Chitin Extraction from Lobster Shell Waste Using Microbial Culture-based Methods

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Abstract

Background and Objective: Lobster shell waste from seafood processing industry was used as the source of raw material to produce the valuable biopolymer chitin. Chemical and biological treatments of lobster shell waste were performed and compared.

Material and Methods: The chemical method required the use of aqueous solutions of HCl and NaOH. Biological treatment included the use of co-cultures with a protease-producing bacterium, either Bacillus megaterium NH21 or Serratia marcescens db11, and an organic acid-producing bacterium Lactobacillus plantarum. The optimal culture conditions, including co-cultivation strategies and glucose concentrations, were identified to improve efficiency of lobster shell deproteinization and demineralization.

Results and Conclusion: Overall, the successive treatment with a combination of Serratia marcescens db11 and Lactobacillus plantarum resulted in the best co-removal of CaCO3 and proteins and chitin yield (82.56%) from lobster shell biomass, with total deproteinization of 87.19% and total demineralization of 89.59%. The results from the proof-of-concept study described here suggest that microbial treatment may be an environmentally friendly alternative to the chemical method of chitin extraction.

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

1. Introduction

The seafood processing industries generate around 6-8 million tons of crab waste, shrimp and lobster shells globally every year [1]. In the processing of crustaceans like lobster, shrimp, and crab for human consumption, about 40-50% of the total mass is waste [2]. Shellfish waste management is a huge problem faced by food industries. Only a small part of the waste is used as animal feed or fertilizers [3-5], while the rest are often dumped in landfills or into the sea and is a potential cause of pollution in coastal areas [6]. Approximately 50-60% of the total weight of the shells in lobster, shrimp, crab, is the non-edible exoskeleton, which is very rich in chitin, and is comprised of other components like calcium, proteins, and pigments [7].

Chitin or poly β-(1→4)-N-acetyl-D-glucosamine, is a polymer that is abundantly found in a number of living organisms [8]. In the native state, chitin occurs as ordered crystalline microfibrils, which is a major component of fungal cell walls, yeast, green algae, and the cuticles of insects [9,10]. Currently, the main commercial sources of chitin are crustacean shells [3,11]. Chitin and its derivative chitosan are biocompatible, non-toxic, biodegradable polymers [2]. These polymers have many potential applications in food and agriculture, medicine, tissue engineering, and as excipients for drug and gene delivery, wastewater treatment, as well as textile and cosmetic industries [12-21]. Chitin can also be depolymerized into its requisite monosaccharide, the amino sugar N-acetyl-D-glucosamine (NAG), which can be a carbon source for the growth of other microorganisms and production of value added products.

In crustaceans, chitin is present as a constituent of a complex network that includes proteins and carbonate deposits, forming the rigid shell [2]. Thus, extraction of chitin requires the removal of the two other major constituents of the shell, proteins and inorganic calcium...
carbonate (CaCO₃) [22]. Conventional methods of chitin extraction from crustacean shells involve a chemical process that consists of two basic steps: (I) protein removal by alkali treatment, and (ii) CaCO₃ removal by acidic treatment [2]. Deproteinization is carried out to depolymerize the biopolymer for the removal of protein and can be achieved by using bases such as NaOH [23]. Demineralization, as the name of the step implies, consists of removing minerals, primarily CaCO₃, and is generally easily achieved because CaCO₃ is converted into water-soluble calcium salts with the release of carbon dioxide [23]. The deproteinization step is challenging mainly due to disruption of covalent bonds between chitin and proteins [4]. The use of harsh chemicals may result in detrimental effects on the molecular mass, which will negatively affect the intrinsic properties of the purified polymer [2].

Dried shrimp shells are valued at a mere $100–120 per ton [24]. However, the chemical method of chitin purifications from waste streams like these has limits set mainly by the production costs, including effluent treatment after acid and alkaline extraction of chitin [2]. As a result, good quality, highly purified chitin can cost up to $200 per kilogram, even though the starting material is comparatively inexpensive [24]. The chemical method of chitin extraction is also an energy-consuming process and gives rise to a large volume of corrosive chemical wastes, which creates a disposal problem and can cause serious ecological damage and environmental pollution [25].

Due to these drawbacks of chemical chitin extraction, there has been growing interest in biological (bio-based) purification [3]. The biological method involves the use of microorganisms, either in monoculture or co-culture that produces protease enzymes and organic acids. Deproteinization can be carried out using protease-producing bacteria like Bacillus (B.) subtilis, Pseudomonas (P.) aeruginosa, Pseudomonas maltophilia, Serratia (S.) marcescens and others that break down proteins into water soluble protein hydrolysates, while the demineralization step can be carried out using organic acid-producing microorganisms like Lactobacillus (L.) paracasei, Lactobacillus pentosus, among others, that produce lactic acid that can remove calcium ions from shells [26-30]. The various biological methods of chitin extraction by microorganisms are of tremendous interest, as enzymes and organic acids can be produced by bacteria at a relatively low cost, and the process is environmentally friendly, allowing for production of high quality chitin, based on degree of acetylation. The biological method also leads to a liquid fraction, rich in proteins, which potentially can be used for human and animal feed [2].

The utilization of shellfish wastes can potentially solve environmental problems, as well as providing economic benefits. It can be an alternative to the disposal of shellfish wastes in landfills or in the oceans [24]. Since these wastes are rich sources of industrially important ingredients like chitin, there is an immense potential to bioprocess them and use chitin as a biotechnological feedstock to generate products of practical application. The sugars from seafood waste can be used as the primary feedstock to produce renewable biodiesel. It is a novel approach that would not require any current landmass in use or that could be potentially used for food crops, allowing the technology to remain out of the food vs. fuel debate, a philosophical barrier for some bio-fuel development efforts utilizing corn, palm oil, and canola oil [31].

The aim of this study is to produce crude chitin from lobster shell waste through the co-culturing of Bacillus megaterium NH21 or Serratia marcescens db11 and Lactobacillus plantarum in the presence shell waste. The optimal culture conditions, including co-cultivation strategies and carbon feedstock (i.e., glucose) concentrations were optimized for small-scale, batch deproteinization and demineralization of lobster shell waste. The chitin obtained was characterized and compared with those produced from using the bacterial cultures individually and the original sample. Much research has been performed on chitin extraction using different techniques and microorganisms. This work describes the use of two species of bacteria in sequential co-culture to remove mineral and protein contents from lobster shells in order to extract chitin.

2. Materials and Methods

2.1 Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Lobster shell waste was a generous gift from Gloucester Seafood Processing Inc. (Gloucester, MA, USA). The shells were washed, dried, ground and sieved (1 mm sieve) and stored at room temperature until used. The protease-producing bacterium Bacillus megaterium strain NH21 is an environmental isolate from Lake Webster, USA. Another protease producer, Serratia marcescens db11 is an unpigmented isolate originally characterized by Flyg and coworkers [32]. The organic acid producer Lactobacillus plantarum was isolated from cellulose waste [33]. All bacterial strains were stored in 20% glycerol at 80°C.

2.2 Inoculum preparation

L. plantarum was grown on Man Rogosa Sharpe (MRS) agar and incubated overnight at 37°C. MRS media composition used in this work is as follows (per liter): 10 g peptone, 15 g yeast extract, 20 g dextrose, 1 g Tween-80 (polysorbate 80), 2 g dipotassium hydrogen phosphate (K₂HPO₄), 5 g sodium acetate, 2 g triammonium citrate,
0.2 g magnesium sulfate, 0.05 g manganese sulfate, balance distilled H2O. For solid media, 15 g agar was added before sterilization. Peptone, yeast extract and agar were purchased from Thermo-Fisher Scientific (Agawam, MA, USA). All other MRS media components were purchased from Sigma-Aldrich (St. Louis, MO, USA). B. megaterium and S. marcescens were grown on plates of Lysogeny Broth (LB) agar (Thermo-Fisher Scientific, Agawam, MA, USA) and incubated overnight at 30°C. To prepare starter cultures, 100 ml of sterile MRS broth was inoculated with L. plantarum and LB broth was inoculated with B. megaterium and S. marcescens separately and incubated with shaking (180 rpm) at 37°C and 30°C, respectively, for 48 h.

To prepare an inoculum for fermentation, 4 ml of the starter culture was transferred into 100 ml of sterile MRS broth or LB broth (2 vol% inoculation) and incubated with 180 rpm agitation at 37°C and 30°C for 48 h, respectively.

2.3 Chemical treatment of the lobster shell waste

The method of Aytekin and Elibol [34] was adopted to chemically extract chitin from lobster shells. Briefly, the removal of protein was achieved by treating the lobster shells with 1M NaOH solution for 3 h, at elevated temperatures (80-90°C) with continuous stirring and a solids-to-solvent ratio of 1:10 (w/v). Demineralization was achieved by constant stirring of the dried, ground, deproteinized shells in 1N HCl for 2 h, at ambient room temperature and a solid-to-solvent ratio of 1:15 (w/v).

Two chemical extraction methods were followed to remove proteins and inorganic matter from shells (Fig. 1): (I) the protein was removed first, followed by removal of inorganic matter, i.e. deproteinization was followed by demineralization (DP-DM); ii) the reverse order was followed, i.e., demineralization was followed by deproteinization (DM-DP).

For all methods, the resulting deproteinized, demineralized chitin was filtered from lobster shells and washed to neutrality with water. The chitin was then dried overnight at 60°C in an oven (Hi-Temp Vacuum Oven, Thermo Scientific).

2.4 Biological treatment of the lobster shell waste

In separate cultures, 10 g of lobster shells were added to 50 ml of water supplemented with 0%, 2%, 5% or 10% glucose and inoculated with 10% B. megaterium, S. marcescens and were incubated at 30°C; and with 10% L. plantarum and incubated at 37°C with shaking (180 rpm) for 96 h.

Three different strategies were applied in the co-culture experiments (Fig. 1): (i) simultaneous inoculation of a protease producing bacterium (either B. megaterium or S. marcescens) and L. plantarum, incubated at 37°C with shaking (180 rpm); (ii) inoculation with a protease producing strain, supplemented with 5% glucose (incubated at 30°C with shaking (180 rpm)). After three days, L. plantarum was added to the culture, followed by incubation at 37°C with shaking (180 rpm); (iii) the reverse order of (ii). After six days incubation time for all co-culture strategies, chitin was harvested, and the culture supernatant was collected for further analysis.

2.5 Analysis of chemical properties of samples

The pH of the supernatant was determined using a potentiometer (Orion Star A211, Thermo Scientific, USA). The ash content of the shell residues was determined by combusting the shell powder in an electric furnace at 500°C for 4 h and measuring ash weight.

The protein content was determined in each sample by checking the nitrate content in the samples using the cadmium reduction method with a spectrophotometer (DR 4000-1 UV-VIS, Hach, USA). The protein content in the untreated samples was considered as the basis for computing the deproteinization percentage, which was expressed by Eq.1:

\[
\text{Deproteinization} (\%) = \frac{(P_O - P_F)}{P_O} \times 100 \quad \text{Eq. 1}
\]

where \(P_O\) and \(P_F\) are the protein concentrations (%) before and after the co-culture treatments, \(O\) and \(F\) represent the mass (g) of original samples and biologically treated residues, respectively on a dry weight basis [35]. The calcium content in the samples was determined by atomic absorption spectroscopy using an air-acetylene flame (AAnalyst 300, Perkin Elmer, USA). Demineralization percentage was calculated using the Eq. 2:

\[
\text{Demineralization} (\%) = \frac{(C_O - C_F)}{C_O} \times 100 \quad \text{Eq. 2}
\]

where \(C_O\) and \(C_F\) represent the calcium content in the original and in the biologically treated residues, respectively on a dry weight basis. All experiments discussed here were conducted in triplicate, and values are presented as the mean ± standard deviation.

The chitin yield was calculated with Eq. 3:

\[
\text{Chitin yield} (\%) = \frac{\text{Chitin in residue (g)}}{\text{Initial chitin in shell waste (g)}} \times 100 \quad \text{Eq. 3}
\]
2.6 Proteolytic activity assay

The proteolytic activities were determined using the substrate casein. A 0.5 ml aliquot of culture supernatants was diluted and mixed with 0.5 ml of 1% (w v⁻¹) casein in 100 mm potassium phosphate buffer (pH 7). The mixture was incubated for 10 min at 40°C. The reaction was terminated by adding 1 ml of 0.4 M trichloroacetic acid and allowed to stand at 40°C for 20 min. The mixture was then centrifuged at 3619.1 g for 5 min to remove precipitate. A 0.5 ml aliquot of the supernatant was mixed with 2.5 ml of 0.5 M sodium carbonate and 0.5 ml of 0.5 M Folin-phenol reagent, and the mixture was then incubated for 20 min at 40°C. A standard curve was made using solutions of L-tyrosine in concentration ranges of 0-100 µg ml⁻¹.

One unit of protease activity is defined here as the amount of enzyme required to release 1 µmol of L-tyrosine min⁻¹. The protease activities were determined from the mean of at least two separate measurements carried out in multiple replicates, and the differences between the values did not exceed 5%.

2.7 Chitin characterization using Thermogravimetric analysis (TGA)

TGA monitors weight change in materials during heating as a function of time and temperature. The acquired measurements provide information about the thermal stability and composition of the material. TGA assays were conducted with a TGA Q500 (TA Instruments) by heating the samples from 10°C to 800°C by ramping temperature input of 5°C per min.

2.8 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray spectroscopy (EDX) analysis of chitin

The dried sample of chitin was coated with a gold layer under vacuum using a sputter coater (Denton Vacuum Sputter, Desk IV, USA). High-resolution images of surface topography of chitin samples were produced using a highly-focused JSM-5610 (JEOL Ltd., Tokyo, Japan) scanning electron microscope operated at 20 kV. Qualitative Energy Dispersive X-ray (EDX) spectra of the deproteinized and demineralized samples were recorded using an EDX detector (EDS 6587, Oxford Instruments, UK) mounted on the scanning electron microscope.

2.9 Statistical analysis

Each preparation and measurement was conducted in triplicate. The experimental data were subjected to an analysis of variance (ANOVA) for a completely random design (CRD) using a Statistical Analysis System (SAS Institute, Inc., 2000). Differences were significant at p≤0.05.

3. Results and Discussion

3.1 Chemical treatment of the lobster shell waste

Removal of proteins and inorganic matter (minerals) may be achieved by treatment of lobster shells using different strategies. Therefore, two chemical extraction methods were followed to remove proteins and inorganic matter from the shells. The chemical compositions of the lobster shells before and after the treatment are shown in Table 1.
It was seen that the protein content in the residues when the DM-DP method followed was lower than that of DP-DM, suggesting more complete deproteinization of the shells. Demineralization levels did not vary extensively between methods.

3.2. Biologically mediated chitin extraction using separate microbial cultures

Biological methods of organic and inorganic matter removal from chitin are an attractive alternative to chemical methods due to the lack of toxic waste products [36]. pH and proteolytic activity were monitored throughout the experiments with respect to glucose concentration. When the concentrations of glucose varied from 0% to 10%, the changes in the pH and proteolytic activity were studied for 6 days of culture, using *B. megaterium* NH21, *S. marcescens* db11 and *L. plantarum*, respectively, as biocatalysts (Fig. 2). The maximum protease activities of 161.3 U ml\(^{-1}\) for *B. megaterium* and 195.13 U ml\(^{-1}\) for *S. marcescens* were obtained from cultures initially supplemented with 5% glucose, and *L. plantarum* was found to produce negligible amounts of protease irrespective of the glucose concentration, as seen in Fig. 2. The latter result was expected, as *L. plantarum* was used in this work for its production of organic acids.

Under culture conditions that fostered maximum protease activity, the deproteinization level of lobster shells by *B. megaterium* was 70.29% and by *S. marcescens* was 76.73% (Table 2), calculated according to equation 2. The deproteinization levels coincided with the trend of proteolytic activity (Fig. 2), as the largest percentage of protein was removed from shells under culture conditions where protease activity was shown to be the strongest in culture supernatants.

### Table 1. Chemical compositions of lobster shell biomass and residues after chemical treatments

<table>
<thead>
<tr>
<th>Shells</th>
<th>Protein (%)</th>
<th>Calcium (%)</th>
<th>Deproteinization (%)</th>
<th>Demineralization (%)</th>
<th>Ash (%)</th>
<th>Chitin yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>23.54 ± 0.78</td>
<td>45.68 ± 0.34</td>
<td>-</td>
<td>-</td>
<td>12.17 ± 1.1</td>
<td>-</td>
</tr>
<tr>
<td>DP – DM</td>
<td>6.67 ± 0.98</td>
<td>4.26 ± 1.02</td>
<td>80.16 ± 0.56</td>
<td>92.54 ± 0.94</td>
<td>2.87 ± 0.83</td>
<td>75.4 ± 1.23</td>
</tr>
<tr>
<td>DM – DP</td>
<td>2.72 ± 1.2</td>
<td>5.09 ± 0.67</td>
<td>89.97 ± 0.87</td>
<td>91.54 ± 1.31</td>
<td>3.94 ± 0.48</td>
<td>89.8 ± 2.5</td>
</tr>
</tbody>
</table>

DP-DM = deproteinization followed by demineralization. DM-DP = demineralization was followed by deproteinization. All the values are means of triplicates, the standard deviations are indicated.

### Table 2. Results obtained from residues after treatment with individual microbial cultures.

<table>
<thead>
<tr>
<th>Microbial treatment</th>
<th>Glucose (%, w/v(^{-1}))</th>
<th>Protein (%)</th>
<th>Calcium (%)</th>
<th>Deproteinization (%)</th>
<th>Demineralization (%)</th>
<th>Ash (%)</th>
<th>Chitin yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>23.54 ± 0.7</td>
<td>45.68 ± 0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12.17 ± 0.76</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus megaterium</em> NH21</td>
<td>0</td>
<td>14.30 ± 0.9</td>
<td>41.32 ± 1.66</td>
<td>51.40 ± 0.4</td>
<td>27.63 ± 1.1</td>
<td>9.73 ± 0.43</td>
<td>38.73 ± 2.11</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>12.58 ± 1.1</td>
<td>34.89 ± 0.5</td>
<td>58.32 ± 2.1</td>
<td>40.42 ± 0.94</td>
<td>7.21 ± 1.17</td>
<td>46.63 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.84 ± 0.67</td>
<td>32.72 ± 0.9</td>
<td>70.29 ± 1.7</td>
<td>43.34 ± 0.7</td>
<td>6.82 ± 0.3</td>
<td>49.73 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>18.57 ± 0.73</td>
<td>32.49 ± 1.3</td>
<td>41.62 ± 0.8</td>
<td>47.36 ± 0.68</td>
<td>8.59 ± 0.62</td>
<td>40.90 ± 1.05</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> db11</td>
<td>0</td>
<td>13.79 ± 1.4</td>
<td>43.88 ± 0.78</td>
<td>56.06 ± 1.03</td>
<td>27.95 ± 1.23</td>
<td>5.64 ± 0.2</td>
<td>39.54 ± 0.90</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>16.86 ± 0.11</td>
<td>38.47 ± 0.84</td>
<td>47.51 ± 0.7</td>
<td>38.26 ± 1.04</td>
<td>6.46 ± 0.84</td>
<td>42.82 ± 2.76</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.17 ± 0.98</td>
<td>35.84 ± 0.7</td>
<td>76.73 ± 1.3</td>
<td>40.05 ± 0.47</td>
<td>7.25 ± 0.37</td>
<td>50.71 ± 3.25</td>
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<tr>
<td></td>
<td>10</td>
<td>14.21 ± 1.23</td>
<td>32.36 ± 0.69</td>
<td>51.88 ± 0.7</td>
<td>43.54 ± 0.75</td>
<td>8.62 ± 0.17</td>
<td>43.48 ± 2.59</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>20.84 ± 0.56</td>
<td>19.05 ± 0.76</td>
<td>17.60 ± 0.17</td>
<td>65.64 ± 0.33</td>
<td>5.74 ± 1.2</td>
<td>29.66 ± 1.09</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>22.79 ± 0.84</td>
<td>15.56 ± 1.54</td>
<td>21.67 ± 2.1</td>
<td>72.83 ± 0.26</td>
<td>3.16 ± 0.6</td>
<td>37.09 ± 2.08</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-</td>
<td>15.68 ± 1.28</td>
<td>-</td>
<td>73.02 ± 1.3</td>
<td>4.88 ± 0.3</td>
<td>-</td>
</tr>
</tbody>
</table>

ND = not determined. All the values are means of triplicates, the standard deviations are indicated after the ±.
Growth of L. plantarum was poor in lobster shell medium without glucose. This shows that the lobster shell waste on its own could not provide enough nutrition for the growth of L. plantarum. Therefore, the protein, calcium, and ash content of residues from these culture conditions were not determined. However, in L. plantarum cultures, the initial concentration of glucose had a significant effect on the production of lactic acid. When the glucose concentration was 5%, the culture pH dropped from 7.9 to 4.6 (Fig. 2b), and the demineralization efficiency increased to 72.83%.

The pH of the medium decreased with the increase in culture time. This change can be related to the conversion of glucose to lactic acid by the microbial activity, which indicates removal of inorganic matters. Lactobacillus strains are known to produce lactic acid, which dissolves the calcium carbonate present in crustacean shells in such a way that significant demineralization had occurred in the shells by the conclusion of a co-culture treatment [33, 37].

The use of individual microorganisms in the deproteinization and demineralization of wastes for chitin production have been previously reported. Bacillus megaterium was used on distillery yeast biomass waste which resulted in 84% and 76% protein removal using culture and crude enzyme extracted from the isolate, respectively [38]. The protein and mineral removal from natural crab shell wastes with S. marcescens FS-3 was 84% and 47% respectively, after 7 days of fermentation [28]. In another study, L. plantarum was used to achieve demineralization and deproteinization of 54 and 45% respectively, at a low substrate concentration (2%) [12]. In this study, from Table 2, it can also be inferred that individual cultures of B. megaterium and S. marcescens facilitated higher levels of deproteinization but were less efficient in demineralization, the reverse of which was true with cultures of L. plantarum. By considering all these aspects, it was hypothesized that co-culture with two distinct strains could be employed for more efficient extraction of chitin from lobster shells.

3.3. Co-cultivation experiments with B. megaterium NH21, S. marcescens db11 and L. plantarum

Chitin extraction was performed by incubating lobster shell biomass with co-cultures of B. megaterium and L. plantarum or S. marcescens and L. plantarum. The pH and proteolytic activity were monitored throughout the co-cultivation experiments in order to determine the optimal co-cultivation strategy. As shown in Fig. 3, the proteolytic activity of B. megaterium increased rapidly to a maximum level of approx. 520 U ml⁻¹ within 24 h, and remained relatively constant almost until day 3, immediately followed by a rapid decline. The pH of the culture broth decreased rapidly from 6.5 to 4.2 in 3 days, as a result of L. plantarum metabolic activity. For S. marcescens, the proteolytic activity peaked at a maximum level of 575 U ml⁻¹ at day 2 and started decreasing significantly from day 4 onwards, whereas the pH of the culture broth decreased rapidly from 6.7 to 4.3 in 3 days as a result of L. plantarum metabolic activity. As discussed before, the increase in acidity with time shows the production of lactic acid, which indicates removal of inorganic material. Therefore, day 3 was chosen to be the appropriate point for inoculating the lobster shell waste culture medium (already inoculated with the respective protease producers) with L. plantarum under the experimental conditions.

Fig. 2. Proteolytic activity amounts (a) and pH (b) of culture broths from B. megaterium NH21, S. marcescens db11 and L. plantarum after incubation for 120 h. Error bars represent standard deviations of triplicate tests.
Three different strategies, as described in section 2.4, were applied in the co-cultivation experiments. The resultant changes in pH and proteolytic activity were followed throughout the culture time and are displayed in Fig. 4. When L. plantarum was employed in the co-cultivation, the pH of the culture supernatant (Fig. 4a) decreased gradually as culture time increased, which indicated a robust production of organic acids throughout the course of cultivation [39]. In culture supernatants where L. plantarum was added 3 days after inoculation with B. megaterium, the proteolytic activity was maximum at almost 515 U ml\(^{-1}\) and then drastically decreased to around 13 U ml\(^{-1}\) on day 6 when the pH decreased to 5.2. For culture supernatants where L. plantarum was added 3 days after inoculation with S. marcescens, the proteolytic activity was maximum at almost 599 U ml\(^{-1}\) on day 3 and then drastically decreased to 20 U ml\(^{-1}\) on day 6 when the pH decreased to 5.6 (Fig. 4 a and b).

Previously, Jung et al. [40] studied the extraction of chitin from red crab shells using a co-fermentation process. They stated that co-fermentation was more efficient than one-step extraction of crude chitin from crab shell waste. Aytekin et al. [34] had also attempted biological extraction of chitin from prawn waste using co-cultivation of Lactococcus (L.) lactis and Teredinobacter (T.) turnirae. They achieved best chitin yield with 15% glucose where T. turnirae was inoculated first and L. lactis was introduced at the end of 4 days.

In this study, for deproteinization, single cultivations with B. megaterium achieved a protein removal efficiency of 70.29%, while higher deproteinization levels of 76.73% were achieved with S. marcescens. For demineralization, single cultivations with L. plantarum achieved a mineral removal efficiency of 72.83%. The co-cultivation system reached a much higher efficiency. Overall, the successive treatment with a combination of S. marcescens and L. plantarum (as in S to L) gave the best co-removal of CaCO\(_3\) (89.59%) and proteins (87.19%) and a chitin yield of 82.56% from lobster shell biomass, as shown in Table 3.

![Fig. 3. Changes in pH and proteolytic activity in the culture liquor from co-cultures of: (a) Bacillus. megaterium NH21 and Lactobacillus plantarum (b) Serratia. marcescens db11 and Lactobacillus plantarum. Error bars represent standard deviations of triplicate tests.](image)

<table>
<thead>
<tr>
<th>Microbial treatment</th>
<th>Deproteinization (%)</th>
<th>Demineralization (%)</th>
<th>Chitin yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L + B</td>
<td>54.54 ± 1.51</td>
<td>60.65 ± 1.13</td>
<td>52.24 ± 0.22</td>
</tr>
<tr>
<td>L to B</td>
<td>65.46 ± 1.22</td>
<td>63.68 ± 0.64</td>
<td>58.33 ± 1.90</td>
</tr>
<tr>
<td>B to L</td>
<td>78.63 ± 0.94</td>
<td>82.90 ± 1.01</td>
<td>74.85 ± 3.98</td>
</tr>
<tr>
<td>L + S</td>
<td>63.46 ± 1.11</td>
<td>66.01 ± 1.02</td>
<td>59.87 ± 1.45</td>
</tr>
<tr>
<td>L to S</td>
<td>76.34 ± 1.74</td>
<td>68.76 ± 1.42</td>
<td>63.19 ± 2.02</td>
</tr>
<tr>
<td>S to L</td>
<td>87.19 ± 0.78</td>
<td>89.59 ± 0.94</td>
<td>82.56 ± 2.76</td>
</tr>
</tbody>
</table>

L + B: simultaneous inoculation of L. plantarum and B. megaterium NH21; L to B: culture started with L. plantarum, B. megaterium NH21 was then added after 3 days of culture; B to L: culture started with B. megaterium NH21, L. plantarum was then added after 3 days of culture; L + S: simultaneous inoculation of L. plantarum and S. marcescens db11; L to S: culture started with L. plantarum, S. marcescens db11 was then added after 3 days of culture; S to L: culture started with S. marcescens db11, L. plantarum was then added after 3 days of culture. All the values are means of triplicates, the standard deviations are indicated after the ±.
Fig. 4. Changes in pH (a) and proteolytic activity (b) of culture supernatants from the different co-cultivation experiments. L + B: simultaneous inoculation of *Lactobacillus plantarum* and *Bacillus megaterium* NH21; L to B: culture started with *Lactobacillus. plantarum*, *Bacillus megaterium* NH21 was then added after 3 days of culture; B to L: culture started with *Bacillus megaterium* NH21, *Lactobacillus plantarum* was then added after 3 days of culture; L + S: simultaneous inoculation of *Lactobacillus plantarum* and *Serratia. marcescens* db11; L to S: culture started with *Lactobacillus plantarum*, *Serratia. marcescens* db11 was then added after 3 days of culture; S to L: culture started with *Serratia. marcescens* db11 *Lactobacillus plantarum* was then added after 3 days of culture. Error bars represent standard deviations of triplicate tests.

3.4 Chitin characterization using Thermogravimetric analysis (TGA)

Thermal decomposition was examined on chitin samples extracted after successive treatments in combination with *B. megaterium* and *L. plantarum* cultures (as in B to L, Fig. 5a) and successive treatment in combination with *S. marcescens* and *L. plantarum* cultures (as in S to L, Fig. 5b). Thermogravimetric analysis was carried out to determine the temperature and weight change of the samples, which allows quantitative composition analysis of the chitin. The thermogravimetric curves were obtained at a heating rate of 5°C per min, in the temperature range of 10-800°C. In the chitin thermograms, mass losses were observed in two steps for all the chitin samples. The observed mass loss in the first step (between 50 and 150°C) was due to the evaporation of water. The second observed mass loss (between 300 and 500°C) can be attributed to the complete degradation of the chitin structures. It was observed that for both the samples, after 500°C, the percentage of residual mass remained constant.

Fig. 5. Thermogravimetric analysis for chitin from lobster shell waste (a) after successive treatment with a combination of *Bacillus. megaterium* NH21 and *Lactobacillus plantarum* (as in B to L), (b) after successive treatment with a combination of *Serratia marcescens* db11 and *Lactobacillus plantarum* (as in S to L).
In TGA results for chitin samples in previous studies, weight loss was observed in two steps, indicating a similarity to the analysis of the results observed here [41, 42]. Based on the findings, it can be said that this technique of using a combination of microorganisms to obtain chitin from lobster shell wastes can be commonly used in future chitin extraction studies.

3.5. Scanning electron microscopy (SEM) and Energy Dispersive Spectroscopy (EDX) analysis of chitin

High-resolution scanning electron micrographs of lobster shells were examined before and after different chitin extraction treatments. Fig. 6a shows the characteristic microstructure of raw lobster shells (without any treatments). The lobster shell fragment appears homogenous and solid as many inorganic components are tightly embedded in the gaps in the chitin structure and flexible protein molecules. Therefore, the inherently arranged and ordered chitin fibers, i.e. the major component of the shell, could not easily be detected. Fig. 6b shows the image of the shell after demineralization with L. plantarum. Individual chitin fiber bundles were observed in contact with each other in certain areas. Fig. 6c and 6d shows the shell after deproteinization with B. megaterium and S. marcescens, respectively. These samples have visible shallow depressions; however, single fibers cannot be discerned. The SEM images of the shell after deproteinization showed less fractured chitin than after demineralization. Fig. 6e and 6f shows the shell after successive treatment with a co-culture of B. megaterium and L. plantarum (as in B to L) and of S. marcescens and L. plantarum (as in S to L), respectively. After these treatments, the shell fragments became highly porous, have several visible depressions with a densely-fractured structure and “swollen” look. The results showed that the treatments markedly altered the lobster cuticle microstructure and the results are in accordance with previous studies [25, 43]. The qualitative EDX analysis of the demineralized shells (Fig. 7a) shows that calcium has been removed considerably, whereas qualitative EDX analysis of deproteinized shells (Fig. 7b) shows strong signals for calcium and magnesium. The smoothness in the structure after the demineralization process in contrast to the blocky or rough appearance of the untreated samples suggests that the minerals were perhaps located between and around chitin-protein fibers, as has been demonstrated in previous studies [44].

3.6 Comparison of chemical and biological methods of chitin recovery

Chemical and biological treatments of lobster shell waste have been achieved for recovery of chitin by applying several strategies. For the chemical method DM-DP, deproteinization level was about 89.97%, which was much higher than the DP-DM method. This could be attributed to the ability of NaOH to penetrate the matrix for breaking the protein bonds, since deproteinization was performed after mineral removal. However, previous studies have reported that the use of harsh acid treatments can cause hydrolysis of the polymer, inconsistent physical properties in chitin can be a source of pollution [40]. Other studies have also shown that high NaOH concentrations, high deproteinization temperatures and use of acids such as HCl can cause potentially undesirable depolymerization of chitin [2]. Thus, the optimal conditions for biological demineralization and deproteinization using organic acid and protease producing microorganisms were also investigated. Previously, Fagberno [45] used L. plantarum to extract chitin from the heads of African river prawn. Lactic acid bacteria have been reported to extract chitin from various sources, such as crayfish, scampi waste, and prawn waste, etc [29,34,42]. Crab shell waste was treated using S. marcescens FS-3, a strain isolated from environmental samples that exhibited strong protease activity [28]. B. megaterium PB 4 has been used for deproteinization of distillery yeast biomass waste [38].

3.7 Comparison of microbiological culture-based chitin recovery methods

In this study, three microorganisms, B. megaterium NH21, S. marcescens db11 and L. plantarum, have been studied, individually and as co-cultures, as biocatalysts for chitin purification. L. plantarum was more effective in removal of minerals as compared to B. megaterium and S. marcescens, while the reverse was true for the protein removal (Table 2). Therefore, it was concluded that the individual treatment with either of these strains individually was not sufficient for chitin extraction. Yet, if the aim is to obtain protein hydrolysate from lobster shell waste, the use of B. megaterium or S. marcescens (or similar bacterial strains) may be a good choice due to higher protease activity. The deproteinization levels coincided with the trend of proteolytic activity (Fig. 2), which indicates that strong proteolytic activity is necessary for achieving efficient protein removal from the shells. An attempt was made to cultivate both the organic acid and protease producing microorganisms together. Previously, the extraction of chitin from red crab shell waste was carried out by co-culture of two different microorganisms, and it was observed that co-culture of the waste using two strains was efficient and applicable for a one-step extraction of crude chitin from the waste shells [40].
Fig. 6. SEM micrographs for (a) raw lobster shell showing its characteristic microstructure, (b) shells after treatment with *Lactobacillus plantarum*, (c) shells after treatment with *Bacillus megaterium* NH21, (d) shells after treatment with *Serratia marcescens* db11, (e) shells after successive treatment with a combination of *B. megaterium* NH21 and *L. plantarum* (as in B to L, according to Fig. 5 legend), (f) shells after successive treatment with a combination of *S. marcescens* db11 and *L. plantarum* (as in S to L, according to Fig. 5 legend). In (c) and (d), arrows point to shallow depressions on the surface of the shell that are likely the result of protease treatment. The shell fragments became highly porous, has and (e) and (f) display several visible depressions with a densely-fractured structure (arrows).

Fig. 7. Qualitative energy dispersive X-ray (EDX) analysis of (a) decalcified and (b) deproteinized samples.
In the present work, the co-cultivation system produced isolated chitin with a greater purity. Overall, the best chitin yield and co-removal of proteins and CaCO$_3$ from lobster shell biomass was achieved by successive treatment with a combination of S. marcescens and L. plantarum (as in S to L) (Table 3). The SEM results also show that the deproteinization and demineralization treatments had a noticeable impact on the lobster shell biomass. The calcium peak (Fig. 7a) area decreased in intensity according to the qualitative EDX analysis, suggesting that the structure is significantly depleted of mineral after removal of calcium chloride. The smoothness of the structure (Fig. 6c and d) without the majority of the calcium carbonate is in contrast to the more “blocky” appearance of untreated and deproteinized samples, indicating that the biominerals were indeed located between and around the chitin–protein fibers, as has been shown from previous studies [43, 45]. The strong signals of calcium obtained by qualitative EDX analysis for deproteinized (but not demineralized) samples confirm the presence Ca$^{2+}$ in the structure (Fig. 7b). The biological treatment resulted in lower deproteinization and demineralization efficiencies as compared to the conventional chemical extraction method. Finally, it should be noted that this work is only a small, flask-scale study.

4. Conclusion

In conclusion, organic acid and protease producing microorganisms can be used as biocatalysts to treat lobster shell waste to reduce the presence of inorganic and proteinaceous matter. The lobster shells used in this study were demineralized and deproteinized to a considerable degree. The optimization of lobster shell degradation by the microorganisms in this study is essentially a proof-of-concept and offers a preliminary glimpse of an industrial scale process for biological chitin extraction. This extraction process is complicated and is affected by many parameters such as the microorganisms used, concentration of carbon source provided, specific strategy applied, etc. The results obtained here suggest that the microbial co-culture has the potential to be a “green” alternative to the chemical method. Thus, for reduction of harsh, corrosive chemicals and also to reduce the costs involved in waste management, the biological method of chitin extraction is a more environmentally friendly option for extraction of chitin. This is a small-scale study under laboratory conditions. Studies are being undertaken to make this method more suitable for large-scale chitin extraction operations. More research must be done in order to overcome the problems that are commonly related to up-scaling any biological process such as mass transfer challenges (O$_2$ transfer to cells, nutrient transfer, etc.), stirred-tank fermenter power requirements with larger amounts of chitin and cellular biomass, the effect of increased metabolic load on the culture’s yield and output, maintaining steady supply of nutrients and removal of wastes, stream recycling, solid-liquid separation. Fundamental studies on lobster shell surface interactions of the microorganisms used here could also be performed in order to explore other frontiers of bioreactors, thus providing stimulating research in this area. This study could potentially be the first step in the process of utilizing sea food wastes to serve as a carbon feedstock for microbial bioconversions to value added products.

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6. Conflict of Interest

The authors declare that they have no conflicts of interest.

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استخراج کیتیئن از ضایعات پوست خرچنگ با استفاده از روش‌های بر پایه کشت میکروبی

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چکیده
سابقه و هدف: ضایعات پوست خرچنگ حاصل از صنعت فراوری غذاهای دریایی به عنوان ماده خام برای استخراج کیتین مورد استفاده قرار گرفت. تیمارهای شیمیایی و زیستی ضایعات پوست خرچنگ انجم و مورد مقایسه قرار گرفت.

مواد و روش‌ها: روش شیمیایی نیازمند استفاده از محلول‌های هلاکتیک اسید و هیدروکسید سدیم بود. تیمار زیستی شامل استخراج کیتین با استفاده از روش‌های زیستی، شامل کشت همزمان با دبیسیو سادیوتروس db1 و سروتروس مارسسن db1، و استخراج کیتین با استفاده از روش‌های شیمیایی، شامل استخراج کیتین با استفاده از محلول‌های هلاکتیک اسید و هیدروکسید سدیم بود. تیمار زیستی بهترین نتیجه را در حذف همزمان کلسیم و پروتئین، هر یک آن با استخراج کیتین با استفاده از روش‌های شیمیایی داشت. تیمار زیستی بهترین نتیجه را در حذف همزمان کلسیم، کربنات و پروتئین، هر یک آن با استخراج کیتین با استفاده از روش‌های شیمیایی داشت.

نتیجه‌گیری: با استفاده از روش‌های زیستی، می‌توان استخراج کیتین با استفاده از روش‌های شیمیایی را جایگزین کرد.

واژگان کلیدی: کشت میکروبی، استخراج کیتین، باسیلوس مگاتریوم، سروتروس مارسسن db1، پوست خرچنگ.