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Chitooligosaccharides and Thermostable Chitinase Against *Vulvovaginal Candidiasis* and Saprophyte Fungi: LC Mass Studies of Shrimp Shell Fermentation by *Bacillus altitudinis*

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Abstract

Some patients with candidiasis seek alternatives drug to treat vaginal yeast infection like herbal preparations and probiotics. However, the effectiveness of such treatments has not received much study. In this research, the unique chitinotrophic *Bacillus* was isolated on shrimp shell from salt lakes and identified as *Bacillus altitudinis* by 16SRNA sequencing. This strain produced a novel chitin-oligosaccharide material and thermostable chitinase (5.1 units/ml) during 4 days incubation on shrimp shell medium; nevertheless, its growth on nutrient agar was negative. The zymogram showed less than 50 kD protein responsible for chitinase activities. The LC/MS detection of concentrate fermented products showed the production of oligosaccharide during chitin fermentation. As results of shrimp shell degradation, 65.6 mg/l protein, 73.4 mg/l *N*-acetyl glucose amine, and oligosaccharide were produced. Synergism activities of chitooligosaccharide and chitinase from this strain against fungi and pathogen candida (staining with methylene blue showed that almost 50% of 10^6 cells were died during 6 h) are promising for new anti-fungal drug with no side effect.

Introduction

Vulvovaginal candidiasis defined as vaginal and vulval infection caused by yeast, most often *Candida albicans* which affects 75% of women. Overgrowth of *Candida glabrata* and *C. albicans* may result as growth of *Vulvovaginal candida* [7]. The topically applied azole drugs are more effective than Nystatin for this infection [3]; however, the resistance is common and also liver damage is reported for using Azol drug [25]. Tea tree oil [9], lemongrass oil [23], and herbal medicinal plants [26] are used for treatment; however, most of them have bad smell and low effect. Therefore, a constant search for new drugs are necessary, and given the fact that fungi are eukaryotes are difficult to find suitable drug without side effect for humans.

Application of bioactive materials for fungi control are important and new [24, 29] since they are not toxic to human and most have anti-cancer, antioxidant and anti-tumor activities [14]. One of these bioactive materials has been obtained from shrimp shell. Shrimp shell waste is important sources of bioactive molecule [18].The major compounds from shrimp shell are chitin and chitosan. There are many microorganism able to degrade chitin by chitinase enzyme. Chitinase enzymes are a kind of glycosyl hydrolase with catalytic power which by breaking the beta 1 and 4 glycosidic linkage between *N*-acetyl glucosamine units can decompose chitin polymer into chitiooligosaccharides [10].

Chitinolytic enzymes are produced in large numbers in the presence of substrates containing chitin, including cranberry shells, peaches and mimics, which are used in agriculture, insect control, medicine, food, and fungi control [19]. Furthermore, bacteriostatic effect on Gram-negative bacteria and the anti-fungal activity of functionalized Chitin nano-crystals in polylactic acid films has been reported by Salaberria and Diaz [20].

Recent studies on chitin have attracted interest for converting them to oligosaccharides. Non-toxicity, biodegradability, and biocompatibility of Chitooligosaccharides

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promote their biological application compared to their precursor polymers. Chitooligosaccharides and their analogues are useful for their solubility and biological activities such as antimicrobial activity, anti-tumor, and immune system enhancer [15]. The Chitooligosaccharides cocktails of the fermented end products of shrimp shells have strong cholesterol lowering effect, antioxidant activity, and resist lipid peroxidation and specifically influence the growth of health beneficial microbes [8].

Shrimp shell is fermented by many bacteria such as *Aeromonas caviae*, *Bacillus* sp., *Serratia plymuthica*, and *Enterobacter agglomerans* [27]. There is no report on chitinase activities on *Bacillus altitudinis*.

In this research, the use of bioactive material derived from shrimp waste fermentation in medical applications and treatment of diseases including Vulvovaginitis is presented and has been used in several ways to obtain high yields bioactive material through environmentally friendly methods from shrimp waste. Here, the unique *Bacillus* isolated on shrimp shell from Iranian salt lakes was identified as *B. altitudinis* which produced a novel bioactive material and thermostable chitinase. The chitooligosaccharide production of this strain and synergism effect with chitinase against fungi is reported for the first time.

Materials and Methods

Isolation and Culture Media

Samples were collected from fish breeding pool, fish waste, shrimp shell waste, and the soil near plant roots from different regions in Iran, inoculated on colloidal chitin agar (CCA) medium containing 1% colloidal chitin (colloidal chitin was prepared according modified method as described by Setia et al. [21]), 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.05%NaCl, 0.1% NH_4Cl, 0.05% $MgSO_4$ $7H_2O$, 0.05% $CaCl_2$ $2H_2O$, 0.05% yeast extract, and 2% agar, and incubated at 30 °C for 3 days. Strains exhibiting a clear zone (degradation of chitin) around the colony were picked and further purified on the same medium. A strong chitinolytic (> 10 mm in diameter of halos zone) strain was selected and examined for anti-fungal activity. Finally, a strain that had high fungal activity and did not grow on the nutrient agar was selected for further examination.

Bacterial Identification

To identify the bacterium, a polymerase chain reaction (PCR) was performed to amplify the 16SrRNA gene from the genomic DNA of selected strain. The forward primer (27F) was 5′-AGAGTTTGATCMTGGCTCAG-3′, and the reverse primer (1492R) was 5′-TACGGYTACCTTACGACT T-3′. The temperature cycle was at 95 °C for 45 s, 56 °C for 45 s, and 72 °C for 45 s for 30 cycles and 7 min at 72 °C for extension. The nucleotide sequence of the 16SrRNA gene was determined and compared with published 16SrRNA sequences using Blast search at Genbank data base of Eztaxon.

Fungi Strains

Six strains *C. albicans* (PTCC 5027), *C. glabrata* and *Candida vulvovaginal* recovered from patients with Vulvovaginitis infection in Al-Zahra Hospital in Isfahan, Iran. Also *Alternaria alternate* (ATCC 5224), *Aspergillus flavus* (ATCC 5004) and *Fusarium solani* (PTCC 5284) were used as saprophyte fungi.

Shrimp Shell Analyses by XRF

The shrimp shells of *Penaeusse misulcatus* were collected from Persian Gulf, boiled, washed, and dried. Total compounds were analyzed by XRF. This also was used as carbon and nitrogen instate colloid chitin.

Sugar and Total Protein Assay in Shrimp Shell

The total reduced sugar and protein were detected by dinitrosalicylic acid (DNS) and Bradford, respectively. However *N*-acetyglucose amine was detected by Schales' reagent.

Semi-solid Fermentation of Shrimp Shell

The solid fermentation media was carried on by the isolate for 3 days incubation on semi-solid plate at 37 °C with 10% shrimp shell. The shrimp shells hydrolyzed were then extracted in pure water and centrifuged. Cell free hydrolysate was obtained by filtration and used as the source of the crude enzyme. Partial purification was performed using the chitin semi-solid medium. The supernatant was harvested after 3 days incubation and concentrated by heat (60 °C for 2 h). At this temperature, some protein was destroyed; however, the chitinase was stable and concentrated.

Chitinase Assay in Fermented Shrimp Shell

The following procedure was performed for determination of chitinase activities: the isolate was grown on colloid chitin or shrimp shell broth (1% shrimp shell, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.05%NaCl, 0.1% NH_4Cl, 0.05% $MgSO_4$ $7H_2O$, 0.05% $CaCl_2$ $2H_2O$, 0.05% yeast extract), and samples were taken at 1, 2, 3, 4, 5, 6, and 7 days. Each sample was centrifuged at 8000×*g* for 5 min and the supernatant was used for enzyme activities. Chitinase activity was assayed by measuring the amount of the reducing end

group of *N*-acetyl-D-glucosamine (GlcNAc). The assay mixture consisted of 0.05 ml of supernatant, 0.5 ml of 0.5% colloidal chitin, and 0.45 ml of 50 mM sodium acetate buffer pH 5.0 at 37 °C for 1 h.

The reaction was terminated by the addition 200 µl of 1 N NaOH. The supernatant was mixed with 1 ml Schales' reagent (0.5 M sodium carbonate and 1.5 mM potassium ferricyanide) and heated at water bath for 15 min. The amount of chitinase produced was measured at 585 nm and the activity was calculated from a standard curve based on known concentrations of *N*-acetyl-D-glucosamine [27]. The supernatant was applied at different temperatures (10 °C to 100 °C) for the enzyme assay [28] for optimum temperature.

The effect of medium initial pH on chitinase production was studied by adjusting the initial pH from 6 to 10 by using 0.1 N HCl–NaOH. To examine the optimum pH of the chitinase, 1% w/v colloidal chitin was prepared as the substrate at different pHs (3–12) by using the following buffers: glycine–HCl buffer (pH 3–5), NaH_2PO_4–Na_2HPO_4 buffer (pH 6–8), borate buffer (pH 8–9), and glycine–NaOH buffer (pH 10–12) and the supernatant chitinase was added to the substrate and then incubated in 30 °C for 30 min. Finally, the remaining activities were determined [31].

SDS-PAGE and Zymogram Analysis for Chitinase

Protein analysis after concentration by heat treatment (60 °C for 2 h) with sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out according to the method described by Laemmli [11]. 100 µl of heated and condensed supernatant were mixed with 100 µl of 2× sample buffer, boiled for 2 min, and loaded on 12% gel. The gel was stained with 0.25% coomassie brilliant blue R-250 (Bio-Rad), and destained with 10% acetic acid and 7% methanol.

Zymogram was performed as followings: protein sample was added into SDS-PAGE sample buffer (60 mM Tris–Cl, pH 6.8, 15% glycerol, and 0.1% bromphenol blue) and heated. The proteins were separated through the 10% polyacrylamide gel containing 0.1% of colloidal chitin. After electrophoresis SDS was removal by buffer (0.4 M Tris–Cl, pH 7, 1% casein, 2 mM EDTA, 0.02% sodium azide) for 2 h with several times washings. The gel was then transferred into 50 mM Tris–Cl buffer (pH 7) twice, each time for 15 min and rinsed with de-mineralized water followed by rinsing in 2-propanol for 5 min under shaking condition. Gel was incubated in digest buffer (50 mM Tris–HCl buffer, pH 7, 1 mM EDTA, and 0.1% of colloidal chitin) at 37 °C for 12 h to allow the enzyme to hydrolyze colloidal chitin substrate. Finally, the gel was stained with 0.1% of Congo Red solution for 15 min followed by washing with 1 N NaCl [31].

Paper Chromatography Analysis for Acetyl Glucosamine

Paper chromatography analysis was performed using Whatman No. 1 and a isopropanol/acetic acid/water (3:1:1) solvent to determine oligomer purity in supernatant the strain grown on colloid chitin followed by incubation for 3 min at 180 °C. *N*-acetylglucosamine (GlcNAc) and Glucose standards (Sigma-Aldrich, St. Louis, MO, USA) were used for reference. Amino sugars were detected by staining with a solution of aniline blue/diphenylamine (1 ml of aniline, 1 g of diphenylamine, 100 ml of acetone, 10 ml of 85% phosphoric acid).

Yeast Growth Inhibition Assay

MIC and MFC determinations was performed in accordance with the guidelines in NCCLS document M27-A [5]. For broth micro dilution method in the first well of sterile 96-well U-shaped plates, 120 µl of heated and condensed supernatant was used as anti-yeast. 60 µl of this well was transferred to the second well and mixed with 60 µl of BSA. These steps were repeated in subsequent wells and so each well contained a solution of supernatant with a density of half of the previous well. 60 µl (of 0.5 McFarland) of each of the three strains was added to wells. Finally 60 µL of sterilized SDB was added to all wells and the plates incubated at 30 °C for 24 h. The MIC was defined as the lowest concentration at which the optical density (OD) measured by spectrophotometer reduced to < 10% of the OD of the growth in the control. Minimal fungicidal concentrations (MFC) were evaluated by transferring 0.1 ml from all clear MIC wells (no growth seen in micro dilution trays) onto SDA plates. The MFC was the lowest concentration that killed 99.9% of cells (with less than five colonies remaining). Inhibition zones were detected for three species of *Candida* and compared with effects of Nystatin (0.3 mg/ml).

Detection of Anti-yeast activities by Methylene Blue

Several colonies of fresh and 24-h candida culture were transferred to get 5×10^8 yeast per milliliter. Cell free supernatants were mixed with yeast and during the 6, 12, and 24 h, the number of live and dead yeasts was evaluated using methylene blue. In this experiment, live yeasts are colorless and the dead cells are blue.

Detection of Anti-saprophyte Fungi with Inhibition Zone

The chitinolytic isolated was assayed for anti-fungal activity against *A. alternate, A. flavus,* and *F. solani*. Fungal spores are spread on the plate PDA and loaded cell free

supernatants in the up well and the down well was loaded with the same volume of buffer. The plates were incubated at 28 °C for five days. The diameter of inhibition zones were measured and experiment was repeated three times [17].

LC Mass of Fermented Shrimp Shell

The LC mass spectroscopy is a specific and sensitive analytical method for detecting chitiooligosaccharides [12]. The LC mass (Shimadzu LCMS 2010 A) was used from culture supernatant of shrimp shell and colloidal chitin with inactivated enzyme (100 °C for 5 min).

Anti-fungal Effect of Chitinase on Polyacrylamide Gel

The zymogram was prepared of the part of the gel containing the sample. Therefore gel was placed in Triton X-100, 25% for half an hour for SDS removal. Then the gel was transferred to a sterile plate and washed several times with distilled water for at least 5 h, transferred to a PDA plate containing the fungus *Candida*, and placed at 30 °C for 24 h. The inhibitory effect of chitinase enzyme on gel was studied.

Results

Isolation and Identification of chitinolytic bacteria: Thirty-eight chitin-degrading bacteria with different morphology of colonies were isolated. A gram positive spore forming bacteria isolated on shrimp shell media from salty lake in Iran selected and identified. Morphological and physiological characteristics of the selected isolate are presented in Table 1. According to Bergey's Manual of Systematic Bacteriology strain was classified as a bacterium belonging to the genus *Bacillus*. Primary identification and biochemical test showed that *B. aerius* and *B. altitudinis* are closed to each other and both are chitinase positive, however the stains are different by reduced nitrate and VP test. The 16S rRNA gene was amplified using universal primers, cloned, sequenced, and analyzed. Alignment of this sequence (1452 bp) through matching with reported 16S rRNA gene sequences in the Eztaxon and biological test showed %100 similarities to *B. altitudinis* and submitted to NCBI data base as Gen bank MK773490, however this strain did not have growth on nutrient agar.

The XRF and atomic adsorption analysis of raw shrimp shell are shown in Table S1. The XRF analyses showed no toxic material in shells therefore anti-fungal activities of fermented shell must be safe to human.

Table 1 Morphological and physiological characteristics of chitinase-producing bacteria isolated strain

Morphological characteristics	
Colony morphology	White round irregular edges
Form	Rod
Gram stain	Positive
Spore	Positive
Flagella	Terminal tuft of flagella
Motility	Positive
Physiological characteristics	
Anaerobic growth	Positive
Acid from glucose	Positive
Gas from glucose	Positive
Hydrolysis of mannose	Positive
Hydrolysis of mannitol	Positive
Hydrolysis of rhamnose	Positive
Hydrolysis of lactose	Positive
Hydrolysis of xylose	Negative
Hydrolysis of sucrose	Negative
Hydrolysis of gelatin	Positive
Hydrolysis of Chitin	Positive
Utilization of citrate	Positive
Hydrolysis of Casein	Positive
Indol formation	Negative
H_2S	Positive
Methyl red test	Positive
VP test	Negative
Urease	Negative
Nitrate reduction	Negative
Growth temperature	25–80 °C

Chitinase Assay

Chitinase enzyme activity was calculated using standard curve of different concentrations of *N*-acetyl glucosamine. According to the obtained values shown in Fig. 1, the range of enzymatic activity was between 1.48 and 4.64 Iu/ml. The highest enzyme activity was observed at 4.64 Iu/ml after 4 days.

The effect of temperature on enzyme activity was analyzed at various temperatures. The optimum temperature of enzyme activity was between 40 to 60 °C (Fig. 2a) and highest activity was observed at 50 °C (5.1 Iu/ml). Thermostability of the enzyme was also measured by incubation at 60–70 °C up to several hours, enzyme remained high active even after heated at 60 °C for 6 h or 70 °C for 2 h (Fig. 2b). The best pH for the production of chitinase are shown in Fig. 3.The crude chitinase exhibited maximum activity between pH 6.8 to 9.2 with an optimum activity at pH 8.4.

The molecular weight of crude concentrated enzyme after heat treatment (60 °C for 2 h) was determined by gel electrophoresis using a standard marker (Fig. 4a).

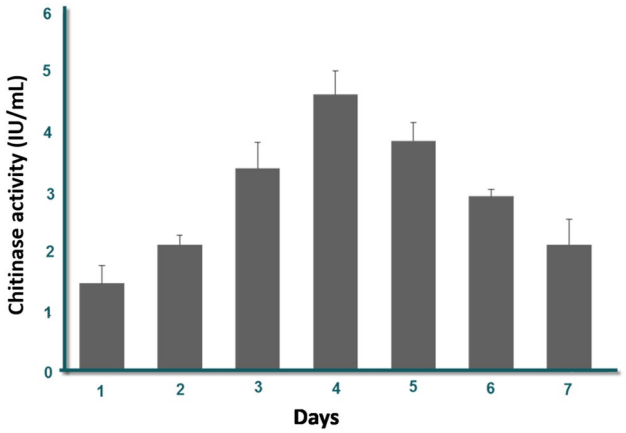

Fig. 1 Determination of chitinolytic activity by *B. altitudinis* in shrimp shell medium at 37 °C for 7 days

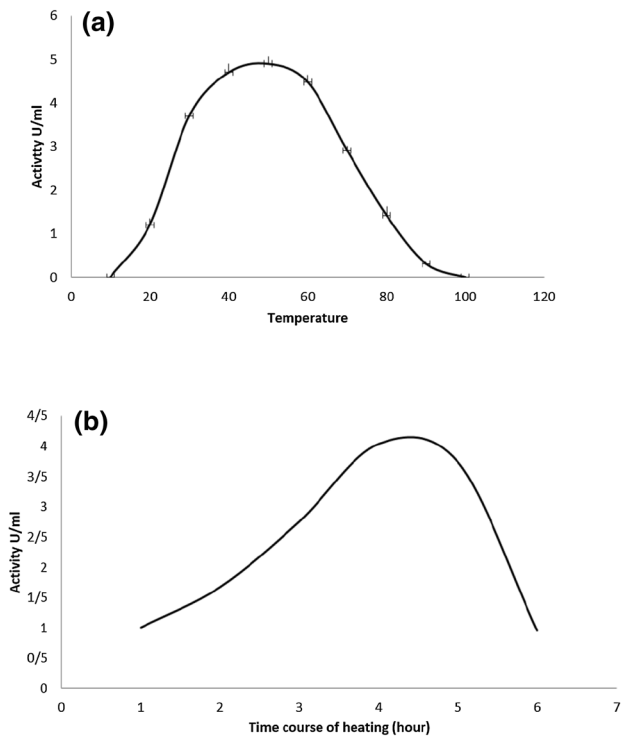

Fig. 2 **a** The effect of temperature upon chitinase *B. altitudinis* activity. The crude enzyme was incubated with the colloidal chitin substrate at the indicated temperature. **b** The effect of heat treatment at 60 °C. To analyze the heat stability, crude enzyme was incubated at 60 °C for 7 h

The Protein sample after heat treatment of isolated strain revealed a single band on 12% SDS-PAGE with an estimate of less than 50 kDa. In the heat enzyme concentration (60 °C for 2 h) the heat stable enzyme retained active while other heat sensitive proteins denature at elevated temperature. We applied this method for purification of chitinase of *B. altitudinis*.

Further, a chitinolytic activity of pure band was confirmed by zymogram (Fig. 4b). The comparison of zymograph and SDS-PAGE showed that a less than 50 kD protein had chitinase activities which is reported here on this strain for the first time (Fig. 4b). This pure band has anti-yeast activities as well.

The total sugar and protein existing in supernatant shrimp shell after fermented with *B. altitudinis* were 73.4 mg/l and 65.6 mg/l, respectively.

The monomers and oligomers with heat-inactivated enzyme (100 °C for 5 min) were visualized by paper chromatography (Fig. 5). Paper chromatogram was preliminary identification for crude enzyme by-products. Here the aniline blue reacts with *N*-acetyl glucosamine and its derivatives (like *N*-acetyl glucosamine phosphate) and give yellow color. Glucose and *N*-acetyl glucosamine derivatives produced from shrimp shells was seen in the paper chromatogram and later the LC/Mass analyzed was used to approve our initial result. The LC/MS spectroscopy is a specific and sensitive analytical method for detecting chitooligosaccharides. The LC/MS spectroscopy from culture supernatant of shrimp shell with *B. altitudinis* inactivated enzyme (100 °C for 5 min) is presented in Fig. 6. Chitooligosaccharides and *N*-acetyl glucosamine were detected by LC mass fragment ions at 829, 626, 423, and 222 m/z.

Yeast Growth Inhibition Assay

Table 2 depicts MIC as well as MFC results of the heated and condensed supernatant obtained with microdilution method for each of the studied *Candida* species. The antifungal activities of chitinase and chitin-oligomer (after enzyme denaturing by boiling) against *Candida* species are summarized in Table 3. The data showed that the supernatant *B. altitudinis* grown on Shrimp shell exhibited action against all *Candida* species, especially *C. albicans* species. According to the results of this study, it has been found that chitinase and chitin-oligomer produced by bacteria has an inhibitory effect on *Candida* fungi growth (Table 3). Antifungal activities of the cell free supernatant of *B. altitudinis* was grown on shrimp shell, *Cadida vulvovaginalis* and compare to Nystatin solution (0.3 mg/ml) (Fig. S1).

Detection of anti-yeast activities by methylene blue is a simple and low-tech technique for detecting live yeasts and dead yeasts one by one under an optical microscope. Because dead yeasts do not have the ability to reproduce, their walls become permeable to methylene blue when they are added to the methylene blue using a microscope. The candida death during 6 h was 50% by methylene blue stain (Fig. S2).

Anti-fungal effects of supernatant on the growth of *A. alternate, A. flavus,* and *F. solani* were investigated by a well diffusion assay on a PDA plate (Fig. S3) and Antifungal effect of chitinase produced by *B. altitudinis* from

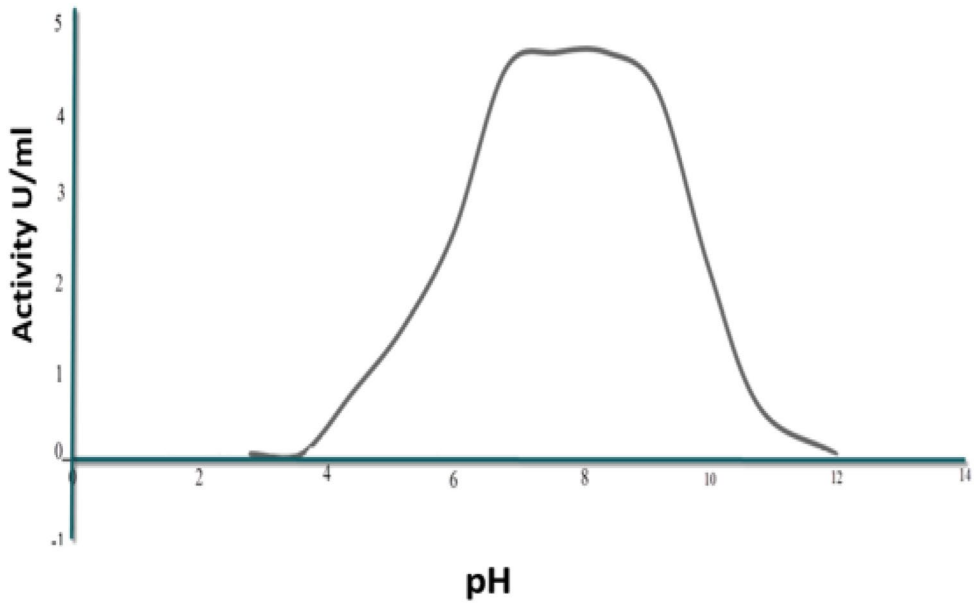

Fig. 3 The effect of pH upon chitinase activity. The crude enzyme was incubated with the colloidal chitin substrate at the indicated pH using 20 mM of citrate buffer (pH 5–6), phosphate buffer (pH 6–8), and Tris–Cl buffer (pH 7–8)

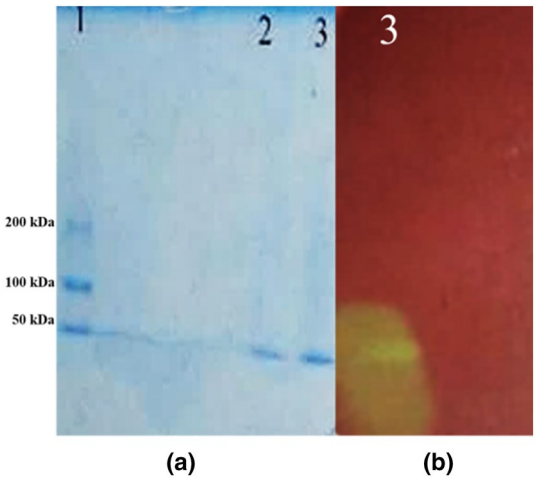

Fig. 4 **a** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and **b** Zymogram analysis of crude chitinase activity after heat treatment. Protein sample was added to SDS-PAGE sample buffer and heated at 60 °C. The proteins were separated with the 8% gel containing 0.1% of glycol chitin. After electrophoresis, the gel was washed by SDS removal buffer

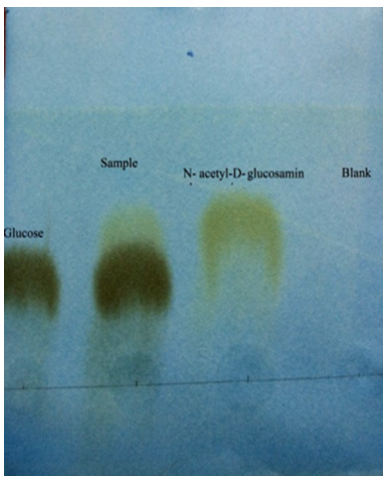

Fig. 5 Paper chromatography analysis of production of *N*-acetyl glucose amine during shrimp shell degradation by *B. altitudinis*, chromatogram paper was stained with aniline blue and showed monomer glucose and *N*-acetyl glucose amine derivatives. Aniline blue reacts with *N*-acetyl glucosamine and its derivatives (like *N*-acetyl glucosamine phosphate) and give yellow to brown color (Color figure online)

polyacrylamide gel against *Candida vulvovaginal* on PDA is shown in Fig. 7

Discussion

In recent years, many studies confirm the importance of chitinolytic bacteria as promising biological control agents for various phytopathogens [27]. Candidiasis, the main opportunistic fungal infection, has gradually increased over the past 30 years [1]. In this study, we investigated the production of *Bacillus* chitinase as effective anti-yeast enzyme from cheap substances followed by chitooligosaccharides production. Enzyme zymogram on gel showed inhibition activities of chitinase on vulvovaginitis as a common and malignant disease in women. The anti-fungal activities by purified chitinase from *A. terreus* and *Bacillus* have been reported previously [6, 28]. However the

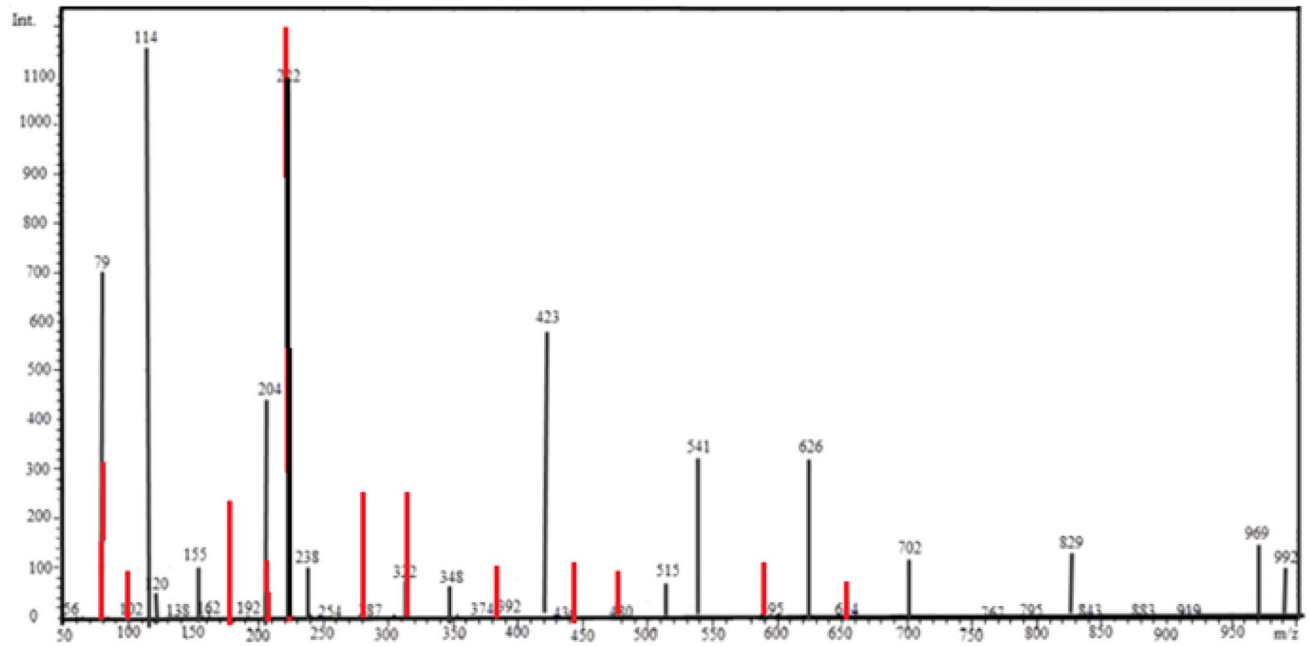

Fig. 6 LC mass spectroscopy from culture supernatant of *B. altitudinis* in shrimp shell. Chitooligosaccharides were detectable fragment ions, at m/z 829,626, 423, and 222. The red lines represent pure and standard *N*-acetyl glucosamine chromatogram (Color figure online)

Table 2 MIC and MFC of supernatant 100 µl (0.13 mg/ml total protein) *B. altitudinis* grown on Shrimp shell for candida strains

Species	MIC (dilution factor)	MFC (dilution factor)
Candida albicans (PTCC 5027)	128	512
Candida glabrata	64	512
Candida vulvovaginit	64	256

synergic effect of termostable chitinase enzyme and chitin-oligosaccharide from *B. altitudinis* on *Candida vulvovaginalis* is reported here for the first time. The data showed that during 12 h treatment all *Candida* were inhibited by these products.

In recent years with the development of water farming, shrimp farming is widespread; disposal of shrimp waste, especially with fish waste, is an environmental crisis and an economic problem [4]. In this way, the application of technology suitable for converting biological materials from shrimp waste to valuable products is essential. The degradation of shrimp shell bio-waste by the chitinolytic bacteria is the cost effective and alternative method for the production of chitooligosaccharides in large scale. The result of LC mass showed that *B. altitudinis* utilized shrimp shell chitin as sole carbon source during solid state fermentation and degraded of chitin into chitooligosaccharides and glucosamine.

The isolated *Bacillus* was unique and did not grow on nutrient agar, or any rich media. However, the growth was positive on chitosan, chitin, and CCB, with peptone as nitrogen sources and shrimp shell as the only sources of nitrogen and carbon. The growth on glucose as only carbon source occurred only at pH 9, yet on shrimp shell grew at pH 7 and evaluated for the production of chitinases. Thermostable chitinase enzymes from the Indonesian *Bacillus* sp. [31], *Bacillus amyloliquefaciens* [13], and *Bacillus licheniformis* have been reported but there are

Table 3 Comparison effects of Nystatin and supernatant *B. altitudinis* grown on Shrimp shell against some *Candida* species

Species	Inhibition zone with Nystatin(mm) 100 µl (0.3 mg/ml)	Inhibition zone with cell free supernatant(mm) 100 µl (0.13 mg/ml total protein)	Inhibition zone with cell free supernatant after heat treatment (100 °C for 5 min) (mm) 100 µl (0.13 mg/ml total protein)
Candida albicans (PTCC 5027)	18	23	9
Candida glabrata	18	25	12
Candida vulvovaginit	14	18	8

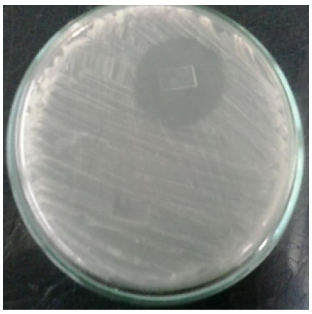

Fig. 7 The effect Anti-fungal of chitinase produced by *B. altitudinis* from zymogram polyacrylamide gel against *Candida vulvovaginal* on PDA. The gel was cut and embedded on PDA

no data about chitinase activities on shrimp shell in *B. altitudinis*. The XRF analyses showed that there are no toxic materials in the shrimp shells; therefore, anti-fungal activities of fermented shell must be safe to human.

Two strategies were used for enzyme concentration, semi-solid culture medium and heat concentration at 60 °C for 2 h. The semi-solid medium has many advantages. It involves comparatively low investment and allows the use of substrate with high dry matter content. Hence it yields a high enzyme concentration and at 60 °C for 2 h and the heat stable enzyme could retain while other heat labile proteins denature at elevated temperature. We applied this technique for purification of chitinase from *B. altitudinis*.

The highest activity for crude chitinase (5.1 IU/ml) was detected at 96 h incubation on shrimp at 55 °C with good agreements on the chitinase produced from *Bacillus circulans* reported by Wiwat et al. [30] and from Bacillus laterosporous MML2270 isolated from rice rhizosphere soil by Shanmugaiah [22].

The maximum temperature was in the range of 40–60 °C with optimal activity at 50 °C. These results are in good agreement with other chitinases from many microbes for example Zeki and Muslim, 2010 [16], Anuradha and Revathi, 2013 [2]. The crude chitinase exhibited maximum activity between pH 6.8 to 9.2 with an optimum activity at pH 8.4. Several workers have reported that purified chitinase exhibited an optimum activity at a pH 6.0 and stable at pH 4.5 to 7.5 for *Enterobacter* sp. KB3 [27]. The bacterial chitinases are functional in broad ranges of pH from 5.0 to 8.0; however, an optimum of pH 5.8 is reported for *Aspergillus terreus* [6].

In conclusion the chitinase and chitin-oligosaccharide produced by quite unique *B. altitudinis* are anti-saprophytes and pathogen fungi would be useful for industrial, medical, argo and food preservative technology. Synergism effect of chitinase and chitin-oligomer could be used successfully in seriously infections. Solubility of chitooligosaccharides in water facilitates their industrial application. Their antimicrobial activities are stable in high temperature and it does not depend on the pH or temperature. Chitooligosaccharides can be used as natural medicine for the advantage of not producing major side effect.

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