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Research Article

Production of High Viscosity Chitosan from Biologically Purified Chitin Isolated by Microbial Fermentation and Deproteinization

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The objective of this study was to produce high viscosity chitosan from shrimp chitin prepared by using a two-step biological treatment process: decalcification and deproteinization. Glucose was fermented with *Lactobacillus pentosus* L7 to lactic acid. At a pH of 3.9 ± 0.1, the calcium carbonate of the shells was solubilized in 48 hours. The amounts of residual calcium in the form of ash (1.4 ± 0.5%) and crude protein (23.2 ± 2.5%) were further eliminated by the activity of proteolytic *Bacillus thuringiensis* SA. After decalcification and deproteinization of the shrimp shells, residual calcium and crude protein of shrimp chitin flakes were 1.7 ± 0.4% and 3.8 ± 1.3%, respectively. Chitin was deacetylated with 50% NaOH at 121°C for 5 hours. After deacetylation, the chitosan had residual calcium, crude protein content, and degree of acetylation of 1.6 ± 0.6%, 0.4 ± 0.3%, and 83.2 ± 1.5%, respectively. The viscosity of chitosan prepared from chitin extracted by this two-step biological process was 1,007 ± 14.7 $mPa \cdot s$, whereas chitosan prepared from chemically processed chitin had a viscosity of 323 ± 15.6 $mPa \cdot s$, indicating that biologically purified chitin gave chitosan with a high quality.

1. Introduction

The main unutilized biomass from the shrimp packaging and processing industries is heads and body carapaces, which constitute 45–50% of the wet weight of fresh shrimps [1–5]. About 20–40% of shrimp biowaste consists of chitin encrusted with calcium carbonate, protein, astaxanthin, and lipid residues [6, 7]. Shrimp biowaste is often treated in landfills or discarded in sea water, resulting in ecological problems in coastal areas, whereas a small part is used as a major component in chicken or fish feed, mixed with other agricultural raw materials [6]. The exoskeletons of crustacean waste from the seafood industry are traditionally used to prepare commercial chitin and chitosan [8, 9]. Chitin, or β-(1, 4)-linked *N*-acetyl glucosamine (GlcNAc), is the most abundant natural polysaccharide on earth after cellulose [10, 11]. Chitin can be converted to chitosan (β-(1, 4)-linked linear polymer of 2-acetamide-2-deoxy-β-D-glucose) by deacetylation with concentrated NaOH. Commercial applications of chitosan are influenced by its viscosity in solution. The viscosity of chitosan depends strongly on the viscosity of the "preproduct" chitin [6].

In commercial chitin extraction, chitin is usually isolated by a simple process which involves the use of alkaline and acid solutions to deproteinize and decalcify the shrimp shells; this is considered to be a non-environmentally friendly process. Chemical chitin extraction has a high efficiency for recovering purified chitin, but the process creates hazardous wastes which are harmful to human health and ecological systems [6, 7, 12, 13]. In addition, the chemical process has a negative effect on the intrinsic and physical properties of purified chitin, leading to a decrease in the viscosity of chitosan [6]. Crude proteins and carotenoids in the extracting solution are useless after deproteinization and decalcification [14, 15].

Continued chitin production by chemical processes without development and utilization of novel technologies cannot solve the problem of achieving environmental sustainability [15]. Currently, strictness in environmental protection has become a basic requirement for waste management in food and agricultural industries.

To overcome the shortcomings of chemical chitin purification, several biotechnological techniques have been developed that are considered to be efficient alternative approaches for recovery of high quality chitin [4, 6, 15–18]. The use of commercial crude enzymes to extract chitin influenced the cost production and also gave the low extraction efficacy [18]. As a substitute for chemical and enzymatic processes, lactic acid fermentation combined with microbial deproteinization warrants further investigation because they are eco-friendly and positive procedures. The bacterial strain *L. pentosus* L7 was found to provide the high acid production, while *B. thuringiensis* SA could produce proteases in a culture medium containing shrimp shells. *B. thuringiensis* is well known as a bacterium that can produce insecticidal protein and is nonpathogenic to humans.

Therefore, these two bacterial strains were used to extract chitin from shrimp shells. The objective of this work was to investigate a novel process for producing high viscosity chitosan from chitin isolated by two-step purification: the first step using the lactic acid bacterium *L. pentosus* L7, and the second step using the protease-producing bacterium *B. thuringiensis* SA. Based on our knowledge, no prior study has reported the preparation of chitosan from biologically purified chitin prepared by using a combination of both strains.

2. Materials and Methods

2.1. Raw Materials. Shells and heads of marine Pacific white shrimp (*Litopenaeus vannamei*) were obtained from a seafood wholesaler in Samut Sakhon province, Thailand. Both biowastes were packed into an ice box for transportation and stored frozen at −20°C in the laboratory until used. Before proximate analysis, both shrimp biowastes were washed thoroughly with tap water and dried in a hot-air oven at 102°C for 12 hours. For chitin and chitosan flake productions, shrimp shells were homogenized in a blender until small sized pieces (10–20 mm) were obtained; these were then kept frozen until used.

2.2. Microorganisms. The lactic acid bacterium *L. pentosus* L7 and proteolytic *B. thuringiensis* SA were obtained from the Department of Microbiology, King Mongkut's University of Technology Thonburi, Thailand. Both strains were kept in a freezer at −80°C for long-term storage and cultured on slants immediately prior to use.

2.3. Preparation of Shrimp Head Extract Solution (SHES). Shrimp head extract solution (SHES) was prepared by boiling shrimp heads in deionized water. Briefly, shrimp heads were mixed with deionized water at a ratio of 1 : 2 (50%, w/v) and boiled for 30 minutes. SHES was obtained by filtering the solution through cotton to remove the sediment and then adding deionized water to obtain the initial liquid volume before heat extraction.

2.4. Determination of L. pentosus L7 Growth in Various Culture Media Having SHES as a Major Component. *L. pentosus* L7 was cultured in de Man, Rogosa, and Sharpe medium (MRS), pH 6.6 (20 g glucose, 10 g peptone, 5 g beef extract, 5 g yeast extract, 5 g sodium acetate, 2 g triammonium citrate, 2 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$, 1 g Tween 80, and 1,000 mL distilled water), and incubated at 37°C 24 hours for use as an inoculum. The growth of *L. pentosus* L7 in the following culture media: (1) MRS, (2) SHES, (3) SHES plus 2% glucose, (4) SHES plus 2% NaCl, (5) SHES plus 2% glucose and NaCl, and (6) modified MRS medium (using SHES to replace peptone, beef extract, and yeast extract), was determined at 37°C using the spread plate technique on MRS agar.

2.5. Determination of B. thuringiensis SA Growth in SHES Compared with Nutrient Broth (NB) and Tryptic Soy Broth (TSB). *B. thuringiensis* SA was cultured at 37°C in nutrient broth (NB), pH 7.0 (5 g peptone, 3 g beef extract, 3 g yeast extract, 5 g NaCl, and 1,000 mL distilled water) for 24 hours and used as an inoculum. The growth of *B. thuringiensis* SA in SHES was determined at 37°C using the spread plate technique on nutrient agar (NA) compared with the growth in NB and TSB.

2.6. Chemical Chitin Extraction. A mixture of 500 g of wet shrimp shells and 2,250 mL of 4% HCl was kept at room temperature for 4 hours to eliminate inorganic components in the shells. The decalcified shrimp shells were then separated and washed several times with tap water. Deproteinization was performed by adding the dried decalcified shrimp shells into 5% NaOH at a ratio of 1 : 10, w/v. The mixture was shaken in a 90°C water bath for 12 hours. Chitin flakes were obtained by washing the solid residue with tap water and drying overnight in an oven at 80°C.

2.7. Biological Chitin Purification. A 24-hour culture of *L. pentosus* L7 in SHES plus 2% glucose was used as a starter in the decalcification step. Shrimp shell flakes (500 g) were mixed with 500 mL of starter (1 : 1, w/v) followed by 10% (w/w) glucose and 1% (w/w) salt in a 1,000 mL conical flask. Fermentation was performed at 37°C for 48 hours; the decalcified shrimp shells were then separated by a sifter. The retentate was washed with tap water and dried in an oven overnight at 80°C. For deproteinization of the decalcified shrimp shells, 10% (v/v) of 24-hour culture of *B. thuringiensis* SA in SHES medium was added to a sterilized mineral solution (0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$) containing 3% (w/v) dried decalcified shrimp shells. The mixture was incubated at 37°C with agitation at 200 rpm for 72 hours. The chitin was washed and dried overnight in an oven at 80°C.

2.8. Chitosan Production. Twenty mL of 50% (w/v) sodium hydroxide solution was added to 1 g dried decalcified and

deproteinized chitin. Deacetylation was performed at 121°C for 5 hours. Chitosan flakes were obtained by washing the solid residue with tap water until the pH was neutral and then dried overnight in an oven at 80°C.

2.9. Analytical Procedures. The pH of the fermenting liquids was determined using a pH meter (Metrohm, Riverview, FL, USA). Total titratable acid (TTA) was estimated by titration with 0.1 M NaOH to neutralize all the total titratable protons. The endpoint was at pH 8.1. The calculation expresses the titratable acidity in terms of g/L of lactic acid. Reducing sugar was determined by the Somogyi-Nelson method [19]. Moisture, protein, and ash contents were considered as the basis for determination of decalcification (DC) and deproteinization (DP) efficiencies. Moisture, fat, and ash contents of samples were determined by AOAC methods [20]. Protein content was analyzed by a modified Biuret method, as described by Gornall et al. [21]. Briefly, 100 mg dried materials were digested with 10 mL 0.5 M NaOH for 4 hours at 40°C; protein in the alkaline solution mixture was collected by centrifugation and used to estimate the protein concentration. DC and DP were calculated as percentages, according to Rao et al. [14].

For determination of proteolytic activity, the supernatant (crude enzyme) was collected from the culture by centrifugation at 10,000 ×g at 4°C for 10 minutes. One hundred twenty μL of a diluted enzyme solution was added to 480 μL of azocasein (0.5% in 50 mM Tris buffer, pH 8.0, containing 5 mM $MgCl_2$) and the mixture was incubated at 37°C for 1 hour. The reaction was stopped by adding 600 μL of 10% trichloroacetic acid; this was mixed and allowed to sit for 30 minutes at 4°C. The liquid was separated from the precipitate by centrifugation at 10,000 ×g at 4°C for 15 minutes. Four hundred μL of 1.8 N NaOH was added to 800 μL of the reaction liquid, mixed, and measured for absorbance at 420 nm (A_{420}) using a microplate reader (Varian Cary 50 MPR; Varian, Medical Systems, Palo Alto, CA, USA). One unit of enzyme activity was defined as the amount which yielded an increase in A_{420} of 0.01 in 60 minutes at 37°C.

The degree of acetylation of chitin and chitosan samples was determined by UV-Vis spectroscopy, as described by Wu and Zivanovic [22].

For color analysis, L^*, a^*, and b^* values were measured by a Hunter Lab UltraScan XE Colorimeter (HunterLab, Reston, VA, USA) using the CIELAB color system, where L^* indicates lightness on a scale ranging from 0 (black) to 100 (white), while positive and negative values of a^* represent red and green, and positive and negative values of b^* represent yellow and blue, respectively. The whiteness index (WI) was calculated based on the following [3]:

$$\mathrm{WI} = 100 - \left[\left(100 - L^*\right)^2 + a^{*2} + b^{*2}\right]^{1/2}. \quad (1)$$

Viscosities of chitin and chitosan samples were determined using a rheometer (Physica MCR 150; Anton Paar, Ashland, VA, USA) according to a modification of the Bajaj method [23]. Chitin samples were dipped into 50% ethanol for 30 minutes at 45°C to remove fat content. The chitin was then crushed into powder. *N*, *N*-dimethylacetamide (DMA) (99.5%, anhydrous, Unilab; Ajax Finechem, NSW, Australia) containing 5% lithium chloride (LiCl) (98.0%, anhydrous; LOBA Chemie, Mumbai, India) was used to prepare 0.1% (w/v) chitin solution. The mixture was stirred for 120 hours at room temperature. Chitosan solutions (1%, w/v) were obtained by dissolving chitosan powder in diluted acetic acid (1%, v/v). Each mixture was stirred for 24 hours at 55°C. A cone and plate geometry with a cone angle of 2.0° and radius of 24.975 mm was used for the measurements, and the gap size was 0.49 μ. The shear rates ranged from 0.1 to 100 s^{-1}. The experiments were conducted at 20°C. High, medium, and low viscosity chitosan represented viscosities of ≥400, 200–400, and ≤200 mPa·s, respectively [23].

Table 1: Chemical properties of shrimp shells and heads.

Component (%, w/w)	Shrimp shells (abdominal)	Shrimp heads
Moisture	80.0 ± 0.3	86.1 ± 2.2
Crude protein	25.9 ± 2.0	40.7 ± 2.7
Lipid	2.4 ± 1.0	13.9 ± 1.1
Ash	27.9 ± 0.9	22.2 ± 0.6
Chitin[a]	43.8	23.2

[a]by calculation.

Table 2: Chemical properties of shrimp head residue after heat extraction.

Component	Residue after heat extraction (%, w/w)
Crude protein	38.0 ± 3.7
Lipid	5.0 ± 0.3
Ash	27.5 ± 1.9

2.10. Data Analysis. All experiments were run in triplicate. Experimental data were expressed as mean ± s.d. ($n = 6$).

3. Results

3.1. Chemical Compositions of Raw Materials. Table 1 shows the composition of shrimp shells (abdominal part) and shrimp heads. The shrimp abdominal shells had less protein and lipid than shrimp heads; therefore, the abdominal part was the preferred raw material for chitin and chitosan preparations. The cost of the culture media for *L. pentosus* L7 and *B. thuringiensis* SA used in the process was a high investment. Shrimp heads are a readily available biowaste material, and SHES obtained by heat extraction process is cheap and easy to prepare. The SHES contained crude protein content of 1.0 ± 0.1%, which could well support the growth of these bacteria. The residue after extraction still had some nutritional value (Table 2), which could be used for other applications such as animal feed production.

3.2. Microorganisms. *Lactobacillus pentosus* L7 was isolated from the Thai traditional fermented sausage, known as "Nham." *L. pentosus* L7 showed high acid production efficacy

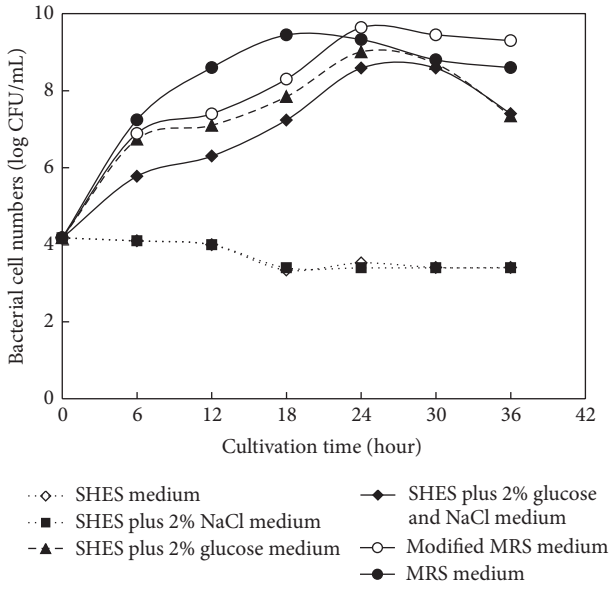

Figure 1: Growth of *L. pentosus* L7 in six different culture media.

when glucose was supplemented as a carbon source for lactic acid production. A protease-producing bacterium, *B. thuringiensis* SA, was screened and isolated from coastline in Bangkhuntien district area, Bangkok, by using the double-layer agar technique. *B. thuringiensis* SA showed strong gelatinase, caseinase, and collagenase activities. Using the combination of both strains to decalcify and deproteinize shrimp shells, purified chitin with high quality could be obtained.

3.3. Growth of L. pentosus L7 and B. thuringiensis SA in Culture Media Having SHES as a Major Component. A 24-hour culture of *L. pentosus* L7 (0.1%) in MRS broth was inoculated into MRS; SHES; SHES plus 2% glucose; SHES plus 2% NaCl; SHES plus 2% glucose and NaCl; and modified MRS medium. The growth curves of *L. pentosus* L7 in these media were performed using the spread plate technique on MRS agar (Figure 1). The SHES could not support the growth of *L. pentosus* L7 unless a carbon source such as glucose was supplemented. The modified MRS medium which used SHES to replace peptone, beef extract, and yeast extract could satisfactorily promote the growth of *L. pentosus* L7 similar to the MRS medium. *L. pentosus* L7 cell numbers reached 9.2 ± 0.2 log CFU/mL after 24-hour cultivation. SHES plus 2% glucose could also promote the growth of *L. pentosus* L7; cell numbers reached 8.5 ± 0.2 log CFU/mL after 24-hour cultivation. SHES plus 2% glucose could save on the cost of inoculum medium; therefore, SHES plus 2% glucose medium was selected as the cultivation medium to prepare the inoculum for lactic acid fermentation of shrimp shells. When a 24-hour culture of 0.1% *B. thuringiensis* SA in NB was inoculated into SHES, NB, and TSB, the three media gave similar growth support for the bacteria. The cell numbers of

Table 3: Residual crude protein and ash content in decalcified shrimp shells and raw chitin flakes during purification by a two-step biological process.

Biological process	Crude protein (%, w/w)	Ash content (%, w/w)
First step: *L. pentosus* L7 fermentation		
24 hours	26.3 ± 1.2	3.8 ± 0.4
48 hours	23.2 ± 2.5	1.4 ± 0.5
Second step: deproteinization with *B. thuringiensis* SA		
24 hours	16.4 ± 2.5	1.5 ± 0.4
48 hours	8.0 ± 1.2	1.5 ± 0.4
72 hours	3.8 ± 1.3	1.7 ± 0.4

B. thuringiensis SA reached 8.6 ± 0.2 log CFU/mL after 24-hour cultivation indicating that SHES would be suitable for use as the culture medium for *B. thuringiensis* SA.

3.4. Two-Step Biological Chitin Purification of Shrimp Shells. Removal of protein and calcium from shrimp shells is critical step in chitin purification. Lactic acid fermentation was selected as the first process in a two-step biological chitin extraction. The fermentation time for calcium removal could be decreased by increasing the inoculum size, but a high inoculum level had a significant effect on the production costs. As noted in the previous section, *L. pentosus* L7 could grow well in SHES plus 2% glucose. Changes in the amount of reducing sugar, lactic acid, number of *L. pentosus* L7 cells, and pH of the fermenting liquid were determined (Figure 2). The initial pH of 7.0 ± 0.1 fell to 3.9 ± 0.1 after 48 hours and continued to decrease slightly upon further incubation. The acidity was the highest after 48 hours and then remained at that level. The reducing sugar in the fermenting liquid remained at only 4.0 ± 0.1 g/L after 72 hours. In the first 24 hours, the number of *L. pentosus* L7 cells increased to 11.0 ± 0.2 log CFU/mL indicating significant growth. Over the first 24 hours, the ash content in shrimp shells sharply decreased from 27.9 ± 0.9% to 3.8 ± 0.4% (Table 3). The results suggested that the fermentation process ended after 48 hours as indicated by constant pH and lactic acid concentration (Figure 2). At the end of fermentation, the decalcified shrimp shells had crude protein and ash contents of 23.2 ± 2.5% and 1.4 ± 0.4%, respectively (Table 3). The decalcified shrimp shells were further processed by a deproteinization step via the proteolytic activity of *B. thuringiensis* SA. The proteolytic activity and soluble protein in the extraction liquid were examined (Figure 3). The highest proteolytic activity, 290.1 ± 15.8 unit/mL, was detected after 48 hours and the highest amount of soluble protein in the extraction liquid was found after 72 hours; this indicated that the deproteinization could be considered finished after 72 hours. At the end of the process, decalcification and deproteinization efficiencies of the two-step biological extraction were 98.1 ± 0.3% and 96.8 ± 0.7%, respectively (Figure 4).

3.5. Properties of Chitin Flakes Obtained from Chemical and Two-Step Biological Processes. The composition of the shrimp

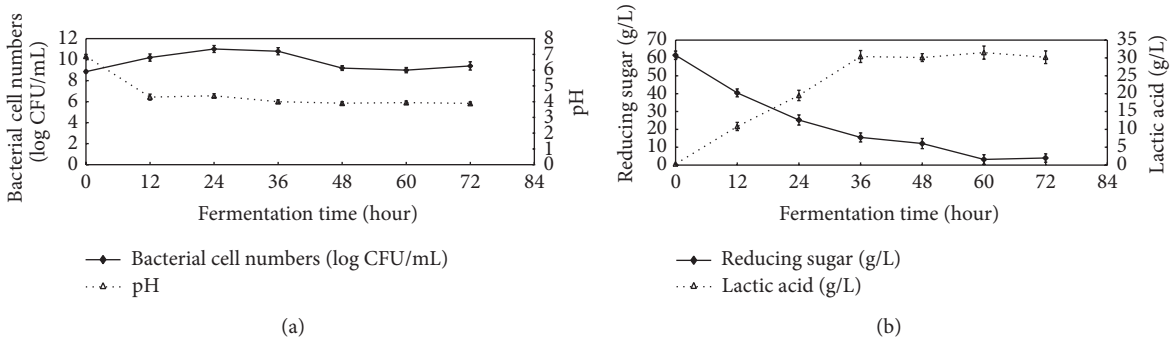

Figure 2: Changes in pH, lactic acid, cell numbers of *L. pentosus* L7, and reducing sugar in the fermenting liquid during decalcification of shrimp shells by *L. pentosus* L7.

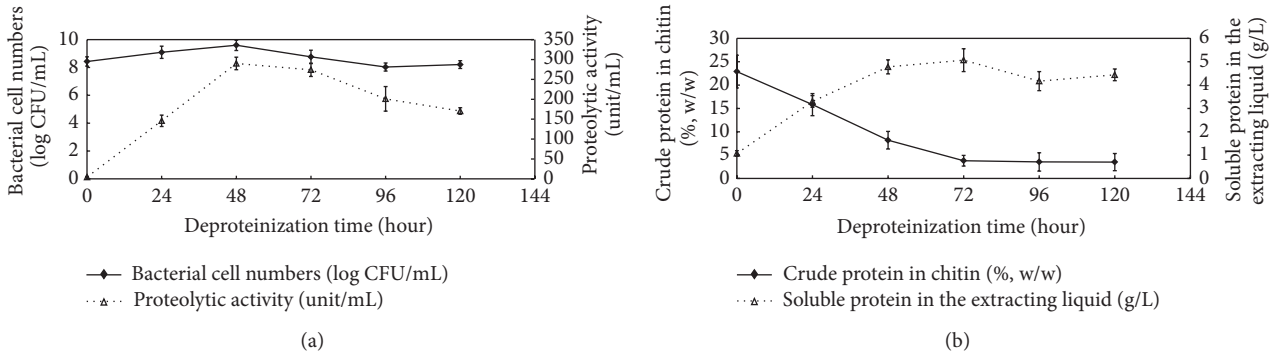

Figure 3: Deproteinization of decalcified shrimp shells by *B. thuringiensis* SA.

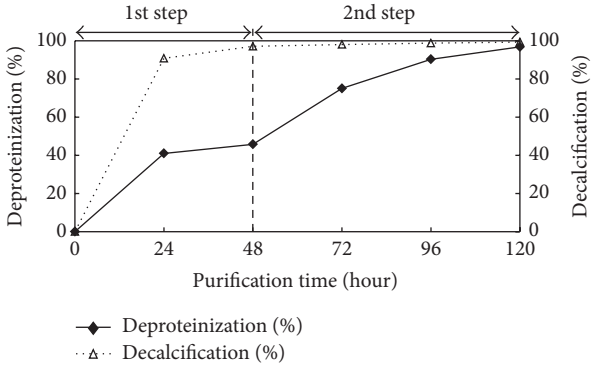

Figure 4: Deproteinization and decalcification efficacies from shrimp shell waste during the first step fermentation with *L. pentosus* L7 and the second step deproteination with *B. thuringiensis* SA.

shells after extraction by chemical or two-step biological treatments is shown in Table 4. The results revealed that the chemical treatment for chitin production was more effective in deproteinization and decalcification than the biological process. However, protein and ash contents are not the only parameters to be considered for chitin quality. Molecular weight distribution, degree of acetylation (DA), grade of polymerization, and viscosity of chitin are also considered important parameters of chitin quality. Among these, viscosity and DA are commonly used as quality indicators [6].

In this study, viscosity measurements were performed to determine the rheological properties of extracted chitin. The viscosity of biologically purified chitin was 110 ± 15.1 mPa·s, whereas the viscosity of chemically processed chitin was 82 ± 13.6 mPa·s (Table 4). In terms of color analysis, the chemically processed chitin had a lighter color than that from the biological process (Table 4). However, both showed a white property, leading to greater consumer preference and acceptability of the product. The biological process could preserve the structure of shrimp chitin better than the chemical process by retaining higher DA and viscosity.

3.6. Properties of Chitosan Flakes Obtained from Chitin Extracted by Chemical and Two-Step Biological Processes. Chitin obtained from shrimp shells after extraction by chemical and two-step biological processes was incubated with 50% NaOH (121°C for 5 hours) for deacetylation to chitosan. Chitosan is a polymer of β-(1-4)-D-glucosamine units, which can be obtained by deacetylation of chitin, a polymer of β-(1-4)-*N*-acetyl-D-glucosamine (NAG) units. Under the selected deacetylation condition, the degree of deacetylation of chitosan was higher than 70% (Table 5), leading to solubility in 1% acetic acid. The chitosan derived from the biologically purified chitin was more viscous than the chitosan manufactured by the chemical process (323 ± 15.6 mPa·s), in which viscosity of 1,007 ± 14.7 mPa·s was obtained from chitosan prepared from biologically purified

Table 4: Properties of chitin flakes obtained from chemical and two-step biological processes.

Properties	Shrimp chitin flake products	
	Chemical process	Biological process
pH	7	7
Appearance	White	White and light yellow
Crude protein (%, w/w)	0.3 ± 0.1	3.8 ± 1.3
Ash (%, w/w)	0.6 ± 0.1	1.7 ± 0.4
Deproteination efficacy (%)	99.7 ± 0.1	96.8 ± 0.7
Decalcification efficacy (%)	99.0 ± 0.1	98.1 ± 0.3
Color properties		
L^*	87.3 ± 1.6	84.7 ± 1.2
a^*	0.3 ± 0.1	0.7 ± 0.2
b^*	7.0 ± 0.1	7.2 ± 1.4
WI[a]	85.5	83.1
Degree of acetylation (%)	96.5 ± 0.3	98.2 ± 0.5
Viscosity[b] (mPa·s)	82.0 ± 13.6	110.0 ± 15.1
Yield (%)	20.0 ± 0.5	25.4 ± 1.2

[a]whiteness index, by calculation.
[b]viscosity determined at shear rate of 20 s^{-1} (25°C).

Table 5: Properties of chitosan flakes obtained from deacetylation of chitin extracted by chemical and two-step biological processes.

Properties	Shrimp chitosan flake products	
	Deacetylation of chemically processed chitin	Deacetylation of biologically purified chitin
pH	7	7
Appearance	White	White
Crude protein (%, w/w)	0.3 ± 0.1	0.4 ± 0.3
Ash (%, w/w)	0.3 ± 0.1	1.6 ± 0.6
Color properties		
L^*	73.1 ± 2.0	77.3 ± 1.4
a^*	0.5 ± 0.1	0.7 ± 0.2
b^*	7.0 ± 0.3	6.4 ± 0.6
WI[a]	72.1	76.4
Degree of deacetylation (%)	82.2 ± 1.2	83.2 ± 1.5
Viscosity[b] (mPa·s)	323.0 ± 15.6	1,007 ± 14.7

[a]whiteness index, by calculation.
[b]viscosity was determined at shear rate of 50 s^{-1} (20°C).

chitin. In terms of color analysis, chitosan prepared from biologically purified chitin had a lighter color than chitosan prepared from chemically processed chitin (Table 5). Chitosan prepared from biologically purified chitin had crude protein and calcium contents of 0.4 ± 0.3% and 1.6 ± 0.6%, respectively. The degree of deacetylation was 83.2 ± 1.5%.

4. Discussion

The applications of microorganisms, proteolytic enzymes, and shrimp shell fermentation for deproteinization or decalcification of seafood industry wastes are a current research trend in the conversion of biowastes into value-added biomass. The biological process is an environmentally friendly alternative for preparation of chitin. In this study, two-step biological chitin production from shrimp shells, using lactic acid fermentation for decalcification followed by bacterial proteolysis for deproteinization, was performed. The abdominal part of the shrimp shell was selected as a raw material for chitin preparation, since it contained low protein and fat (Table 1).

The high cost of media for cultivating lactic acid bacteria and *B. thuringiensis* could be overcome by protein extraction of shrimp head waste (SHES). The price of synthetic culture medium (broth) for lactobacilli is about 400 baht per L; whereas the cost for preparation of culture medium made from SHES plus 2% glucose is about 9 baht per L. Also, the solid residue after SHES preparation still had some nutritional value and can be used as an animal feed supplement.

A fast removal of proteins from shrimp abdominal shells is necessary to avoid spoilage and the development of a bad smell. Shrimp shell fermentation by lactic acid bacteria for decalcification and deproteinization would be an alternative approach for inhibition of spoilage microorganisms. By fermentation of shrimp shells with lactobacilli, a decalcification efficiency of up to 86% has been reported [6, 14, 16, 24–27]. In the present study, decalcification efficacy with *L. pentosus* L7 reached 97% but required 10% (w/w) glucose. This is in accordance with previous reports [6, 24, 27]: for example, 15% (w/w) was the optimum glucose concentration for *Pediococcus acidilactici* fermentation to remove calcium from shrimp shells [27], while 2.5 g crab shells were decalcified with 50 mL of 10% glucose (solid to liquid ratio of 1 : 20) [24]; and for decalcification of *P. monodon* shells, 0.54 g glucose per g of dried shells was added to decrease pH to 4.6 [6]. The decrease of residual proteins in shrimp shells after fermentation indicated that deproteinization of the shrimp shells occurred spontaneously, together with decalcification by the proteolytic activity of *L. pentosus* L7 or by the in situ proteases in the biowaste [6]. The presence of the lactic acid bacterium *L. pentosus* L7 (9.0 ± 0.2 log CFU/mL) in the protein and calcium rich liquor portion indicates that it could be used as a food supplement for humans, animals, or microorganisms [28].

In the deproteinization step, *B. thuringiensis* SA produced high protease activity (290.1 ± 15.8 unit/mL) during deproteinization of decalcified shrimp shells. The liquid fraction should be collected and concentrated to obtain the crude protease. *B. thuringiensis* SA was found to produce parasporal crystal proteins during the sporulation stage; the crystal protein might be recovered from the extracting liquid after deproteinization of decalcified shrimp shells.

Quality criteria of chitosan are viscosity, molecular weight, or distribution of molar masses. The viscosity of chitosan is strongly dependent on the viscosity of the "preproduct" chitin; the biological processes have been reported to be an effective way to obtain a high quality of chitin [6, 7, 17–19].

Figure 5: Chitosan flakes obtained from biologically purified chitin.

The chitosan obtained from biologically purified chitin had a high viscosity compared with chitosan prepared from chemically processed chitin (the same lot of shrimp waste in the present study, Table 5). Commercial chitosan purchased from a supplier in Samut Sakhon province, Thailand, had a viscosity of 331 mPa·s. Bajaj et al. [23] reported that commercial chitosan with high viscosity had 442.4 mPa·s, while the medium and low viscous grades had much lower viscosities; this indicated that the quality of the chitosan used in the present study was good. Although the highest chitosan viscosity was reported by Bajaj et al. [23], a chemical process was used to prepare chitin, together with an N_2 atmosphere for deacetylation.

The production of chitosan from chitin isolated by a two-step biological purification process for decalcification and deproteinization is not commercially used, but the process has good potential to create biologically purified chitin with a high grade of purity and provides a high viscosity chitosan after deacetylation (Figure 5). High viscosity chitosan has various applications, for example, as an emulsifying agent, or dietary ingredient, and for metal reduction, scaffolds (tissue engineering), enzyme immobilization, and drug delivery [28]. The disadvantages of a two-step biological process are overall longer purification time (5 days) and higher costs due to the requirement of sterilize process and a carbon source in deproteinization and decalcification steps. However, the wastes from the biological processing are not harmful to humans or the environment, and useful by-products such as protein hydrolysate, calcium lactate, astaxanthin, crude protease, and parasporal crystal proteins during purification could be obtained from the methods used in the present study, whereas chemical process creates toxic waste and causes some depolymerization of the chitin, which influences its molecular weight and viscosity, leading to lower viscosity chitosan after deacetylation.

5. Conclusions

Chitin purification procedures play an important role in obtaining a high viscosity chitosan after deacetylation. Compared with the chitosan product made from chemically processed chitin, high viscosity chitosan can be successfully prepared by a biological process.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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