrimp bio-waste (**b**)

adequately be used to describe the factors for chitinase (Y1) and GlcNAc (Y2) production under a wide range of operating conditions. Of the four independent variables studied, incubation temperature had a significant ($p \leq 0.05$) linear and quadratic effect on both chitinase and GlcNAc production. Initial moisture content of the substrate had a significant ($p \leq 0.05$) quadratic effect on GlcNAc production. Similarly, incubation time had a significant ($p \leq 0.05$) linear effect on GlcNAc production. At the same time, inoculum level had a significant ($p \leq 0.05$) linear effect on both chitinase and GlcNAc production. Incubation temperature (X1) and incubation time (X3) both had a significant ($p \leq 0.05$) level of interactive effect on both chitinase and GlcNAc production. Initial moisture content of the substrate (X2) and inoculum level (X4) had a significant ($p \leq 0.05$) level of interactive effect on chitinase production. The usefulness of fit of the regression equation was evaluated by the determination of coefficient (R^2). The values of R^2 recorded as 0.8298 for chitinase and 0.9129 for GlcNAc mean that the response model can explain 82.98 and 91.29 % of the total variations for chitinase and GlcNAc, respectively and indicate the accuracy of the model (a value of $R^2 > 0.75$ indicates the correctness of the model).

The relationship between the four variables was determined by constructing three-dimensional surface and counter plots, which explain the interaction between response and the experimental data as a function of the levels of two variables with the other two variables at their central value. Figure 5 represents the plots of interaction of variables in chitinase production by *Vibrio* sp. CFR173 M. From Fig. 5a–f, it was inferred that the enzyme activity increased along with an increase in incubation temperature up to a certain point beyond which the enzyme activity decreased. The quadratic effect of incubation temperature was very significant in comparison to that of all other variables. A significant ($p \leq 0.05$) interactive effect between incubation temperature and incubation time, as well as between initial moisture content of the substrate and inoculum level was observed.

The plots of interaction of variables in GlcNAc production by *Vibrio* sp. CFR173 M represented in Fig. 6a–f show a well defined quadratic effect for incubation temperature and initial moisture content of the substrate on GlcNAc production. The amount of GlcNAc was increased along with an increase in incubation temperature up to certain point beyond which the amount of GlcNAc decreased. It also showed a significant interactive effect between incubation temperature and incubation time (Fig. 6b).

The optimum levels of independent variables were determined by the least square method using the desirability graph (Online Resource 4) and the optimum levels of four variables predicted for maximum chitinase activity were; 25 °C of incubation temperature, 63 % (w/w) of initial moisture content of substrate, 98 h of incubation and 0.5 ml of inoculum for 5 g substrate (Online Resource 4a). Whereas for GlcNAc production the predicted optimum levels of incubation temperature, initial moisture content of substrate, incubation time and inoculum level were, respectively; 35 °C, 53 % (w/w), 98 h and 3.9 ml for 5 g substrate, (Online Resource 4b). The optimum levels of independent variables varied deeply with respect to the production of maximum response of variables viz. chitinase and GlcNAc. An overall twofold increase in chitinase (10.07 U/g IDS) and 3.7 fold increase in GlcNAc (21.29 μmol/g IDS) production was achieved after RSM optimization when compared to the medium optimized after 'one factor at a time' approach (5.13 U/g IDS chitinase and 5.78 μmol/g IDS GlcNAc).

Validation of the model

The good agreements between the predicted and experimental results obtained in fractional factorial design were validated using random combinations of four independent

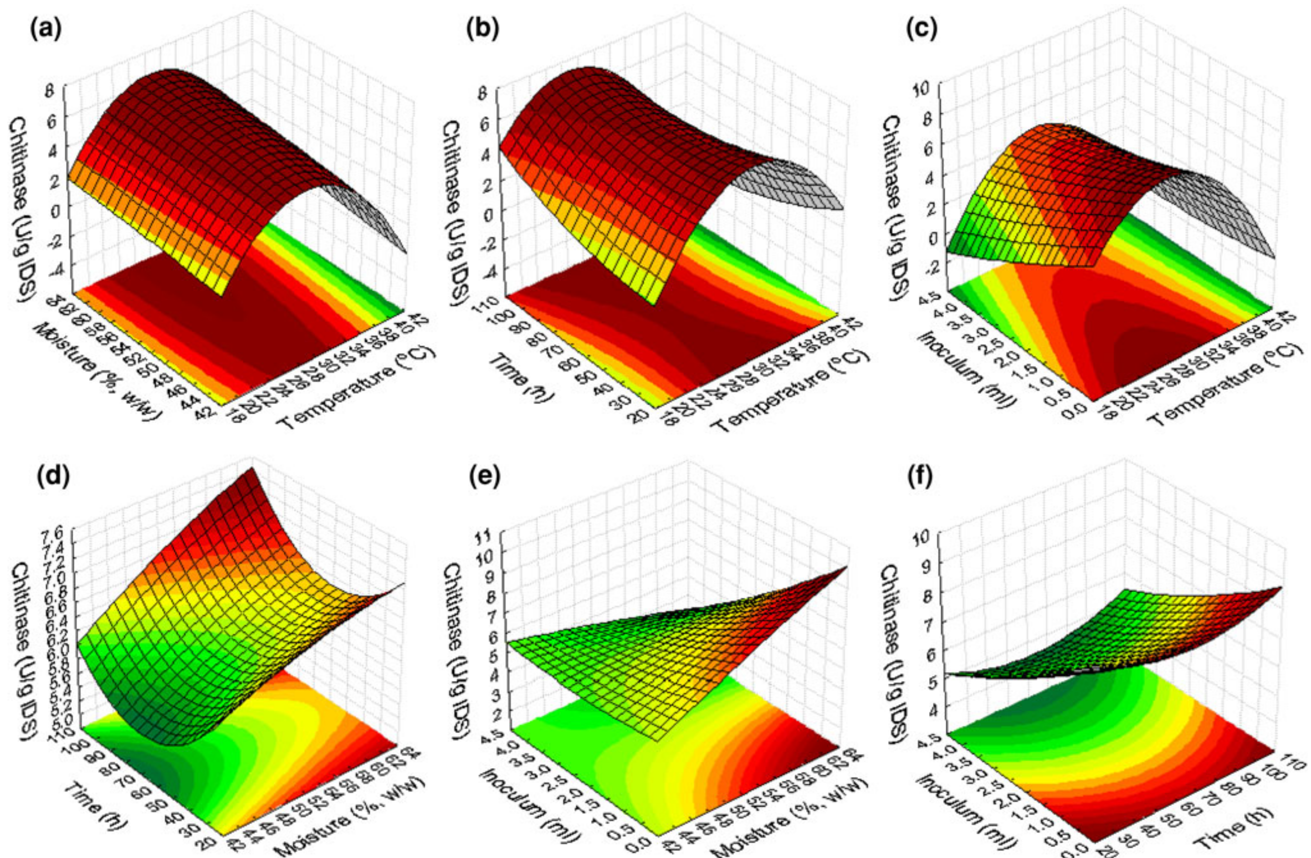


Fig. 5 Response surface and counter plots of chitinase production by marine *Vibrio* sp. CFR173 M in solid state fermentation as a function of incubation temperature and initial moisture content of substrate (**a**); incubation temperature and incubation time (**b**); incubation temperature and inoculum concentration (**c**); initial moisture content of the substrate and incubation time (**d**); initial moisture content of the substrate and inoculum concentration (**e**); incubation time and inoculum concentration (**f**) at a central value of all other variables

variables (X1–X4) (Table 5). The R^2 values for experimental versus predicted response were 0.9358 for chitinase activity and 0.8739 for GlcNAc yield.

Discussion

Enrichment, isolation, screening and identification of chitin hydrolyzing marine microorganisms

Screening of natural sources such as seawater and marine sediment is useful in the isolation of chitin hydrolyzing microorganisms that degrade shrimp bio-waste to produce bioactive compounds. In this study all 250 chitinolytic isolates obtained from 68 different water and sediments samples from marine environment were found to be bacteria. In fact, in marine environments chitin degradation and recycling is mainly carried out by chitinolytic bacteria (Gooday 1990; Keyhani and Roseman 1999; Suginta et al. 2000; Claudiana et al. 2011). Generally, chitinolytic fungus favors easily degradable chitin as compared to the raw shrimp shells and chitinolytic marine fungi are rarely reported (Gooday 1990; Suresh and Chandrasekaran1998). This possibly explains the reason why only chitinolytic bacteria were obtained from the enriched marine samples. A similar observation was reported earlier by Frank et al. (2005). Interestingly, the five cultures belonging to different species of bacteria namely *Stenotrophomonas* sp. CFR221 M, *Vibrio* sp. CFR173 M, *Phyllobacteriaceae* sp. CFR16 M, *Bacillus badius* CFR198 M and *Bacillus* sp. CFR188 M were found to have potential for degradation of shrimp bio-waste under SSF and exhibited both high chitinase activity and GlcNAc yield. These particular observations demonstrate the novel properties of marine bacteria that actually mineralize the vast quantities of crustacean shells that are disposed into the marine environment. Among the five bacterial isolates, 2 (*Bacillus badius* CFR198 M and *Bacillus* sp. CFR188 M) were related to phylogenetic class Bacilli and one (*Vibrio* sp. CFR173 M) was related to phylogenetic class Vibrio. In fact these two classes of bacteria have been frequently recovered during enrichment of chitin degrading microorganisms from

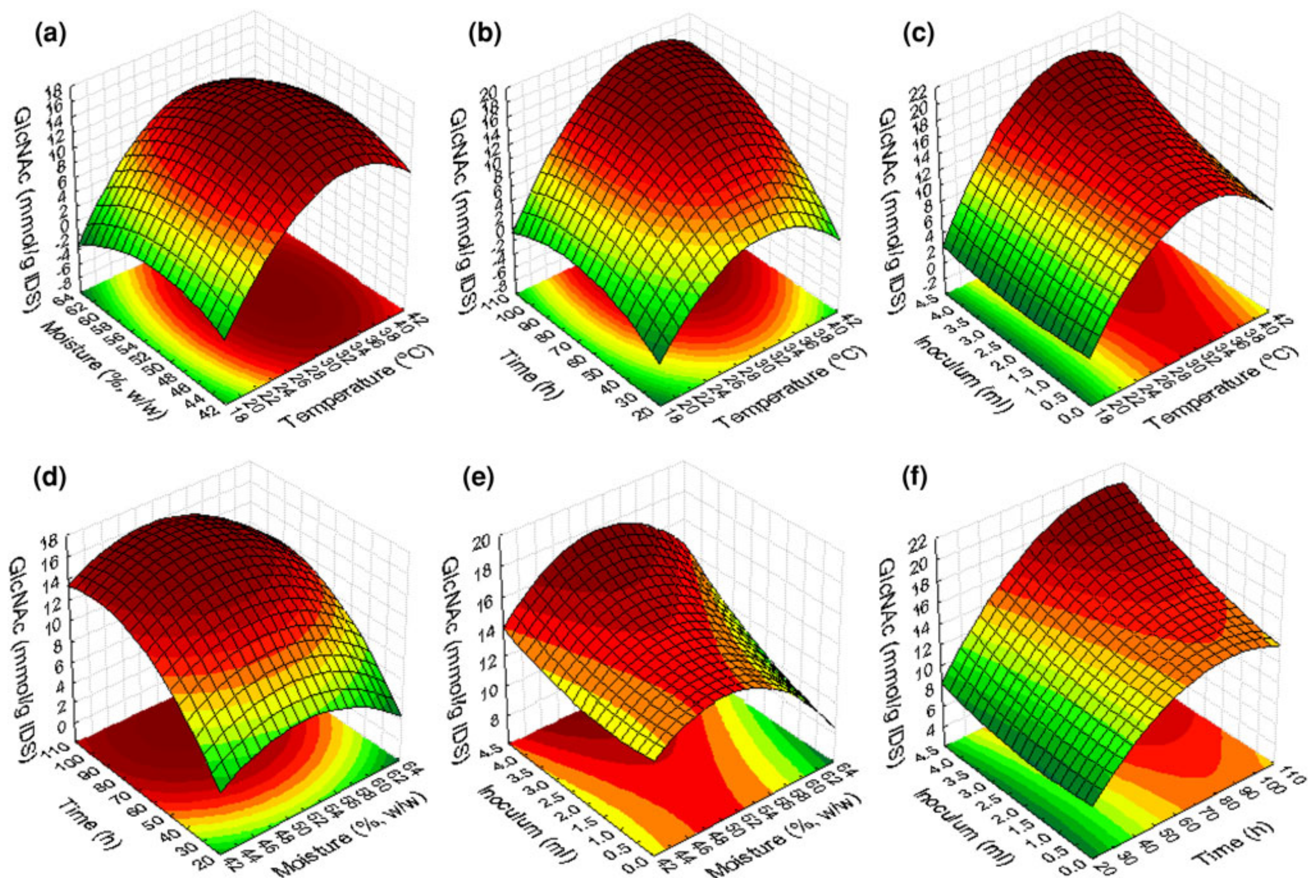


Fig. 6 Response surface and counter plots of biodegradation of shrimp bio-waste and production of *N*-acetyl-D-glucosamine (GlcNAc) by marine *Vibrio* sp. CFR173 M in solid state fermentation as a function of incubation temperature and initial moisture content of substrate (**a**); incubation temperature and incubation time (**b**); incubation temperature and inoculum concentration (**c**); initial moisture content of the substrate and incubation time (**d**); initial moisture content of the substrate and inoculum concentration (**e**); incubation time and inoculum concentration (**f**) at a central value of all other variables

marine samples (Gooday 1990; Svitil et al. 1997; Suginta et al. 2000; Frank et al. 2005; Shiro et al. 2007). However, to the best of our knowledge chitinolytic *Stenotrophomonas* sp. CFR221 M and *Phyllobacteriaceae* sp. CFR16 M have not been reported from marine environment.

SSF of shrimp bio-waste and optimization of parameters by 'one factor at a time' approach

Incubation temperature is characteristic of an organism and profoundly affects the production of enzymes and other metabolites, the duration of synthesis phase, and also the stability of products in SSF (Suresh and Chandrasekaran 1998, 1999; Pankaj et al. 2005; Suresh et al. 2011a). Results of the present investigation indicated that 37 ± 2 °C was the optimum temperature for maximal chitinase production for all the bacteria except *Vibrio* sp. CFR173 M. Prabhu and Chandrasekaran (1999) reported that 35 °C was the optimum temperature for maximal L-glutiaminase production under SSF by *V. costicola* isolated from the marine environment. At the same time the optimum temperature for maximum production of GlcNAc was 27 ± 2 °C by almost all the cultures except *Bacillus* sp. CFR188 M. This result partially explains that chitin might be optimally hydrolyzed by the chitinases produced by the organisms at this temperature. Perhaps this differential preference for varied optimal temperatures with respect chitinase and GlcNAc production could be attributed to the fact that each microorganism has its own requirement of special conditions for maximum growth and metabolites production (Suresh et al. 2011a).

The particle size, and therefore the specific area, of the substrate for adsorption of microorganisms are both of importance in the successful production of desired reactions or products in SSF. With smaller particle size, the available surface area for microbial growth is larger but poses difficulty in aeration/respiration due to limitation in inter-particle space availability. With larger substrate particles, the condition is the opposite. These two opposing

Table 5 Random combination of independent variables of X1, incubation temperature (°C); X2, initial moisture contentment of the substrate (%, w/w); X3, incubation time (h) and inoculum concentration (ml) for validation run for *N*-acetyl-D-glucosamine (μmol/g IDS) and chitinase (U/g IDS) production by marine *Vibrio* sp. CFR173 M in solid state fermentation

Run	Independent variables (actual)				Response variable	
	X1	X2	X3	X4	Observed	Predicted
N-acetyl-D-glucosamine*						
1	15	45	60	0.25	0.29	−0.90
3	37	60	120	1.50	23.81	13.82
4	15	60	90	0.25	0.56	−1.30
5	37	60	60	1.50	10.16	9.60
6	37	45	60	0.75	13.92	11.11
7	22	52	60	0.25	12.84	8.95
8	22	60	90	1.50	8.82	6.77
9	22	45	60	1.50	9.16	7.71
10	22	60	90	3.00	16.09	8.91
*Chitinase***						
1	15	45	60	0.25	0.23	0.62
2	22	52	90	0.75	7.22	7.39
3	37	60	60	1.50	3.59	4.33
4	35	45	90	3.00	2.27	2.39
5	45	60	90	1.50	0.06	−0.17
6	22	60	90	1.50	6.61	7.16
7	15	60	120	3.00	0.58	1.74
8	22	45	60	1.50	5.92	4.97
9	22	60	90	3.00	4.23	5.08

* $R^2 = 0.8739$

** $R^2 = 0.9358$

factors probably interact together and thus determine optimum growth and activity of microorganisms (Suresh and Chandrasekaran 1998; Prabhu and Chandrasekaran 1999). In the present study, almost all the cultures showed preference for optimum substrate particle size of <22 mesh for maximal chitinase production (Fig. 3). The medium particle size (<22 mesh) might facilitate a high aeration rate and consequently influence the growth and high enzyme synthesis by the organisms.

Concentration of inoculum is a critical factor and its importance in SSF has been emphasized (Suresh and Chandrasekaran 1999). Too much or too little of inoculum was found to be ineffective for good growth and enhanced product synthesis in SSF. An increase in the number of viable cells in the inoculum would ensure a rapid proliferation and enhanced biomass synthesis. At the same time, after a certain limit, competition for nutrients resulted in the decreased metabolic activity of the organism (Nampoothiri et al. 2004). In the present study high levels (2.5–3.5 A_{600}) of inoculum concentration supported maximum production of enzyme and GlcNAc as indicated by the results obtained for the study. This could be partially attributed to the increased number of bacterial cells in the inoculum which might have contributed to a rapid proliferation of biomass and high products formation. Since optimum concentrations of inoculum required for each species of bacteria vary in terms of their biosynthetic abilities, rate of cell division, and generation time no generalizations can be drawn. Moreover, at optimum inoculum concentrations there is a balance between proliferating biomass and the availability of nutrients that regulates the rate of enzyme synthesis and metabolite production (Suresh and Chandrasekaran 1999; Nampoothiri et al. 2004; Suresh et al. 2011a).

The initial moisture content of the solid substrate medium is a critical factor that determines microbial growth and product yield in SSF (Pandey et al. 1999; Suresh and Chandrasekaran 1998, 1999; Nampoothiri et al. 2004, Suresh et al. 2011a). Moisture is reported to cause swelling and thereby facilitates utilization of the substrate by the microorganisms. Higher than optimum moisture level causes reduced porosity, loss of particle structure, development of stickiness, reduction in gas volume, and decreased gas exchange (Prabhu and Chandrasekaran 1999; Suresh and Chandrasekaran 1999; Suresh et al. 2011a). Similarly, a moisture level lower than optimum develop higher water tension, lower degree of swelling, and reduced solubility of the nutrients of the solid (Suresh and Chandrasekaran 1998, 1999; Nampoothiri et al. 2004). Moreover, the optimum moisture content required for maximal growth and activity varies for different species of bacteria and mold. However, in the present study maximum chitinase activity and GlcNAc yields were obtained for all five bacterial strains studied when the initial

moisture content of the substrate was adjusted to 55–60 % (w/w). Furthermore, it was observed that the chitinase activity was reduced when the moisture content of the substrate was changed to a level that was above or below the optimum. Similar observations were reported earlier for production of microbial chitinase in SSF (Suresh and Chandrasekaran 1998, 1999; Binod et al. 2005).

Incubation time of fermentation strongly influences the rate of growth and synthesis of enzyme/metabolites. In the present study, it was found that the optimum incubation time required for maximal chitinase and GlcNAc production (Online Resource 2) varied for the different marine bacteria studied. This variation could be attributed to the differences in the physiological functions, the rate of growth, and product formation by the respective species of bacteria during SSF. Data obtained in the study confirmed that the maximum chitinase activity and GlcNAc yield obtained after optimization by 'one factor at a time' approach varied significantly ($p \leq 0.05$) with respect to time and organisms. In general, marine bacteria produce different types of chitinases for chitin degradation for their nutritional purposes (Gooday 1990) and optimization of the process conditions led to an increase in the growth and enzyme production.

Scanning electron microscopy (SEM) and HPLC

Significant degradation of chitin microfibers observed in the SEM micrograph of fermented shrimp bio-waste substrate by *Vibrio* sp. CFR173 M compared to non-fermented shrimp bio-waste substrate show the degradation of chitin by the chitinase producing bacteria during SSF. In fact, the unusual ability of marine bacteria to degrade chitinous material, as well as their role in the recycling of chitinous material in the marine environment was reported earlier (Gooday 1990; Claudiana et al. 2011). Further SEM analysis also confirmed the adsorption of these marine bacteria on solid shrimp bio-waste substrate. Marine bacteria are reported to be highly stable for use in SSF due to their remarkable ability to adsorb on solid particles (Chandrasekaran 1997; Prabhu and Chandrasekaran 1999). Thus, the results of the present study strongly advocate the use of *Vibrio* sp. CFR173 M for use in SSF of solid shrimp bio-waste by virtue of their capability to adsorb and degrade the chitinous substrates to produce high yields of chitinase and GlcNAc.

Fractional factorial design

After the selection of one best organism and identification of factors by 'one factor at a time' approach, a fractional factorial design of RSM was developed for the biodegradation of shrimp bio-waste and concomitant production of chitinase and GlcNAc by *Vibrio* sp. CFR173 M in SSF based on our previous experience of using the same design for the optimization of *β*-*N*-acetyl-D-glucosamine production by *Penicillim monoverticillium* in SSF (Suresh et al. 2011a). The results obtained for the studies indicated an interaction of a variety of variables. Thus, initial moisture content of the substrate, incubation time and inoculum level had significant linear and/or quadratic effect. Interaction between incubation temperature and incubation time, as well as between initial moisture content of the substrate and inoculum level had significant effect on GlcNAc yield. With respect to chitinase activity, inoculum level had significant linear effect. Interaction between incubation temperature and incubation time, as well as between initial moisture content of the substrate and inoculum level had significant effect on chitinase activity. These results were in agreement with previous studies on chitinase production in SSF (Neetu et al. 2005; Suresh et al. 2011a). The high values of R^2 obtained for chitinase (0.8298) and GlcNAc (0.9129) production by *Vibrio* sp. CFR173 M indicate the suitability of the RSM models. The R^2 is a proportion of variability in response values explained or accounted for by the model (Mantgomery 1984). In the present study an overall twofold increase in chitinase and 3.7 fold increase in GlcNAc production was achieved after RSM optimization as compared to the medium optimized after 'one factor at a time' approach. These observations strongly advocate the advantage of media and process optimization using statistical modeling using RSM for enhancing the capabilities of the biocatalyst towards obtaining maximal amounts of enzyme as well as the metabolite of interest.

Enhanced syntheses of chitinase after statistical optimization have been reported by several investigators. For instance; a 141 % increase in *Alcaligenes xylosoxydans* (Vaiday et al. 2003), a 4.21 fold increase in *Pantoea dispersa* (Vipul et al. 2006), a 29 % increase in *Streptomyces* sp. NK1057, a 9.3 % increase in *Streptomyces* sp. NK528, a 28 % increase in *Streptomyces* sp. NK 951 (Nawani and Kapadnis 2005), and from 616 to 1475 (U/IDS) increase in *Enterobacter* sp. NRG4 (Neetu et al. 2005) have all been reported. Recently, Suresh et al. (2011a) reported a 4.5 fold increase in *N*-acetylhexosaminidase production by *P. monoverticillium* CFR2 under SSF after statistical optimization. To the best of our knowledge the present study is the first of its kind on fermentative production of GlcNAc from shrimp bio-waste and its optimization by RSM. It is very difficult to compare, in terms of magnitude, the results of chitinolytic activity observed during SSF using shrimp bio-waste with other types of solid substrates used by investigators as well as those reported in the literature since the chemical compositions of the solid substrates vary diversely and may strongly influence the rate of solid state

fermentation. Further the differences in chitinase yields could be related to the type of organism, culture vessel, and methods of chitinase assays.

Even though the enzymatic degradation shrimp shells are eco-friendly, it is more complex since it involves both the production of the enzyme and the digestion of the substrate (Bruno et al. 2003). Concomitant production of enzymes and antioxidant materials from seafood processing chitin-rich bio-materials such as squid pens, shrimp, and crab shells have been attempted employing SmF (Wang et al. 2010; Annamalai et al. 2011). Recently, enzymatic degradation of shrimp shells and production of GlcNAc has been reported (Suresh and Anil Kumar 2012) and the present study and previous studies conducted by the author strongly indicate the scope for the concomitant production of chitinase and bio-active molecules such as GlcNAc by marine bacteria employing the economical SSF process using inexpensive and easily available shrimp bio-waste.

Conclusions

Based on the results obtained from the present investigation it is concluded that marine bacteria are ideal biocatalysts by virtue of their chitinase producing ability for possible bio-degradation and reclamation of the abundant chitinous shrimp shell bio-waste in addition to the concomitant production of chitinase and GlcNAc. Five different species of marine bacteria with potential for industrial production of chitinase and GlcNAc were also recognized. Further SSF could be the ideal bioprocess not only for effective utilization of these bio-wastes but also for economic production of chitinase and GlcNAc in a large scale setting since shrimp bio-wastes have demonstrated their suitability as solid substrates for fermentation by bacteria. There is no doubt this concomitant approach of simultaneous production of an industrial enzyme and a value added bioproduct could turn into an important element in the efficient management of this abundant bio-waste in the future. This attractive eco-friendly integrated biotechnological solution will not only minimize the environmental problems due to shrimp bio-waste disposal, but also promote the economic value of this marine bio-material.

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