Bioextraction of chitin from vannamei shrimp shell using a mixture Latic Acid Bacteria

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Abstract. Chitin, a cationic polymer derived from shrimp shell, has gained widespread recognition for its potential applications in medicine, environmental solutions, and agriculture. This study was aimed to investigate the biological extraction of chitin from shrimp shells using a mixed bacterial starter comprising *Lactobacillus plantarum* SK (5), *Lactobacillus plantarum* NS (9), and *Pediococcus pentosaceus* BP (20), with varying fermentation durations of 0, 5, 10, and 15 days. The effects of fermentation time on protein and ash content were examined, and FTIR analysis was conducted on the 15th day. Fermentation reduced protein content by 31.29%, demonstrating effective deproteinization. However, ash content increased due to challenges in separating chitin extract from bacterial biomass. FTIR analysis on day 15 identified functional groups (N-H, O-H, C-H, C=O, C-O-C) in the extracted chitin, which had a deacetylation degree of 36%. This research however underscores the protein and ash content in the chitin extract obtain still do not meet the quality standards for chitin based on SNI (Indonesia National Standard).

1 Introduction

Indonesia boasts extensive maritime potential, with shrimp emerging as a prominent resource. The Ministry of Fisheries and Maritime Affairs highlights shrimp as the principal export commodity in Indonesia's fisheries sector, making the most significant contribution to the country's foreign exchange earnings from fishery product exports. Shrimp stands as a cornerstone in Indonesia's fisheries, positioned for substantial export growth, with national shrimp production estimated to reach 2 million tons by 2021 [1]. Various types of shrimp from pond cultivation and catches include tiger prawns, pink prawns, white cat prawns, and giant prawns.

The shrimp freezing industry generates substantial by-products, including shells, heads, and tails. Notably, remaining shrimp heads contain high protein levels and essential amino acids. However, unutilized by-products lead to environmental pollution and unpleasant odors. To address this, further processing and utilization of these by-products are crucial for reducing environmental impact and increasing added value. The utilization of shrimp shells

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in Indonesia is currently limited, mainly employed in producing crackers, paste, shrimp paste, and animal feed [2]. To enhance their economic value, efforts are underway to process shrimp shell into chitin and chitosan [3, 4, 5, 6], which hold higher economic potential compared to their current low-value applications.

Chitin, a complex organic compound found in the shells of crustaceans, ranks as the second most abundant polysaccharide after cellulose. It typically dissolves in organic acids, particularly within a pH range of approximately 4 to 6.5 and remains insoluble at lower or higher pH levels [7]. The chitin extraction process involves two crucial stages: demineralization, which utilizes acid, and deproteinization, which employs a high base concentration. Structurally like cellulose but featuring additional acetyl groups, chitin can be converted into chitosan through a deacetylation process. Chitin and chitosan have widespread applications, serving various purposes such as adsorbing heavy metals and dyes, acting as preservatives, antifungals, cosmetics, pharmaceuticals, flocculants, anticancer agents, and antibacterials [8, 9, 10, 11]. Chitin extraction conventionally employs chemical methods involving NaOH for deproteination and HCl for demineralization [13]. While chemical processes effectively reduce protein and mineral levels, they pose side effects, including potential harm to the body and environmental damage due to strong acid and base chemicals. As an alternative, biological methods utilizing lactic acid bacteria offer a more sustainable approach. Biological extraction involves utilizing lactic acid bacteria, such as Lactobacillus for demineralization [12], and proteolytic bacteria, such as Bacillus for deproteinization [13] In this study, we utilized a mixture of lactic acid bacteria isolated from fermented fish products (bekasam), including *L. plantarum SK* (5), *L. plantarum NS* (9), and *P. pentosaceus BP* (20) [14] for chitin extraction. Our previous reports demonstrated that these isolates produce organic acids and possess proteolytic activity [14,15], so it is hoped that these three LABs can carry out the demineralization and deproteinization processes in chitin extraction biologically. However, the capability of our isolated lactic acid bacteria to extract chitin biologically remains unknown. Thus, the objective of this study aimed to assess the impact of adding a mixture of bacteria including *L. plantarum SK* (5), *L. plantarum NS* (9), and *P. pentosaceus BP* (20) for biological chitin extraction during a 15-day fermentation period on pH value, protein content, and ash.

2 Method

2.1 Materials

The vannamei shrimp shells was purchased from PT Ocean Indo Shrimp, Tangerang, Banten. The isolates of lactic acid bacteria, namely *L. plantarum SK (5), L. plantarum NS (9),* and *P. pentosaceus BP (20* were obtained from the collection of Dr. Desniar. Some of the microbiological and chemical analysis materials used include: deMan Rogosa and Sharpe Agar (MRSA, Oxoid, England), deMan Rogosa and Sharpe Broth (MRSB, Merck, Germany), distilled water, Sulfide Indole Motility (SIM, Oxoid, England), Lugol (Merck, Germany), alcohol (Merck, Germany), safranin (Himedia, India), crystal violet (Himedia, India), Nutrient Broth (NB, Oxoid, England)), glucose (Merck, Germany), Plate Count Agar (PCA, Oxoid, England)). Various tools were essential for the research, such as an incubator (Thermolyne type 4200 incubator, City, Country), autoclave (Yamato SM52, Japan), pH meter (WalkLab, Singapore), microscope (Olympus CX23, City, Japan), HPLC, Erlenmeyer analytical balance (Sartorius), and Whatman 42 paper.

2.2 Method

The research methodology encompassed several stages, beginning with the preparation of bacterial cultures (*L. plantarum SK* (5), *L. plantarum NS* (9), and *P. pentosaceus BP* (20)). Following this, raw material preparation was conducted, followed by a 15-day biological extraction of chitin through fermentation. Observations were diligently recorded on days 0, 5, 10, and 15. Proximate analysis was conducted on the raw material of shrimp shell, including moisture, ash, protein, fat, and carbohydrate content. During the fermentation process of shrimp shell with LAB mixture, pH condition and total acidity analysis were conducted. Additionally, analyses of protein content and ash content were carried out on chitin extract obtained at different fermentation times. Functional groups of chitin extract on the 15th day of fermentation were analysed using an FTIR spectrophotometer.

2.2.1 Preparation of raw material from shrimp shell

The vannamei shrimp shell, including the head and body shell, was washed with running water until clean. The shrimp shells were then drained to remove excess water and dried using an oven at a temperature of 40-55 °C for 48 hours. Dried shrimp shells were finely ground using a chopper, filtered for uniformity, and subsequently stored at room temperature. Proximate analysis, encompassing assessments of protein, ash, water content, and carbohydrate [16], was systematically carried out to ascertain the chemical properties of the processed carapace.

2.2.2 Preparation of lactic acid bacteria starter

The preparation of the bacterial starter culture commenced with refreshing the isolate, followed by the verification of bacterial cultures through Gram staining, catalase, and motility tests. Isolate refreshing involved introducing one isolate into sterilized slant agar (MRSA) media and subsequent incubation for 48 hours at 37 °C. Bacteria growing on MRSA media were cycled and placed in MRS - B media, followed by incubation at 37 °C for 24 hours. A 10% inoculum was then introduced into 90 mL MRS-B media and incubated at 37 °C for 24 hours. The starter underwent testing for total lactic acid bacteria using the pour method [15].

2.2.3 Bio-extraction of chitin from vannamei shrimp shell using mixture lactic acid bacteria

Chitin bio-extraction from shrimp was conducted following the protocol of Duan et al. [17] with modifications. Initially, 10 g of shrimp shell powder was mixed with 6.5% w/w glucose and 90 mL of distilled water. This mixture was then sterilised using an autoclave at 121 °C and 1 atm for 15 minutes. Subsequently, fermentation was initiated by adding a 10% v/w mixture starter containing lactic acid bacteria, namely *L. plantarum SK (5), L. plantarum NS (9),* and *P. pentosaceus BP (20)*. The fermentation process lasted for 15 days at a temperature of 37 °C, with observations made on days 0, 5, 10, and 15. Throughout the fermentation process, pH measurements were taken. Following filtration using nylon mesh, the fermentation residue was rinsed with distilled water until reaching a pH of 7. Samples achieving pH 7 were then dried in an oven at 55-65 °C for two days. The dried residue underwent testing for protein content, ash content, degree of deacetylation, and functional group analysis.

2.2.4 Functional group analysis using Fourier Transform Infrared FTIR

The functional groups in the chitin extract were analyzed using FTIR spectrophotometry, where the functional groups of commercial chitins derived from shrimp shell were also analyzed as a comparative standard. The analytical procedure involved grinding solid samples with a potassium bromide salt mixture (ratio of 2:98 w/w), shaping pellets with a 7 mm diameter, and then measuring absorbance in the 4000-400 cm^{-1} range using FTIR spectroscopy. The percentage of deacetylation degree of chitin extract from vannamei shrimp shell can be calculated using the results from FTIR analysis, referring to the study conducted by Domszy and Robert [18].

2.3 Data analysis

The experimental design used in this study is a completely randomized design, where the fermentation duration (days) serves as the factor with 5 treatment levels: 0, 5, 10, 15 days. The response parameters observed include pH level, ash content, and protein content. The experiment was replicated 4 times. The data obtained were analyzed using analysis of variance (ANOVA) with a confidence interval of 95%. If the treatment significantly affects the observed response parameters ($p < 0.05$), Duncan's test is conducted as a post hoc test. Differences between treatments are depicted using different letters on the bar graph.

3 Results and Discussion

3.1 Characteristics of vannamei shrimp shell

The raw materials employed in this study consisted of the heads and carapace of vannamei shrimp (*Litopenaeus vannamei*), sourced from PT Ocean Shrimp Indo Tangerang Banten. The raw materials used in this study are classified as fresh, characterized by their bright and shiny ash-colored shells, fresh aroma, as well as solid and compact texture. The shrimp shells are cleaned from entrails, then dried using an oven and crushed to obtain a powdered form. The chemical composition of the vannamei shrimp shell powder is presented in Table 1.

Table 1. Chemical composition of vannamei shrimp shell powder

Chemical composition	Content (%)	Content (%)
Moisturizer	9.51±0.08	12.55
Protein	33.89±0.35	21.5*
Ash	34.2±0.46	47.18**
Carbohydrate	22.34±0.9	22.09***

Note = * [19],** [20], ***[21]

The vannamei shrimp shell powder exhibits varying chemical compositions, with the highest concentration being in protein content, followed by ash content, while water content records the lowest value. The vannamei shrimp shell presents a relatively low water content of 9.51±0.08%. This reduction is attributed to the drying process in an oven at 55 °C for two days. In addition, ash content serves as an indicator of mineral composition, with the vannamei shrimp shell exhibiting a relatively high ash content due to its rich mineral content. The vannamei shrimp shell boasts an ash content percentage of 34.2±0.46%, exceeding the findings of Ravichandran et al. [19] at 21.5%. Discrepancies in ash content are likely influenced by habitat and environmental differences.

Protein content in vannamei shrimp shell reached 33.89±0.35%, a result consistent with research by Suptijah et al. [20], where powdered shrimp shell demonstrated a protein content

of 34.69%. This elevated protein level is attributed to the feed provided to vannamei shrimp, often characterized by a high protein content due to their cultivation in pond environments. Carbohydrate levels in this study were determined using the by-difference method, a technique for approximating carbohydrate content in food ingredients, including crude fiber. The vannamei shrimp shell, analyzed using this method, revealed a carbohydrate content of 22.34±0.9%. These findings align closely with the research of Suptijah et al. [21], who reported a carbohydrate content by difference in vannamei shrimp shell at 22.09%.

3.2 The pH level changes during the fermentation process of shrimp shell

The fermentation process of shrimp shell utilizing bacteria is subject to various influencing factors, including temperature, pH, and duration. As the acid concentration in the solution increases, the pH value decreases. The alterations in pH values throughout the fermentation process for vannamei shrimp shell are depicted in Figure 1.

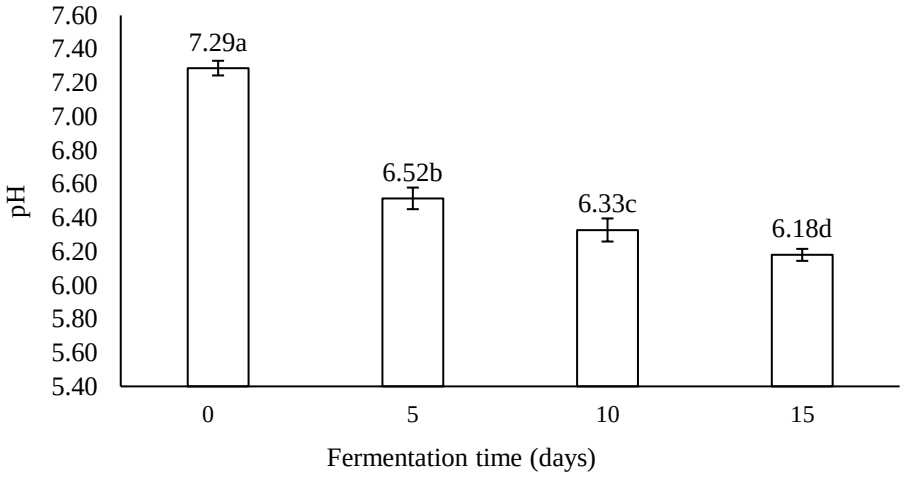

Fig. 1. pH values during fermentation with mixture lactic acid bacteria

Fermentation duration of shrimp shell significantly influences pH conditions ($p<0.05$) (Figure 1). pH conditions at each observation time during fermentation differ significantly, with pH values ranging from 7.29 to 6.18. The fermentation of shrimp shell for chitin extraction, employing lactic acid bacteria, resulted in a noticeable decrease in pH values over the 15-day observation period. This decrease in pH level in the study indicates an active fermentation process conducted by the LAB mixture. However, these pH conditions differ from those observed in the study by Duan et al. [17], which used *L. acidophilus* in the fermentation of shrimp shell. They reported that pH values ranged from 4.26 at the outset to 3.80 at the culmination of fermentation. The differences in pH conditions during shrimp shell fermentation are likely attributed to variations in bacterial cultures and differing fermentation durations.

Duan et al. [17] reported that the pH decrease led to an increase in hydrolyzed protein due to the breakdown of mineral content in crustaceans, such as calcite and calcium carbonate crystals, surrounded by protein fibers and chitin. An augmentation in lactic acid bacteria (LAB) contributes to pH reduction, as LAB produces endoproteinase enzymes during lactic acid fermentation from shrimp by-products. The higher the lactic acid concentration, the greater the potential for pH reduction. Lowering the pH inhibits the growth of harmful microbes in fermented products and imparts a sour taste. Pratomo et al. [22] asserted that the

rise in titrated acid and pH decrease during fermentation is a result of the acidification activity by lactic acid bacteria.

3.3 Characteristic of chitin extract from vannamei shrimp shell

3.3.1 Ash content

The extraction of chitin from shrimp shells involves two crucial stages: deproteinization and demineralization. Demineralization is the process of eliminating mineral content from shrimp shell by-products, which can be indicated by the ash content in the extract. The ash content of chitin extracted from vannamei shrimp shells at different fermentation durations is illustrated in Figure 2.

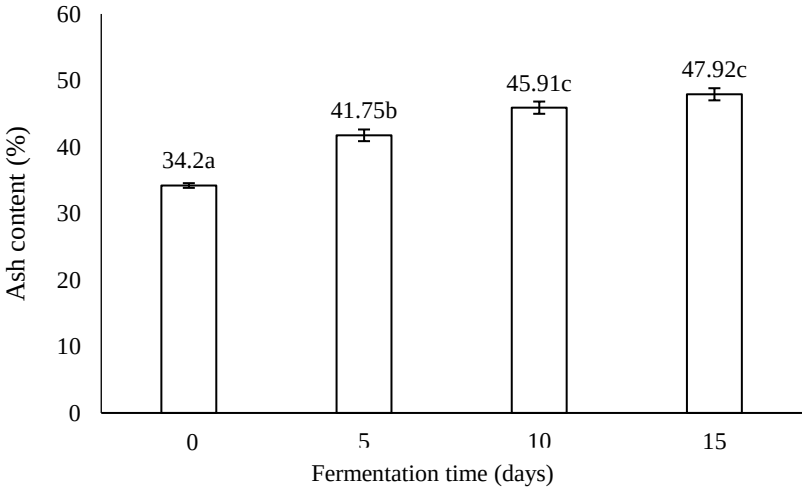

Fig. 2. The ash content of chitin extracted at different fermentation durations

The ash content in chitin extract from vannamei shrimp shells underwent a significant change during fermentation ($p<0.05$). It increased during the 15-day fermentation period, rising from 34.2% to 47.92%. However, there was no significant difference in ash content between the chitin extracts obtained from fermentations lasting 10 and 15 days. These results contradict the findings of Rao and Stevens [12], who showed that the use of *L. plantarum* strain A6 bacteria could reduce the ash content of shrimp shell carapace to 60-80%. The increase in ash content in this study can be attributed to two factors: the presence of adhering lactic acid bacteria cells to the chitin extract and the powdered form of the shrimp shell as raw material making it challenging to distinguish between released minerals and the chitin-protein complex of the shrimp shell. Dead bacterial cells may contribute to the increase in ash content. Dead bacterial cells, when damaged and detached, increase ash content by up to 5-10% of the dry weight [23]. According to Smith et al. [24], this increase is due to damage to cellular components.

Lactic Acid Bacteria (LAB) are known to reduce ash levels by producing lactic acid during the fermentation process. The reaction between lactic acid and calcium carbonate in shrimp shells forms calcium lactate, which settles on the shell's surface [12]. However, in this research, the powdered form hinders the washing process from effectively removing the precipitated minerals/calcium, resulting in an increase in ash content. The ash content parameter specified in SNI 7948:2013 [25] sets a maximum limit of 5% for tin quality requirements. However, the chitin obtained from bioextraction using lactic acid bacteria in this study exceeded this limit, reaching 47%.

3.3.2 Protein content

Biological deproteination relies on protease enzymes produced by proteolytic microbes. Protease enzymes hydrolyze proteins into simpler compounds, specifically peptides from amino acids [26]. This deproteination has a significant impact on the protein content in the shrimp shells. The protein content of chitin extracted at different fermentation durations is illustrated in Figure 3.

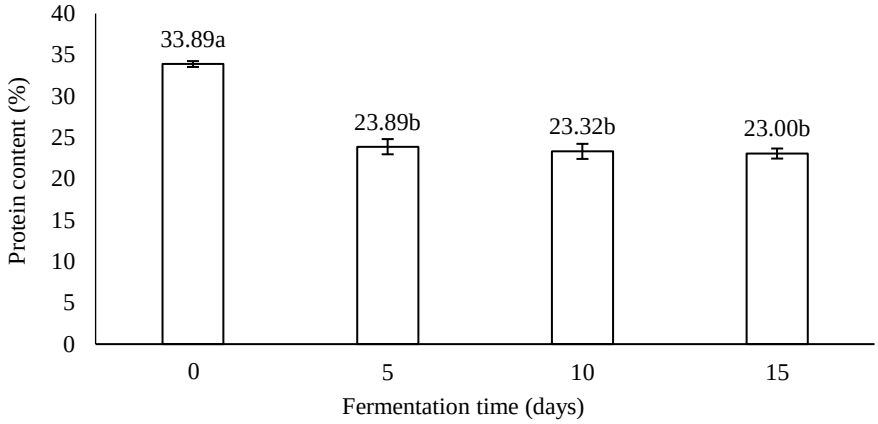

Fig. 3. The protein content of chitin extracted at different fermentation durations.

Fermentation of vannamei shrimp shell can decrease the protein content in the resulting chitin extract ($p<0.05$) (Figure 3). However, the protein content in the chitin extract produced from fermentations lasting 5, 10, and 15 days did not differ significantly. The reduction in protein content in shrimp waste reached 31.29% after 15 days of fermentation. This indicates a deproteinization process during fermentation using lactic acid bacteria. These results are consistent with the findings of Rao and Stevens [12], where fermentation of shrimp waste using *L. plantarum* strain A6 bacteria achieved a deproteinization result of 45.6%. In contrast, Duan et al. [27] reported a significantly higher deproteination value of 97.8% at the end of fermentation using *L. acidophilus* bacteria for chitin extraction from fermented shrimp waste. The success of deproteinization in chitin bioextraction from shrimp shell can be attributed to variations in bacterial cultures, the mass of shrimp waste in fermentation, and the duration of fermentation.

Proteins in shrimp shells can form physical and covalent bonds, with physical bonds removable through size reduction and washing, while covalently bound proteins can be eliminated via chemical or biological methods. Biological deproteination relies on protease enzymes generated by bacteria during fermentation, which hydrolyze peptide bonds in proteins, breaking them down into simpler molecules. The pH value plays a crucial role in this process, influencing protease enzyme activity. *Lactobacillus plantarum*, for instance, exhibits optimal enzymatic activity within a pH range of 5.5 to 6.5 [12]. Deviations from this optimal pH range can result in decreased protease enzyme activity, affecting the efficiency of the deproteination process.

3.3.3 Functional group and degree of deacetylation

Functional groups in the chitin extract from vannamei shrimp shell were analyzed using Fourier Transform Infrared (FTIR) spectroscopy. Additionally, commercial chitin derived from shrimp shell was analyzed as a comparative standard. The FTIR spectra of both samples can be seen in Figure 4.

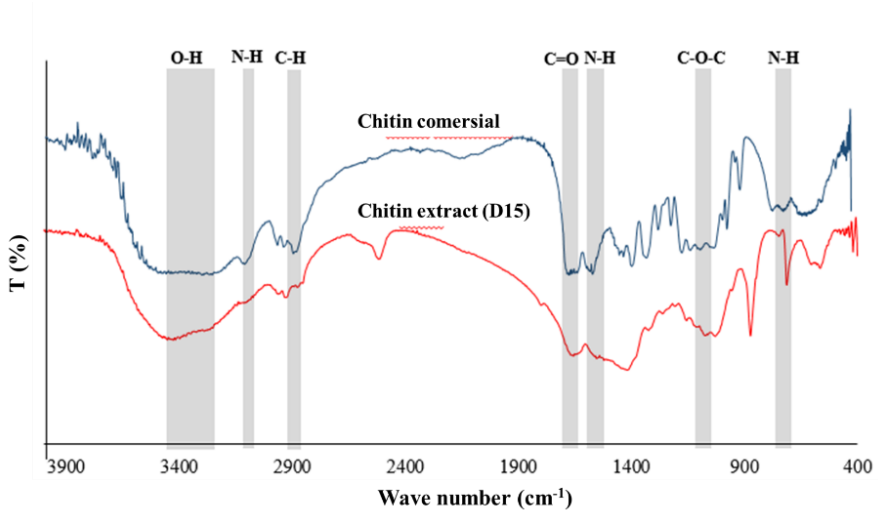

Fig. 4. FTIR spectrum of commercial chitin and chitin extract

The chitin extract exhibits absorption peaks at slightly different wavenumbers compared to commercial chitin (Figure 4). Although, characteristic functional groups of chitin polymer were detected in the chitin extract from this study, including vibrations of O-H, C-H, CH3, C=O, C-O-C, and N-H deformation. In addition, the SH group was detected in the FTIR spectrum of the chitin extract from vannamei shrimp shell at wavenumber around 2500 cm^{-1}. The thiol (-SH) group detected in the chitin extract is likely due to bacterial cell contamination in the extract. Bacteria are known to produce sulfides as a metabolic byproduct [28]. The absorption peaks of wavenumbers in the chitin extract and commercial chitin are summarized in Table 2.

Table 2. Functional group of chitin derived from shrimp

Wave number (cm^{-1})		Group	Group name	Wave number (cm^{-1})
Chitin*	**Chitin extract**			
3575	3453	NH	Amino	3500-3300
3298	3426	OH	Hydroxyl	3600-3200
2891	2890	CH	Hydrocarbons	2840-3000
1660	1655	C=O	Amide 2	1820-1630
1117	1073	COC	Ether	1150-1000

Description: *commercial chitin

Natural chitin primarily forms as α-chitin and β-chitin. Based on the FTIR spectrum, it is known that the type of chitin from shrimp shell is α-chitin. According to Burner et al. (2009), for α-chitin, the amide I band is split into two components at 1660 and 1630 cm^{-1} due to hydrogen bonding effects or the presence of an enol form of the amide part, whereas β-chitin exhibits a band at 1630 cm^{-1}. The amide II band is observed in both chitin spectra: at 1558 cm^{-1} for α-chitin and 1562 cm^{-1} for β-chitin. Moreover, based on the absorption of the amide groups in the FTIR spectrum, it is known that the chitin extract from vannamei shrimp shell in this study has a deacetylation degree of 36%. This result is close to the findings of Dompeipen [29], where the deacetylation degree of the produced chitin was 37.88%. According to the Indonesian National Standard, a degree of deacetylation below 60% classifies the polymer as chitin, while values above 60% categorize it as chitosan.

4 Conclusion

Chitin bioextraction from vannamei shrimp shell using a mixture of lactic acid bacteria, including isolates (*Lactobacillus plantarum SK (5), Lactobacillus plantarum NS (9),* and *Pediococcus pentosaceus BP (20)*), was successfully conducted for a fermentation period of 15 days. The LAB isolates contributed to the decrease in pH and protein content during the fermentation process. The extract obtained from this fermentation exhibited characteristic functional groups of chitin polymer, including vibrations of O-H, C-H, CH_3, C=O, C-O-C, and N-H deformation, with a deacetylation degree of 36%. However, the protein and ash content in the chitin extract obtained still do not meet the quality standards for chitin based on SNI (Indonesian National Standard).

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