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Shrimp Digestive Bacteria Performance for Shrimp Waste Chitin Extraction

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Abstract

Generally, chitin is isolated chemically. An environmentally friendly alternative to chitin extraction is enzymatic extraction. The research aims to determine the effectiveness and optimum conditions for enzymatic chitin extraction of shrimp waste (heads, shells, and tails) using lactic acid bacteria (LAB) and proteolytic bacteria from the shrimp's digestive tract (faeces and intestines). The research stages were isolation, enrichment, and determining shrimp digestion's bacterial colonies, microscopic identification of shrimp digestive bacteria, and determining the effectiveness and optimal conditions for shrimp waste's chitin isolation. Aerobic and Anaerobic Total Plate Count (TPC), LAB, and proteolytic bacteria from shrimp's digestive tract per gram of sample were respectively 5.52 and 5.59 log CFU; 4.76 and 4.56 log CFU; 6.46 and 6.20 log CFU. Microscopic morphology shows that LAB and proteolytic bacteria of shrimp digestive are gram-positive as cocci or rods. The deproteinization value of shrimp waste reached 31%, especially for treatment with a pH of 5.0–7.0 (during proteolytic bacteria fermentation) and pre-treatment of boiling, drying and coarse grinding of shrimp waste (PP2) before extraction. However, the demineralization value of shrimp waste is only 4.5% (PP1) and 3% (PP2), with LAB as the primary fermentation agent because proteolytic bacteria have been unable to reduce the mineral content in shrimp waste further.

Keywords: chitin; enzymatic extraction; digestive tract; lactic acid and proteolytic bacteria; shrimp waste.

Abbreviations: Total Plate Count (TPC), lactic acid bacteria (LAB), Plate Count Agar (PCA), De Man, Rogosa, and Sharpe (MRS).

INTRODUCTION

Chitin, a copolymer composed of N-acetylglucosamine (GlcNAc) units (>50%) and glucosamine (GlcN), is the main component of crustacean (shrimp, crabs and lobsters) shells, insect skeletons, and cell walls of fungi, diatoms, bacteria, and algae with stiff and rigid characteristics as a protective function from the outside world (Bastiaens et al., 2020; Ehrlich, 2010; Zargar et al., 2015). The application of chitin and its derivatives is widely used in various industries, including food, health, cosmetics, agriculture, paper, biotechnology, and others (Nainggolan, 2023; Perez & Wertz, 2022). The global market for chitin and its derivatives in 2020 reached 106.9 thousand tons and is estimated to increase to 281.7 thousand tons in 2027 with a monetary value of US$ 2,900 million (Perez & Wertz, 2022).

The inedible portion of the shrimp reaches 44-63%, including the head, skin and tail, which are considered waste (Adeyeye & Aremu, 2016). Those shrimp waste themselves contain chitin (15–40%), protein (20–40%), minerals (30–60%), and astaxanthin (reddish pigment-carotenoid, a potent antioxidant) (Bastiaens et al., 2020; El-Bialy & Abd El-Khalek, 2020; Hu et al., 2019).

Generally, chitin and its derivatives are isolated chemically with a concentrated strong acid (usually HCl) and a strong base (generally NaOH) to remove minerals and proteins in the chitin raw material (Bastiaens et al., 2020; Kumari & Kishor, 2020). Besides removing the other essential ingredients in shrimp shells, chemical extraction also creates chemical residue pollution and affects the physico-chemical characteristics of the product (Duan et al., 2012; Kaur & Dhillon, 2015). Another alternative method is by isolating chitin using proteolytic enzymes or microbes and lactic acid bacteria to digest the protein and mineral content in the raw material, leaving chitin as the main product (Cahú et al., 2012; Perez & Wertz, 2022). However, on the other hand, the price of enzymes is relatively high, and the low level of extraction efficacy is a challenge for the method (Kim & Park, 2015), so it is necessary to look for alternative enzymes or microbes that produce robust, stable and economical enzymes.

Research indicates that the shrimp digestive tract is a rich source of proteolytic bacteria and lactic acid bacteria (Holt et al., 2021; Kongnum & Hongpattarakere, 2012). Shrimp waste also harbours chitinolytic bacteria (Masri et al., 2021; Setia & , 2015). These findings suggest that the bacteria naturally present in the raw material could be harnessed for enzymatic extraction of chitin, presenting a promising avenue for research. However, the effectiveness of chitin extraction from shrimp waste using these specific groups of bacteria still needs to be explored, underscoring the need for further investigation.

The main aim of this study is to determine the effectiveness and identify the optimum conditions for extracting chitin from shrimp waste using bacteria from shrimp digestion. This research is crucial in advancing our understanding of chitin extraction methods and could potentially contribute to the development of more efficient and sustainable processes in the future.

MATERIALS AND METHODS

Isolation, enrichment, and bacterial colonies determination of shrimp digestion

Shrimp for digestive tract bacteria isolating were obtained from the Flamboyan market, Pontianak City, West Kalimantan Province, without selecting a particular type of shrimp, weighing around 20-30 per kg. The method for isolating microbes from the shrimp digestive tract (faeces and intestines) was adapted from the work of (Kongnum & Hongpattarakere, 2012) with several notable modifications. These included thoroughly washing the shrimp with distilled water, homogenising the intestines and faeces with sterile saline solution (NaCl 0.85% w/v), and diluting to 10^{-5}. The incubation and colony counting was performed with Plate Count Agar (PCA) (temperature 30°C, 24–48 hours, aerobic and anaerobic conditions); De Man, Rogosa, and Sharpe (MRS) agar (lactic acid bacteria, temperature 30°C, 24–48 h, aerobic and anaerobic conditions) (Kongnum & Hongpattarakere, 2012); and calcium M-Protein agar (proteolytic bacteria, temperature 30°C, five days), followed by a clear zone test with 5–10% acetic acid solution for 1 minute (Erkmen, 2021).

The number of bacterial colonies was calculated using a formula based on SNI 2332.3:2015 (Badan Standardisasi Nasional, 2015a).

$$N = \frac{\sum C}{[(1 \times n1) + (0{,}1 \times n2)] \times (d)}$$

With N, ∑C, n1, n2, d, respectively, the number of sample colonies (CFU/mL or colonies/g), the number of colonies on all plates counted, the number of plates in the first and second dilution counted, and the first dilution calculated.

Microscopic identification of shrimp digestive bacteria

Shrimp digestive bacteria incubated on MRS agar and M-Protein calcium agar were subjected to gram staining. The successive gram staining process begins by taking one of the colonies from the agar medium using a sterile tube needle, placing it on a glass slide and then fixing it with methanol. Next, the colonies were given a solution of crystal violet for 1 minute, iodine for 1 minute, 90% ethanol for 30 seconds, and safranin for 1 minute. Rinsing with distilled water was carried out at each change of solution administration. Bacterial cell colonies morphology (colour and shape) was observed using a microscope.

Determination of the effectiveness and optimum conditions for chitin isolation

The raw materials for chitin extraction, namely shrimp head, shell, and tail waste, were sourced from the Flamboyan market in Pontianak City, West Kalimantan. The shrimp waste was obtained without any sorting based on the type and size of the shrimp.

The preparation of the raw materials followed a modified version of the method outlined by (Hamdi et al., 2017). This method involved two treatment models. In the first treatment, the shrimp waste was thoroughly washed, cooked with distilled water (1:2 w/v) for 30 minutes at 100°C, and then left to drain at room temperature for approximately 2 hours. It was then incubated with LAB from shrimp digestion. The second treatment was similar to the first, with the addition of a drying stage in an oven at 60ºC for 48 hours. This was followed by coarse grinding of the shrimp shells with a blender before further treatment with LAB.

Chitin extraction was carried out by incubating 250 g of chopped shrimp waste (in 750 mL of distilled water) with 25 ml of LAB inoculum (the number of colonies was counted turbidimetrically) plus 12.5 g of glucose at room temperature for 24 hours using an orbital shaker (speed 60 rpm) for the demineralization process. The reaction was stopped by increasing the fermentation temperature to 90°C for 20 minutes. Next, the solution was filtered, and the chitin solids were rinsed with distilled water and dried in an oven at 50°C for about one and a half days. The yield was calculated, followed by the analysis of proximate.

Dry filtrate (100 g) resulting from LAB extraction plus 10 g of glucose and 300 ml of buffer solution based on pH treatment was further incubated with proteolytic bacterial culture (taken from 2 culture Petri dishes). Four pH treatments were prepared (5.0, 6.0, 7.0, 8.0). For pH 5.0, 0.1 M acetate buffer is used (5.772 g of sodium acetate and 1.778 g of concentrated acetic acid are mixed in 800 mL of distilled water, then the pH was adjusted with concentrated HCl, then distilled water was added to a volume of 1000 mL); pH 6.0 used 0.1 M citrate buffer (24.269 g of sodium citrate and 3.358 g of concentrated citric acid were mixed in 800 mL of distilled water, then

the pH was adjusted to 0.1 N HCl, then distilled water was added to a volume of 1000 mL); For pH 7.0 and 8.0, 1 M Tris-HCl buffer was used (121.14 g of Tris base was mixed in 800 mL of distilled water, then the pH was adjusted with HCl, then distilled water was added to a volume of 1000 mL). Fermentation was carried out at room temperature for 6 hours using an orbital shaker (speed 60 rpm). Incubation was stopped again by increasing the fermentation temperature to 90°C for 20 minutes. Afterwards, the fermentation solution was filtered, and the solids were washed with distilled water until neutral and dried in an oven at 50°C overnight. The extraction results were then calculated and tested proximately.

Proximate tests include ash content-based on SNI 2354.1.2010 (Badan Standardisasi Nasional, 2010), water-based on SNI 2354.2.2015 (Badan Standardisasi Nasional, 2015b), fat-based on SNI 01-2354.3-2006 (Badan Standardisasi Nasional, 2006a), and protein-based on SNI 01-2354.4-2006 (Badan Standardisasi Nasional, 2006b), which are all carried out in duplicate.

Determination of LAB colonies turbidimetrically for chitin extraction

Faeces and intestines from the shrimp digestive tract were taken sterilely and then enriched for bacteria using MRS agar and MRS broth at a dilution of 10^0 to 10^{-7}. They were then incubated at 30°C for 48 hours. Next, the number of colonies on the agar media was counted at each dilution. In contrast, the absorbance at each broth media dilution was measured by spectrophotometry at a wavelength of 600 nm (OD600).

The number of colonies on the agar media and the absorbance value on the broth media were then compared to obtain a standard curve. The equation of the standard curve is then used to calculate the number of LAB colonies used for chitin extraction.

RESULTS AND DISCUSSION

Type and number of bacterial colonies in the shrimp digestive tract

The total bacterial colonies (TPC), LAB, and proteolytic bacteria from the shrimp digestive tract, both aerobic and anaerobic, are depicted in Figure 1.

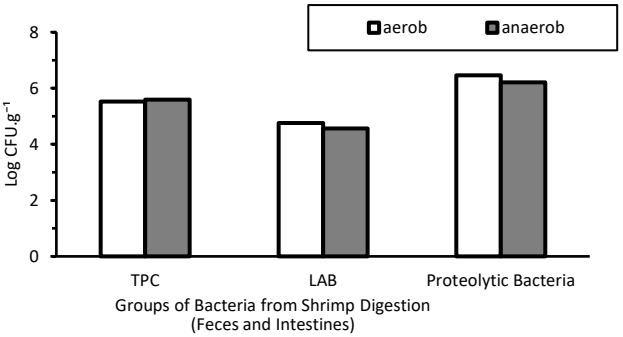

Figure 1. Bacterial colonies from the digestive tract (faeces and intestines) of shrimp incubated aerobically and anaerobically.

Figure 1 shows that aerobic and anaerobic TPC, LAB, and proteolytic bacteria from the shrimp digestive tract were 5.5 and 5.6 log CFU/g, 4.8 and 4.6 log CFU/g, and 6.5 6.5 and 6.2 log CFU/g. The presence of LAB and proteolytic bacteria in the shrimp digestive tract is in line with the results of several other studies (Bhowmik et al., 2015; Fitriadi et al., 2023; Holt et al., 2021; Kongnum & Hongpattarakere, 2012; Mulyati et al., 2023; Omont et al., 2020; Zhao et al., 2018). (Kongnum & Hongpattarakere, 2012) found that the digestion of some wild shrimp (including *Metapenaeus brevicornis* and *Penaeus merguiensis*) and cultivated shrimp (*Litopenaeus vannamei*) contained total bacteria ranging from 6.9–8.1 log CFU/g and LAB ranging from 3.2–4.1 log CFU/g. Furthermore, (Mulyati et al., 2023) found that the population of several proteolytic bacterial isolates from the digestive tract of *L. vannamei* ranged from 5.7–7.4 log CFU/g. This value range is still in line with the results of this research.

Figure 1 also reveals an interesting observation. The total number of LAB and proteolytic bacteria exceeds the TPC value, indicating that the TPC content alone cannot fully capture the presence of these bacteria. This discrepancy is likely due to the different content of PCA media for TPC and selective media for LAB and proteolytic bacteria.

PCA is a standard medium to estimate the bacterial population in a food or liquid sample. However, it cannot depict the entire bacterial population's presence or indicate differences in the various types of bacteria present in a sample (Mendonca et al., 2020). Meanwhile, selective media contains complete nutrients to support certain microbial groups' growth optimally. As a result, the population size in a sample can be estimated (Corry et al., 2011).

The morphology of LAB and proteolytic bacteria from the shrimp digestive tract based on gram staining is shown in Figure 2.

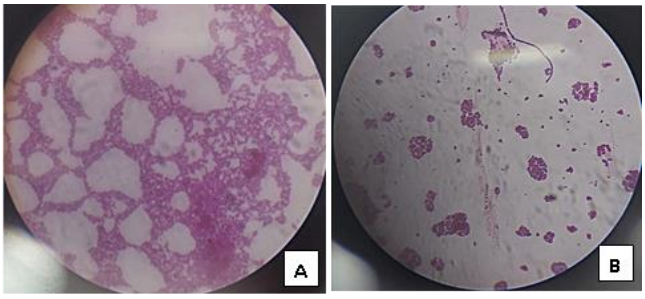

Figure 2. Microscope morphology of shrimp digestion (faeces and intestines) at 400x magnification: (A) LAB; (B) proteolytic bacteria

Figure 2, illustrates that LAB and proteolytic bacteria in shrimp digestion are gram-positive bacteria, taking the form of cocci or rods.

Morphologically, LAB emits a purplish colour, characteristic of the group of gram-positive bacteria, is in

the form of cocci or rods, does not form spores, and is very tolerant of low pH (Mokoena, 2017). Meanwhile, proteolytic bacteria produce proteases (protein-breaking down enzymes) with a broader morphological scope because there are groups of bacteria that belong to a gram-positive group (such as the Bacillus), whereas others are gram-negative (such as Pseudomonas) (Solanki et al., 2021). Furthermore, BAL can also digest protein to meet its amino acid needs for life (Hutkins, 2019; M. Liu et al., 2010). One source of LAB protein is casein (Hutkins, 2019), which is available in calcium M-Protein agar. The higher values of proteolytic bacterial colonies compared to the TPC and LAB (Figure 1) could be due to LAB and some other groups of bacteria having the ability to digest protein in calcium M-Protein agar.

Some LAB genera found to be quite dominant in shrimp digestion are Lactobacillus and Streptococcus (Zhao et al., 2018). Gram-positive and negative bacteria in the cocci or rod shaping were found in proteolytic bacterial isolates from the intestines of tiger shrimp (*Penaeus monodon*) (Bhowmik et al., 2015).

It's important to note that our study did not detect gram-negative bacteria. This could be because our microscopic analysis only involved one general colony, without examining and isolating each variant shape of proteolytic bacteria growing on selective agar media. Therefore, further analysis, such as PCR via the 16S rRNA gene sequencing method, is necessary to identify the specific types of LAB and proteolytic bacteria in the shrimp digestive tract. This method is widely used for identifying the genus and species of microbes in a sample (Johnson et al., 2019).

Number of LAB colonies for chitin extraction

The turbidimetric standard curve of BAL at a wavelength of 600 nm (OD600) for chitin extraction is depicted in Figure 3.

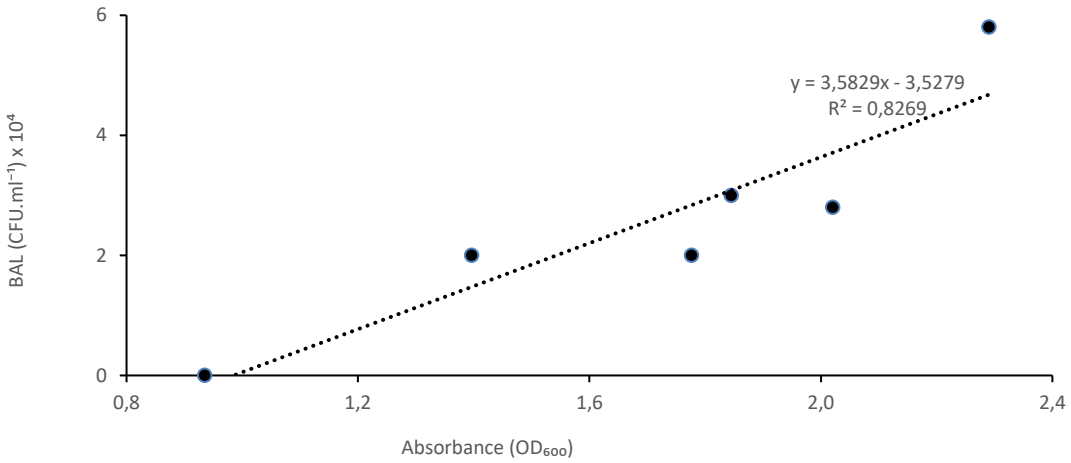

Figure 3. Standard curve for LAB colony measurements at OD600.

The number of LAB colonies used for chitin extraction based on the standard curve equation (figure 3) is described in Table 1.

Table 1. Number of LAB colonies for chitin extraction.

Equation of standart curve	Preliminary treatment	Absorbance (x)	BAL (CFU/ml)
y = 3,5829x - 3,5279	PP1	2,101	4x10⁴
	PP2	1,891	3,2x10⁴

Note: y (bacterial colony); x (absorbance); PP1 (preliminary treatment of shrimp waste: washed, boiled for 30 minutes, drained); PP2 (PP1 followed by oven drying at 60ºC 48 hours and coarse blending).

The calculation based on the standard curve implies that the number of LAB colonies used for fermentation in PP1 was 4x10⁴ CFU/ml (OD_{600} 2.1), while in PP2, it was 3.2x10⁴ CFU/ml (OD_{600} 1.9). The value is much smaller compared to (Ruangwicha et al., 2024), which found five LAB strains (four *Lactobacillus sp.* and *Streptococcus thermophilus*) with OD_{600} 0.8–1.0 equivalent to 10^8 CFU/ml. On the other hand, (Lu et al., 2023) used *Streptomyces sp.* SCUT-3 strain to extract amino acids, oligopeptides, calcium and chitin from *L. vannamei* shrimp shell waste with an inoculum size of OD_{600} 9.0. However, there is no information on the estimated colonies amount of Streptomyces sp. at the OD_{600} value.

Changes in shrimp shells' chemical composition during the extraction with LAB and proteolytic bacteria

The shrimp shells' chemical composition changes during extraction with LAB and proteolytic bacteria can be seen in Figure 4.

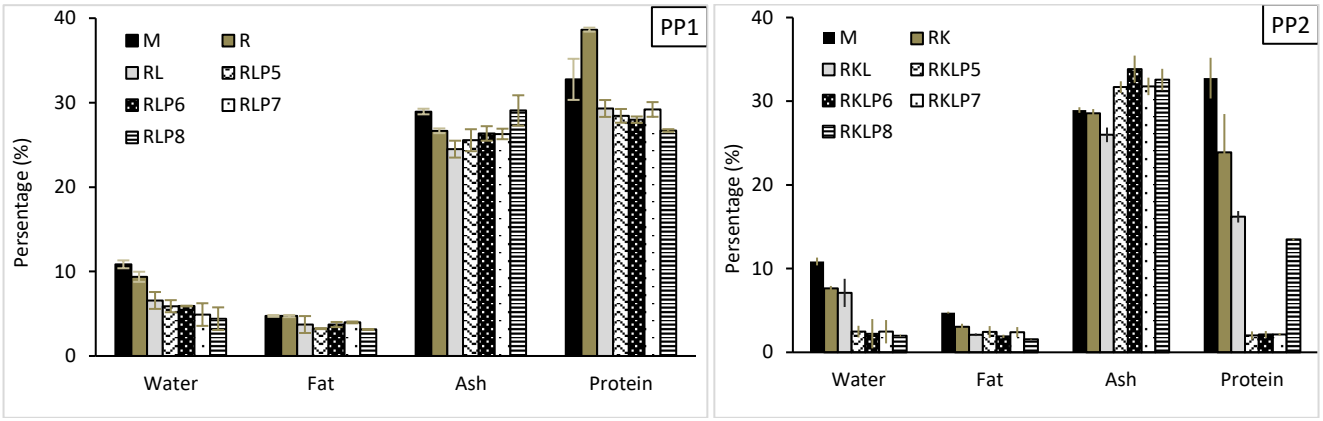

Figure 4. Changes in the chemical composition of shrimp waste (skin and head) during fermentation with LAB and proteolytic bacteria of shrimp digestion (faeces and intestines). Extraction stage: M (raw shrimp waste); R (shrimp waste with PP1); RL (PP1 after fermented with BAL); RK (shrimp waste with PP2); RKL (PP2 after fermented with BAL). Pretreatment: PP1 (shrimp waste washed, boiled, drained); PP2 (PP1 followed by oven drying and coarse blending). P5–P8: treatment of pH (5.0–8.0) during proteolytic bacteria fermentation. Proximate analysis was carried out in duplicate.

Figure 4 presents a detail breakdown of the composition of shrimp waste during fermentation process. It reveals that shrimp waste primarily comprises 10.9% water, 4.8% fat, 29.0% ash, and 32.8% protein. The remaining 22.5% is suspected of chitin and other compounds such as astaxanthin. Further data demonstrates that LAB and proteolytic bacteria, through fermentation, can reduce the fat and protein content of shrimp waste. Notably, the percentage reduction of proximate content in PP2 was higher than in PP1, with the most significant decline observed in PP2 protein levels, reaching 31% (pH treatment of 5.0–7.0). However, it is essential to note that the ash content of PP1 and PP2 increased after extraction with proteolytic bacteria.

Available studies indicate variations in the shrimp waste content. The waste (head, shell, tail) of five shrimp species (*L. vannamei*, *Macrobrachium rosenbergii*, *P. monodon*, *Fenneropenaeus chinensis*, *Penaeus japonicus*) is reported to consist of 66.2–73.9% water, 1.8–5.7% fat, 5.0–8.5% ash, 6.8–10.1% protein (Z. Liu et al., 2021). Another study states that the waste of *Pandalus borealis* shrimp from the Lagos Atlantic Sea comprises 4.2% water, 0.8% fat, 29.5% ash, and 19.1% protein (Adeyeye & Aremu, 2016). In contrast, the shell of *P. borealis* from the Barents Sea (Norway) with a dry matter of 22±2% consists of 0.3–0.5% fat, 32–38% ash, and 33–40% protein. The rest is chitin (17–20%) and astaxanthin (wet weight 14–39 mg.kg^{-1}) (Rødde et al., 2008). Moreover, data on *L. vannamei* shrimp shells (dried basis) has 4.0% of fat and astaxanthin, 21.7% minerals, 54.6% protein, and 19.7% chitin (Lu et al., 2023). These data imply that regardless of water content, the most significant composition of shrimp waste is protein and minerals, followed by chitin.

The pretreatment of washing and boiling, followed by oven drying and coarse grinding of PP2, influences the effectiveness of deproteinization. It is reported that the boiling of shrimp waste itself at 80ºC for an hour can dissolve the fat, ash, and protein content of shrimp waste respectively 0.1–0.5%, 0.3–0.6%, 2.0–2.2% (Fadhallah et al., 2023). Although the numbers are different, a decrease in the fat, ash and protein content of shrimp waste after pretreatment (PP1 and PP2) can be seen in Figure 4, even if there are some distinction data (see R in PP1).

Pretreatment and BAL fermentation can decrease shrimp waste's fat, ash, and protein content in PP1 by around 1%, 4.5%, and 3.5% (note that protein content increases by around 5.9% after PP1 pretreatment). Meanwhile, in PP2, the reduction is around 3% for fat and ash content and 17% for protein content. The low reduction content of ash was not expected because the preliminary hypothesis that lactic acid from LAB would effectively bind calcium in shrimp waste, which then sharply reduced the mineral content of shrimp waste as reported in several studies (Rao et al., 2000; Sixto-Berrocal et al., 2023; Xu et al., 2008). Furthermore, the shrink of protein level implies the role of LAB in digesting the shrimp waste's protein.

As information, after the 24-hour PP1 extraction process with BAL, there was a change in the aroma and condition of the shrimp waste, leading to a putrefaction process. Then, the shrimp waste was immediately washed thoroughly, filtered, and dried in an oven at 50°C for about one and a half days to ensure the extracted shrimp waste was dry enough. The process continues with coarse grinding of the shrimp waste extract before extraction with proteolytic bacteria. Next, a sterilisation process was carried out (121ºC, 15 minutes) on the PP1 medium before incubating proteolytic bacterial inoculum in the shrimp waste medium. For PP2, sterilisation was applied before each fermentation step. Signs of shrimp waste spoilage after 24 hours of fermentation with *Lactobacillus plantarum* were also reported (Rao et al., 2000). Sterilisation of the fermentation medium before inoculum incubation to shrimp waste has been mentioned in several studies (Lu et al., 2023; Ruangwicha et al., 2024).

The further fermentation process with proteolytic bacteria did not affect the fat content of PP1 shrimp waste (still at 3–4%). Additionally, the protein content of PP1 shrimp waste only decreased by around 0.1-3%. The relatively similar result also occurred in the fat content of PP2 shrimp waste. On the contrary, the protein content of PP2 shrimp waste decline up to around 14% (except for pH treatment of 8.0). While for ash content as states before, it increases for both pretreatments, after the proteolytic bacteria fermentation.

Proteolytic bacteria are suspected to be less able to digest minerals in shrimp waste. In addition, a decrease in water content and other compounds after fermentation causes mineral levels to appear increases. (Huang et al., 2022) reports increased mineral levels after fermentation of shrimp waste with a mixture of LAB, proteolytic, and chitinolytic bacteria. The lack of proteolytic bacteria effectiveness in digesting minerals is implied in some research (Lu et al., 2023; Waldeck et al., 2006). However, the preliminary deproteinized process increases shrimp waste's demineralisation degree (Lu et al., 2023).

The overall protein content decrease of 19 and 31% (PP2) and 4–6% (PP1) is relatively low compared to other studies (Lu et al., 2023; Ruangwicha et al., 2024; Sixto-Berrocal et al., 2023). However, all of these studies were carried out with a reasonably long fermentation time (3–5 days) and a relatively high substrate-to-solution ratio (1:10 to 1:20). In this study, the ratio of substrate-to-solution was only 1:3. Interestingly, there is a report of quite intense enzyme activity of the gram-positive proteolytic bacteria from the digestion of tiger shrimp (*P. Monodon*) at pH 5–7 (Bhowmik et al., 2015), which is in line with the result of PP2. Unfortunately, PP1 does not confirm the data.

Yield changes during chitin isolation from shrimp waste

Figure 5 describes the yield of chitin from shrimp waste during the extraction process with LAB and proteolytic bacteria from shrimp digestion.

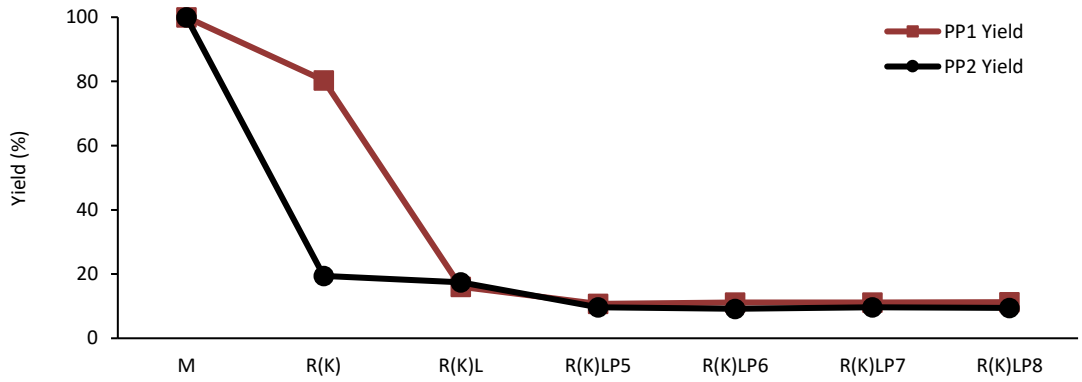

Figure 5. Yield changes during chitin isolation from shrimp waste with shrimp digestion bacteria. Extraction stage: M (raw shrimp waste); R/RK (after pretreatment); R(K)L (after BAL fermentation); R(K)LP (5–8) (after proteolytic bacteria fermentation at pH of 5.0–8.0).

As shown in Figure 5, the initial drying process removes more than eighty per cent of the shrimp waste weight, which contrasts with the low water content of raw shrimp waste (only 10.9%). It is suspected that the actual water content is much higher than the data. It is assumed that before proximate analysis, the water in the shrimp waste evaporates because of the relatively high room temperature, and there is a humidity difference between the shrimp waste and the surrounding air. Moreover, some of the filtrate is accidentally thrown away during the washing process, reducing the yield.

Overall, the final yield of shrimp waste filtrate after the isolation process was 10.7–11.3% (PP1) and 9.2–9.6% (PP2). It's worth noting that some research reports higher chitin yield from shrimp waste isolated enzymatically, ranging from 17-46% (Ghorbel-Bellaaj et al., 2013; Lu et al., 2023; Rødde et al., 2008; Ruangwicha et al., 2024; Xu et al., 2008). However, the yields still contain some other impurities.

Discussion and future recommendation

Although the results of this research do not meet initial expectations regarding deproteinization and demineralization, the potential of shrimp digestion bacteria to isolate chitin from shrimp waste can be noticed. Generally, some studies show high deproteinization and demineralization results are obtained using one or several specific microbes. Furthermore, fermentation conditions (amount of inoculum, pH, temperature, time, sugar sources and concentrations, substrate-to-liquid ratio, and process stages) (Lu et al., 2023; Rao et al., 2000; Ruangwicha et al., 2024; Sixto-Berrocal et al., 2023) also influence fermentation effectiveness. Several studies have even

succeeded in isolating several other compounds besides chitin, such as proteins or peptides and free amino acids, calcium and pigments through fermentation (Lu et al., 2023; Ruangwicha et al., 2024).

Further analysis needs to be conducted to determine the quality of chitin produced through fermentation. FTIR (Fourier-transform infrared spectroscopy), XRD (X-ray diffraction) and SEM (Scanning electron microscope), which can detect the purity and structural morphology of chitin, can support the analysis (Ruangwicha et al., 2024; Triunfo et al., 2022).

This study fermented using free bacterial cells. Free microbial cells tend to be vulnerable to environmental pressures (Mehrotra et al., 2021), which decreases their numbers due to their inability to survive in certain conditions. One possible development of a fermentation method is by using immobilised microbial cells. The cells of the microbe are immobilised by binding/trapping them to a specific carrier/support.

Some studies say immobilised cells have several advantages over free cells. The immobilised cells have higher metabolic activity and cell density, so the cells are more productive compared to free cells. Moreover, immobilisation provides the cells better protection against surrounding environmental conditions, improving the cells' survival ability. Then, immobilised cells can be reused without losing their activity. Besides, separating immobilised cells from extracts is easier (Mehrotra et al., 2021; Zur et al., 2016). Compared to using pure enzymes, applying immobilised cells can be cheaper because there is no need for enzyme isolation and purification stages. Moreover, microbial cells generally produce more than one type of enzyme, which can lead to a series of chemical reaction catalysation, not just one type of chemical reaction like pure enzymes (Lu et al., 2023).

The immobilized whole-cell biocatalysts have been used to produce fine chemicals, biofuels (Polakovič et al., 2017), biosurfactants (Subsanguan et al., 2020), therapeutic drugs (Anteneh & Franco, 2019), winemaking (Genisheva et al., 2014), and wastewater treatments (An et al., 2008). However, no information is available about the production of chitin and its derivatives using these biocatalysts.

CONCLUSIONS

The number of bacterial colonies shows that proteolytic bacteria dominate the shrimp digestive tract. Microscopic morphology shows that LAB and proteolytic bacteria are gram-positive as cocci or rods. Furthermore, LAB and proteolytic bacteria from shrimp digestion can reduce the protein content (deproteinization) of shrimp waste by up to 31%, especially for treatment with a pH of 5.0–7.0 (during fermentation with proteolytic bacteria) and pre-treatment of boiling, drying and coarse grinding of shrimp waste. However, the reduction in mineral content (demineralization) of shrimp waste was only 4.5% (PP1) and 3% (PP2), with LAB as the main contributor because proteolytic bacteria were not able to further reduce the mineral content in shrimp waste.

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Authors' Contributions: Kristina Novalina Nainggolan designed the study and wrote the manuscript. Kristina Novalina Nainggolan, together with Yudha Perdana Putra and Evi Fitriyani, conducted the laboratory work and analysed the data. All authors read and approved the final version of the manuscript.

Competing Interests: The authors declare that there are no competing interests.

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REFERENCES

Adeyeye, E. I., & Aremu, M. O. (2016). Chemical Composition of Whole Shrimp, Flesh and Shell of Pandalus borealis from Lagos Atlantic Ocean. *FUW Trends in Science & Technology Journal*, *1*(1), 26–32. https://www.researchgate.net/publication/305642149

An, T., Zhou, L., Li, G., Fu, J., & Sheng, G. (2008). Recent Patents on Immobilized Microorganism Technology and Its Engineering Application in Wastewater Treatment. *Recent Patents on Engineering*, *2*(1), 28–35. https://doi.org/10.2174/187221208783478543

Anteneh, Y. S., & Franco, C. M. M. (2019). Whole cell actinobacteria as biocatalysts. *Frontiers in Microbiology*, *10*(FEB), 1–15. https://doi.org/10.3389/fmicb.2019.00077

Badan Standardisasi Nasional. (2006a). SNI 01-2354.3-2006 Cara uji kimia - Bagian 3: Penentuan kadar lemak total pada produk perikanan. In *Jakarta: Badan Standardisasi Nasional*. https://idoc.pub/queue/sni-uji-kadar-lemak-pnxkq7ykg94v

Badan Standardisasi Nasional. (2006b). SNI 01-2354.4-2006 Cara uji kimia - Bagian 4: Penentuan kadar protein dengan metode total nitrogen pada produk perikanan. In *Jakarta: Badan Standarisasi Nasional*.

Badan Standardisasi Nasional. (2010). *SNI 2354.1:2010 Cara uji kimia - Bagian 1: Penentuan kadar abu dan abu tak larut asam pada produk perikanan*.

Badan Standardisasi Nasional. (2015a). SNI 2332.3:2015 Cara uji mikrobiologi - Bagian 3: Penentuan Angka Lempeng Total

(ALT) pada produk perikanan. In *Jakarta: Badan Standardisasi Nasional*.

Badan Standardisasi Nasional. (2015b). SNI 2354.2:2015 Cara uji kimia – Bagian 2: Pengujian kadar air pada produk perikanan. In *Jakarta: Badan Standarisasi Nasional.*

Bastiaens, L., Soetemans, L., D'Hondt, E., & Elst, K. (2020). Sources of chitin and chitosan and their isolation. In L. A. M. Van Den Broek & C. G. Boeriu (Eds.), *Chitin and Chitosan: Properties and Applications* (1st ed., pp. 1–34). John Wiley & Sons Ltd. https://doi.org/10.1002/9781119450467.ch1

Bhowmik, S., Islam, S., Ahmed, M. M., Belal Hossain, M., & Hossain, M. A. (2015). Protease producing bacteria and activity in gut of tiger shrimp (Penaeus monodon). *Journal of Fisheries and Aquatic Science*, *10*(6), 489–500. https://doi.org/10.3923/jfas.2015.489.500

Cahú, T. B., Santos, S. D., Mendes, A., Córdula, C. R., Chavante, S. F., Carvalho, L. B., Nader, H. B., & Bezerra, R. S. (2012). Recovery of protein, chitin, carotenoids and glycosaminoglycans from Pacific white shrimp (Litopenaeus vannamei) processing waste. *Process Biochemistry*, *47*(4), 570–577. https://doi.org/10.1016/j.procbio.2011.12.012

Duan, S., Li, L., Zhuang, Z., Wu, W., Hong, S., & Zhou, J. (2012). Improved production of chitin from shrimp waste by fermentation with epiphytic lactic acid bacteria. *Carbohydrate Polymers*, *89*(4), 1283–1288. https://doi.org/10.1016/j.carbpol.2012.04.051

Ehrlich, H. (2010). Chitin and collagen as universal and alternative templates in biomineralization. In *International Geology Review* (Vol. 52, Issues 7–8). https://doi.org/10.1080/00206811003679521

El-Bialy, H. A. A., & Abd El-Khalek, H. H. (2020). A comparative study on astaxanthin recovery from shrimp wastes using lactic fermentation and green solvents:an applied model on minced Tilapia. *Journal of Radiation Research and Applied Sciences*, *13*(1), 594–605. https://doi.org/10.1080/16878507.2020.1789388

Erkmen, O. (2021). *Microbiological Analysis of Foods and Food Processing Environments*. Elsevier Inc. https://doi.org/https://doi.org/10.1016/C2021-0-01219-0

Fadhallah, E. G., Koesoemawardani, D., & Indraningtyas, L. (2023). Chemical Properties of Liquid Broth Extracted from Freshwater and Marine Shrimp Shells Waste. *Biology, Medicine, & Natural Product Chemistry*, *12*(2), 437–440. https://doi.org/10.14421/biomedich.2023.122.437-440

Fitriadi, R., Setyawan, A. C., Palupi, M., Nurhafid, M., & Kusuma, R. O. (2023). Isolation and molecular identification of proteolytic bacteria from vaname shrimp (Lithopenaeus Vannamei) ponds as probiotic agents. *Iraqi Journal of Veterinary Sciences*, *37*(1), 161–170. https://doi.org/10.33899/ijvs.2022.133468.2251

Genisheva, Z., Teixeira, J. A., & Oliveira, J. M. (2014). Immobilized cell systems for batch and continuous winemaking. *Trends in Food Science and Technology*, *40*(1), 33–47. https://doi.org/10.1016/j.tifs.2014.07.009

Ghorbel-Bellaaj, O., Hajji, S., Younes, I., Chaabouni, M., Nasri, M., & Jellouli, K. (2013). Optimization of chitin extraction from shrimp waste with Bacillus pumilus A1 using response surface methodology. *International Journal of Biological Macromolecules*, *61*, 243–250. https://doi.org/10.1016/j.ijbiomac.2013.07.001

Hamdi, M., Hammami, A., Hajji, S., Jridi, M., Nasri, M., & Nasri, R. (2017). Chitin extraction from blue crab (Portunus segnis) and shrimp (Penaeus kerathurus) shells using digestive alkaline proteases from P. segnis viscera. *International Journal of Biological Macromolecules*, *101*, 455–463. https://doi.org/10.1016/j.ijbiomac.2017.02.103

Holt, C. C., Bass, D., Stentiford, G. D., & van der Giezen, M. (2021). Understanding the role of the shrimp gut microbiome in health and disease. *Journal of Invertebrate Pathology*, *186*(April 2020), 107387. https://doi.org/10.1016/j.jip.2020.107387

Hu, J., Lu, W., Lv, M., Wang, Y., Ding, R., & Wang, L. (2019). Extraction and purification of astaxanthin from shrimp shells and the effects of different treatments on its content. *Revista Brasileira de Farmacognosia*, *29*(1), 24–29. https://doi.org/10.1016/j.bjp.2018.11.004

Huang, C. H., Lin, C. H., Huang, H. H., & Tsai, G. J. (2022). Development of Fermented Shrimp Shell Product with Hypoglycemic and Hypolipidemic Effects on Diabetic Rats. *Metabolites*, *12*(8). https://doi.org/10.3390/metabo12080695

Hutkins, R. W. (2019). Microbiology and Technology of Fermented Foods: Second Edition. In *Wiley-Blackwell*. IFT Press, John Wiley & Sons, Inc. https://doi.org/10.1002/9780470277515

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., Leopold, S. R., Hanson, B. M., Agresta, H. O., Gerstein, M., Sodergren, E., & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications*, *10*(1), 1–11. https://doi.org/10.1038/s41467-019-13036-1

Kaur, S., & Dhillon, G. S. (2015). Recent trends in biological extraction of chitin from marine shell wastes: A review. *Critical Reviews in Biotechnology*, *35*(1), 44–61. https://doi.org/10.3109/07388551.2013.798256

Kim, Y., & Park, R. D. (2015). Progress in bioextraction processes of chitin from crustacean biowastes. *Journal of the Korean Society for Applied Biological Chemistry*, *58*(4), 545–554. https://doi.org/10.1007/s13765-015-0080-4

Kongnum, K., & Hongpattarakere, T. (2012). Effect of Lactobacillus plantarum isolated from digestive tract of wild shrimp on growth and survival of white shrimp (Litopenaeus vannamei) challenged with Vibrio harveyi. *Fish and Shellfish Immunology*, *32*(1), 170–177. https://doi.org/10.1016/j.fsi.2011.11.008

Kumari, S., & Kishor, R. (2020). Chitin and chitosan: origin, properties, and applications. In S. Gopi, S. Thomas, & A. Pius (Eds.), *Handbook of Chitin and Chitosan: Volume 1: Preparation and Properties*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-817970-3.00001-8

Liu, M., Bayjanov, J. R., Renckens, B., Nauta, A., & Siezen, R. J. (2010). The proteolytic system of lactic acid bacteria revisited: A genomic comparison. *BMC Genomics*, *11*(1), 5–8. https://doi.org/10.1186/1471-2164-11-36

Liu, Z., Liu, Q., Zhang, D., Wei, S., Sun, Q., Xia, Q., Shi, W., Ji, H., & Liu, S. (2021). Comparison of the proximate composition and nutritional profile of byproducts and edible parts of five species of shrimp. *Foods*, *10*(11), 1–16. https://doi.org/10.3390/foods10112603

Lu, D. L., Zhang, M. S., Deng, J. J., Lu, W. J., Yang, Z. D., Li, Z. W., Chen, Y. C., & Luo, X. C. (2023). Highly efficient shrimp shell recovery by solid-state fermentation with Streptomyces sp. SCUT-3. *Chemical Engineering Journal*, *458*(December 2022), 141256. https://doi.org/10.1016/j.cej.2022.141256

Masri, M., Sukmawaty, E., & Aditia, L. (2021). Novel chitinolytic bacteria from the shrimp shell processing waste. *Biodiversitas*, *22*(5), 2672–2681. https://doi.org/10.13057/biodiv/d220527

Mehrotra, T., Dev, S., Banerjee, A., Chatterjee, A., Singh, R., & Aggarwal, S. (2021). Use of immobilized bacteria for environmental bioremediation: A review. *Journal of Environmental Chemical Engineering*, *9*(5), 105920. https://doi.org/10.1016/j.jece.2021.105920

Mokoena, M. P. (2017). Lactic acid bacteria and their bacteriocins: Classification, biosynthesis and applications against uropathogens: A mini-review. *Molecules*, *22*(8). https://doi.org/10.3390/molecules22081255

Mulyati, Suryati, Baga, I., Ardiansyah, Hamal, R., & Rusli. (2023). Proteolytic Bacteria of Digestive Tract Vanname Shrimp.pdf. *Asian Journal of Fisheries and Aquatic Research*, *25*(3), 113–122. https://journalajfar.com/index.php/AJFAR/article/view/671

Nainggolan, K. N. (2023). Ekstraksi Enzimatik Kitin dan Kitosan dari Limbah Udang. *MANFISH JOURNAL*, *4*(1), 50–71. https://doi.org/https://doi.org/10.31573/manfish.v4i1

Omont, A., Elizondo-González, R., Quiroz-Guzmán, E., Escobedo-Fregoso, C., Hernández-Herrera, R., & Peña-Rodríguez, A. (2020). Digestive microbiota of shrimp Penaeus vannamei and oyster Crassostrea gigas co-cultured in integrated multi-trophic aquaculture system. *Aquaculture*, *521*, 735059. https://doi.org/10.1016/j.aquaculture.2020.735059

Perez, S., & Wertz, J.-L. (2022). *Chitin and Chitosans in the Bioeconomy* (1st ed.). Taylor & Francis Group. https://doi.org/https://doi.org/10.1201/9781003226529

Polakovič, M., Švitel, J., Bučko, M., Filip, J., Neděla, V., Ansorge-Schumacher, M. B., & Gemeiner, P. (2017). Progress in biocatalysis with immobilized viable whole cells: systems development, reaction engineering and applications. *Biotechnology Letters*, *39*(5), 667–683. https://doi.org/10.1007/s10529-017-2300-y

Rao, M. S., Muñoz, J., & Stevens, W. F. (2000). Critical factors in chitin production by fermentation of shrimp biowaste. *Applied Microbiology and Biotechnology*, *54*(6), 808–813. https://doi.org/10.1007/s002530000449

Rødde, R. H., Einbu, A., & Vårum, K. M. (2008). A seasonal study of the chemical composition and chitin quality of shrimp shells obtained from northern shrimp (Pandalus borealis). *Carbohydrate Polymers*, *71*(3), 388–393. https://doi.org/10.1016/j.carbpol.2007.06.006

Ruangwicha, J., Cheirsilp, B., & Suyotha, W. (2024). Green biorefinery of shrimp shell waste for α-chitin and high-value co-products through successive fermentation by co-lactic acid bacteria and proteolytic fungus. *Bioresource Technology*, *393*, 1–12. https://doi.org/10.1016/j.biortech.2023.130106

Setia, I. N., & S. (2015). Chitinolytic Assay and Identification of Bacteria Isolated from Shrimp Waste Based on 16S rDNA Sequences. *Advances in Microbiology*, *05*(07), 541–548. https://doi.org/10.4236/aim.2015.57056

Sixto-Berrocal, A. M., Vázquez-Aldana, M., Miranda-Castro, S. P., Martínez-Trujillo, M. A., & Cruz-Díaz, M. R. (2023). Chitin/chitosan extraction from shrimp shell waste by a completely biotechnological process. *International Journal of Biological Macromolecules*, *230*(October 2022). https://doi.org/10.1016/j.ijbiomac.2023.123204

Solanki, P., Putatunda, C., Kumar, A., Bhatia, R., & Walia, A. (2021). Microbial proteases: ubiquitous enzymes with innumerable uses. *3 Biotech*, *11*(10), 1–25. https://doi.org/10.1007/s13205-021-02928-z

Subsanguan, T., Khondee, N., Nawavimarn, P., Rongsayamanont, W., Chen, C. Y., & Luepromchai, E. (2020). Reuse of Immobilized Weissella cibaria PN3 for Long-Term Production of Both Extracellular and Cell-Bound Glycolipid Biosurfactants. *Frontiers in Bioengineering and Biotechnology*, *8*(July), 1–14. https://doi.org/10.3389/fbioe.2020.00751

Triunfo, M., Tafi, E., Guarnieri, A., Salvia, R., Scieuzo, C., Hahn, T., Zibek, S., Gagliardini, A., Panariello, L., Coltelli, M. B., De Bonis, A., & Falabella, P. (2022). Characterization of chitin and chitosan derived from Hermetia illucens, a further step in a circular economy process. *Scientific Reports*, *12*(1), 1–17. https://doi.org/10.1038/s41598-022-10423-5

Waldeck, J., Daum, G., Bisping, B., & Meinhardt, F. (2006). Isolation and molecular characterization of chitinase-deficient Bacillus licheniformis strains capable of deproteinization of shrimp shell waste to obtain highly viscous chitin. *Applied and Environmental Microbiology*, *72*(12), 7879–7885. https://doi.org/10.1128/AEM.00938-06

Xu, Y., Gallert, C., & Winter, J. (2008). Chitin purification from shrimp wastes by microbial deproteination and decalcification. *Applied Microbiology and Biotechnology*, *79*(4), 687–697. https://doi.org/10.1007/s00253-008-1471-9

Zargar, V., Asghari, M., & Dashti, A. (2015). *A Review on Chitin and Chitosan Polymers : Structure , Chemistry , Solubility , Derivatives , and Applications*. *00*, 1–24. https://doi.org/10.1002/cben.201400025

Zhao, Y., Duan, C., Zhang, X. X., Chen, H., Ren, H., Yin, Y., & Ye, L. (2018). Insights into the gut microbiota of freshwater shrimp and its associations with the surrounding microbiota and environmental factors. *Journal of Microbiology and Biotechnology*, *28*(6), 946–956. https://doi.org/10.4014/jmb.1710.09070

Zur, J., Wojcieszyńska, D., & Guzik, U. (2016). Metabolic responses of bacterial cells to immobilization. *Molecules*, *21*(7). https://doi.org/10.3390/molecules21070958

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