A novel and improved microbial-chemical process to obtain chitin, chitosan and chito-oligosaccharides from shrimp waste

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Abstract
In this research, the obtention of products with a high added value such as chitin, chitosan and chito-oligosaccharides from a protein-chitinous by product (shrimp waste) was explored, such process also considered to reduce the use of aggressive treatments with acids and alkalis as usually is done; such practices generate serious environmental problems, as well as, resulting products may content dangerous metallic substances. We used 2 % shrimp waste suspended in tap water, as a culture medium for growing *Bacillus thuringiensis* strain Bt-LBM1 during 96 h at 28 °C. The extracellular protease secreted during this fermentation process reduced 73 % of the protein contained in the shrimp waste. The remaining material was then recovered by centrifugation at 5000 rev/min for 10 min, and dried overnight at 40 °C. The resulting solid was then treated with NaOH at 50% for 4 h at 60 °C with shaking to chitin deacetylation and to obtain chitosan. The chito-oligosaccharides were finally produced by using the extracellular chitosanase of *B. thuringiensis* (LBM2) produced when culturing such bacterium in a medium with colloidal chitosan suspended at 2 % in tap water during 96 h at 28 °C. The chitosan obtained in this process was 78.26 % deacetylated, while the chito-oligosaccharides were at least 6 compounds including mono, di, three and tetrasaccharides.

Keywords

B. thuringiensis, shrimp waste, chitin, chitosan, chito-oligosaccharides.

Introduction

Since 1998 to 2019, the volume of shrimp captured in México has varied within 80 to 220 K tons where 40-50 % of such volume represents crustacean wastes [1]. In Mexico, a minimal proportion of such material is used to manufacture chicken feed and food flavors, while in other countries the main use is for the obtention of chitin and chitosan. Both polymers are widely used in industries such as medical, pharmaceutical, food and effluent treatments [2]. Unfortunately, the obtention of chitin-chitosan require that the crustacean wastes being subjected to aggressive chemical processes with acids and alkali, for removing both minerals and proteins and deacetylation [3]. Besides, the use of hydrochloric acid (HCl) may alter the physical-chemical properties of both chitin and chitosan and is also a source of environmental pollution [4],[5]. Therefore, other alternatives have been proposed such as the use of lactic acid produced by several microorganisms such as *B. subtilis, Lactobacillus helveticus, Pseudomonas aeruginosa, Lactobacillus paracasei,Lecanicillium fungicola* and *Penicillium chrysogenum* for demineralization. For deproteinization, some microbial proteolytic enzymes as that secreted by *Lactobacillus plantarum* have been proposed [4]. It is interesting, therefore, that exploitation of shrimp waste by microbial means has had few relevance, in view that its chemical composition consists of 22-54 % protein, 13-35 % chitin and 18-38 % minerals [6]. Based on previous information, the aim of the present study was to produce chitin, chitosan, and chito-oligosaccharides from chitosan, through microbial processes using both *B. thuringiensis* strains LBM1 and LBM2 reducing the use of chemical treatments.

Methods

Bacterial strains

The protease-producing *B. thuringiensis* (LBM1) and chitosanase-producing *B. thuringiensis* (LBM2) were used. Both cultures were provided by the microbial culture collection of the Microbial Biotechnology Laboratory at the Faculty of Biological and Agricultural Sciences, Universidad Veracruzana. México. Bacterial cultures were reactivated by seeding lyophilized spores in nutrient broth and culturing at 180 rev/min, 28 °C for 18 h [7].

Substrate

Shrimp waste (*Penaeus* sp.) was collected at Puerto de Alvarado, Veracruz, México, where shrimps are captured for commercial purposes and hand peeled. The wastes were hand cleaned under water and then sun dried. Afterwards, they were ground-up and passed through a #40 mesh [8].

Medium and culture conditions

Culture medium was prepared by suspending the milled shrimp waste in tap water, at the concentration of 2 %. Bacterial incubation was carried out at 28 °C, 180 rev/min for 80 h. In some experimental sets, the values of variables were modified to study the optimal conditions for the production of both protease and chitinase. For each 25 ml of fresh media, 1 ml of inoculum (third sub-culture) was grown for 18 h in the same media and under the same conditions [9].

Evaluation of fermentation

Batch fermentation at *B. thuringiensis* LBM1 was carried out in a Fernbach 2L flasks (working volume 500 mL) with 2 % of shrimp waste suspended in tap water and incubated in shaker at 180 rev/min, 28 °C for 96 h.

Protease production

Protease production was monitored by measuring the absorbance at 280 nm of soluble products released by the enzymatic activity where casein was used as substrate [10]. For this purpose, 0.5 ml of supernatants from cultures grown in shrimp waste medium, and 1 ml of 1% casein in 0.2 M glycine-NaOH buffer (pH 9.0) were mixed and incubated for 30 min at 37 °C. Then, 3.5 ml of cold trichloroacetic acid (7 %) were added and then, the mixture was incubated for 30 min at 5 °C. Tubes were centrifuged at 6,000 rev/min for 10 min and then the absorbance at 280 nm corresponding to digested casein was measured. Absorbance data were interpolated in a standard curve prepared with a series of dilutions of the amino acid tyrosine (0 to 300 μM/ml). One protease unit (PU) was defined as the amount of enzyme required to produce 1μg of tyrosine/min. [9].

Chitinase activity

Chitinase activity was evaluated by measuring the reduction of 3,5-dinitrosalicylic acid by the amino sugar N-acetyl-D-glucosamine (NAG) released by enzymatic hydrolysis of chitin [11]. One ml of 10 % wet colloidal chitin suspended in 0.2 M phosphate buffer pH 6.5, and 1 ml of supernatants from cultures were mixed and incubated for 60 min at 50 °C. Enzymatic action was stopped by adding 1 ml 1% NaOH and the mix was then centrifuged at 6000 rev/min for 10 min. One ml supernatant and 1 ml of 1% 3,5- dinitrosalicylic acid (dissolved in 30% sodium potassium tartrate in 2 M NaOH) were mixed, to measure soluble NAG

produced by chitinases present in culture supernatants. Tubes were incubated for 5 min in boiling water, and their absorbance at 535 nm was recorded. Readings were interpolated in a standard curve prepared with a series of dilutions of NAG (0 to 10 µM/ml) and 3,5-dinitrosalicylic acid. A chitinase activity unit (CU) was defined as the amount of enzyme required to produce 1 µmol of NAG in 1 h [12].

Bromatological analysis

Both the original shrimp wastes as the residual solid obtained after the culture of Bt-LBM1 were studied for knowing their composition by the conventional techniques used in food analysis. The total nitrogen was determined by the Kjieldahl method. Ash were determined by calcination. The lipid content was determined by continuous extraction at reflux of the sample. The crude fiber was evaluated by digesting the sample with H_2SO_4 and NaOH, both at 1.25 % [13].

Obtention of chitosan by deacetylation of chitin

The chitin deacetylation was carried out with 50 % NaOH (w/w) for 6 h at 90°C under shaking. The resulting residue was filtered and washed with water until neutralization and dried overnight at 40 ºC.

Determination of the deacetylation degree

The deacetylation degree (DD) was verified with the potentiometric titration method using chitosan samples of 0.05 g, which were dissolved in 10 mL of 0.1 M HCl and stirred for 30 min [14]. When material was fully dissolved, 1% phenolphthalein indicator (two drops) was added and then titrated with 0.1 M sodium hydroxide using a burette, until color had changed. The deacetylation of chitosan was determined using the following equation:

$$\%DD = 2.03\left(\frac{V1 - V2}{m + 0.0042(V2 - V1)}\right)$$

m = sample mass;
V2, V1 = volumes of 0.1 M of NaOH used for titration
2.03 = coefficient resulting from the weight of the monomer chitin.
0.0042 = coefficient resulting from the difference in molecular weights of chitin and chitosan monomers.

Colloidal chitosan production

The solid chitosan sample was mixed with phosphoric acid in a ratio of 1:10 (w:v) and left at 5 °C for 24 h. Subsequently, it was resuspended in a water ethanol solution 1:2 (previously cooled to 20 °C) and placed under refrigeration for 12 h. The resulting material was collected by centrifugation and washed with cold ethanol up to pH 7.0. The obtained colloidal chitosan was used both as substrate for enzyme induction of bacterial chitosanase and as well as raw material for Chito-oligosaccharides production.

Obtention of extracellular chitosanase of Bacillus thuringiensis (Bt-LBM2)

The colloidal chitosan samples obtained in this experimentation were used to make three separate growing media, to produce three different batches of chitosanase. For enzyme

production in submerged culture, the basal medium was [15] (0.625 g/L^{-1} of $(NH_4)_2C_6H_6O_7$; 0.250g/ L^{-1} NaCl; 0.375g/ L^{-1} $NaHCO_3$; 0.375g/ L^{-1} K_2HPO_4; 0.275g /L^{-1} de $MgSO_4$; 1 g /L^{-1} yeast extract; 1g/ L^{-1} of hydrolysate amino acid, and 6.75 mL/ L^{-1} glycerol) was added to 20 g/L^{-1} of colloidal chitosan. The pH was adjusted to 7.0 and autoclaved. The production of different enzymes was undertaken in Erlenmeyer flasks, incubated under shaking (180 rev/min) for 80 h at 28 °C, inoculated with 0.1 mL of a culture of Bt, grown under the same conditions and cultured for 45 h. The contents of the flasks were centrifuged, and the supernatants were recovered and then sterilized with Millipore filtration units (0.22 μm) [16].

Enzymatic hydrolysis of colloidal chitosan for chito-oligosaccharides production

Five hundred microliters of colloidal chitosan (15% w/v) in 0.1 M phosphate buffer of pH 7.0 was mixed with an equal volume of the sterile enzyme solution, and incubated at 50 °C for 9 h. The reaction was quenched with 1 mL of 1 N NaOH solution and centrifuged. Reducing sugars were determined by the DNS method [17] and proteins [18] were measured every 1 h until the end of the experiment. The hydrolysis kinetic was reported as mmol of glucosamine per milliliter per microgram of protein (mmol of GlcN. mL^{-1}. mg of $protein^{-1}$). For the production of chito-oligosaccharides, equal volumes (100 mL) of 15% of colloidal chitosan solution and sterile enzyme extract were mixed and incubated under the same conditions until the maximum of reducing sugar concentration was observed. Subsequently, the sample containing the chito-oligosaccharides were subjected to Chromatographic separation in Sephadex G-15

Thin layer chromatography (TLC)

The two fractions obtained of chito-oligosaccharides were concentrated by lyophilization in a Labconco Model 451 F equipment with a vacuum pressure of 50–100 mm Hg and a temperature of 100 °C.

The dried chito-oligosaccharides sample was resuspended in one-tenth of the original volume with distilled water. For TLC analysis, 80 μL samples of the concentrate were applied on silica gel plates. For chromatographic separation, a mixture of propanol–water–ammonium hydroxide (7:3:1) was used as mobile phase [19]. After that, dried chromatograms were sprayed with a methanolic solution of 4 % trifenyltetrazolium chloride and heated at 110 °C [20]. Reference samples (30 μL) of amino sugars were placed on the same silica gel plates at a concentration of 100 μg/mL.

Results

The fermentation of shrimp wastes by BT (Fig.1) showed the production of high levels of proteolytic activity (350 U/ml), and very low levels of chitinolytic activity (0.12 U/ml). Also amino sugars evaluated as N-acetyl-D-glucosamine (NAG 0.135 mg/ml) were accumulated in the medium. The bacterial biomass and a residual solid (rich in chitin) also were accumulate in the broth (data not shown). Table 1 summarizes the material balance of fermentation after 96 h of bacterial incubation at 28 ºC with a shaking of 180 rev/min.

Fig. 1 Proteolytic and chitinolytic activity of Bacillus *thuringiensis* LBM1 in media with shrimp waste.

Table 1. Material balance after fermentation of shrimp waste by *Bacillus thuringiensis* LBM1*

Parameter	Solid remaining	Biomass	Protease	N-acetil-D glucosamine	Chitinase
Result	35.73 % of initial solid (chitin)	0.240 g (dry weight by 100 ml of medium)	350 PU/ mg of protein	0.135 mg/ml	0.12 QU/ml

*96 h, 28°C, 180 rpm.

Table 2 shows the results of the bromatological analysis of raw material at initial time of fermentation and up to 96 h. The data analysis shows variations that resulted in each of the parameters evaluated where there is a low moisture content of the raw material at the beginning of the fermentation (4.35 %); however, at 96 h an even greater moisture loss is observed. Likewise, a decrease in the percentage of protein is observed at the end of the fermentation time, due to the fact that LBM1 strain is highly proteolytic (350 UP/mg protein), while the crude fiber (chitin) remains constant throughout the process, this is due to the fact that this microorganism produces chitinases in a very low proportion (0.2 UQ/mg chitinase) which prevents the degradation of the substrate of interest. Finally, there is also an increase in the concentration of ashes (calcium salts), which can be easily removed from media by wash out.

Table 2. Bromatological analysis of the raw material at different fermentation times

Time (hour)	Humidity (%)	Ashes (%)	Protein (%)	Ethereal extract (%)	Raw fiber (%)
0	4.35	32.88	26.31	1.74	34.72
24	1.97	36.88	22.87	4.14	34.13
48	1.85	38.08	20.25	4.63	35.20
72	1.80	39.09	17.98	5.12	34.29
96	1.77	50.10	7.29	5.11	35.73

Once fermentation was finished, the resulting material was washed and recovered by centrifugation at 5000 rev/min for 15 min and washed with distilled water three times and then dried at 40 °C overnight and subsequently a single chemical treatment was treated with 50% NaOH for 4 h at 60 °C under stirring conditions to deacetylate chitin to obtain chitosan. Such chitosan was characterized by a potentiometric titration finding a degree of deacetylation of 78.26 %. Finally, the chito-oligosaccharides were produced by enzymatic saccharification of colloidal chitosan, with the crude chitosanase produced by the Bt LBM2 strain grown in a medium with 2% chitosan suspended in a synthetic medium for 90 h at 50 °C (Fig. 2).

Fig. 2 Production of chitosanase when Bt-LBM2 was grown in a medium with colloidal chitosan as the only carbon and nitrogen source.

When the chito-oligosaccharides mixture was analyzed by exclusion chromatography in a column Sephadex G-15, two major fractions were observed (Fig. 3).

Fig. 3 Chromatographic analysis of the chito-oligosaccharides contained in a mixture produced by enzymatic hydrolysis of colloidal chitosan by the extracellular chitosanase of Bt-LBM2.

Results indicated in figure 4, shows that a mixture of about 6 oligosaccharides were produced, whose molecular weight corresponded mainly to 3 or more monosaccharide subunits which were mainly contained in the peak 1. The peak 2 corresponds to low concentrations of some compounds, although a low amount of the monosaccharide NAG was produced when the enzymatic treatment was extended.

Fig. 4 Chito-oligosaccharides present in the two fractions obtained by exclusion chromatography in Sephadex G-15 of the enzymatic hydrolysate of colloidal chitosan by the extracellular chitosanase of Bt-LBM2. (NAG, N-acetil-D-glucosamine; Gluc, glucosamine; QB, chitobiase).

Conclusions

The results showed that, *B. thuringiensis* LBM1 was able to deproteinize shrimp shell. The resulting chitin, as the desired product was easily converted to chitosan by deacetylation in a 50 % NaOH solution at 60 °C during 4 h. We got a chitosan with a good degree of deacetylation about 78.26 %. Finally, the saccharification the colloidal chitosan with the chitosanase from *B. thuringiensis* LBM2 produced about 6 chito-oligosaccharides, which included mono, di, tri and tetrasaccharides.

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