Novel biological and chemical methods of chitin extraction from crustacean waste using saline water

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Abstract

BACKGROUND: Several techniques have been conventionally proposed to recover chitin from shrimp waste using large amount of freshwater and chemicals. The standard chemical recovery of chitin was first compared by replacing fresh water with seawater. In addition, biotechnological process with *Bacillus subtilis* (B1) and *Bacillus licheniformis* (B2) using seawater during all steps of chitin extraction was also studied.

RESULTS: The demineralization rate (DM) was statistically significant when using seawater (p=0.000020) by chemical recovery in comparison to deproteinization (DP). Increasing HCl concentration (from 1 to 1.28 M) and reaction time (from 60 to 90 min) resulted in DM similar to fresh water (p>0.05). Highest DP rates were obtained with crude protease (B1; DP ≈74%) and (B2: DP ≈84%), when fermentation was carried out for 24 h at an enzyme/substrate ratio of 2. Maximum DP was reached (≈79% for B1 and ≈82% for B2) after 15 days, while DM ranged between 55-60%.

CONCLUSION: Combined enzymatic DP (with B2) followed by chemical DM process was used to produce chitin (DP≈84%, DM≈94%) which on transformation to chitosan showed a degree of deacetylation equivalent to ≈71%. Combined approach could transform crustacean waste using sea water into chitin product of commercial value.

Key words: crustacean waste, chitin, demineralization, deproteination, fermentation, seawater

INTRODUCTION

Chitin is the most abundant renewable resource after cellulose and lignin. Every year around 10^{11} tonnes of chitin is produced by crustaceans, molluscs, insects and fungi. Crustacean wastes are the major wastes produced from industrial processing of sea foods, such as shells of crab, shrimp, prawn, krill and lobster.[1, 2] The annual worldwide production of crustaceans for 2008 was around 10,795,000 in comparison to 6,091,869 tonnes in 2005 (FAO, 2008 and 2010).[1, 3]

The crustacean waste causes unpleasant odors, and environmental pollution posing a problem for processing plants requiring immediate treatment.[4] Seafood processing and consumption generate tonnes of shrimp waste annually, such as in 1989, the total amount of crustacean wastes produced in Québec was 9,587 tonnes.[5] Mexico accounted for 80,000 tonnes (1998), Germany around 22,616 tonnes (2005), and Indonesia processed 514,589 tonnes (2005).[3, 6] The seafood processing waste consists of 45% shrimp waste, which accounts for 50-70% of the weight of raw material.[1] About 5% of shrimp waste is used for animal feed and the rest is left as such leading to decomposition representing its limited utilization.[6]

Crustacean wastes are the most important source of chitin (20-30%) along with protein (30-40%) and mineral content (30-50%) at varying percentage, depending upon the species and seasonal variations.[2] The compact matrices of chitin fibres in crustacean shells are interlaced with proteins and these matrices are reinforced with the deposition of calcium salts. To achieve high accessibility of chitin, they must be quantitatively removed at the highest purity to be used for various applications.[7] Chitin with its chitosan (deacetylated derivative) has applications in food, biotechnological products, agriculture, wastewater treatment plant, pharmaceutical products, cosmetics, and textiles.[1]

Current industrial methods rely on chemical process for isolation of chitin involving: 1) grinding; 2) demineralization (DM) with strong acids; 3) deproteination (DP) with alkali medium at 90-100 ºC followed by; 4) pigment removal using solvent extraction or chemical oxidation.[1, 8] Furthermore, the chemical steps are generally performed under high temperature conditions requiring higher energy input. Moreover, the washing steps give rise to very large volumes of polluted effluents with high concentrations of mineral acid, which are technically difficult and expensive to recycle. These conventional chemical extraction processes are extremely hazardous to the environment, costly and consume high concentrations of mineral acids with large quantity of freshwater consumption during each washing step.[1]

To overcome the harsh chemical treatments, biotechnological processes, such as microbial fermentation and application of proteolytic enzymes (enzymatic extracts or isolated enzymes) have been carried out for demineralization and deproteinization by different researchers and these reports are summarized in the Table 1.

The flow sheet of biological and chemical treatment method of chitin extraction from crustacean waste is presented in Fig. 1. The highest rate of DM and DP can be reached by carrying out simple fermentation but level of hygiene during production restricts its human or animal consumption.[1] The rate of DM and DP obtained by using crude protease or pure industrial enzymes needs additional chemical treatment step for better yield. However, several chemical and biological treatments were limited to freshwater conditions and there is no study as yet reported using seawater conditions. Therefore, cost-effective, easily controllable industrial process for chitin extraction of high purity and degree of acetylation are required for a satisfactory level of specific applications. In the present research, the standard industrial recovery process of chitin is carried out by replacing fresh water with sea water. To overcome the problem

of harsh chemical treatment, biotechnological process of using microorganisms with *in-situ* fermentation was also carried out. As a novel method, application of sea water for DM and DP of crustacean waste for chitin extraction has been optimized using statistical tools.

MATERIAL AND METHODS

Crustacean waste, reagents and analyses

The crustacean waste was obtained from seafood processing plant located at Gaspesie, Quebec. The seafood waste (shells of crab, shrimp, prawn, krill and lobster) was oven dried, powered using a grinder and stored at 4 ±1ºC.

Chemicals, such as nutrient broth (NB), glucose, NaOH, HCl, were purchased from Fisher scientific, Canada. Yeast extract used in the present study was a gift from Lallemand, Canada. The moisture and ash content of the crustacean waste were determined by AOAC (Association of Official Analytical Communities) standard methods 930.15 and 942.05. The residue was dried at 105 ±1 ºC and cooled at room temperature for moisture content calculation by weight difference before and after the drying process. The dried residue was separated into two portions with one part placed in oven at 600 ±1ºC for 4 h (AOAC 1984) in a muffle furnace (Fischer Scientific model 182A) to quantify ashes for DM analysis. The other part of the dried residue for its total nitrogen content measured by (AOAC 984.13) Kjeldahl was placed in an automated apparatus (Buchi, Switzerland) for DP analysis.[11]

Chemical method

Demineralization step

Demineralization was carried out at room temperature using 1 M hydrochloric acid. Each test was performed with 60 mL of acid solution and 6 g of raw material under agitation (800 rpm) for 60 min. The tests were performed, in triplicate, in Erlenmeyer flasks of 250 mL capacity. In the

same flask, the filtrate was mixed with 150 mL (3×50 mL) of fresh water for 15 min to remove the maximum residual HCl and other impurities. The treated crustacean waste was oven dried at 60 ±1°C for 18 h and weighed for DM analysis to quantify ashes and rate of DM was calculated using (Eq.1) below. [7, 14]

$$\text{DM}\ (\%) = \frac{(A_0 \times O) - (A_R \times R)}{A_0 \times O} \times 100 \qquad \text{(Eq. 1)}$$

Where, A_O and A_R are ash contents (g/g) of crustacean waste before and after treatment, and O and R are dry mass (g) of original sample and treated residue, respectively.

Deproteination step

Following the demineralization step, the demineralized shrimp shells were deproteinated with 1.25 M NaOH under vigorous stirring at 90 ±1°C using 60 mL of solution per gram of demineralized shells.[15, 16] After 2 h of reaction, the solid samples were washed with 150 mL (3×50 mL) of fresh water. Later, treated crustacean waste was dried and weighed for nitrogen analysis and rate of DP was calculated using (Eq.2) below. [7, 14]

$$\text{DP}\ (\%) = \frac{(P_0 \times O) - (P_R \times R)}{(P_0 \times O)} \times 100 \qquad \text{(Eq. 2)}$$

Where, P_O and P_R are protein contents (g/g) of crustacean waste before and after treatment, and O and R are dry mass (g) of original sample and treated residue, respectively.

Effects of sea water on chitin extraction

The previously elaborated extraction protocol with fresh water was studied using sea water and artificial seawater (at 17.5 and 35% salinity) was prepared according to Lyman-Fleming formula.[17] The effect of seawater tests on chitin extraction were performed in triplicate for DM and DP steps. The comparison between the two seawater salt concentrations and the protocol with fresh water was carried out by using ANOVA and Tukey post hoc tests.

Optimization of DM for chitin extraction

An optimization was carried out with sea water using factorial design (2 factors, 3-levels and 3 blocks). The raw material was first mixed with 60 mL of hydrochloric acid (HCl) at varying concentrations of 0.5, 1.5 and 1 M. The reaction flasks were stirred at 800 rpm at room temperature (20 ±1°C) for 1, 2 and 3 h of reaction in triplicates followed by washing step with 150 mL (3×50 mL) of seawater. Demineralization (DM %) was calculated at the end of each experiment in order to optimize the concentration of HCl with reaction time. A Box Behnken Design (BBD) with 9 runs (three levels and two factors) was used to assess the effect of acidic hydrolysis conditions, such as concentration of HCl (0.5, 1, 1.5 M) and reaction time (1, 2, 3 h) on rate of demineralization. Prediction using results obtained was determined for final optimum condition to be carried out in triplicates.

Statistical analyses and the design of experiments were carried out by using STATISTICA, STAT SOFT version 7. Responses surface methodology was used as a tool for the analyses and the difference was considered significant at p-value < 0.05 (α was fixed to be 0.05).[18]

Biological method

Bacterial strains and inoculum preparation

The strains, *Bacillus licheniformis* NRS-1264 (B1) and *Bacillus subtilis* B-59994 (B2) were collected from ARS, USDA (U.S Department of Agriculture). Inoculum for B1 and B2 were routinely grown in the nutrient broth (NB) medium composed of (g L^{-1}): peptone 5.0, yeast extract 2.0, NaCl 5.0 and pH were maintained at 7.0, before sterilization.[13] The sterilized medium volume was 100 mL in a 250 mL conical flask which was inoculated at 5% (*v/v*) with B1 and B2 separately and incubated for 12 h at 150 rpm at 40 ±1ºC in an incubator shaker (INFORS HT–multitron standard). For determination of colony forming unit (CFU), about 1 mL

of this inoculated media was taken to determine the inoculum density for each of the bacterium, which was around 4.3×10^6 CFU/mL for B1 and 3.9×10^6 CFU/mL for B2.

Fermentation conditions for DM and DP

The fermentation set-up was performed in 250 mL flasks containing 100 mL of seawater medium composed of 18 g L^{-1} crustacean waste, supplemented with 10 g L^{-1} of glucose and with initial yeast extract of 0.1%. To increase the rate of DM with enhanced microbial growth and acid production, readily available fermentable sugar (glucose) and nitrogen source (yeast) were chosen.[13] Initial pH of the fermentation medium was set to 7.0. After sterilization, 10% (*v/v*) of inoculum was added and fermentation was monitored for 5, 10 ad 15 days at 40 ±1ºC and 150 rpm on a rotary shaker. Soon after the fermentation, the treated crustacean waste was separated by centrifugation at 3810 *x g* for 20 min and the supernatant was collected for enzyme analysis. The residue was washed with 150 mL (3×50 mL) of seawater (35% salinity) and dried at 60±1 ±1ºC for 24 h and weighed before carrying out DM and DP analyses.

Crude protease harvesting method for DM and DP

Bacillus species are industrially well-established microorganisms for the production of extra cellular proteins.[19] For enzyme production, fermentation with each of the strain was carried out for 5 days. The fermentation mixture was centrifuged and the supernatant collected was further vacuum filtered using 0.45 µm filter paper. The supernatant was used to determine the crude protease activity and accordingly used for DP and DM (as detailed in previous section) of chitin.

Analysis method

Protease activity assay

The protease activity was measured using casein as a substrate.[20, 21] The filtered supernatant i.e. crude enzyme was diluted 1:1 with distilled water and 0.5 mL of this dilution was mixed with 0.5

mL of 0.1 mM sodium carbonate buffer containing 1% (*w/v*) azocasein and incubated for 15 min at optimal temperature. The optimal temperature and pH for B1 (70 ºC, 10.0) and B2 (60 ºC, 8.0) were set. The reaction was stopped by addition of 0.5 mL of trichloroacetic acid (20% *w/v*). The mixture was allowed to stand at room temperature for 15 min and later centrifuged at 7656 *x* g for 20 min to settle the precipitate. The supernatant was collected and absorbance was measured at 280 nm using spectrophotometer (Carry 100 Bio®, Varian USA). A standard curve was plotted using the standard solution of azocasein 0-50 mg L^{-1} and one unit of protease activity was defined as the amount of substrate converted per minute under standard assay conditions.[20, 21]

Deacetylation experiment and determination of the degree of *N*-deacetylation

Deacetylation was achieved by treatment of extracted chitin with sodium hydroxide (NaOH) solution (50%) at 100 °C using a solid to solvent ratio of 1:5 and a reaction time of 5 h as described by [22]. The final material produced was washed with 150 mL (3×50 mL) of water/seawater (35% salinity) and dried at 60±1 °C in a vacuum oven. The degree of deacetylation (DDA) was determined using Fourier-transform infrared (FT-IR) spectroscopy. The DDA of chitosan was calculated from the absorbance 1658 cm^{-1} (amide-I) and 3450 cm^{-1} (OH group) according to the following equation (Eq.3).[23, 24]

$$\mathrm{DDA}(\%) = 100 - [(\mathrm{A}_{1658}\ /\mathrm{A}_{3450})/1.33] \times 100 \qquad \text{(Eq.3)}$$

RESULTS AND DISCUSSION

Chemical composition of crustacean waste

The crustacean waste used in this study had moisture content around 40%, mineral content was around 41% and protein content was around 35%. The overall percentage of protein and mineral content of these wastes was comparable (30-40% and 30-50%, respectively) as reported by Arbia

et al.[2] Mostly, exact composition of crustacean wastes varies with species, seasons and many other factors. The water content in the raw material was 7 ±1.0% (*w/v*).

Chitin extraction

Chemical extraction

Investigation of the effect of seawater on DM and DP

The chemical treatment across different concentrations of sea water salinity for both demineralization and deproteination rate is represented in Fig. 2. Extraction with fresh water, seawater with 17.5 and 35% salinity resulted in demineralization rate as high as 98 ±0.3, 93 ±1.1 and 89 ±0.7%, respectively. The deproteination rate for chemical treatment across different sea water concentrations was less than 80%, indicating that higher concentration of proteins in the crustacean waste requiring harsh chemical treatment. The results showed that the use of seawater affected DM in comparison to DP. There was a significant difference in the DM if fresh water, diluted seawater at 17.5 and 35% were used (p=0.000020). A comparison across the DM rates using the Tukey rank test showed that the DM significantly decreased with increasing salts concentration. Results of DM with fresh water (98 ±0.3%), under same conditions were comparable with 98.5 ±0.1% obtained by Le Roux *et al*.[28] The same test showed that there was no significant difference in the DP, whether it was fresh water or sea water ($p>0.05$). The harsh chemical treatment requiring higher NaOH concentration with high temperature for increased deproteination can cause undesirable deacetylation and alter depolymerization of chitin.[2] Increased deproteination rate can be achieved by adding exo-protease and/or proteolytic bacteria there by eliminating extreme chemical treatment conditions.[1, 2] Further, to increase demineralization rate using chemical treatment, optimization studies were carried out.

Optimization of the operating conditions for DM in sea water

The objective was to maximize DM, finding the right combination of HCl and duration of reaction which would result in maximum DM rate. DM was performed with sea water (35% salinity). The 2D response surface plots across different concentrations of HCl over reaction time are presented in Fig. 3. A fitted response profile was obtained based on the time of reaction against the variation of HCl concentration (M). The DM percentage can be calculated from the equation of the second degree (Eq.4).

$$DM\ (\%) = -11.8152 + 162.6035\ HCl - 63.1167\ HCl^2 + 0.6038\ Time + 0.6038\ Time^2 - 0.9387\ HCl.\ Time \quad (Eq.4)$$

The statistical data collected from analysis of variance (ANOVA) for optimization studies are presented in Table 2. Analysis of variance showed that HCl concentration had both quadratic and linear influence on DM%. However, the linear and quadratic effects of reaction time have no statistically significant effect on DM% (+0.6038). The statistical analysis of regression coefficient showed an acceptable coefficient (R^2>0.9) which meant that approximately more than 90% of the variability could be explained by the model. The proposed optimal values according to desirability (maximize DM); these values were 1.28 M (HCl concentration) and 1 h 33 min (reaction time). This optimum condition was studied in triplicate and the average of the results was 93 ±1.6%, in the confidence interval of the theoretical maximum for the DM (84.71-99.44%). Conventional use of more than 1.5 N HCl resulted with undesirable effect on molecular weight, negative effect on degree of deacetylation and detrimental effect on purified chitin properties.[25] Present finding with optimization of extraction process minimized the experimental runs with relatively diluted acid for increased DM rate, which is comparable across DM rates

obtained using freshwater. Therefore, in terms of demineralization efficiency, compared to biological extraction, the present method presented higher DM rates.

Effect of incubation time on biological extraction

The rates of DM and DP for B1 and B2 species across 5^{th}, 10^{th} and 15^{th} day have been presented in Fig. 4. Addition of the nitrogen supplement along with glucose was used to support the growth of microbial biomass with simultaneous production of acids and to check the rate of DM and DP corresponding to increased fermentation time.[13] ANOVA showed that the rate of DM for B1 and B2 was statistically comparable (p>0.05) across time and between the two species of bacteria (Fig. 4) and mainly ranged between 54.5 and 62.5%. DM was mainly due to the acid produced during fermentation as reported by Rao *et al* and and Ghorbel-Bellaaj *et al*.[13, 26] In this study, it seemed that lower acid production was mainly achieved during fermentation for the two species, B1 and B2. At 5, 10 and 15 days, DM values were almost comparable. This result was in concordance by Ghorbel-Bellaaj *et al*, who observed lower acid production during fermentation of *Bacillus licheniformis* (DM=59.4 - 55.5%).[13] DM values in this study were also comparable to co-culture results of *Bacillus licheniformis* + *Lactobacillus acidophilus* with 50.2% DM[11] and slightly lower than co-culture results of *Lactobacillus salivarius* + *Enterococcus faecium* + *Pediococcus acidilactici* with 68.5% DM.[10] Acid produced is directly responsible for DM of crustacean waste.[13] Inability to produce acid by the two species (B1 and B2) resulted in decreased DM rates in comparison to lactic acid produced by *Lactobacillus* species.

In the case of DP, ANOVA showed significant increase during the fermentation time (p=0.0054). A Tukey post hoc test was carried out to compare DP values across fermentation time and for two *Bacillus* species. By increasing the fermentation time, a significant increase in DP value was observed at 15 days for B1 (p=0.00962) and B2 (p=0.00703) at 82±4.9% and 79±2.8%,

respectively. *Bacillus* exhibited comparable activity during the stationary phase of growth and using seawater in the medium influenced production of proteolytic activity by 150%.[27] The efficiency of fermentation depends on various parameters, such as inoculum, substrate concentration, pH variations, fermentation time and moreover, the fermented wastes impart strong and pungent acidic smell.[1]

Therefore, fermentation using *Bacillus* sp. is a time consuming approach and addition of proteases will be a viable commercial way to increase DP rate. The enzyme activity of both *Bacillus* species in the presence of crustacean waste (Table 3) was 137.5 and 178.75 U/mL for B1 and B2, respectively. This indicated that B1 and B2 can derive its source of carbon and nitrogen from crustacean waste leading to production of protease activity for increased rate of protein removal.

Effect of Protease on DP

Since the rate of DP is not only related to the amount of protease produced, but also to the efficiency and the accessibility of enzymes to the substrates[13], direct enzymatic process of protein removal was used. Enzymatic DP process was applied in seawater using a crude protease from B1 and B2 across incubation periods and enzyme/substrate ratios (E/S) (Fig. 5). Increasing incubation time and E/S (*v/v*) ratio resulted in higher protein removal. The maximum DP reached was 84 ±1.5% (B2) and 74 ±2. %3 (B1) with an incubation time of 24h and E/S=2: for the two *Bacillus* species, DP followed a linear increase across time. The maximum protein removal was carried out by crude B2 enzymes and which is due to its higher protease activity (+30%). For B2, DP increased from 59 ±1.34% (8h) to 65 ±2.1% (12h) and 84 ±1.5% (24h); forB1, DP increased from 61 ±1.7% (8h) to 63 ±2% (12h) and 74 ±2.3% (24h). The rate of DM was also evaluated

and it ranged from 55-60% for all E/S (*v/v*) ratios and incubation time studied which was in accordance to Ghorbel-Bellaaj *et al* and results obtained with fermentation experiments.[13]

The effectiveness of DP depends on the presence of residual small peptides and amino acids, which can be digested by enzymes with production of protein hydrolysate having required amino acid index.[1] The lowest DP values were recorded at E/S=1 for B1 and ranged between 53-57% while it increased from 56 ±1.34 % (8h) to 67 ±1.6% (24h) for B2. Increasing concentration of enzymes resulted in an accelerated protein removal which can be clearly observed when comparing DP for E⁄S=1 and 2 at different incubation time and for same *Bacillus* species. For example, DP was 65 ±2.1% (B2, 12h) for E⁄S=2 while 24h was required to reach 67 ±1.6% when E⁄S=1 was used. [29] observed the same finding and demonstrated that increasing E⁄S from 1 to 5 resulted in an additional 15% protein removal with crude enzymes from *Bacillus mojavensis.* Younes *et al* reported comparable DP (around 76%) at higher E/S (E⁄S=20, 3h incubation) using the same crude enzymes[7] from *Bacillus subtilis* and *Bacillus licheniformis* as the current study but produced in standard synthetic media. The enzyme activities matched[13] the activities of strains grown in synthetic medium containing (g L^{-1}): shrimp waste powder 30, KCl 1.5, K_2HPO_4 0.5, and KH_2PO_4 and justify the high rate of protein removal (DP) (≈ 80%) which was relatively comparable to the results of DP matched[12] with *Bacillus cereus* (DP= 78.6%) and to the results of *Bacillus subtilis* (DP=91.2%) [13] and higher than results of[11] co-culture (*Bacillus licheniformis* + *Lactobacillus acidophilus)* (DP=47.4%) (Table1).

The combined enzyme digestion followed by acid treatment will be a cost-effective, environmental friendly and commercial approach for treatment of crustacean waste.

Proposed treatment method for chitin extraction

It was demonstrated that the crude protease from the both *Bacillus* species and especially B2 could be effective to obtain high rate of DP when sea water was used instead of fresh water. However, to obtain maximum mineral removal and hence purified chitin, an additional step of chemical DM was necessary. For this purpose, a combined enzymatic deproteination (with B2 crude protease, E/S=2, for 24h) step following chemical demineralization was carried out by using sea water, the deproteinated shrimp waste (DP≈84±1.5%) was subjected to demineralization at (as optimized in section 3.2.1: 1.28 M HCl and reaction time of 1h 30 min). Results showed chitin with DP ≈84 ±1.5% and DM ≈ 94%. Chitosan obtained after deacetylation of this chitin showed a degree of deacetylation ≈71% using seawater and 81% using fresh water. The degree of acetylation (DA) in presence of fresh water across commercial alcalase with Na sulphite along with NaOH treatment resulted in 77.6% and using *Lactobacillus* sp. along with HCl followed with NaOH treatment resulted in 81.7% of DA.[9,30] The degree of acetylation suggested that demineralization and deproteination seemed to be effective[9] and the values were within the satisfactory level of specific application.[31-33] Hence, an efficient method of chitin extraction using seawater with a combined chemical and biological treatment method can be used for industrial purpose. Use of seawater as a replacement of freshwater will be advantageous for the industrial treatment of crustaceous wastes. Most of the crustacean processing industries are located near to the sea, and thus, it will minimize the transportation of sea water from the source. The amount of freshwater utilization during chemical method in DM and DP for washing steps was around 250 L to process 1 kg of crustacean waste. With this proposed treatment step, maximum rate of DM (94%) and DP (84%) can be reached and it will be able to extract chitin with highest purity.

The proposed biochemical approach is able to attain highest DP and DM rate across the studies carried out using biological treatment in presence of salt (6% *w/v*) with 59.8% and 81.4% respectively.[34] Traditional chemical treatments give rise to very large volumes of polluted effluents, which are technically challenging, expensive to recycle and can no longer be used as animal feed. However, with biochemical approach, the use of chemical loads is reduced, crustacean bio-waste can be recycled and utilized in an efficient manner as animal feed or protein powder for human consumption.[35] According to our estimation for industry producing 20 tonnes of chitin per year, the cost of using pure water (175 tonnes) will be around ≈27,000 $ (water price in Quebec: ≈1.51$/m^3), in case of seawater the total production cost will be reduced by 10-13%.[36] The biochemical approach can be considered as a viable commercial approach with high rates of DM and DP, alternatively producing high purity of chitin. The biological treatment requires use of crude protease, which can be produced in-situ using crustacean waste as a cheap carbon source. This approach will illustrate a suitable crustacean utilization, crude protease production, crustacean treatment, animal feed and chitin production, altogether with improved in-situ sea-food processing industries. The proposed bio-chemical approach will find whole new applications in converting crustacean wastes into commercial products for industrial application.

CONCLUSION

In the present study, crustacean waste fermentation was carried out using two *Bacillus* strains in seawater. The proteolytic activity of *Bacillus* species increased in seawater used in the preparation of fermentation media, which resulted in high rate of deproteinization (≈84% for B2 and ≈74% for B1). The modification in the chemical treatment, by using sea water instead of fresh-water resulted in a demineralization rate of around 94%. Hence, considering the industrial requirements of DM and DP, a combined chemical and biological method of treatment using

seawater for chitin extraction from crustacean waste was proposed. The proposed method of using seawater for chemical washing and biological treatment of crustacean wastes to extract chitin is an alternative saving freshwater. The proposed biochemical treatment is advantageous over traditional chemical treatment in biotransformation of crustacean wastes into improved in-situ application for sea-food processing industries.

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Table 1. Reports on fermentation and enzymatic hydrolysis of crustacean wastes.

Microorganisms/Enzymes	Fermentation/Bioprocess	Source	DP Treatment	DP (%)	DM Treatment	DM (%)	Ref.
Lactobacillus pentosus 4023	500 g of chitinous with whey, nitrogen source, using immobilized-cell reactor at pH 5.5, 30 ºC for 2 days	Crayfish (chitinous fraction)	with 0.25 M NaOH in 1:5 (w/v) ratio for 6 h at room temp	99.4 ± 0.2	with 0.5 M HCl in 1:5 (w/v) ratio for 6 h at room temp	98.2 ± 0.4	[9]
Lactobacillus salivarius + *Enterococcus faecium* + *Pediococcus acidilactici*	500 g of prawn shell with glucose solution at 35 ºC for 5 days at 250 rpm	Prawn shell (Nephrops norvegicus)	with 1 M NaOH in 1:10 (w/v) ratio for 3 h at 65 ºC	49.37	with 1 M HCl in 1:15 (w/v) ratio for 2 h at room temp	68.39	[10]
Bacillus licheniformis F11-1 + *Lactobacillus acidophilus*	300 g of shrimp shell waste with glucose, yeast at 37 ºC, pH 7.0 for 2 days at 50 rpm	Shrimp shells (Penaeus vannamei)	at 55 ºC, 250 rpm for 60 h	47.37	at 30 ºC, 50 rpm for 48 h	50.23	[11]
Bacillus cereus 8-1	In 12 L of 10% shrimp shell waste at pH 7.0, 37 ºC, for 14 days	Shrimp shells	with fermented residue	78.6	with fermented residue	73	[12]
Bacillus licheniformis RP1	In 100 mL fermentation medium, 5% (w/v) shrimp shell waste with glucose at pH 7.0, 37 ºC for 5 days at 200 rpm	Shrimp shells (Metapenaeus monoceros)	with fermented residue	94.4[a] - 90.8[b]	with fermented residue	59.4[a] - 55.5[b]	[13]
Bacillus subtilis A26	In 100 mL fermentation medium, 5% (w/v) shrimp shell waste with glucose, at pH 7.0, 37 ºC for 5 days at 200 rpm	Shrimp shells (Metapenaeus monoceros)	with fermented residue	91.5[a] - 91.2[b]	with fermented residue	37[a] - 79.9[b]	[13]
Crude protease (*Bacillus cereus* SV1)	Shell waste with water (1:2) cooked for 20 min at 90 ºC, homogenized with pH adjusted to 8.0, enzyme/substrate ratio (20) with	Shrimp shell (Metapenaeus monoceros)	pH 8.0 at 40 ºC	88.8	1.5 M HCl in 1:10 (w/v) at 25 for 6 h	99.5	[14]

Microorganisms/Enzymes	Fermentation/Bioprocess	Source	DP Treatment	DP (%)	DM Treatment	DM (%)	Ref.
	incubation for 3 h at 40 ºC, enzyme inactivation at 90 ºC for 20 min						
Crude protease (*Bacillus licheniformis* NH1, *B. subtilis* A26)	Shrimp waste homogenate with water (1:3) at pH and temp adjusted, incubation for 6 h, enzyme inactivation at 90 ºC for 20 min	Shrimp shell (Metapenaeus monoceros)	pH 10.0 at 50 ºC, pH 8.0 at 40 ºC	65± 3,	1.5 M HCl in 1:10 (w/v) ratio at 50 ºC for 6 h	-	[7]
Crude protease : *Bacillus subtilis and Bacillus licheniformis*	Shrimp shell waste medium supplemented with glucose	Shrimp shell (thoraces and appendices)	DP: 60 ºC for 20 min at pH 7.0	84%	DM: at 25 ºC for 48 h	94%	This study

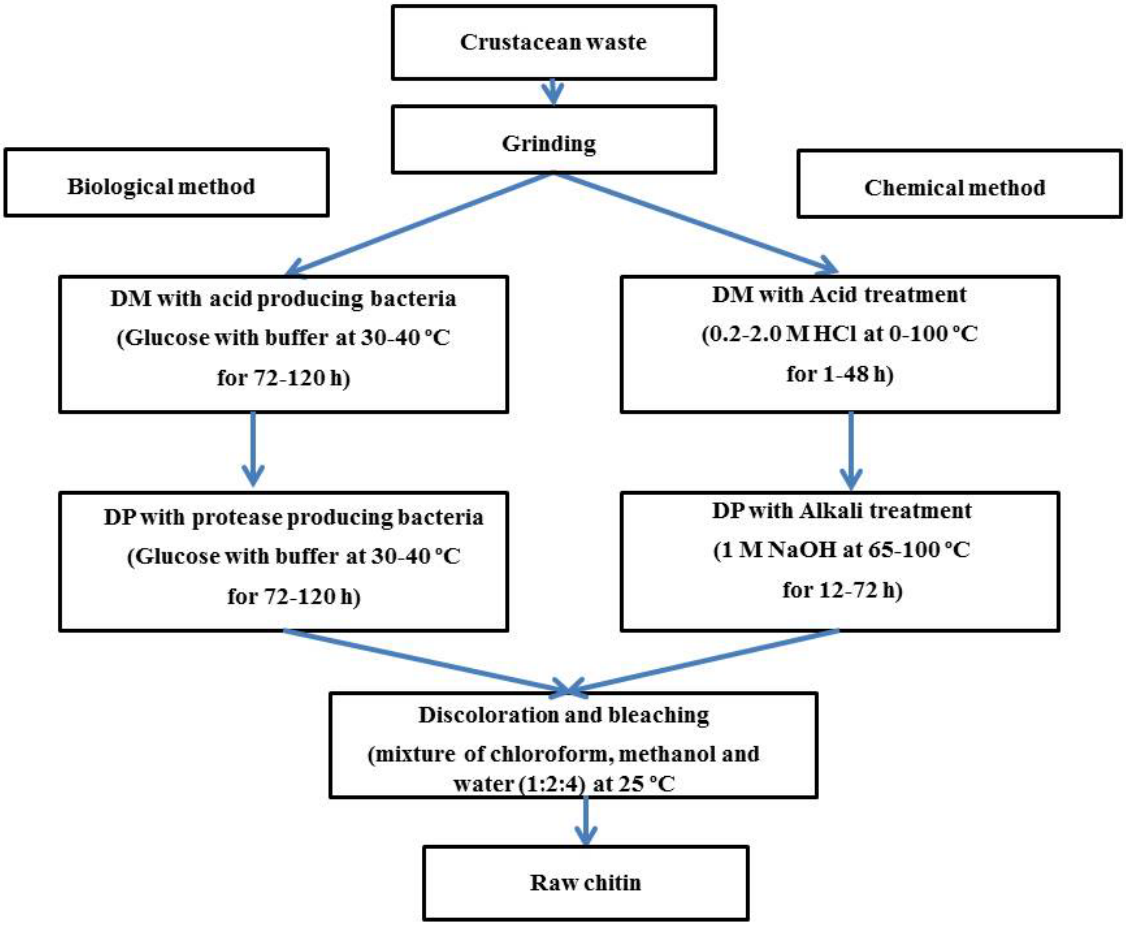
[a]Shrimp shell waste medium, [b]Shrimp shell waste medium supplemented with glucose

Table 2. Analyses of variance (ANOVA) for optimizing the chemical operating conditions for DM.

ANOVA, R^2= 0.95835					
	SS	df	MS	F	p
(1)HCl(M)(L)	1517.58	1	1517.58	73.72	0.001011
HCl(M)(Q)	337.38	1	337.38	16.39	0.015495
(2)Time(h)(L)	30.37	1	30.37	1.47	0.291293
Time(h)(Q)	9.15	1	9.15	0.44	0.541421
Error	82.34	4	20.58		
Total SS	1976.83	8			

Table 3. Comparison of proteolytic activity of *Bacillus* strains across media containing crustacean waste supplemented with synthetic components.

Strain	Media	Proteolytic activity	pH	Temperature	Ref.
Bacillus mojavensis A21	Luria–Bertani broth	7.72 U/mg	9.0	60 °C	[8]
Bacillus subtilis A26 *Bacillus licheniformis* RP1	Shrimp waste	193 U/mL 713 U/mL	8.0 10.0	60 °C 70 °C	[4]
Bacillus subtilis TEB1030 *Bacillus licheniformis* H402	Luria–Bertani broth	8.9 U/mL 59.7 U/mL	8.6	37 °C	[1]
Bacillus licheniformis LBBL-11	Nutrient broth	18.00 U/mL	8.0	37 °C	[28]
Bacillus subtilis B-59994 *Bacillus licheniformis* NRS-1264	Crustacean waste	137.5 U/mL 178.7 U/mL	8.0 10.0	60 °C 70 °C	Our study



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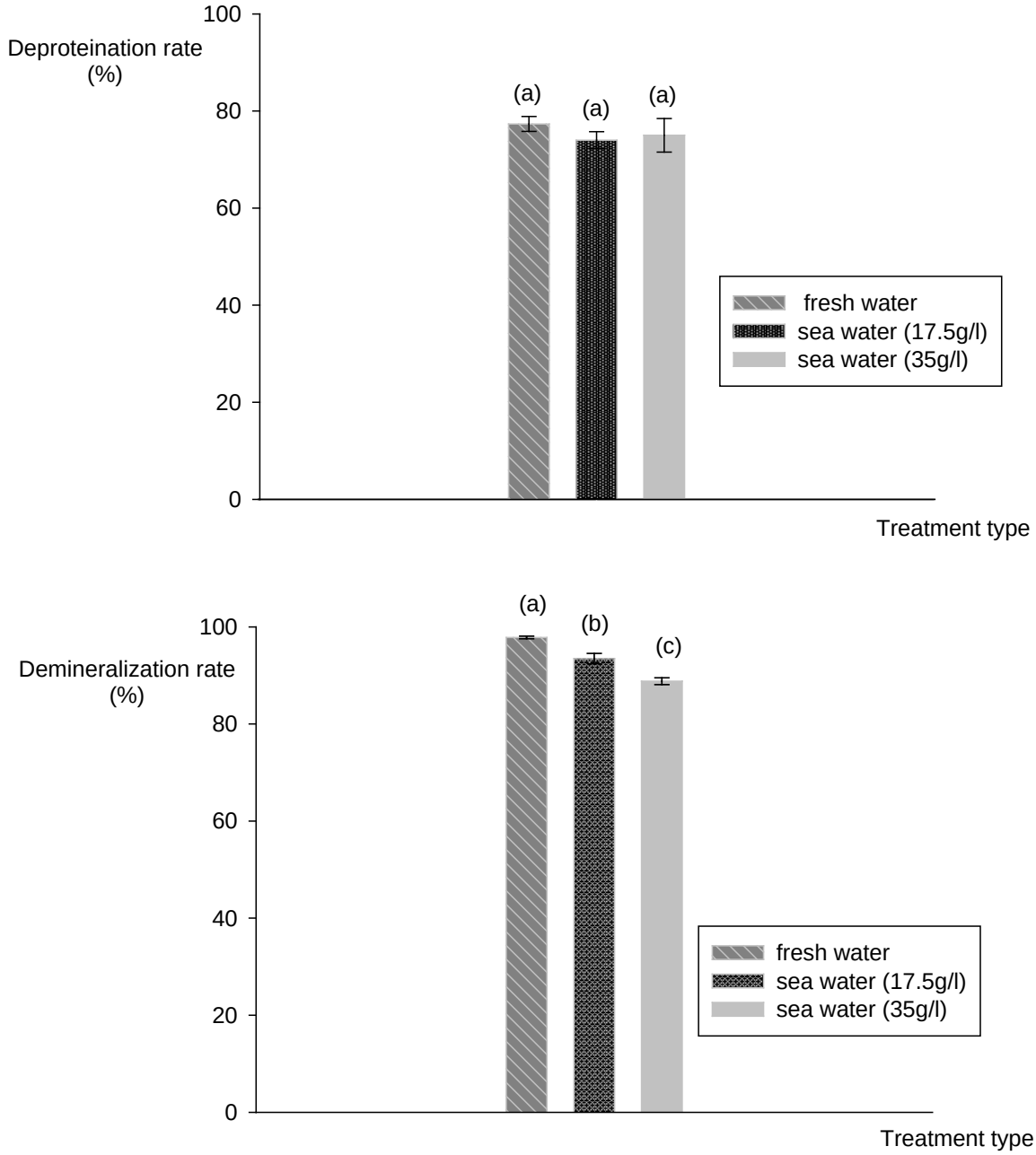


Figure 2. Demineralization (DM) and deproteination (DP) rates obtained by chemical extraction. Values denoted with different lower case letter differ significantly at $P<0.05$ by one-way ANOVA and Tukey's post hoc analyses. Each value is the mean of three independent replicates.

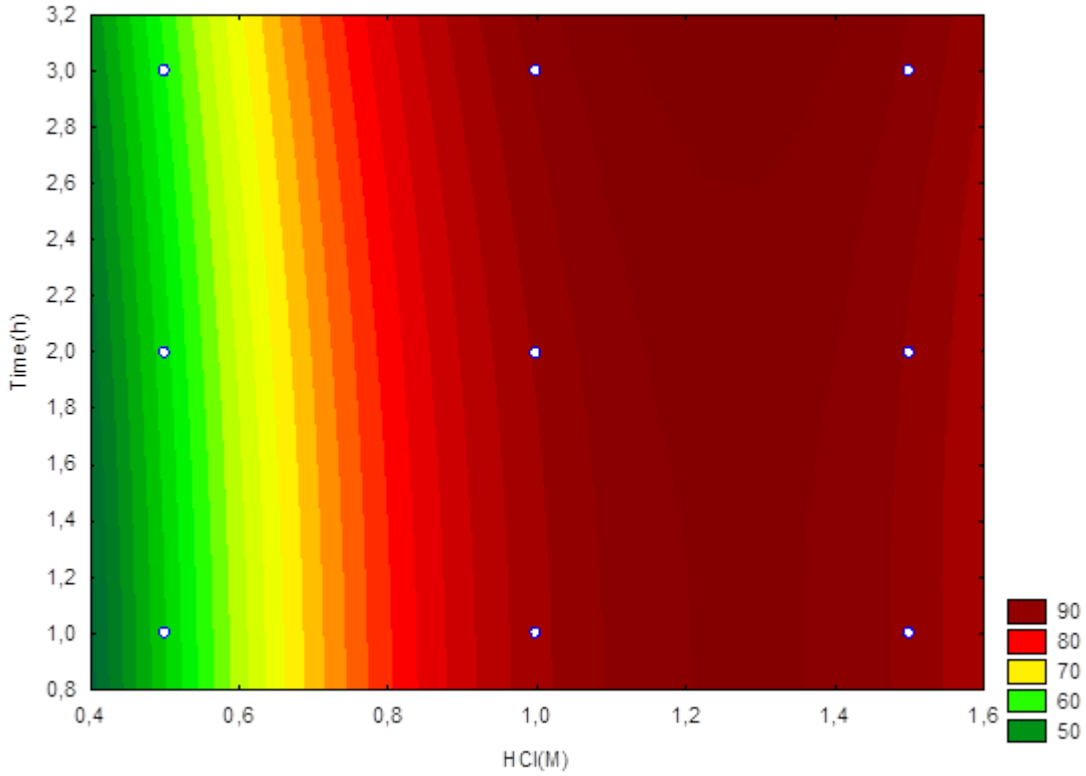
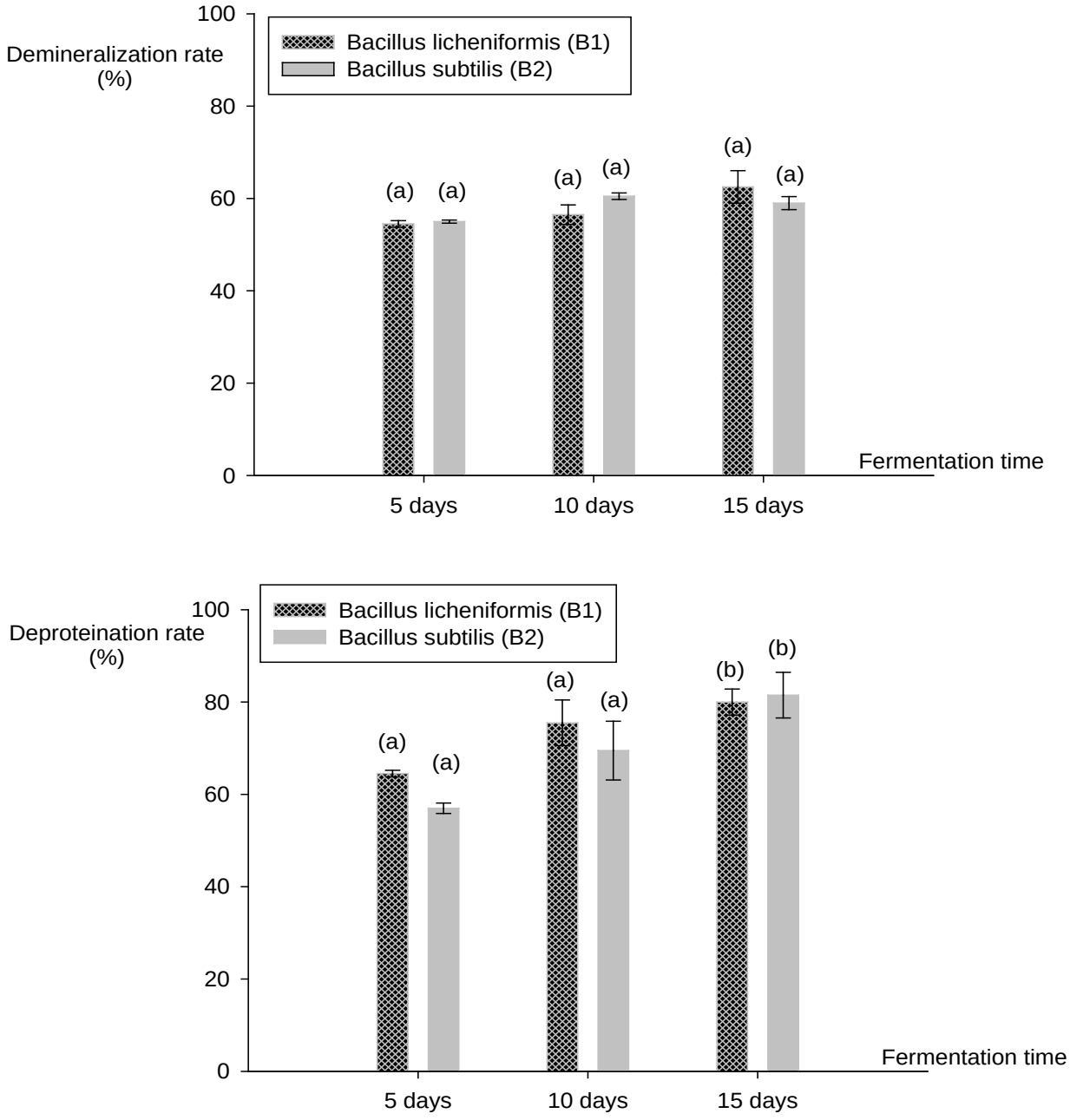


Figure 3. Optimization of the operating conditions for DM using sea water (35 g L^{-1})



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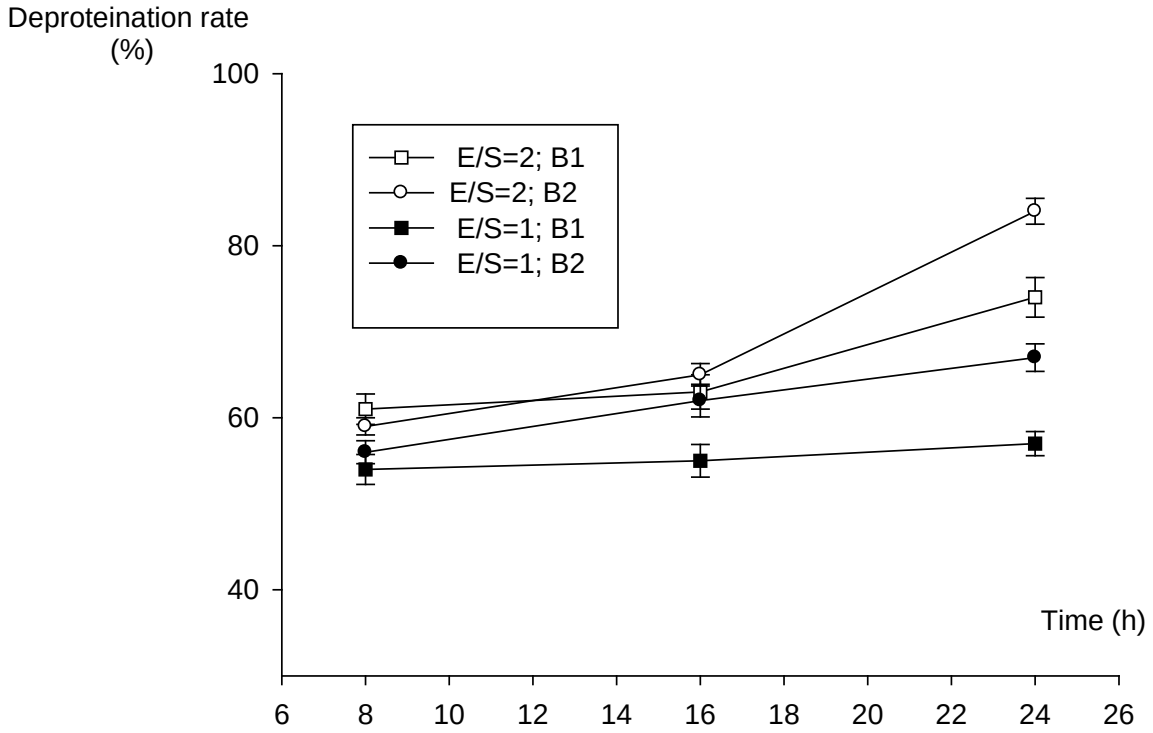

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