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Production of Chitin and Chitosan from Shrimp Shell Wastes Using Co-Fermentation of *Lactiplantibacillus plantarum* PTCC 1745 and *Bacillus subtilis* PTCC 1720

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Abstract

Background and Objective: Disposal of fishery wastes is one of the environmental challenges. Converting wastes into valuable products is an economical solution to solve this environmental problem. One of the wastes of fishery products is shrimp shell waste, which contains large quantities of chitin. Chitin and its derivative, chitosan, include several uses in various industries, especially the food industry. The aim of the present study was to extract chitin from shrimp shell wastes using co-fermentation with mixed microbial cultures. Chitosan production by deacetylation of chitin using various concentrations of NaOH was another aim of this study.

Material and Methods: Batch fermentation was carried out to extract chitin from the shrimp shell using mixed cultures of two microorganisms, *Lactiplantibacillus plantarum* PTCC 1745 and *Bacillus subtilis* PTCC 1720. The Logistic model was used to assess the microbial growth rate. To prepare chitosan from chitin, deacetylation was carried out using NaOH solution. Morphological structures of the chitin and chitosan were studied using scanning electron microscopy. Functional groups of the synthesized chitin and chitosan were assessed using Fourier transform infrared spectroscopy.

Results and Conclusion: High levels of demineralization and deproteinization were achieved using co-fermentation with the mixed microbial populations at 60 $g.l^{-1}$ of glucose concentration. Chitin was produced with high purity and the protein and ash contents included 1.43 and 1.26%, respectively. Data predicted by the Logistic model were fairly matched the experimental data. A maximum cell growth rate of 0.065 (h^{-1}) was achieved at 60 $g.l^{-1}$ of initial glucose concentration at 35 °C. The optimal value of deacetylation (88.2%) was achieved using 50% NaOH solution at 100 °C. The results showed that the use of mixed culture of acid-producing and proteolytic microorganisms is highly effective for extracting chitin from shrimp shell waste.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

One of the most important natural polymers is chitin, which includes a linear chain molecule [1]. There are various natural resources of chitin. Followed by the fungal cell walls and exoskeleton of arthropods, the highest chitin content is found in fishery wastes, especially crab, lobster and shrimp shell wastes [2,3]. Chitinous wastes contain large quantities of proteins, lipids and minerals. All of these materials should be removed to achieve high purity of chitin [4]. The most important derivative of chitin is chitosan. Chitosan is commonly synthesized via the deacetylation of chitin [5]. In deacetylation process, acetyl groups are converted to free amine groups [6]. Chitin and chitosan include various uses in food industries due to their unique biocompatibility, non-toxicity, biodegradability and antimicrobial characteristics

[2,7]. These compounds can be used as functional food ingredients, additives [7], prebiotics [8], food packaging materials, edible films [9] and coating materials for the encapsulation of probiotics [10].

Chitin is extracted from the fishery wastes via deproteinization and demineralization, often carried out using harsh chemical processes. Chemical processes include disadvantages such as waste disposal problems, high operation costs and possibility of short chitin oligosaccharides productions [11,12]. To solve these problems, other methods have been used to remove the mineral contents and proteins such as use of enzymes and microorganisms. However, use of enzymes increases production costs and includes low extraction efficiencies [13]. Use of microorganisms is not only a low-cost process in comparison to purified enzymes but also is much more efficient. In addition, microbial fermentation residues contain high protein contents, which can be used as culture media to decrease costs of biological wastewater treatments [13,14]. Through the fermentation process, microorganisms produce organic acids and proteases, which lead to demineralization and deproteinization, respectively [15,16]. Various microorganisms have been used to extract chitin from fishery wastes, including *Pseudomonas* spp., *Bacillus* spp. and *Lactobacillus* spp. [15,17,18]. Acid-producing microorganisms produce further acid and are further effective in removing minerals [19]. Proteolytic micro-organisms produce further proteases and therefore are further effective in removing protein from chitin. Moreover, fermentation of shrimp shell wastes using proteolytic microorganisms leads to the production of chitin with high molecular weight [19,20].

One of the major problems in research conducted on fermentation using single microorganism is that the yield of chitin extraction is low. In this type of fermentation, efficiency of demineralization or deproteinization is low depending on the microbial species; hence, chemical treatments are needed in addition to microbial fermentation to achieve high purity chitin. Therefore, the aim of this study was to investigate that if fermentation of mixed cultures of acid-producing and proteolytic microorganisms could increase the efficiency of deproteinization and demineralization in the process of chitin production from shrimp shell wastes with no use of acidic and alkaline chemical treatments. For this aim, chitin was extracted from shrimp shell wastes using two microorganisms of *Lactiplantibacillus* (*Lp.*) *plantarum* and *Bacillus* (*B.*) *subtilis.* Growth rates of the mixed microbial population were assessed using kinetic model. The specific growth rate was predicted by the logistic model. For chitosan production, chitin was deacetylated using various concentrations of NaOH solution at various temperatures.

2. Materials and Methods

2.1 Materials and culture media

Shrimp wastes consisting of heads and shells were provided by a fishery product processing factory located at Ahvaz, Iran. Shells were stored at -20 °C to prevent spoilage. Shrimp shells were washed with water and thoroughly dried at 65 °C for 48 h using dryer oven. Then, dried shells were ground and powdered. All chemicals were purchased from Merck, Germany. Aqueous solutions were prepared using double distilled water (DW). The *Lactiplantibacillus (Lp.) plantarum* PTCC 1745 and *Bacillus (B.) subtilis* PTCC 1720 were supplied by the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The was inoculated into a 250-ml flask containing 100 ml of de Man, Rogosa and Sharpe (MRS) broth media and incubated at 37 °C for 24 h using shaking incubator (150× rpm) [21]. The *B. subtilis* inoculum was prepared in 100 ml of media containing peptone (5 $g.l^{-1}$), meat extract (3 $g.l^{-1}$) and NaCl (5 $g.l^{-1}$) and incubated at 30 °C for 24 h using shaking incubator (150× rpm).

2.2 Chitin extraction

Batch fermentation was used to extract chitin from the shrimp shell powders. Experiments were carried out using *Lp. plantarum*, *B. subtilis* and a combination of the two bacteria (1:1). A quantity of 5% (wt) shrimp shell powder was poured into a 250-ml flask containing 100 ml of the culture media. A quantity of 5% (v v^{-1}) inoculum was added into media containing glucose and shrimp shells. The inoculated media were incubated at various temperatures (25, 30, 35 and 40 °C) for 150 h at 150 rpm using shaking incubator. The culture media were supplemented with various glucose concentrations (20, 40, 60, 80 and 100 $g.l^{-1}$).

2.3 Chitosan production

To prepare chitosan, chitin was deacetylated at 120 rpm for 5 h using NaOH solution. First, deacetylation was carried out at various temperatures of 80, 90, 100 and 110 °C and then effects of various concentrations of NaOH (30, 40, 50 and 60%) was studied. Residues were filtered and washed with DW to neutral pH and then completely dried at 60 °C for 12 h.

2.4 Analytical methods

Ash content was assessed based on ASTM D5142 method by dry oxidation at 550 °C for 4 h using furnace [22]. This was transferred into an oven and heated at 105 °C and then dry weight of the samples was measured. Demineralization (%) was expressed as the percentage of decreases in the ash content of the samples. Nitrogen content of the samples was assessed using Kjeldahl method based on the AOAC standard method no. 984.13 [23]. Since most proteins contain 16% nitrogen, the quantity of protein was estimated by the following equation of protein (%) = nitrogen (%) × 6.25 [24].

Cell dry weight was assessed using 0.25-µm filters. Optical density of the samples was measured at 600 nm using spectrophotometer (UNICO, USA). Biomass concentration was calculated using correlations between the cell dry weight and optical density. Fourier transform infrared spectra (FTIR) were recorded using FTIR spectrometer (Tensor 27, Bruker, Germany). The prepared chitin and chitosan were ground using mortar. Then, 1 mg of each chitin and chitosan powders was mixed with 100 mg of KBr and pressed into a KBr pellet. The FTIR spectra were recorded at 400-4000 cm^{-1}. Surface morphology of the prepared chitin and chitosan was studied using scanning electron microscope (SEM) (Phenom-ProX, the Netherlands) after samples were coated with gold using sputtering coater (Emitech K575X, UK). Deacetylation degree (DD) of the samples was assessed using alkalimetry method reported by Liu [25]. Chitosan (0.3 g) was dissolved in 30 ml of HCl solution (0.1 M). Two drops of methyl orange-aniline blue indicator were poured into the solution. Then, solution was titrated using NaOH solution (0.1 M) until the solution color changed from purple to blue-green. The DD was calculated using Eq. 1:

$$DD\ (\%) = \frac{(C_1V_1 - C_2V_2)\times 0.016}{m(1-w)\times 0.0994} \times 100 \qquad \text{Eq. (1)}$$

Where, C_1 and C_2 were the HCl and NaOH concentrations (M), respectively; V_1 and V_2 were the volumes of HCl and NaOH solutions (ml), respectively; m was mass of the chitosan sample (g); and w was the sample water content. Moreover, 0.016 was the amino content equivalent to 1 ml of HCl solution (1 M) and 0.0994 (16/161) was the theoretical amino content of chitin.

3. Results and Discussion

In this study, comparison between *Lp. plantarum*, *B. subtilis* and a combination of the two bacteria during fermentation was carried out. Cell growth of each microbial culture was studied by measuring biomass concentration during the fermentation process. Cell dry weight was depicted as a function of time (Figure 1a). Co-fermentation with the mixed culture of *Lp. plantarum* and *B. subtilis* provided the highest cell growth. Considering increases in growth of the microbial mass, it can be concluded that good symbioses occurred between *Lp. plantarum* and *B. subtilis*. It could be interpreted that the beneficial effects of *B. subtilis* on *Lp. plantarum* were created through the activation of heme-dependent catalase and improvement of reactive oxygen species [26].

Figure 1b presents deproteinization and demineralization of shrimp shells against time for each microbial culture.

It can be seen that *Lp. plantarum* was more effective in demineralization. This was because *Lp. plantarum* was an acid-producing bacterium and organic acid produced by this bacterium played effective roles in removing minerals. Furthermore, *B. subtilis* was further potent in deproteinization because it was a proteolytic bacterium and the proteases produced by this bacterium included significant functions in protein removal. Use of *Lp. plantarum* and *B. subtilis* culture media includes high demineralization ability of *Lp. plantarum* and high deproteinization ability of *B. subtilis*. Maximum demineralization and deproteinization were obtained 44.7 and 60.4%, respectively, after 150 h of fermentation using mixed culture media. Cell growth of the mixed microbial population was assessed during batch fermentation. Cell dry weight at various temperatures of 25, 30, 35 and 40 °C was depicted as a function of time (Figure 2a). As seen in the figure, the highest cell growth was achieved at 35 °C. At the optimal temperature, the exponential phase was completed within 120 h and cell dry weight reached to a maximum value of 4.25 $g.l^{-1}$.

Figure 2b shows deproteinization and demineralization of the shrimp shell wastes against time at temperatures of 25, 30, 35 and 40 °C.

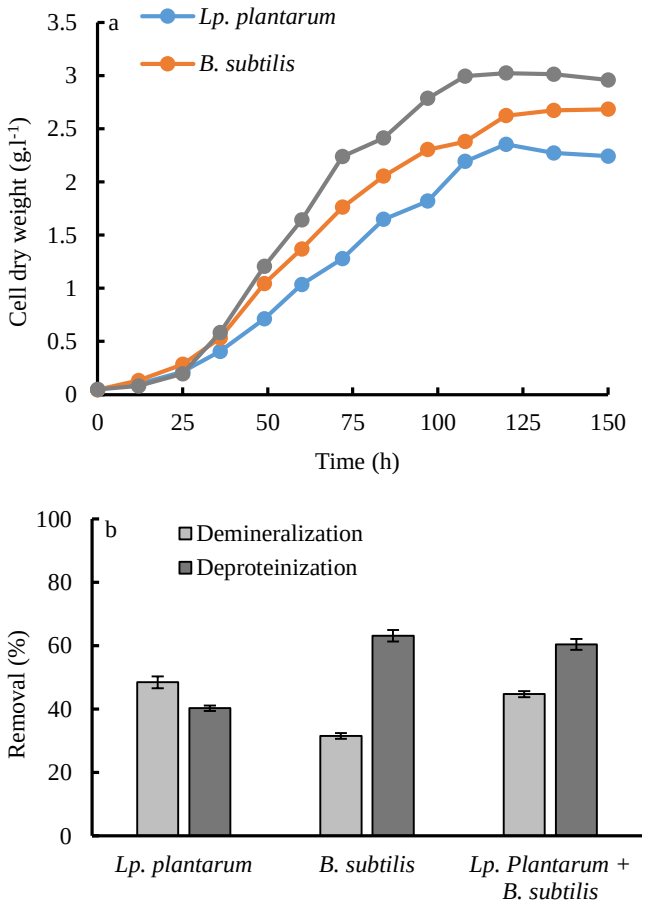

Figure 1. (a) Cell growth of *Lactiplantibacillus plantarum*, *Bacillus subtilis*, and *Lactiplantibacillus plantarum* and *Bacillus subtilis* against time; and (b) effects of culture media on demineralization and deproteinization of the shrimp shells (after 150 h of fermentation)

It can be observed that the maximum quantity of demineralization was achieved at 35 °C. Regarding small differences in the quantity of deproteinization at 30 and 35 °C, the latter temperature was selected as the optimum temperature. At this temperature, demineralization and

deproteinization respectively reached to 64.1 and 72.3% after 150 h. Based on these results, the optimum temperature for chitin extraction from the shrimp shells was 35 °C.

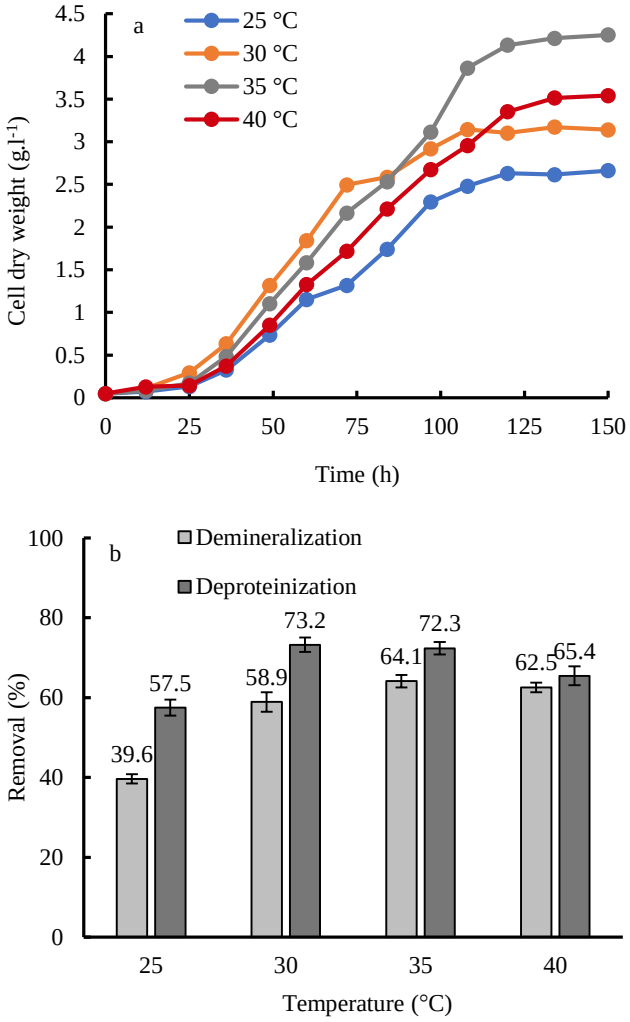

Figure 2. Effects of fermentation temperatures of 25, 30, 35 and 40 °C (a) on the microbial cell growth and (b) on the deproteinization and demineralization of the shrimp shells

Cell dry weight for various glucose initial concentrations of 20, 40, 60, 80 and 100 g.l^{-1} was assessed at 35 °C. The cell growth rate was studied using kinetic model. The specific growth rate predicted by the logistic model was as Eq. 2:

$$\mu = \mu_{max}(1 - \frac{x}{x_{max}}) \quad \text{Eq. (2)}$$

Where, μ_{max} and x_{max} were the maximum specific growth rate (h^{-1}) and the maximum cell concentration (g.l^{-1}), respectively. By substitution of the highlighted equation into the Malthus equation (dx / dt = μx) and carrying out integration, Eq 3 was achieved for the cell dry weight [27]:

$$x = \frac{x_0 \exp(\mu_{max} t)}{1-(\frac{x_0}{x_{max}})(1-\exp(\mu_{max} t))} \quad \text{Eq. (3)}$$

Where, x_0 was the initial microbial concentration. The logistic model was fairly fitted to the experimental data (Figure 3a). Kinetic parameters defined by logistic model for the mixed microbial populations in batch experiments are summarized in Table 1. As seen in the figure, specific growth rate increased with the increases in glucose concentrations. The highest specific growth rate of 0.065 h^{-1} was achieved at an initial glucose concentration of 60 g.l^{-1}. With increases in glucose concentration to values higher than 60 g.l^{-1}, the specific growth rate decreased due to the inhibitory effects of high glucose concentrations.

Figure 3b shows deproteinization and demineralization of the shrimp shell wastes at various glucose concentrations of 20, 40, 60, 80 and 100 g.l^{-1}. By increasing the glucose concentration from 20 to 60 g.l^{-1}, proportions of demineralization and deproteinization increased.

Table 1. Kinetic parameters defined by the logistic model for the mixed cultures of *Lactiplantibacillus plantarum* and *Bacillus subtilis*

Initial glucose concentration, g.l^{-1}	μ_{max}, h^{-1}	x_{max}, g.l^{-1}	R^2
20	0.061	4.24	0.988
40	0.061	6.97	0.984
60	0.065	8.35	0988
80	0.063	8.37	0.981
100	0.057	8.31	0.987

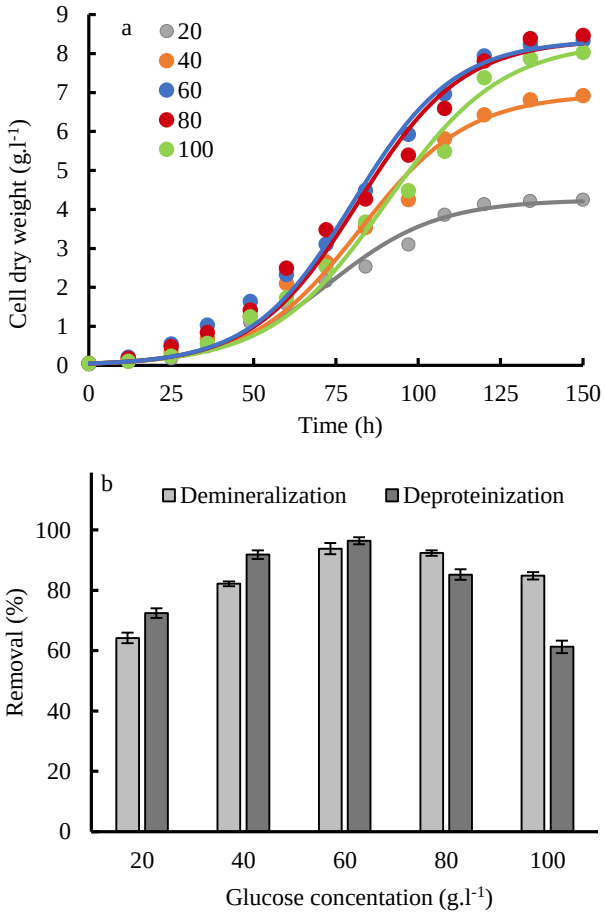

Figure 3. Effects of glucose concentrations of 20, 40, 60, 80 and 100 g.l^{-1} (a) on the microbial cell growth (experimental data and logistic model) and (b) on the deproteinization and demineralization of shrimp shells

By providing sufficient carbon sources, microorganisms grew faster and thus consumed further proteins and minerals. By increasing the glucose concentration to more than 60 $g.l^{-1}$, demineralization and deproteinization decreased. This was because high glucose concentrations could include inhibitory effects on the microbial growth. Decreases in deproteinization were more significant than decreases in demineralization. This might occur because protease enzymes produced by the microbial population decreased at high concentrations of simple carbon sources such as glucose [28], resulting in less hydrolysis of the proteins. After 150 h of fermentation, concentration of lactic acid (mostly by *Lp. plantarum*) was 42.85 g l^{-1}. Protease activity was 173.47 U ml^{-1}. It is clear that higher enzyme activities belonged to *B. subtilis* because of its high proteolytic activity. At 60 $g.l^{-1}$ of glucose concentration, the maximum extents of demineralization and deproteinization were 93.77 and 96.40%, respectively. Under optimal conditions, protein and ash contents in prepared chitin were 1.43 and 1.26%, respectively. These quantities were lower than those reported in the literature for proteins (e.g. 1.5%) and ash (e.g. 2.7%) in commercial chitin [19]. Results of the recent study on the extraction of chitin from shrimp wastes using microbial fermentation are presented in Table 2.

Temperature and sodium hydroxide concentration are most important affecting parameters in chitin deacetylation. In the present study, effects of temperature and NaOH concentration on the chitin deacetylation were investigated. First, deacetylation of chitin was carried out at various temperatures of 80, 90, 100 and 110 °C using 30% NaOH solution (Figures 4a and 4b).

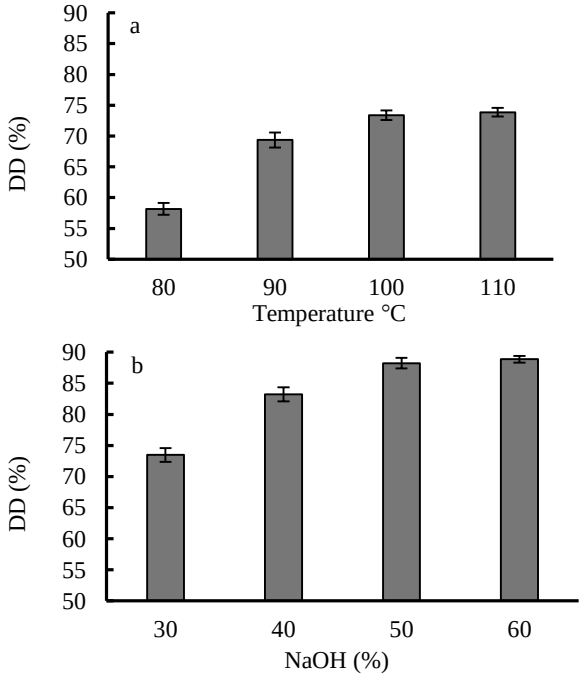

Figure 4. Effects of (a) temperature and (b) NaOH concentration on the degrees of deacetylation

The DD increased with increasing temperature. As removing the acetyl group and breaking the C-N bond was endothermic, the deacetylation process needed higher temperatures to provide the activation energy for the reaction [32]. Since no significant increases were seen in DD for temperatures higher than 100 °C, this value was selected as the optimum temperature. In the following experiments, effects of NaOH concentration was studied at 100 °C and 120 rpm. Deacetylation of chitin is a heterogeneous reaction in the solid-liquid phase that first occurs on the surface of chitin particles. Increasing the concentration of NaOH causes more contact of NaOH molecules with acetyl groups and thus increasing the reaction efficiency [33]. As seen in Figure 4b, the optimum value of 88.2% deacetylation was achieved with 50% NaOH solution.

In general, SEM is one of the most important techniques to study the surface morphology and microstructure of materials. To investigate the surface morphology of shrimp shell wastes, chitin and chitosan, SEM images were recorded at a magnification of 20000× (Figure 5). Figure 5a shows a relatively rough surface with many impurities on shrimp shell wastes, the predominant components of which was a mixture of protein and minerals. Figure 5b shows that fermentation by *Lp. plantarum* and *B. subtilis* effectively removed protein and minerals from the shrimp shells, resulting in slightly smoother and uniform chitin surfaces. High levels of demineralization and deproteinization achieved from the shrimp shell fermentation verified the SEM images. Based on the SEM images (Figure 5c), the surface of chitosan was smooth with the lowest possible quantity of impurities. Chemical treatment with NaOH at high temperatures, in addition to deacetylating chitin, removed small quantities of proteins in chitin and resulted in further homogeneous surfaces of the chitosan.

In this study, functional groups of chitin and chitosan were investigated using FTIR (Figure 6).

The IR spectrum of chitin showed absorption bands at 3480 due to the stretching vibration of O–H bands. Peaks near 3271 and 3097 cm^{-1} were attributed to the stretching vibration of N-H groups [33].

The stretching vibrations of C-H groups were observed at 2869 and 2919 cm^{-1} [34]. The vibration bands at 1636 and 1665 cm^{-1} are linked to the C=O stretching of amide I [35]. The peak at 1561 cm^{-1} corresponded to N-H bending and C-N stretching vibrations of amide II [36]. The peak at 1318 cm^{-1} was assigned to amide III bands and CH_2 wagging [33,37]. The characteristic bands of saccharide structure were as follows: The peak at 1160 cm^{-1} was due to anti-symmetric stretching of the C–O–C bridge and peaks at 1037 and 1083 cm^{-1} were due to C–O stretching vibrations [25,34].

The IR spectra of chitosan showed a peak at 3460 cm^{-1} due to overlapping of the stretching vibrations of O–H and N-H bands [38]. Peaks at 2880 and 2924 cm^{-1} were associated to the asymmetric and symmetric stretching vibrations of -CH groups [39].

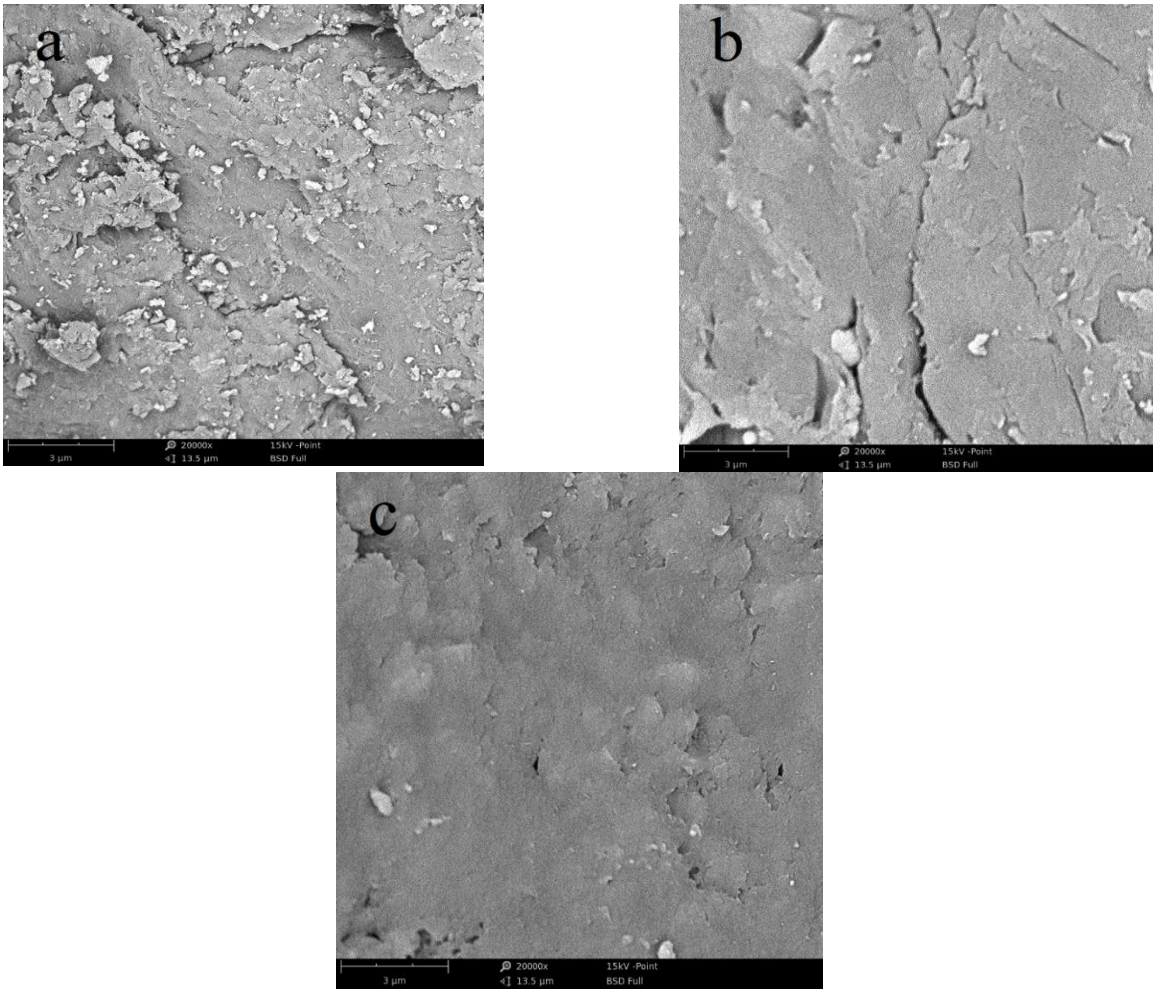

Figure 5. Scanning electron micrographs of (a) shrimp shell wastes, (b) chitin and (c) chitosan (20000×)

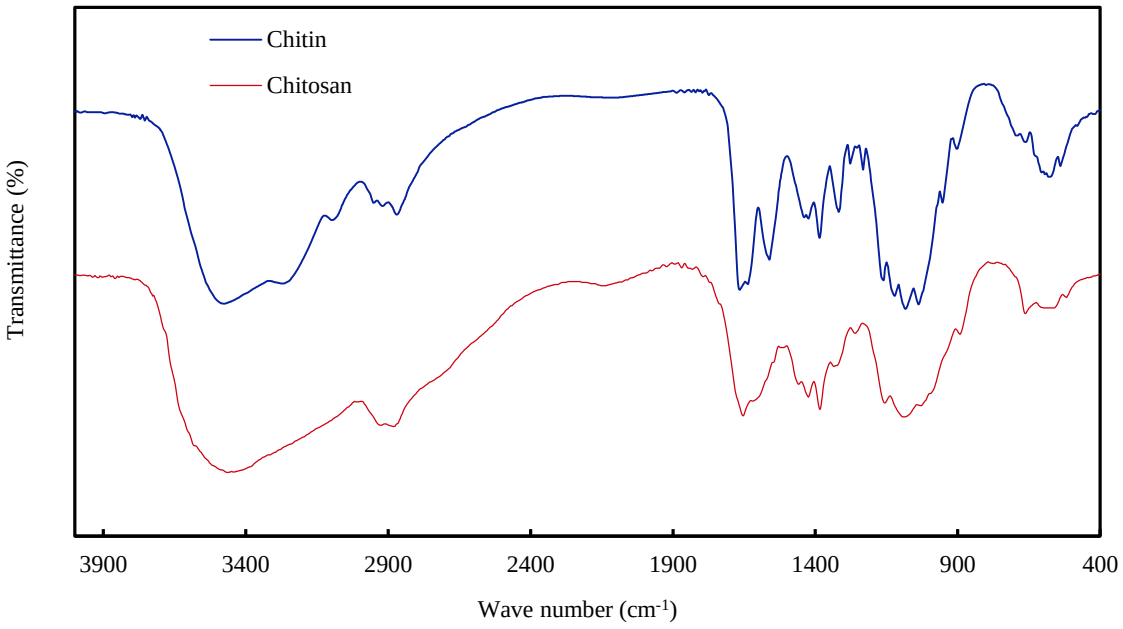

Figure 6. Fourier transform infrared spectra of the prepared chitin and chitosan

Table 2. Comparison of the demineralization and deproteinization of shrimp wastes by various microorganisms reported in recent literatures

Microorganism	Demineralization (%)	Deproteinization (%)	Reference
Bacillus Pumilus	88.0	94.0	[29]
Brevibacillus parabrevis	21.3	95.0	[30]
Lactobacillus acidophilus	90.8	76.0	[15]
Pseudomonas aeruginosa	82.0	91.9	[18]
Paenibacillus elgii	-	94.1	[16]
Bacillus cereus	53.8	97.4	[17]
Lactobacillus rhamnoides and *Bacillus amyloliquefaciens*	78.0	48.3	[31]
Bacillus subtilis and *Acetobacter pasteurianus*	92.0	94.5	[19]
Lp. plantarum and *B. subtilis*	93.8	96.4	This study

The peak at 1653 cm^{-1} was attributed to the stretching vibration band of amide I [40] and that at 1334 cm^{-1} belonged to amide III bands [41]. Moreover, the peak at 1651 cm^{-1} linked to carbonyl stretching of the amide I band significantly decreased, verifying that chitosan was significantly deacetylated [25].

4. Conclusion

In general, chitin was extracted from the shrimp shell wastes of a fishery product processing plant via microbial fermentation. Co-fermentation using *Lp. plantarum* and *B. subtilis* showed good performances for demineralization and deproteinization of the shrimp shells. Results showed that efficiency of the chitin extraction from shrimp shell wastes using mixed cultures of acid-producing and proteolytic microorganisms was high enough that no chemical treatments were needed. The logistic model was adequately fitted to the experimental data for the microbial cell growth. Furthermore, the optimum conditions of chitosan deacetylation were reported as 50% NaOH solution and 100 °C temperature.

5. Acknowledgements

The authors report no conflict of interest.

6. Conflict of Interest

The authors report no conflicts of interest.

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تولید کیتین و کیتوزان از ضایعات پوسته میگو با استفاده از تخمیر همزمان *لاکتی‌پلانتی باسیلوس پلانتاروم* PTCC 1745 و *باسیلوس سوبتیلیس* PTCC 1720

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چکیده

سابقه و هدف: دفع ضایعات شیلات یکی از چالش‌های زیست محیطی است. تبدیل ضایعات به محصولات با ارزش راه حلی اقتصادی برای مقابله با این مشکل زیست محیطی می‌باشد. پوست میگو یکی از ضایعات شیلات است که حاوی مقادیر زیادی کیتین می‌باشد. کیتین و مشتق آن، کیتوزان، کاربردهای زیادی در صنایع گوناگون، به ویژه صنایع غذایی دارند. هدف از مطالعه حاضر بررسی استخراج کیتین از ضایعات پوست میگو توسط تخمیر همزمان با استفاده از کشت مخلوط میکروبی بود. همچنین تولید کیتوزان توسط استیل‌زدایی کیتین با استفاده از غلظت‌های گوناگون سدیم هیدروکسید مورد مطالعه قرار گرفت.

مواد و روش ها: تخمیر ناپیوسته برای استخراج کیتین از پوسته میگو با استفاده از کشت مخلوط دو ریزاندامگان *لاکتی‌پلانتی باسیلوس پلانتاروم* PTCC 1745 و *باسیلوس سوبتیلیس* PTCC 1720 انجام شد. برای ارزیابی نرخ رشد میکروبی از مدل لجستیک استفاده شد. برای تهیه کیتوزان از کیتین، استیل‌زدایی با استفاده از محلول سدیم‌هیدروکسید انجام شد. ساختار ریخت‌شناسی[1] کیتین و کیتوزان با استفاده از میکروسکوپ الکترونی روبشی مورد مطالعه قرار گرفت. گروه‌های عملکردی کیتین و کیتوزان ساخته شده با استفاده از طیف‌سنجی مادون قرمز تبدیل فوریه[2] ارزیابی شدند.

یافته‌ها و نتیجه‌گیری: سطوح بالایی از کانی‌زدایی[3] و پروتئین‌زدایی با استفاده از تخمیر همزمان با جمعیت‌های میکروبی مخلوط در غلظت $g.l^{-1}$ ۶۰ گلوکز به‌دست آمد. کیتین با خلوص بالا تولید شد و محتوای پروتئین و خاکستر به‌ترتیب ۱/۴۳ و ۱/۲۶درصد بود. داده‌های پیش‌بینی‌شده توسط مدل لجستیک با داده‌های تجربی کاملاً مطابقت داشتند. حداکثر سرعت رشد سلولی h^{-1} ۰/۰۶۵ در غلظت اولیه گلوکز $g.l^{-1}$ ۶۰ و ۳۵ درجه سلسیوس به‌دست آمد. مقدار بهینه استیل‌زدایی (٪ ۸۸/۲) با استفاده از محلول ۵۰ درصد سدیم هیدروکسید در دمای ۱۰۰ درجه سلسیوس به‌دست آمد. نتایج نشان داد که استفاده از کشت مخلوط ریزاندمگان‌های تولید کننده اسید و پروتئین کافت برای استخراج کیتین از ضایعات پوسته میگو بسیار موثر است.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] Morphological
[2] Fourier transform infrared spectroscopy; FTIR
[3] Demineralization