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ORIGINAL ARTICLE

Solid-state cultivation of *Bacillus thuringiensis* R 176 with shrimp shells and rice straw as a substrate for chitinase production

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Abstract In this study, shrimp shell powder, prepared by treating shrimp-processing waste by boiling and crushing, was used as a substrate for isolation of chitinase-producing microorganism. These organisms may have an important economic role in the biological control of rice and other fungal pathogens. Two hundred strains of bacteria with the ability to degrade chitin from shrimp shell waste were isolated from paddy soil, and of these, 40 strains showed chitinase activity in a solid state cultivation. One of the most potent isolates (strain R 176) was identified as *Bacillus thuringiensis*. Identification was carried out using morphological and biochemical properties along with 16S rRNA sequence analysis. This strain was able to produce high levels of extracellular chitinase in solid media containing shrimp shells as sole carbon source [1.36 U/g initial dry substrate (IDS)], which was 0.36-fold higher than the productivity in a liquid culture with colloidal chitin. The effects of medium composition and physical parameters on chitinase production by this organism were studied. The optimal medium contained shrimp shell mixed with rice straw in 1:1 ratio added with ball-milled chitin 0.5 % (w/v) and ammonium sulfate 0.5 % (w/v). The highest enzyme production (3.86 U/g IDS) by *B. thuringiensis* R 176 was obtained at pH 7, 37 °C after 14 days growth. With respect to the high amount of chitinase production by this strain in a simple medium, this strain could be a suitable candidate for the production of chitinase from chitinous solid substrates, and further investigations into its structure and characteristics are merited.

Keywords Shrimp shell waste · Chitinase · *Bacillus thuringiensis* · Medium optimization · Solid-state cultivation

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Introduction

Chitin, a homopolymer of N-acetyl-D-glucosamine (GlcNAC) residues linked by β-1,4 bonds, and its derivatives, hold great economic value because of their versatile biological activities and agrochemical applications (Muzzarelli et al. 2012). Marine crustacean wastes are very rich in chitin and are major environment pollutants. Currently applied methods to purify and modify chitin from this material, and to derive useful carbohydrate products from it, involve harsh chemical treatments accompanied by uncontrollable hydrolysis and chemical modification that eventually result in the formation of undesired by-products. Moreover, the above chemical process involve purification costs for the removal of protein and calcium carbonate (Muzzarelli et al. 2012). Many bacteria and fungi produce extracellular chitinolytic enzymes, known as chitinases (E.C. 3.2.1.14), able to convert chitin into compounds that can be of industrial interest, including N-acetyl-D-glucosamine. Chitinase has also received attention due to its use as a biocontrol agent (Zhu et al. 2008; Fenice and Gooday 2006). These enzymes

have been used in biological research for the generation of fungal protoplasts to degrade the fungal cell wall, and are also used in human health care such as making ophthalmic preparations with chitinases (Narayana and Vijaualakshimi 2009).There is an increasing interest in the use of chitinases for the control of plant diseases caused by various phytopathigenic fungi, insects, nematodes, and the production of different chitin oligomers (Huang et al. 2005; de la Vega et al. 2006; Chang et al. 2007). The main drawback is the high cost of chitinase production, and this enhances the necessity to search for high yielding enzyme-productive strains and inexpensive cultivation media (Mabuchi et al. 2000; Muzzarelli et al. 2012). Several species of fungi such as *Trichoderma harzianum* (Felse and Panda 2000) and *Aspergillus* sp. (Rattanakit et al. 2002), and with bacteria and actinomycetes such as *Bacillus subtilis* (Wang et al. 2006), *B. cereus* (Chang et al. 2007), *B. licheniformis* (Waldeck et al. 2006) and *Streptomyces* (Akagi et al. 2006), have shown a chitinase producing ability. The use of microorganisms to process crustacean shell waste offers a waste management solution and commercial rewards.

Solid-state cultivation is thus a more attractive method of enzyme production because it has the advantages of low effluent generation, a requirement for simple fermentation equipment, and direct application of the fermented product. Moreover, submerged culture has previously been favored for the production of several industrial enzymes such as amylase, cellulases, hemicellulases, protease, and xylanase (Chang et al. 2007).

The present study describes the isolation of bacteria achieving high levels of chitinase production by solid-state cultivation. In this study, one of the most potent isolates was identified as *Bacillus thuringiensis* R 176, and the physicochemical parameters for the enhanced production of extracellular chitinase in solid-state culture with shrimp shells were optimized.

Materials and methods

Shrimp shells, rice straw and chemicals

Clean and fresh shrimp shells were collected in Chiang Mai province, Thailand. They were air-dried and broken into pieces in a blender. Raw rice straw was obtained from local farmers in Chiang Mai Province. It was cut to nominal lengths of 0.5–1.0 cm and washed thoroughly with tap water until the washings were clean and colorless, and then air-dried for further treatment. The main composition of this rice straw was as follows: moisture 12.8 %, cellulose 38.6 %, lignin 13.6 %, and hemicelluloses 19.7 %.

Chitosan, chitioligosaccharide, and ethylene glycol chitin were purchased from Sigma (UK). All other chemicals were of analytical grade.

Preparation of colloidal chitin

Colloidal chitin was prepared from shrimp shells according to the method of Roberts and Selitrennikoff (1988) in which 10 g of shrimp shells was added slowly into 100 ml of concentrated HCl and 20 ml of acetone under vigorous stirring for 2 h. The mixture was added to 600 ml of ice-cold 50 % ethanol under vigorous stirring for 1 h and kept overnight at 4 °C. The precipitant was collected by centrifugation and washed with 0.1 M sodium phosphate buffer (pH 7) until the colloidal chitin become natural (pH 7) and used for further applications.

Preparation of ball-milled chitin

Ball-milled chitin was prepared by milling practical-grade chitin in H_2O for 3 days following the method described for the preparation of ball-milled filter paper (Leschine and Canale-Parola 1983). Final particle size of the ball-milled chitin was 180–200 μM.

Preparation of swollen chitin

Swollen chitin (practical grade chitin) was prepared according to the method described by Hackman (1962). Briefly, swollen chitin was prepared by washing the chitin with acetone to form a chitin paste, and then the paste was slowly added to 7–9 volumes of concentrated HCl cooled in an ice bath to 4 °C to arrest hydrolysis. The syrupy liquid was filtered in a glass column through a sintered glass plate filled with glass wool. The chitin-containing filtrate was dropped into aqueous 50 % ethanol solution to precipitate the chitin. The colloidal residue was centrifuged at 8,000 rpm for 20 min and resuspended in water three to five times sedimented by gravity and washed several times with 100 mM potassium phosphate buffer containing 1 mM $CaCl_2$ (pH 6) to remove excess acid and alcohol. Finally, the swollen chitin solution was dialyzed against 100 mM potassium phosphate buffer until a pH of 5–6 was maintained.

Preparation of soluble chitosan

The powdered chitin (180–210 μM) obtained after ball-milling was alkaline-hydrolyzed with 47 % (w/w) sodium hydroxide solution at 25 °C for 56 h. The sample was neutralized by successive washing with deionized water (Berger and Reynolds 1958).

Screening and isolation on chitin agar

Around 100 rhizosphere soil samples were collected from paddy fields in Chiang Mai province. Screening of the chitinolytic bacteria was performed by plating sample solutions from samples on chitin agar plates (shrimp shells powder 10 g; yeast extract 0.5 g; $(NH_4)_2SO_4$ 2 g; KH_2PO_4 0.7 g; Na_2HPO_4 $7H_2O$ 0.2 g; $FeSO_4 \cdot 7H_2O$ 1 mg; $MnSO_4 \cdot 5H_2O$ 1 mg). The medium for the chitin agar plate was examined for the formation of a clear zone around the colony for 5 days. The size of the clear zones and colony size were both measured. After that, the bacterial isolates were checked for thermotolerance by cultivation on the agar plates from 30 to 60 °C for 48 h, and organisms capable of growth at the higher temperatures (45–60 °C) were regarded as thermotolerant. They were maintained on chitin agar slants for further studies.

Primary screening of chitinase production using solid state cultivation

A solid-state medium was prepared in a 250-ml Erlenmeyer flask according to the previous paper: 5 g shrimp shell powder was mixed with 10 ml basal medium containing 0.2 % $(NH_4)_2SO_4$, 0.1 % yeast extract, 0.028 % KH_2PO_4, 0.025 % $MgSO_4 \cdot 7H_2O$, 0.007 % $CaCl_2 \cdot 2H_2O$ (pH 7). After sterilization, the medium was inoculated with 100 μl bacterial suspension (3×10^8 colony forming units, CFU/ml) and incubated at 100 rpm in a rotator shaker at 30 °C for 14 days. The culture was extracted in 100 ml 0.1 M phosphate buffer (pH 7) by stirred for 1 h in an ice bath and then centrifuged at 6,000 rpm for 30 min. The supernatant was filtered to remove bacterial cells and used for measuring chitinase activity. For comparison, each isolate was cultivated in liquid medium containing 1 % colloidal chitin from shrimp shells, 0.03 % polypeptone, 0.03 % yeast extract, 0.07 % K_2HPO_4, 0.03 % KH_2PO_4, and 0.05 % $MgSO_4 \cdot 7H_2O$. After incubation, the culture broth was centrifuged at 6,000 rpm for 30 min, and the supernatant was used for measuring chitinase activity.

Chitinase assay

Chitinase activity was also assayed with colloidal chitin as the substrate. Enzyme solution (0.5 ml) was added to 0.5 ml of substrate solution, which contained 1 % colloidal chitin in a sodium phosphate buffer (10 mM, pH 7). The reducing sugar released was measured by the DNS method (Miller 1959) at 575 nm using GlcNAC as a standard. One unit of enzyme activity was defined as the amount of enzyme capable of liberating 1 μmol of GlcNAC under the conditions.

Identification of selected bacterial isolates

Morphological, physiological, and biochemical characteristics of the chitinolytic bacteria were studied according to the Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) and 16S rDNA gene sequencing with eubacteria specific primer set 16F27N (5'-CCAGAGTTT GATCMTGGCTCAG-3') and 16R1525XP (5'-TTCTGCAGTCTAGAAGGAGGTGWTCCAGGC-3') (Van Overbeek et al. 1997). PCR amplification and sequencing of PCR product for analysis of 16S rRNA were conducted according to Marchesi et al. (1998). A similarity search for the nucleotide sequence of 16S rRNA was carried out using a Blast search at NCBI (www.ncbi.nlm.nih.gov/Blast.cgi).

Optimization of culture condition

The optimum culture conditions for the production of chitinase by *Bacillus thuringiensis* R 176 were examined. The parameters studied include (1) different carbon and nitrogen source, (2) incubation temperature, (3), initial pH of medium, (4) substrate supplement, and (5) duration of incubation. For each parameter optimization, three independent experiments were carried out and the average values are reported.

(1) Different carbon and nitrogen source.

The effects of various carbon source (0.5 %w/v) such as different chitinolytic substrate (ball-milled chitin, colloidal chitin, soluble chitosan, flake chitosan, N-acetyl-D-glucosamine and swollen chitin) replaced shrimp shell powder in growth media for detecting initial on chitinase production. Sets of flasks containing 5 g of one of the above chitinous substrates powder and 10 ml basal medium were inoculated with 100 μl of R 176 bacterial suspension (3×10^8 CFU/ml) and incubated at 100 rpm in a rotary shaker at 30 °C for 14 days. After incubation, the culture was extracted in 100 ml 0.1 M phosphate buffer (pH 7) by stirring for 1 h in an ice bath and then centrifuged at 6,000 rpm for 30 min. The supernatant was filtered to remove bacterial cells and used for measuring chitinase activity.

To select the best nitrogen source, the shrimp shell was supplemented with different nitrogen sources in the basal medium: ammonium sulfate, sodium nitrate, peptone, and urea were tested for chitinase production. Sets of flasks containing 5 g of shrimp shell powder, 10 ml basal medium, and one of the above nitrogen sources in turn (0.5 %w/v) were inoculated with 100 μl bacterial suspension (3×10^8 CFU/ml) and incubated at 100 rpm in a

rotary shaker at 30 °C for 14 days. After incubation, The extracted enzyme was measured for chitinase activity as described previously.

(2) Effect of temperature.

The selected bacteria were grown in Erlenmeyer flasks (250 ml) containing 5 g of shrimp shell powder and 10 ml basal medium, inoculated with the bacterial strain (100 μl inoculum was approximately 3×10^8 CFU/ml) and incubated in culture over a temperature range of 25–60 °C in the optimal condition. After incubation, the extracted enzyme was estimated for chitinase activity.

(3) Initial pH of medium.

The selected bacteria were grown in Erlenmeyer flasks (250 ml) containing 5 g of shrimp shell powder and 10 ml basal medium, inoculated with the bacterial strain (100 μl inoculums was approximately 3×10^8 CFU/ml) and incubated in culture over a pH range of 3–9 in the optimal condition. After incubation, the extracted enzyme was estimated for chitinase activity.

(4) Substrate supplement.

The substrate supplement for chitinase production was carried out in solid-state medium. The selected bacteria were grown in Erlenmeyer flasks (250 ml) containing 5 g of shrimp shell powder mixed with 5 grams of substrate supplement (straw, soy bean, coconut waste, and nut peal) and 10 ml of basal medium inoculated with the bacterial strain (100 μl inoculums with approximately 3×10^8 CFU/ml) and incubated for 14 days at 30 °C. Samples were extracted and the culture supernatant was collected for chitinase activity.

(5) Duration of incubation.

Incubation times were evaluated for chitinase production. The selected bacteria were grown in Erlenmeyer flasks (250 ml) containing 5 g of shrimp shell powder and 10 ml of basal medium inoculated with the bacterial strain (100 μl inoculums with approximately 3×10^8 CFU/ml) and incubated at various times, ranging from 1 to 16 days at 30 °C. Samples were extracted and the culture supernatant was collected for chitinase activity.

Statistical analysis

One-way analyses of variance (ANOVA) followed by LSD tests ($P<0.01$) (Sokal and Rohlf 1995) were used to detect treatment difference on chitinase activity and the optimization of chitinase production. The ANOVA and LSD were performed with the computer program SYSTAT™ v.5.05, for Windows™.

Results

Isolation of the chitinolytic bacteria

In a primary screening experiment, 220 bacterial strains were isolated from different soil samples; they were capable of using colloidal chitin from shrimp shell as a sole carbon source and formed halos on chitin-enriched agar. Forty strains whose colonies formed large and clear zones (>1 cm) were purified and tested for chitinase activity with the DNS method after growth in chitin liquid medium and solid-state cultivation. Most of the strains tested exhibited chitinase activity and *B. thuringiensis* R 176 gave the highest chitinase productivity in solid-state culture [1.36 U/g initial dry substrate (IDS)] and higher than in liquid culture (1.00 U/g IDS).

Bacillus thuringiensis R 176 was therefore used for further studies (Table 1). It was subjected to taxonomic analysis based on Bergey's Manual of Determinative Bacteriology; moreover, the 16S rDNA gene sequencing with eubacteria specific primer set confirmed that the bacterium was *Bacillus thuringiensis*.

Optimization of culture conditions

Growth was carried out in a minimal synthetic medium (M9) and gradually supplemented with the various ingredients that were investigated. The parameters optimized earlier were incorporated into subsequent experiments.

Table 1 Productivity of chitinase in liquid medium and solid-state medium of some isolate bacteria

Isolates	Chitinase activity (U/g IDS)	
	Liquid medium	Solid-state medium
R 74	0.15±0.15 f	ND
R 75	0.23±0.25 f	ND
R 88	4.12±0.10 a	ND
R 108	0.35±0.05 e	ND
R 110	0.80±0.15 d	1.25±0.23 c
R 111	0.95±0.23 c	1.18±0.10 c
R 113	0.32±0.05 e	0.65±0.05 d
R 117	3.65±0.35 b	0.60±0.05 d
R 118	0.14±0.05 f	ND
R 171	0.35±0.05 e	ND
R 176	1.00±0.15 c	1.36±0.25 c
R 181	0.25±0.05 f	ND
R 190	0.87±0.15 d	0.35±0.05 e

The results are the mean of three replicates. Different letters indicate the difference between chitinase activity results ($P\geq0.01$)

ND not detected

Carbon source

The effects of various carbon sources (0.5 %w/v) such as different chitinous substrates were tested on chitinase production. In the control flask, a medium lacking chitin, no chitinase production was observed. It was found that ball-milled chitin was the best carbon source for chitinase production by *B. thuringiensis* R 176. However, when swollen chitin and colloidal chitin were included in the medium, chitinase activity was also detected at 37 °C. Addition of chitinous substrates increased the chitinase production compared with control (Fig. 1).

Nitrogen source

To select the best nitrogen sources, the chitin medium was supplemented with different nitrogen sources (0.5 %w/v). Among the various nitrogen sources in the basal medium, ammonium sulfate was the best nitrogen source for chitinase production (Fig. 2). To evaluate the effect of ammonium sulfate, different concentrations were added to the production medium (0.3–1.0 %w/v). Maximum chitinase production (1.57 U/g IDS) by *B. thuringiensis* R 176 was found in the medium with 0.5 % (w/v) ammonium sulfate.

Incubation time

The production of extracellular chitinase was monitored during the incubation time (Fig. 3). The organism started enzyme production at 6 days and produced maximum enzyme at 14 days.

pH, temperature and substrate supplement

The optimum pH and temperature for the production of chitinase were determined in the optimal medium by varying the pH of the medium and incubation temperature. The results indicated that extracellular chitinase activity appeared less at pH 3 and achieved its maximum level at pH 7 (Fig. 4). It was proved that both acid and alkaline conditions had a negative effect on the production of chitinase. *Bacillus thuringiensis* R 176 gave the highest yield of chitinase at 37 °C. The chitinase yield of isolate at this temperature was more than 2-fold higher than at 30 °C (Fig. 4b).

The highest chitinase activities were detected in the supernatants when *B. thuringiensis* R 176 was cultured in inducing broth at pH 7 (Fig. 4a). The rice straw was the best substrate supplement under solid-state fermentation, followed by shrimp shells and soy bean (Fig. 5).

Discussion

Microbial production of chitinase has captured the worldwide attention of industrial and scientific researchers, because of its wide spectrum of application. In this study, 220 chitinolytic bacteria were isolated from the rhizosphere paddy soil, demonstrating the presence of high numbers of chitin-degrading bacteria in agricultural fields (Nawani and Kapadnis 2003). Among the 220 isolates, 40 bacteria produced clear zones (> 1.0 cm) by hydrolyzing shrimp shell powder, which accounts for 18 % of the total chitinolytic bacterial isolates. The presence of chitinolytic bacteria in the crop rhizoshere soil is highly beneficial as they could suppress plant pathogenic fungi near the root zone and provide sustainable plant protection against root diseases (Shanmugaiah et al. 2008). Further, we evaluated all the 40 chitinolytic bacteria in secondary screening using their culture filtrates, and selected an isolate designated as R 176 as it hydrolyzed shrimp shell powder at the maximum in the qualitative assay performed with solid-state cultivation and on chitin agar. The ratio of the zone of chitin hydrolysis to the colony diameter was high, indicating a better diffusibility of the chitinolytic enzymes.

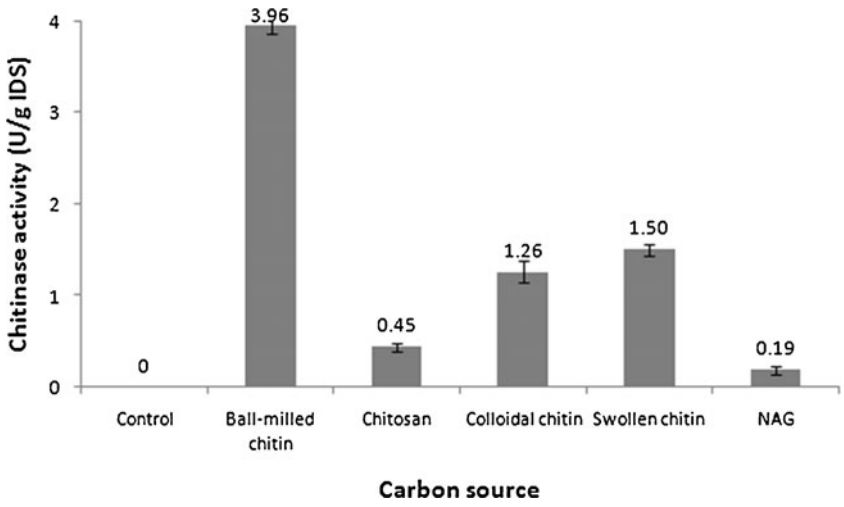

Fig. 1 Effects of different carbon sources on the chitinase production by *Bacillus thuringiensis* R 176 (values are means of three replicates ± SD)

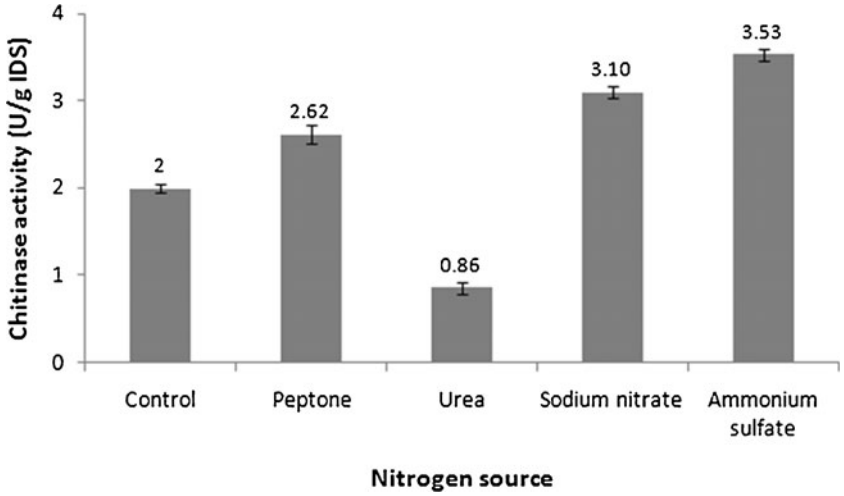

Fig. 2 Effects of different nitrogen sources on chitinase production by *Bacillus thuringiensis* R 176 (values are means of three replicates ± SD)

Identification by biochemical tests and 16S rDNA sequencing showed that R 176 belonged to *Bacillus thuringiensis*. *Bacillus* sp. is one of the most efficient bacteria for the degradation of chitin (Chang et al. 2007).

The culture medium is a key factor for the growth as well as the metabolite production by microorganisms. Among the carbon sources tested, ball-milled chitin was the best carbon source for chitinase production in solid medium. Investigation of the effects of various nitrogen sources showed that ammonium sulfate (0.5 %w/v) was the most favorable nitrogen source for chitinase production, while urea had a repressive effect. Results from the study are supported by previous reports (Rattanakit et al. 2002; Jami al Ahmadi et al. 2008), wheres among the nitrogen sources added to the basal medium, ammonium sulfate was the most effective in increasing the amount of chitinase production by *Aspergillus* sp. SI-13, whereas peptone, yeast extract, and urea had repressive effects.

The temperature and initial pH of the culture medium plays an important role in chitinase production. Maximum chitinase production of strain R 176 was achieved at pH 7 and 37 °C. The majority of the bacteria have been reported to produce maximum levels of chitinase at neutral or slightly acidic pH, whereas fungi mostly secret it in acidic conditions (Sharaf 2005). This is in agreement with the chitinase producer *Bacillus thuringiensis* sp. *kurstaki* HD-1(G) as reported by other researchers (Wiwat et al. 2000; Liu et al. 2002) with maximum chitinase production at 37 °C. *Bacillus thuringiensis* R 176 can produce chitinase in solid-state cultivation at pH 7.0, an alkaline pH, and these strains are likely to have potential for usage as synergists of *B. thuringiensis* crystal proteins, as insects tend to be alkaline. Some previous reports have indicated that the synergistic effect of chitinase and the insecticidal crystals of *B. thuringiensis* may support the idea of improving the insecticidal products by combining both factors in one strain (Liu et al. 2002). The effect of cultivation time on the production of chitinase was investigated by monitoring the enzyme production on solid medium from 3 to 16 days. The maximum chitinase production was found after incubation for 14 days. The optimum temperature of the enzyme was 37 °C (Fig. 4b) and the chitinase maintained stability in the range of temperature 30–60 °C (1.10 U/g

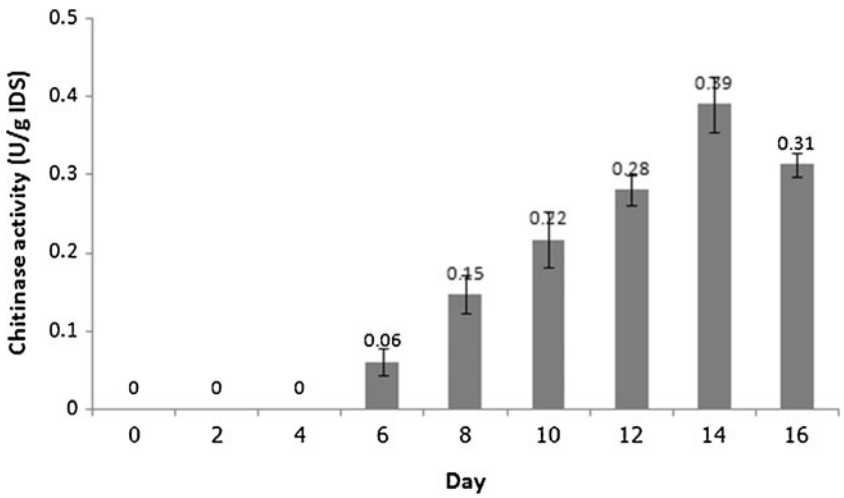

Fig. 3 Effects of incubation times on chitinase production by *Bacillus thuringiensis* R 176 (values are means of three replicates±SD)

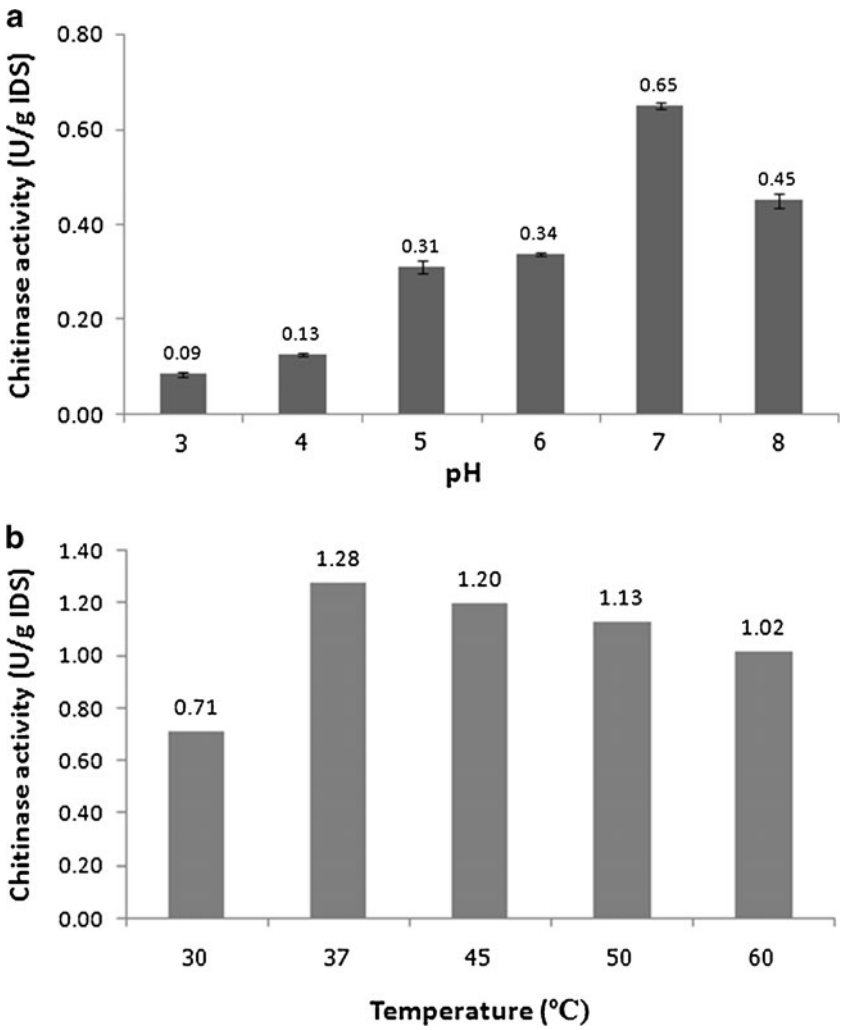

Fig. 4 Effects of pH (**a**) and temperature (**b**) on chitinase production by *Bacillus thuringiensis* R 176 (values are means of three replicates ± SD)

IDS), which coincides with that of thermostable chitinases from *B. thuringiensis*. The thermostability of chitinase of *B. thuringiensis* R 176 is also 10-times higher than that reported for *B. thuringiensis* LBIT-52, *Bt*. LBIT-59, and *Bt*. LBIT-82 chitinases (0.11, 0.12, and 0.15 U/ml, respectively) (Barboza-Corona et al. 1999).

In this study, rice straw was the best substrate supplement under solid-state fementation followed by shrimp shells and

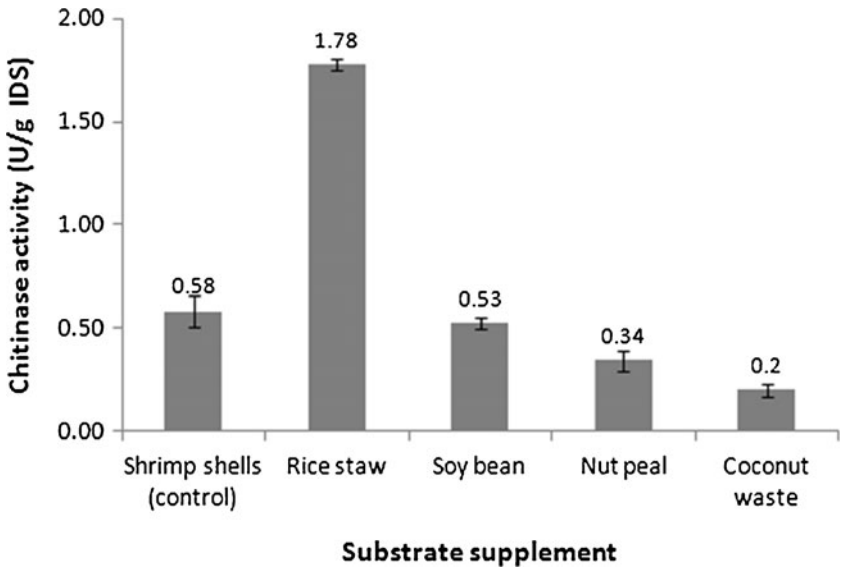

Fig. 5 Effects of substrate supplement on chitinase production by *Bacillus thuringiensis* R 176 (values are means of three replicates ± SD)

soy bean. Our results agree with others. e.g., Sudhakar and Nagarajan (2010) reported that rice agricultural residue was the best substrate supplement for chitinase production by *Serratia marcescens*. Agricultural residue is inexpensive, abundant, and easily available, and supplies the microorganism with better nutrition. Moreover, the use of purified chitin enhances the cost of enzyme production which is a major limitation to the economic feasibility of the bioconversion and utilization of lignocellulosic materials.

Bacillus thuringiensis R 176 has the capability for the production of chitinase on solid cultivation using shrimp shell waste as substrate. These bacteria may be useful for treatment of chitinolytic waste and could be an ideal candidate for biological control of rice pathogens. Further research should concentrate on the purification and characterization of chitinolytic enzymes in order to study their role in the control of rice pathogens.

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