Scaling Up Chitin Extraction from Shrimp Shell Using Symbiotic Co-Lactic Acid Fermentation and Proteolytic Fungal Fermentation in Stirred Tank Bioreactor

Jariya Ruangwicha
International Program of Biotechnology, Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Faculty of Agro-Industry, Prince of Songkla University

Benjamas Cheirsilp
International Program of Biotechnology, Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Faculty of Agro-Industry, Prince of Songkla University

Wasana Suyotha
International Program of Biotechnology, Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Faculty of Agro-Industry, Prince of Songkla University

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Jariya Ruangwicha, Benjamas Cheirsilp*, Wasana Suyotha
International Program of Biotechnology, Center of Excellence in Innovative Biotechnology for Sustainable Utilization of Bioresources, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla, 90110, Thailand
*Corresponding author email: benjamas.che@psu.ac.th

Abstract: *This study aimed to extract chitin from shrimp shell powder (SSP) by demineralization (DM) using symbiotic co-lactic acid fermentation (Co-LAF) by Lactobacillus plantarum and Streptococcus thermophilus and deproteinization (DP) using successive fermentation by protease producing fungus, Rhizopus oligosporus. The process was scaled up in a 5 L-stirred tank bioreactor. DM by Co-LAF and DP by protease producing fungus led to greater DM and DP of 91.92±1.45% and 89.50±1.71%, respectively. The chitin extraction yield was 40.00±2.01%. The scaled-up performance was better than that of the flask-scale, particularly in terms of LA and protease production, and DP, likely due to better heat transfer and aeration provided for the fungi. These results show the possibility of scaling up of biological extraction of chitin from SSP.*

Keywords: Demineralization; deproteinization; lactic acid bacteria; protease

1. INTRODUCTION

Chitin consists of N-acetylglucosamine (GlcNAc) units connected by β-(1 → 4) bonds which is biopolymer obtained from crustacean shells, exoskeletons of insects and fungi. It has applications across food, pharmaceuticals, cosmetics, textiles, agriculture, and wastewater treatment [1]. Shrimp shell waste represents a significant reservoir of chitin, containing approximately 30–35% of this biopolymer, along with calcium carbonate, protein, and pigments. However, extracting pure chitin from this waste necessitates the separation of these additional components through removal and purification processes [2]. Currently, industrial-scale production commonly involves extraction steps such as demineralization (DM) using strong acids to remove inorganic materials, and deproteination (DP) with alkalis at high temperatures (90–100ºC) for protein removal [3]. However, these processes can lead to chitin depolymerization, reducing its molecular weight, viscosity, and degree of acetylation. Moreover, they pose environmental hazards, consume high energy, and result in the loss of valuable proteins [4].

To overcome these issues, alternative biotechnological approaches have been explored, including microbial fermentation and enzymatic methods using proteolytic enzymes. These methods offer potential advantages in terms of efficiency and environmental impact. They not only aim to recover chitin effectively but also preserve other valuable compounds such as pigments, protein hydrolysates, amino acids, and fatty acids, enhancing overall profitability and sustainability across various industries [5].

Lactic acid fermentation (LAF) using lactic acid bacteria (LAB) has emerged as a promising biotechnological approach for chitin extraction. LAF utilizes the acidic pH and lactic acid produced by LAB to facilitate DM of shrimp shell waste while inhibiting pathogens and spoilage microorganisms [6].

Recently, co-lactic acid fermentation (Co-LAF) has been employed to improve LA production by LAB. Co-LAF mutually benefits from their symbiotic relationship, leading to the promotion of their growth and LA production. Ruangwicha *et al*. [9] reported Co-LAF by *L. plantarum* and *S. thermophilus*, highlighting their symbiosis and synergistic effects on the DM and DP of SSP. In the symbiotic relationship, *S. thermophilus* may provide formic acid, pyruvic acid, folic acid, and carbon dioxide to *L. plantarum*, and *L. plantarum* supports *S. thermophilus* growth by providing peptides or amino acids produced from the protein-containing SSP.

LAB requires carbon and nitrogen sources for growth. Pure sugars, such as glucose and sucrose, are commonly used for lactic acid production, but these sugars are not cost-effective for industrial-scale production. Therefore, finding sugar-based agro-industrial waste as a low-cost nutrient source is a challenging strategy to reduce cost, increase profitability and align with Sustainable Development Goals (SDGs) [7,8]. Utilizing these wastes promotes economic, social, and environmental sustainability through waste reduction, bioenergy production, and responsible consumption (Goals 2, 6, 7, 9, 12-14, 17).

Successive two-step fermentation presents advantages that suppress of spoiled microorganism and improve DM and DP efficiency due to the best removal of $CaCO_3$ and proteins from shrimp shell. DM process broke up the calcium-protein-chitin complex which caused the microfibers in SSP to swell thus promoting the DP process [9]. These organic acids make an acidic environment and suppress the growth of spoilage microorganisms by rapid acidification of the medium along with DM [10,11].

Proteases of fungal origin can be produced cost effectively, have an advantage faster production, the ease with which the enzymes can be modified, and mycelium

can be easily removed by filtration. Proteases of fungal origin can be produced cost effectively, have an advantage faster production, the ease with which the enzymes can be modified, and mycelium can be easily removed by filtration. Therefore, food-grade protease-producing fungi, *Rhizopus oligosporus*, which can secrete significant amounts of acidic protease and easily grow on agro-industrial waste substrates, were employed. This fungus is expected to grow under LAF in acidic conditions [12].

This study aimed to scale up Co-LAF and fungal fermentation for chitin extraction using cheap nutrient sources like mature coconut water (MCW), a by-product of the coconut milk industry. MCW is rich in sugars and vitamins suitable for LAB cultivation, lactic acid, and protease production for sustainable chitin extraction. It is expected that this research will contribute to evaluating the feasibility of industrial-scale production and validating fermentation from flask scale, advancing sustainable practices in the shrimp processing industry by emphasizing efficient waste utilization and environmental friendliness.

2. MATERIALS AND METHODS

2.1 Materials

Litopenaeus vannamei shells were obtained from KIANG HUAT SEA GULL TRADING FROZEN FOOD PUBLIC CO., LTD and stored at -20 °C. To prepare shrimp shell powder (SSP), the shrimp shells were defrosted and then dried at 60°C for 48 h in a hot air oven (Binder FD-115, Tuttlingen, Germany). The dried shells were subsequently ground using an electric blender (Mixer Grinder HL7810/00 | Philips) and sieved to obtain a particle size of 0.5-1.0 mm (Fig. 1.)

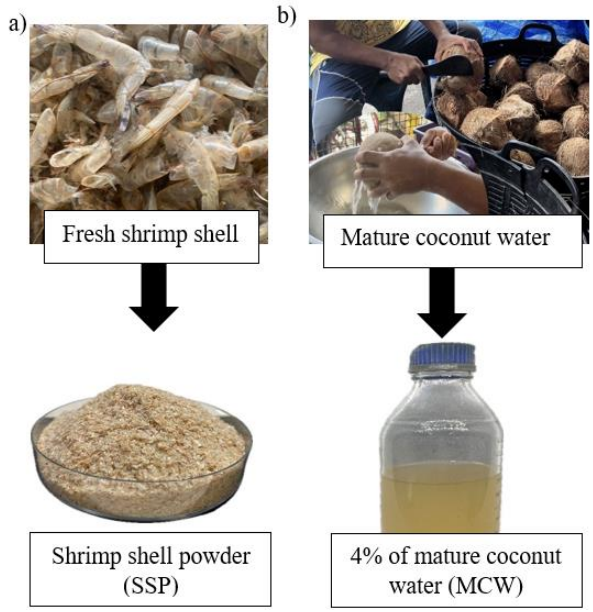

Fig. 1. Material preparation for chitin extraction (a) fresh shrimp shell to shrimp shell powder (SSP) (b) mature coconut water (MCW).

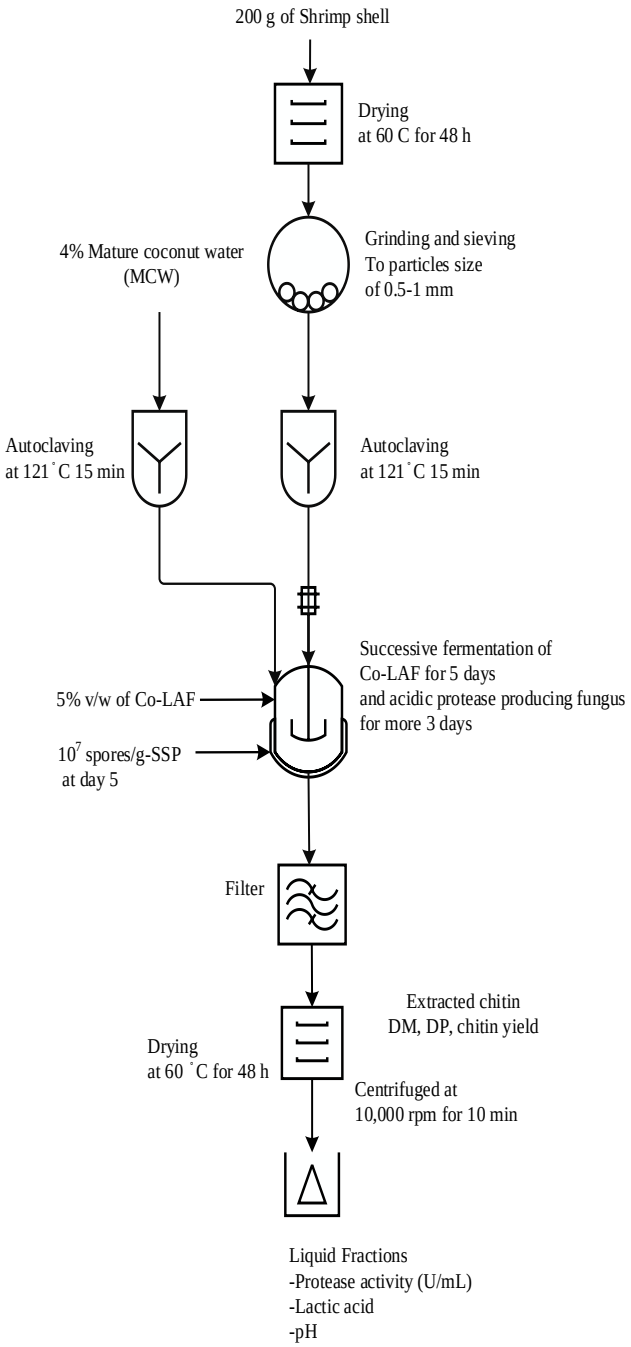

Fig. 2. Chitin extraction from SSP of Co-LAF under anaerobic conditions and successive fermentation with *R. oligosporus* under aerobic condition (a) Anaerobic condition (b) Aerobic condition in serum bottle at working volume 50 mL (c) STB at working volume 2000 mL.

2.2 Microorganisms and inoculum preparation

Lactic acid bacteria, *L. plantarum* TISTR 1465 and *S. thermophilus* TISTR 894 were obtained from Thailand Institute of Scientific and technological Research, Bangkok, Thailand. The cultures were maintained in 30% glycerol and frozen at -20 °C. The inoculum was prepared by transferring 1 mL of active culture to 9 mL of MRS broth and then incubated at 37 °C for 24 h to obtain LAB equivalent to 10^8 CFU/mL. *Rhizopus oligosporus* was cultivated on potato dextrose agar (PDA) slants and kept at 4 °C before using. The spore suspension was prepared at conidia count of 10^9 spores/mL which was added and mixed thoroughly with 0.1% of tween 80.

2.3 Media preparation

Man-Rogosa Sharpe (MRS)

The MRS medium (HiMedia, India) contained proteose peptone 10 g, peptone 10 g, yeast extract 5 g, glucose 20 g, polysorbate 80 1 g, ammonium citrate 2 g, sodium acetate 5 g, magnesium sulfate 5 g and dipotassium hydrogen phosphate 2 g and 1 L deionized water, with pH adjusted to 6.0.

Potato Dextose Agar (PDA)

To prepare potato infusion, unpeeled potatoes 200 g were boiled in 1 L distilled water for 30 min and filtered through cheesecloth. The filtrate is potato infusion. The filtrate was added with dextrose, agar and water and boiled to dissolve all prior to autoclave for 15 min at 121°C. The pH was 5.6±0.2.

2.4 Scale up in bioreactor

Co-LAB was inoculated into the medium with 5% (v/w) of the starters and anaerobically incubated at 37°C, initial pH 6.0 and stirred at 150 rpm for 72 h in a 5-L stirred tank bioreactor (STB, model MDFT,Bemarubishi Co., Ltd (Japan)) with 2 L working volume. Thereafter, additional MCW was added into the culture and then *R. oligosporus* was then inoculated at 10^7 spores/g-SSP to perform successive fermentation at 30 °C, for more 72 h under aerobic fermentation using aeration at 1 volume air per volume medium per min (vvm) during fungal fermentation.

2.5 Determination of pH and organic acids

The fermented sample was measured for pH using a pH meter. Organic acids were analyzed with an HPLC system (Agilent 1260 Infinity), a Refractive Index (RI) detector, and an Aminex HPX-87H column (300 mm × 7.8 mm) by flow rate of 0.6 mL/min and a column temperature of 40°C. Mobile phrase was prepared using 5 mM H_2SO_4.

2.6 Determination of reducing sugar and total sugar

To determine reducing sugar, sample 0.1 mL was added with 0.3 mL DNS reagent and mixed in the test tube. The test tube was boiled for 10 min and cooled at room temperature before adding 1.6 mL of water and measured by spectrophotometer at 540 nm. Total sugar was analyzed by phenol sulfuric method. Five percent of phenol solution (0.5 mL) were added to 0.5 mL appropriate diluted sample and subsequently as much as 1.25 mL of 98 % sulfuric acid was added. The mixture was vortexed for 5 s then incubated at 30 C for 20 min and measured by spectrophotometer at 490 nm.

2.7 Protease activity assay

Neutral and acidic protease activity was measured using a modified method. The substrates used for neutral and acidic protease assays were casein and BSA, respectively. Neutral protease activity was assayed by Sigma's non-specific protease assay method described by Cupp-Enyard [13] with some modifications. Casein solution (0.65%) was prepared in 50 mM Tris-HCl buffer pH 7 and used as substrate. The reaction mixture was made up of 0.130 mL of the casein substrate and 0.25 mL of the culture supernatant. After 30 min incubation at 37°C, the reaction was terminated by adding 0.13 mL of 110 mM trichloroacetic acid (TCA). Then, 0.25 mL of the reaction supernatant was added with 0.625 mL of 0.5 M sodium carbonate and 0.125 mL of 0.5 M Folin and Ciocalteau reagent and the mixture was incubated at 37°C for 30 min. The color produced was measured with UV spectrophotometer (Hitachi U-1900, Tokyo, Japan) at 660 nm. Standard curve of tyrosine was obtained in the range of 10-100 µg/mL. One unit of protease activity was defined as the amount of the enzyme resulting in the release 1 µg/mL of tyrosine per min under the assay conditions. Acid protease activity was assayed as described by Ikasari and Mitchell (1994) with some modifications. Bovine serum albumin (10 mg/mL) as substrate was prepared in 50 mM citrate buffer pH 3. The reaction mixture was mixed with 0.130 mL of substrate and 0.25 mL of appropriate enzyme concentration. After 10 min incubation at 40°C, the reaction was determined by adding 0.13 mL of 10% trichloroacetic acid (TCA). The color development and measurement and activity calculation were the same as mentioned above. The soluble protein concentration was estimated though Lowry's method.

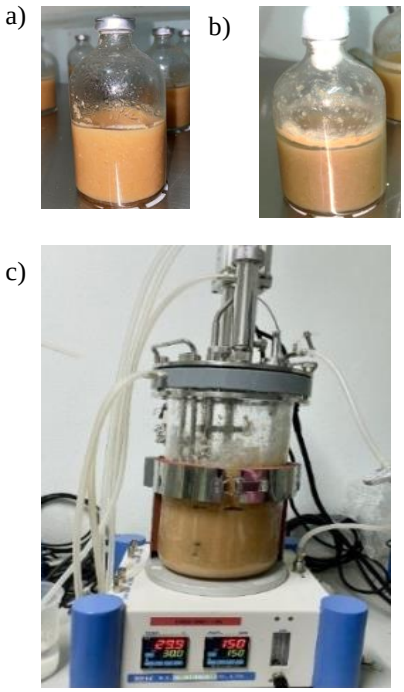

Fig. 3. Chitin extraction from SSP of Co-LAF under anaerobic conditions and successive fermentation with *R. oligosporus* under aerobic condition (a) Anaerobic condition (b) Aerobic condition in serum bottle at working volume 50 mL (c) STB at working volume 2000 mL.

Table 1. Parameters for chitin extraction studied in serum bottle and STB.

Parameters	Serum bottle	STB
Working volume (mL)	50	2000
SSP (g)	5	200
Initial sugar content of MCW (g)	0.2	80
Mixing (rpm)	150	150
Aeration day 5-8 (vvm)	-	1
Inoculum size (mL) of Co-LAB at 10^8 CFU/mL	0.25	100

Parameters	Serum bottle	STB
Inoculum size (mL)of fungus at 10^7 spores/g	5	200
Initial pH	6.5	6.5

2.8 Determination of soluble protein

Protein hydrolysate concentrations were determined as the soluble protein in the liquid fractions by Lowry-Peterson [14]. Alkali copper solution was prepared by mixing 50 mL of 2% $(Na)_2CO_3$ in 0.1 N NaOH and 1 mL of 1% copper sulphate in 1% sodium potassium tartrate. Folin- Ciocalteau reagent was prepared by diluting Folin reagent with distilled water in ratio of 1:1. One of alkali copper solution was added and mixed with 0.1 mL of sample. The mixture was left at room temperature for 10 min and the solution was added and mixed with 0.1 mL Folin-Ciocalteau reagent and left at room temperature for 30 min. The absorbance was measured at 650 nm. Protein concentration was calculated compared to a standard curve of bovine serum albumin (BSA) in several concentrations of 100-700 µg/mL.

2.9 Quantification of protein in solid fraction

The protein content of crude chitin was measured and compared with dried native shells. For this analysis, 0.05 g of dried shell material was mixed with 10 mL of 5% (w/v) NaOH and heated at 95°C for 2.5 h [11]. The supernatant was then collected, and the protein concentration was estimated using Lowry's method [14]. Deproteinization efficiency (%DP) was reported using the following equation:

$$\text{DP (\%)} = \frac{(Po \times O) - (P_R \times R)}{Po \times O} \times 100$$

where P_O and P_R are the protein contents (%) of SSP and crude chitin, respectively. For O and R represent the masses (g) of SSP and extracted chitin, respectively.

2.10 Quantification and ash in solid fraction

Ash content was measured by A.O.A.C. method. The sample was heated at 550 °C for 5 h, and then cooled down in a desiccator to ambient temperature.

$$\text{DM (\%)} = \frac{(Ao \times O) - (A_R \times R)}{Ao \times O} \times 100$$

where Ao and A_R represented the ash content in the SSP and the extracted chitin, respectively. For O and R represent the masses (g) of SSP and crude chitin, respectively.

2.11 Chitin content

The chitin content was measured using the method described by Tokatlı and Demirdöve [16]. The weight difference between SSP and extracted chitin was used to compute the chitin yield. Equations were used to compute the chitin yields:

$$\text{Chitin yield} = \frac{a}{b} \text{ x } 100$$

where a is weight of chitin, b is the weight of SSP.

3. RESULTS & DISCUSSION

The optimal conditions for Co-LAF process from the previous study [12] were used for scale-up process in STB. STB was filled up with 2 L of the culture medium (Fig. 2.). Co-LAF process was performed under anaerobic condition. After fungal inoculation, successive fermentation was performed under aerobic conditions using constant-aeration rate of 1 vessel volume per minute (vvm) for 3 more days. The chitin extraction yield, DP, and DM of SSP were compared with the results using 50 mL serum bottle (Table 2).

Table 2. Percentage of DM, DP, and chitin of extracted chitin and LA beside protease production

Parameters	Serum bottle	STB
DM (%)	94.57±0.83	91.92±1.45
DP (%)	86.08±1.05	89.50±1.71
Chitin yield (%)	38.4±0.56	40.00±2.01
LA (g/L)	57.0±8.32	65.40±1.12
Acidic protease (U/mL)	4.32±0.53	5.03±0.47

The fermentation process was performed for 8 days in total. The pH decreased gradually from pH 6.5 to 4.53 after 24 h, which was faster than that of serum bottle scale (Fig. 1a). The LA production in STB (65.40±1.12 g/L) was also higher than that of serum bottle scale (57.08 g/L). This was possibly due to preferable heat transfer by thoroughly mixing of impeller in STB. The highest protease activity during fungal fermentation was observed at day 7 of fermentation (5.03±0.47 U/mL) possibly due to better oxygen transfer in STB. There are several researchers reported that *Rhizopus* stains achieved maximum production of metabolizes (biocatalyst, organic acids, and biomass) in STB. STB is a reactor that can control impeller speed lead to achieve a high oxygen transfer rate and homogeneous mixing. Proper mixing and shear application are necessary to ensure uniform temperature, pH, and adequate mass transfer of oxygen and nutrients throughout the fermenter contents.

This study also showed higher protease production than that of *Aspergillus tamarii* MTCC5152 (1.51 U/mL) in an STB [17]. Fig.1c. shows residual ash and protein content of SSP during successive fermentation for 8 days. Protein content decreased from 29.07±0.09% to 13.76±0.29% at day 3 before rapidly decreased to 7.79±0.43% at day 7 after fungal inoculation. Similarly, ash content also reduced from 31.7±1.77% to 6.38±0.79%. At the end of fermentation residual protein and ash content were only 6.15±1.97% and 4.99±1.75%, respectively. DM, DP and chitin extraction yield were 91.92±1.45%, 89.50±1.71%, 40%.

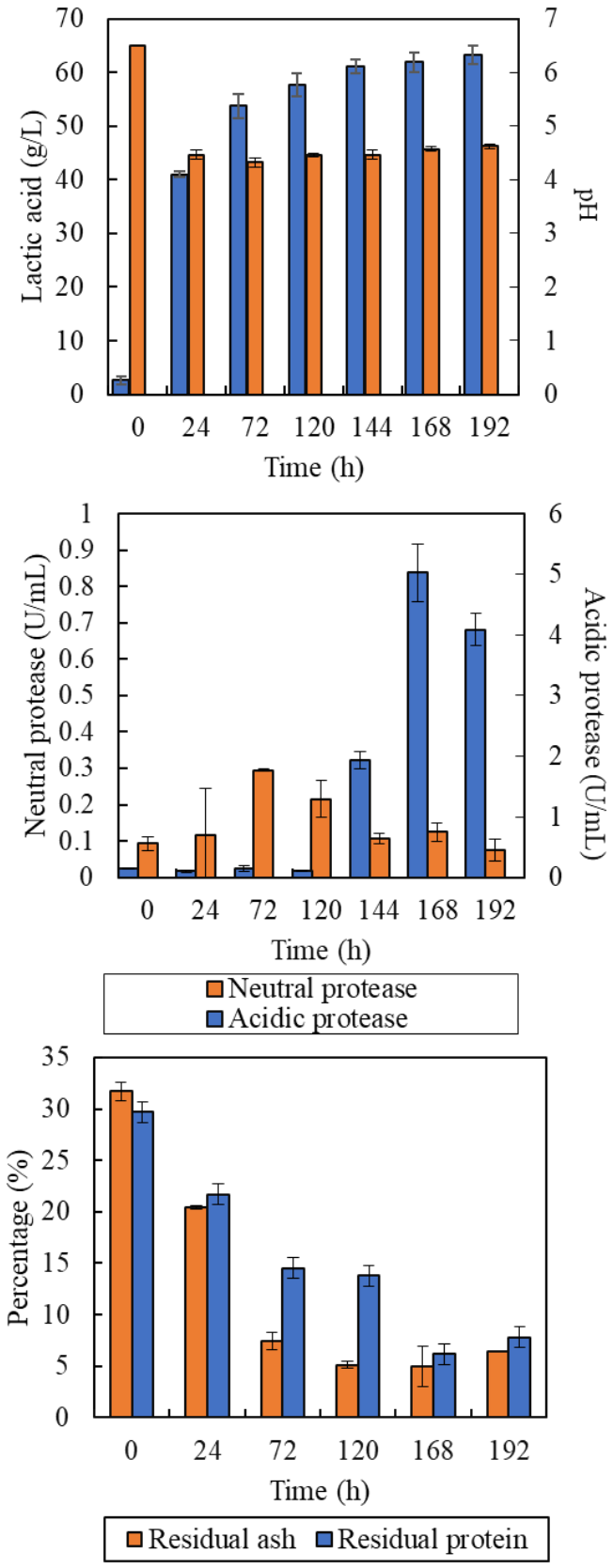

Fig. 4. Time courses of (a) pH (orange bar), LA production (blue bar), (b) acidic and neutral protease activities and (c) percentage of residual ash and protein in STB.

respectively which DM and DP were higher than those reported by Ghaly *et al.* [18], who conducted chitin extraction by *Aspergillus niger* in a drum bioreactor.

The pH of the SSP medium initially decreased due to acidic protease production, then increased after fermentation progress. During fermentation, calcium was solubilized by acid which cause buffering capacity from the SSP. Protease activity ranges from 0.71 U/g to 1.77–1.85 U/g, while the protein content in residual solids decreased from 30.84% to 20.77–25.30% with 20.42–21.99% chitin yield. Bajaj *et al.* [19] scaled up chitin extraction from shrimp shells by proteolytic LAB in 0.25 L (F1), 10 L (F2), and 300 L (F3) fermenters. F1, F2, and F3 preceded protein removal in parallel within 40 h at DP efficiency of 89– 91%. The DM was in the range of 85-90%. Although the microbial fermentation was improved in STB, the conditions for fermentation should be verified to be suitable. To improve the effectiveness of removing protein and calcium from SSP, for illustration, solid loading, and impeller speed should be optimized. Furthermore, it has been reported that additionally adjusting the stirring speed and agitation pattern was necessary to prevent foaming during DM caused by the generation of CO_2 [20].

4. CONCLUSIONS

This study has demonstrated biological approach for chitin extraction from SSP using symbiotic co-lactic acid fermentation (Co-LAF) by *Lactobacillus plantarum* and *Streptococcus thermophilus*, followed by successive fermentation by an acid-active proteolytic fungus, *Rhizopus oligosporus*, in a 5 L stirred tank bioreactor. DM by Co-LAF and DP by protease producing fungus led to greater DM and DP of 91.92±1.45% and 89.50±1.71%, respectively. The chitin extraction yield was 40.00±2.01%. The process showed that Co-LAF effectively produced lactic acid and protease, leading to improved demineralization (DM) and deproteinization (DP) of SSP. The results indicated that scaling up in bioreactor gave better performance than the flask-scale production.

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