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# Effect of Soaking and Proteolytic Microorganisms Growth on the Protein and Amino Acid Content of Jack Bean Tempeh (*Canavalia ensiformis*)

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**Abstract:** Soaking is an important step in making tempeh. Tempeh fermentation normally involves the natural presence of proteolytic bacteria capable of producing protease enzymes to break down peptide bonds in protein molecules. This study evaluated the protein and amino acid content of Jack bean tempeh (*Canavalia ensiformis*) soaked in distilled water during natural fermentation for 12, 24, 36, and 48 h. In this study, the crude proteins were determined using the Kjeldahl technique, amino acids were determined from protein hydrolysis, and proteolytic bacteria were enumerated for Total Plate Counts and further identified using Vitek 2.0 Compact System. The results showed that soaked Jack beans have higher protein and amino acid content, with sixteen essential and non-essential amino acids required for human bodies. The protein content of soaked Jack beans varied from 35% at 12 h to 32% at 24 and 36 h and 33% at 48 h. Soaking for 12 h yielded the highest amino acid concentration of 38,000 mg/kg L-glutamate, and the lowest of 14,000 mg/kg L-proline. Seven isolated bacteria showed proteolytic activity on Skim Milk Agar with a clear zone of 3.00 mm to 10.65 mm surrounding the colony. The bacteria identified were *Pediococcus pentosaceus*, *Stenorophomonas maltophilia*, *Cronobacter sakazakii*, and *Klebsiella pneumonia* ssp. In summary, Lactobacillaceae and Enterobacteriaceae were the predominant bacteria during tempeh fermentation, indicating the synergistic interaction between these microflorae during soaking conditions as part of their survival in this hostile environment.

**Keywords:** Jack bean tempeh; protein; proteolytic bacteria; soaking process



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## 1. Introduction

Tempeh is a fermented food that is very popular in Indonesia. Tempeh, a traditional fermented food originating from Indonesia, is primarily made from soybeans, and is known for its high protein content, desirable amino acid profile, and various health benefits [1,2]. Tempeh can greatly reduce total cholesterol, LDL cholesterol, and triglycerides levels in the blood [3]. Tempeh is also known for its high protein content and digestibility. Tempeh is a fermented food with high protein content, and may contain enzymes, particularly protease [4,5]. Tempeh is prepared in many steps, including soaking, peeling, heating, inoculating with mold, and fermenting at 30 °C for 36 h [6]. Each stage has a purpose, and helps to increase the nutritional content of the product [7]. Furthermore, tempeh fermentation has been shown to reduce the levels of antinutritional factors such as phytic acid and trypsin inhibitors, enhancing the nutritional quality of the final product [1].

Tempeh is a fermented bean product made by mixing with *Rhizopus oligosporus*. Jack beans are a type of nut that can be used to make tempeh [8]. Jack beans have properties similar to soybeans. Since Jack beans are cheaper than soybeans, tempeh producers

benefit from using this raw material. Jack beans (*Canavalia ensiformis*) have gained interest for tempeh production due to their potential nutritional benefits, affordability, and ability to diversify food sources [9]. Jack bean tempeh contains 29.04% protein, and all necessary and non-essential amino acids [10,11]. It has excellent protein quality during fermentation, becoming a partially hydrolyzed protein with high digestibility and reduced allergenicity [12].

Soaking Jack beans can improve their nutritional value by reducing the levels of antinutrients, and making more of the beneficial nutrients available for absorption [13]. Soaking can also help to break down nutrients, making the beans easier to digest [14]. Typically, the soaking process for Jack bean tempeh takes between 12 and 36 h. The multiplication of microorganisms is represented by foam on the water surface during the soaking phase. Lactic acid bacteria dominate the bacterial community, with a concentration of $10^6$–$10^9$ CFU/g and a pH of 4.5–5.0 [15,16]. Their ability to lower pH through the formation of protease enzymes has proven beneficial for fermented foods.

Microorganisms require water and low relative humidity to survive. Foaming on the wet surface and a pH range of 4.5–5.0 indicate bacterial proliferation during the soaking process. Soaked legumes have been found to contain lactic acid bacteria, Enterobacteriaceae, and a dominant yeast [17]. Proteolytic bacteria can generate protease enzymes and convert proteins to polypeptides, oligopeptides, and amino acids through hydrolysis [18]. As a result of this process, the nutritional content of the raw materials becomes more easily digestible in the body [19].

This study focuses on soaking and proteolytic microorganism growth effects on Jack bean tempeh's protein and amino acid content. Soaking is an essential step in tempeh production, as it helps soften the beans, removes antinutritional factors, and facilitates the growth of microorganisms [20–22]. However, the optimal soaking duration to maximize the nutritional benefits of tempeh remains unclear, and the impacts of various soaking durations on the growth of proteolytic microorganisms have not been thoroughly investigated. To address this knowledge gap, this study investigated Jack bean tempeh's protein and amino acid content, and examined proteolytic bacterial growth's impacts during the soaking process. The soaking experiments with varying durations of 12, 24, 36, and 48 h were carried out to determine when microbial growth increased. After the soaking process, the bacteria were removed, and their effects on Jack bean tempeh's protein and amino acid content were analyzed.

This research aims to contribute to understanding the relationship between soaking duration, proteolytic microorganism growth, and the nutritional properties of Jack bean tempeh. The findings may have practical implications for tempeh producers and consumers seeking to optimize the nutritional benefits of this versatile and nutritious food source, particularly in regions where soybean production is limited, or where alternative legumes could contribute to increased food security and diversification [1,2,9].

## 2. Materials and Methods

### 2.1. Materials

The study was conducted from September 2019 to February 2020 at the Food Microbiology Laboratory, Faculty of Agro-Industrial Technology, Universitas Padjadjaran, Bandung, Indonesia. It required four to six months post-harvest Jack beans (Central Java, Indonesia), distilled water, NaCl (Merck, Darmstadt, Germany), de Man Rogosa and Sharpe (MRS) Agar (Merck, Germany) with 0.8% Calcium Carbonate (Merck, Germany), Yeast and Mold Agar (Merck, Germany), Plate Count Agar (PCA) (Merck, Germany), Nutrient Agar (Merck, Germany), Skim Milk Agar (Merck, Germany), a pH meter, and a Vitek 2.0 Compact System (Laboratory of Biofarma, Bandung, Indonesia).

### 2.2. Crude Protein

Analysis of crude protein was performed using the Kjeldahl technique [23]. An amount of 10 milliliters of the sample was added to a Kjeldahl flask containing 0.15 g $CuSO_4$,

5 g $Na_2SO_4$, and 20 mL of concentrated $H_2SO_4$. The flask was then placed in a distillation device, and 50% NaOH was added. The two liquid layers were boiled until they combined, and the distillate was collected in an Erlenmeyer flask containing 10 mL of 0.1 N standard hydrochloric acid and 3 drops of methyl red indicator. Distillation was continued until the volume reached 75 mL, and then titration was performed with 0.1 N NaOH standard solution until the solution turned yellow. Protein conversion, % Protein, was calculated using the conversion factor of % Nitrogen *5.71.

### *2.3. Determination of Amino Acids*

The ACQUITY UPLC system (Waters, Milford, MA, USA) analyzed 17 amino acids. The system consisted of a thermostat, autosampler, high-pressure binary pump, and photodiode array detector (PDA). The samples were separated chromatographically using the AccQ-Tag Ultra C-18 Column (2.1 mm × 100 mm; 1.7 µm).

### *2.4. Isolation of Proteolytic Microorganisms*

#### 2.4.1. Sample Preparation

Jack beans were obtained for the study from four to six months post-harvest in Central Java, Indonesia. The samples were soaked in distilled water for 0, 12, 24, 36, or 48 h at 30 °C and 60% relative humidity.

#### 2.4.2. Total Bacterial Counts for Microbiological Assessments

Approximately 25 mL of bean-soaking water was added to 225 mL of 0.85 percent (*w/v*) sodium chloride (Merck, Germany) solution. The mixture was diluted 1:9 in NaCl, and dilutions from $10^{-4}$ to $10^{-6}$ were tested. A 100 mL aliquot of each dilution was plated on de Man Rogosa and Sharpe Agar (MRSA) with 0.8% calcium carbonate (Merck, Germany) to determine Lactic Acid Bacteria Count, on Yeast and Mold Agar media (YMA) (Merck, Germany) to determine Yeast and Mold Count, and on Plate Count Agar media (Merck, Germany) to determine the Total Plate Counts of mesophilic bacteria, yeast, and mold (log $CFUg^{-1}$). Each assay was performed in triplicate, and the plates were incubated at 30–37 °C for 24–48 h to allow bacteria to grow, and 5 days at 25 °C to allow yeast and mold to grow [24].

#### 2.4.3. Bacteria Subculture and Purification

After 72 h of incubation, the bacterial colonies were removed from the plates using an illuminated loop [25]. Subsequently, a fresh nutrient agar plate was prepared for sub-culturing the bacterial colonies, while various-colored mycelial colonies were transferred from the YMA plate to a new YMA plate with the blades illuminated. All plates were then placed in an incubator at a constant temperature for an extended period. The bacterial colonies were cultured at 37 °C for 18–48 h on Nutrient Agar plates, and at 25 °C for 3–5 days on YMA plates. Purified colonies and mycelia were then transferred to an agar slant and appropriately stored for subsequent microorganism screening and characterization.

#### 2.4.4. Screening Proteolytic Microorganisms by Qualitative Assay

The microbes isolated from the previous steps were subcultured, purified, and screened for proteolytic activity using Skim Milk Agar [26,27]. Skim Milk Agar plates were prepared by combining autoclaved agar ingredients with 1% pasteurized skim milk, and the isolated bacteria were swabbed onto them. The proteolytic candidates were incubated at 37 °C for 48 h, and the resulting clear zone around each well was measured to quantify proteolytic activity.

The Gram staining procedure was conducted to identify bacteria based on color, shape, and appearance under a microscope. The process involved treating bacterial samples with crystal violet, Lugol's iodine, 95% alcohol, and safranin. After each staining step, the

samples were washed with distilled water. The stained bacterial colonies were observed under oil immersion at 1000× magnification under a light microscope.

### *2.5. Phenotypic Identification of Proteolytic Bacteria Using the Vitek 2.0 Compact System*

The Vitek 2.0 Compact System, located at the Laboratory of Biofarma, Indonesia, was used to identify and characterize the extracted bacteria with proteolytic potential. This system utilizes phenotypic characterization to determine the types and species of microorganisms. Special cards detect microorganisms, with GP cards indicating Gram-positive bacteria, and GN cards indicating Gram-negative bacteria.

### *2.6. Statistical Analysis*

Data analysis was determined by one-way analysis of variance (ANOVA) to assess the significant difference among the samples. Statistical analysis using SPSS software version 25 was used, and $p$-value < 0.05 indicated the significant differences.

## 3. Results and Discussion

### *3.1. Crude Protein*

Jack beans are high-protein food, with protein content comparable to soybeans, with a crude protein level of between 28% and 35%. The crude protein content of raw Jack beans is 29.71%. The protein content of Jack beans was determined using each soaking technique from 0 to 12 h, except for Jack beans without an epidermis, due to the growth of lactic acid bacteria during soaking [28,29]. After 12 h, the number of bacterial populations in whole Jack beans increased, but they were not used to make tempeh. The epidermis of the beans was removed after soaking, and *Rhizopus oligosporus* was used to ferment them to make tempeh [17]. The result of the Jack beans' protein content according to soaking time are shown in Figure 1.

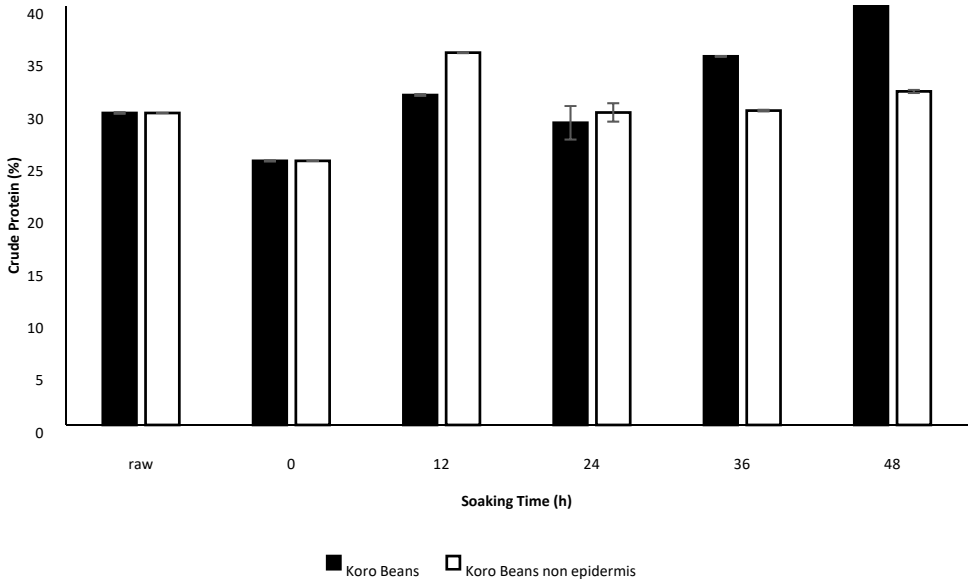



**Figure 1.** Crude protein content in soaked dehulled Jack beans.

Jack beans have a high protein content, which makes them a good source of essential nutrients, especially when consumed in moderation [30]. Consuming these seeds can significantly increase one's diet's overall amount of protein. Previous findings suggest cooking and soaking beans can substantially change their nutritional profile [28]. After 24 h of soaking, Jack beans had 30% protein. In contrast, dehulled Jack beans contained 32% protein, indicating a small reduction in protein concentration compared to Jack beans soaked for 12 h. According to a study by Kanetro et al. (2021) [31], after 36 and 48 h of soaking, the protein content of Jack beans increased. However, Jack seed germination

lowered protein content after 24 h of soaking, but consistently increased it after 72 h. During germination, there are food mobilization reserves that undergo a degradation process, and the composition of substances favors that required to grow seeds into new plants [32]. Protein levels are also increased during the 18-h germination period of horse gram (*Macrotyloma uniflorum* (Lam.) [33]. Carbohydrates are the principal energy source throughout the germination process. As the protein will be utilized in the late stages of embryonic development, the rise in protein content after germination was driven by a quicker drop in carbohydrate and fat percentages than protein. During germination, protein synthesis and breakdown of bean components might increase the protein concentration.

There was a significant increase in protein over the 48-h soaking period. This increase can be attributed to higher protein solubility, as measured by increased free amino acid levels [34]. During germination, the activity of protease enzymes that hydrolyze proteins increases. Protein hydrolysis results in the formation of short peptide chains, or a decrease in the molecular weight of the protein, thereby increasing its solubility. Hydrolysis of proteins affects hydrogen bonds and hydrophilic interactions, contributing to protein solubility. Germination occurs when seeds are soaked in water, causing the protein structure to break down, and increasing the solubility of the protein.

### *3.2. Amino Acid Content in Raw Jack Beans and Jack Beans Soaked for 12 h*

The amino acid content of raw Jack beans and dehulled Jack beans after 12 h of soaking is displayed in Figure 2. Jack beans from Central Java contain all the necessary amino acids and several non-essential amino acids. The soaking procedure reduced amino acid content, making the protein more water-soluble [11]. Tryptophan, methionine, histidine, threonine, proline, leucine, lysine, arginine, valine, isoleucine, and phenylalanine are all necessary amino acids. Non-essential amino acids include tyrosine, aspartate, glycine, alanine, glutamine, and serine. In terms of non-essential amino acids, the highest-scoring essential amino acids are leucine and glutamate. Leucine is required for the development of the human body, particularly that of children [35,36]. Tryptophan and lysine amino acids are markers of child stunting.

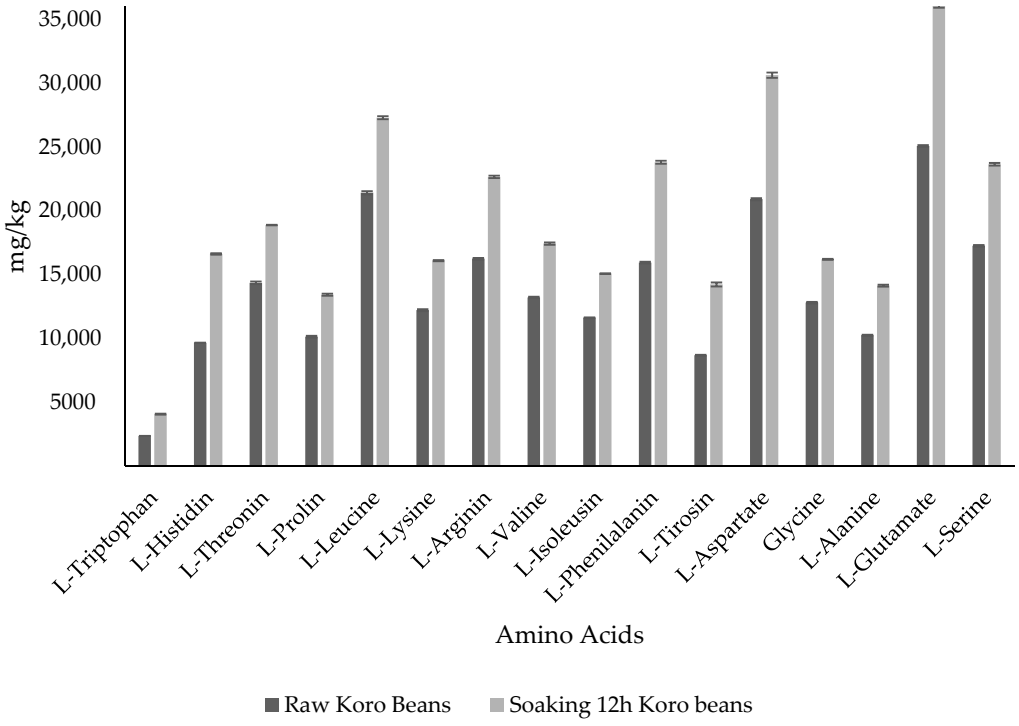



**Figure 2.** Amino acid content in raw Jack beans and 12 h soaking Jack beans.

The germination process requires amino acids to promote growth, leading to significant changes in amino acid levels compared to Jack beans [31]. Similar to germinated Jack beans, soybean germination also alters the number and types of amino acids, affecting the functional properties of seed flour. As mentioned in the previous section, these properties include oil absorption, water absorption, foaming, and emulsifying capacity of seeds. The improvement in foaming capacity of growing in Jack bean flour for 72 h compared to seed flour is supported by a significant increase in some hydrophobic amino acids in germinated seed flour.

In general, soaking can increase the availability of amino acids in certain foods by breaking down anti-nutrients such as phytic acid and tannins [37]. These anti-nutrients can bind to minerals and proteins, making them less available for absorption in the body. Soaking can help reduce these compounds' levels, thereby increasing the bioavailability of amino acids and other nutrients [38].

Figure 2 illustrates the results of the 12-h soaking process, which showed an increase in certain amino acids, consistent with a study by Wongsiri et al. (2015) [39], where essential amino acids, including leucine, lysine, and valine, increased while other essential amino acids decreased. The amino acid profile of Jack bean is particularly notable for its high content of lysine, which is an essential amino acid that is often limited in other plant-based protein sources [40]. Lysine is important for protein synthesis, tissue repair, and producing hormones, enzymes, and antibodies. In addition to lysine, Jack bean also contains significant amounts of other essential amino acids such as methionine, threonine, and tryptophan, which are also important for various biological processes in the body. Similar research by Sujak et al. (2014) [41] found that soaking Jack beans for 24 h increased their lysine content by up to 11%, and their methionine content by up to 13%. Another study published by Ogunsina et al. [42] found that soaking Jackbeans for 12 h increased their protein digestibility and the availability of certain essential amino acids, including lysine and threonine. An increase in leucine levels during soaking process can improve the nutritional value of protein and the potential of Jack beans as a functional food to prevent diabetes, since leucine is the primary amino acid that can increase insulin [43].

Soaking can be a beneficial practice for increasing the availability of amino acids in certain foods [44]. The increased levels of foaming capacity, essential amino acids, and insulin-stimulating amino acids observed in the 72-h germination of Jack bean flour suggest that germinated flour can be used as an additive to improve food products.

### *3.3. Microbial Communities from Soaked Jack Beans*

Table 1 depicts the growth of microorganisms during the soaking process. Soaking beans in water can increase the growth of microorganisms such as bacteria, yeast, and fungi, which can proliferate easily in a humid environment [45]. The formation of bubbles or foam on the soaked beans indicates the proliferation of microorganisms. Moreover, the growth of microorganisms affects the pH of the soaking water. As shown in Table 1, the pH of the soaked Jack beans decreased as the soaking time increased. After the soaking phase, the pH was 5.89. The decrease in pH indicated the presence of a colony of microbes during the soaking process that produced lactic acid [46]. The breakdown of nutrients by microbes during the soaking phase increased lactic acid.

During the soaking process of legumes, a diverse range of microorganisms, including bacteria, fungi, and molds, can proliferate in the aqueous environment. As depicted in Table 1, these microorganisms thrive due to the presence of nutrients, moisture, and favorable conditions for growth. The proliferation of these microorganisms can have both positive and negative impacts on the nutritional properties and safety of the final product, making it essential to monitor and control their growth during the tempeh production process. The total plate count method was used to analyze the aerobic and mesophilic microorganisms. MRS Agar is a selective medium which is commonly used to isolate lactic acid bacteria [47], PCA for mesophilic organisms, and YMA for yeast and mold organisms [48]. As depicted in Table 1, the optimal growth conditions for mesophilic yeast

and mold organisms were aerobic conditions, and moderate temperatures ranging from 20–45 °C, which led to increased development of these microorganisms on PCA and YMA during the soaking phase. Low microbial count of TPC and YMA indicate good sanitation and hygienic practices have been implemented during food production [49].

**Table 1.** Microorganism colony counts in various culture media and pH Value.

| Soaking Time (h) | Plate Count Agar ($log_{10}$ CFU $g^{-1}$) | Yeast and Mold Agar ($log_{10}$ CFU $g^{-1}$) | de Man Rogosa and Sharpe Agar ($log_{10}$ CFU $g^{-1}$) | pH |
|---|---|---|---|---|
| 0 h | $5.50 \pm 0.07^{a}$ | $5.68 \pm 0.06^{a}$ | $3.22 \pm 0.06^{a}$ | $7.00 \pm 0.00^{c}$ |
| 12 h | $6.58 \pm 0.06^{b}$ | $6.68 \pm 0.00^{b}$ | $4.18 \pm 0.14^{b}$ | $6.42 \pm 0.23^{b}$ |
| 24 h | $7.66 \pm 0.01^{c}$ | $7.62 \pm 0.03^{c}$ | $5.04 \pm 0.00^{c}$ | $5.96 \pm 0.25^{a}$ |
| 36 h | $7.37 \pm 0.13^{d}$ | $7.97 \pm 0.09^{d}$ | $6.35 \pm 0.50^{d}$ | $5.93 \pm 0.30^{a}$ |
| 48 h | $9.70 \pm 0.06^{e}$ | $9.65 \pm 0.08^{e}$ | $7.66 \pm 0.43^{e}$ | $5.89 \pm 0.27^{a}$ |

Notes: Treatment means labeled with the same lowercase letters were not significantly different at the 5% Duncan's multiple distance test level.

According to Table 1, the total amount of lactic acid bacteria increased during the soaking process, which reduced pH, as reported by previous studies [16,24]. The presence of lactic acid bacteria can be used to assess the quality of fermented products [47,48]. The pH value indicates whether fermentation can proceed without the addition of other microorganisms to control the fermentation process. As shown in Table 1, the growth of lactic acid bacteria increased with longer soaking times. This suggests that the 48-h soaking period produces a superior fermented product compared to the non-soaking technique.

### *3.4. Isolation and Screening of Proteolytic Microorganisms by Qualitative Assay*

#### 3.4.1. Screening of Proteolytic Microorganisms Using Skim Milk Agar (SMA)

The screening process involved the use of selective medium—Skim Milk Agar (SMA)—as a protein source. Microbial identification was performed to determine the type of microbes that grew in Jack beans [50]. SMA medium is commonly used to isolate proteolytic bacteria, and is composed primarily of lactose (as a carbon source) and casein (as a nitrogen source) [51].

The formation of a clear zone surrounding the colony defined the growth of proteolytic bacteria. These bacteria degraded casein in the SMA medium, resulting in a distinct-colored region of organic acids [52,53]. Seven bacterial isolates recovered from the Jack beans soaking procedure were able to create extracellular protease, as evidenced by the clear zone generated surrounding their growth on Skim Milk Agar [54]. Based on the results presented in Table 2, seven isolates were obtained and subjected to morphological examination to establish their features. The clear zone diameters for each isolate were also measured, with isolate 1 displaying the highest proteolytic activity ($22.4 \pm 2.82$ mm), followed by isolate 2 ($21.95 \pm 0.99$ mm). Isolates 5 and 7 exhibited the lowest proteolytic activity, with clear zone diameters of $3.8 \pm 0.57$ and $5.15 \pm 1.77$ mm, respectively.

#### 3.4.2. Morphological Characteristics of Bacterial Isolates

Seven bacterial isolates were obtained from the complex colonies and characterized using microscopic and colonial morphology measures, including Gram stain, shape, color, texture, form, and elevation (Table 3). All seven isolates exhibited a smooth texture, with isolates 5 and 7 being white, and isolates 1, 2, 3, 4, and 6 being cream-colored. The absence of chromogens in a bacterial colony can be identified by its white color, while bacteria with chromogens can display various colors. The expansion of the colonies can also reveal distinct colors, as seen in this study, where the colonies appeared cream-colored. Bacteria with chromogens can create pigments that can dissolve and diffuse into the medium, thereby changing the color of the medium [55].

**Table 2.** The Qualitative Proteolytic Assay by Clear Zone (mm).

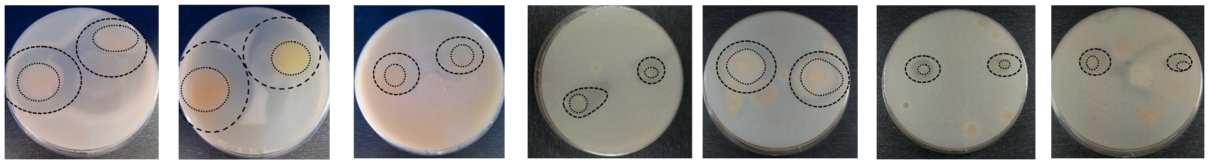
| No | Isolate | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| Soaking Time | 0 h | 12 h | 12 h | 12 h | 12 h | 24 h | 48 h |
| All diameter ◌ (mm) | 35.6 ± 0.14 | 32.7 ± 0.57 | 22.7 ± 0.85 | 25.55 ± 3.18 | 23.0 ± 0.70 | 29.65 ± 1.20 | 10.2 ± 0.14 |
| Colony's diameter ◌ (mm) | 13.2 ± 2.97 | 10.75 ± 0.35 | 11.4 ± 0.14 | 14.9 ± 2.55 | 1.92 ± 0.14 | 20.1 ± 0.14 | 5.05 ± 1.91 |
| Clear zone diameter (mm) | 22.4 ± 2.82 | 21.95 ± 0.99 | 11.30 ± 0.99 | 10.65 ± 0.63 | 3.8 ± 0.57 | 9.55 ± 1.06 | 5.15 ± 1.77 |
| |  | | | | | | |

**Table 3.** Seven bacterial isolates by microscopic and colonial morphology measures.

| No. | Isolate | Soaking Time | Texture | Color | Shape | | Elevation | Gram Staining |
|---|---|---|---|---|---|---|---|---|
| | | | | | **Rod** | **Coccus** | | |
| 1 | Isolate 1 | 0 h | Smooth | Cream | √ | X | Convex | negative |
| 2 | Isolate 2 | 12 h | Smooth | Cream | √ | X | Convex | negative |
| 3 | Isolate 3 | 12 h | Smooth | Cream | √ | X | Convex | negative |
| 4 | Isolate 4 | 12 h | Smooth | Cream | x | √ | Flat | positive |
| 5 | Isolate 5 | 12 h | Smooth | White | x | √ | Flat | positive |
| 6 | Isolate 6 | 24 h | Smooth | Cream | x | √ | Flat | positive |
| 7 | Isolate 7 | 48 h | Smooth | White | x | √ | Flat | positive |

Key: √ = yes; x = no.

The bacterial isolates identified in Table 3 were primarily coccus- and rod-shaped. Isolates 4–7 had a coccus shape and belonged to the Gram-positive bacteria as determined by Gram staining. Rod-shaped bacteria were present in isolates 1–3, which were found to be Gram-negative bacteria.

In addition, isolates 1–3 exhibited a convex elevation, while isolates 4–7 had a flat elevation, according to Table 3. Elevation refers to the colony's degree of flatness or convexity on the surface agar media. This can be observed by looking at the Petri dish's side: if the colonies form a mound (convex), it indicates that the bacteria are stacked on top of one another; in contrast, if the colony's height is flat, and bacterial growth is seen at the margins, it indicates a different growth pattern.

### *3.5. Identification of Proteolytic Microorganisms by Biochemical Tests*

In order to determine the potential of proteolytic bacteria in the isolates, biochemical activity was analyzed using the Vitek 2.0 compact cased system [56]. The Vitek 2.0 system employs identification cards for Gram-positive (GP) and Gram-negative (GN) bacteria. In this study, 43 biochemical tests were conducted to measure the utilization of carbon sources, enzymatic activity, and bacterial resistance.

The identification cards were used to determine the confidence level of the selected bacteria [57]. A confidence level close to 99% indicates a closer match to the typical pattern of a particular bacterium. The level of acceptance is categorized based on the probability score: 96–99% probability indicates an excellent confidence level of selected bacteria,

93–95% probability is classified as very good, 89–92% probability is considered good, and 85–88% probability is considered an acceptable level of confidence [58].

The Analytical Profile Index or a manual kit can quickly identify a microorganism. The Manual Kit includes 20 microtubules and a dehydrated substrate. When the bacteria are inoculated and cultured, a color shift can occur upon applying a reagent to the wells. The color change indicates the similarity of a bacterium's metabolic features to those of a particular bacterium stated in an identification table [59].

#### 3.5.1. Gram-Negative (GN) Bacterial Isolates

Table 4 shows the identification of Gram-negative bacteria with reaction performance, including those identified correctly and those mis- or unidentified in GN well test. Gram-negative bacteria are microorganisms with a thin layer of peptidoglycan that stain pink during staining [60].

**Table 4.** Gram-Negative Card Bacterial Identification using Vitek 2.0 system.

| Isolate | Isolate 1 | Isolate 2 | Isolate 3 |
|---|---|---|---|
| Soaking Time | 0 h | 12 h | 12 h |
| Result | *Stenorophomonas maltophilia* | *Cronobacter sakazakii* | *Klebsiella pneumonia* spp. |
| Degree of accuracy in identification | 99% excellent | 99% excellent | 97% excellent |

The microorganisms identified by the Gram-negative (GN) card were isolates that had originally stained Gram-negative, namely, isolate 1, isolate 2, and isolate 3. The GN identification card can identify these three isolates with a 99% probability confidence level. The Gram-negative organism was identified with the GN Identification Card, a single-use, disposable card based on established biochemical methods [61]. GN well contains carbon sources (D-Maltose, D-Mannitol, D-Mannose, etc.), enzyme-substrates (Ala-Phe-Pro-Arylamidase, D-Cellobiose, β-galactosidase, etc.), and substrates for hydrolysis compounds. The GN well contains specific Gram-negative bacteria, which will yield a positive sign (+) with 95–100% compatibility, and a negative sign (−) with 0–5% compatibility [61]. Isolate 1 was identified as *Stenorophomonas maltophilia* with a 99% probability of accuracy, indicating excellent identification organism of the organism. *Stenorophomonas maltophilia* belongs to the Proteobacteria phylum, the Gammaproteobacteria class, and the Xanthomonadales family [62].

Isolate 2 was identified as *Cronobacter sakazakii* using the GN card with a 99% probability and excellent identification confidence. *Cronobacter sakazakii* belongs to the Proteobacteria phylum, Gammaproteobacteria class, and Enterobacteriaceae family. Isolate 3 was identified as *Klebsiella pneumoniae* ssp. *pneumoniae* with a 97% probability and excellent identification confidence. *Klebsiella pneumoniae* ssp. *pneumoniae* is also a member of the Proteobacteria phylum, Gammaproteobacteria class, and Enterobacteriaceae family [16,63].

According to Table 4, all isolated bacteria belonged to Proteobacteria phylum, with an excellent confidence level of identification. All bacterial isolates showed positive reactions (+) to β-N-Acetyl-Glucosaminidase, β-Glucosidase, Citrate (Sodium), and L-Lactate Alkalinization.

#### 3.5.2. Gram-Positive (GP) Bacterial Isolates

Gram-positive bacteria are microorganisms that have thick peptidoglycan cell wall layers. They can retain the violet color (violet crystals) during staining analysis [61]. The results of GP cards used to test Jack beans are shown in Table 5. The microorganisms identified by the Gram-positive (GP) card were isolates that had originally stained Gram-positive, namely, isolate 4, isolate 5, isolate 6, and isolate 7. The 64 wells in the GP test were used to identify Gram-positive bacteria in the soaking Jack beans.

The GP supplement test included carbon sources (D-Maltose, D-Mannitol, D-Raffinose, etc.), enzyme-substrates (β-glucosidase, α-L-Fucosidase, β-D-Fucosidase, etc.), and a substrate for hydrolysis compound.

**Table 5.** Gram Positive Card Bacteria Identification using Vitek 2.0 Systems.

| Isolate | Isolate 4 | Isolate 5 | Isolate 6 | Isolate 7 |
|---|---|---|---|---|
| Soaking Time | 12 h | 12 h | 24 h | 48 h |
| Result | *Enterococcus durans* | *Staphylococcus aureus* | *Enterococcus faecium* | *Pediococcus pentosaceus* |
| Degree of accuracy in identification | 99% excellent | 99% excellent | 95% very good | 88% acceptable |

Table 5 identifies Gram-positive microorganisms with reaction performance, including those identified correctly and those mis- or unidentified in GP well test. Four isolates were identified using the GP card due to their purple stain, which indicated Gram-positive staining. These isolates were *Staphylococcus aureus*, *Enterococcus faecium*, *Enterococcus durans*, and *Pediococcus pentosaceus*. The identification of *Enterococcus durans* with a 95% probability indicated excellent identification confidence in isolate 4. *Staphylococcus aureus* was identified using Vitek 2.0 with a 99% probability, indicating excellent identification confidence in isolate 5. These isolates belong to the Firmicutes phylum, Bacilli class, and Staphylococcaceae family.

*Staphylococcus aureus* is a candidate proteolytic bacterium because it produces several major extracellular proteases, including Staphylococcal serine protease, cysteine protease, metalloprotease, and staphopain. *Staphylococcus aureus* is a pathogenic bacterium that is commonly found in soil and on the skin. During the soaking process of the beans, it was found that *Staphylococcus aureus* was able to survive in acidic conditions, with the ability to grow and survive at pH $\geq$4.6 [1,17].

Isolate 6 was identified as *Enterococcus faecium* with a 95% probability, indicating very good identification, and belonged to the Firmicutes phylum, Bacilli class, and Enterococcaceae family [17]. Isolate 7 was identified as *Pediococcus pentosaceus* with an 88% probability, which is an acceptable confidence level. *Pediococcus pentosaceus* belongs to the Firmicutes phylum, Bacilli class, and Lactobacillaceae family [1].

The total lactic acid bacteria showed high variability during the soaking process, including *Enterococcus pseudoavium*, *Enterococcus sulfureus*, *Enterococcus italicus*, and *Pediococcus pentosaceus*. *Enterococcus* is a predominant bacterial population during the tempeh process [24]. *Enterococci* are lactic acid bacteria that are important in the food and dairy product industry because of their proteolytic and lipase activities, as well as their citric metabolism, probiotic activity, and bacteriocin production [64]. Bacteriocin production of *Enterococcus* sp. is used to extend the shelf life of tempeh. *Enterococcus durans* and *Enterococcus faecium* are used as starters in food fermentation due to their enzymatic and proteolytic activities, which act as probiotics [65].

During the back-slop soaking of soybeans for making tempeh, the predominant microbe found was *Pediococcus* sp. [1]. Upon analysis, *Pediococcus pentosaceus* was found to be the predominant bacteria. *Pediococcus pentosaceus* stabilized the pH value and titratable acidity later in fermentation [66]. *Pediococcus pentosaceus* produced the highest proteolytic activity as a lactic acid bacterium, particularly in acidic conditions. Proteolytic activity in all the studied LAB may contribute to producing potential amino acids [67]. *Pediococcus pentosaceus* contained isoleucine, proline, glutamate, and glycine, with proline amino acid being the highest percentage. The proteolytic activity of *Pediococcus* sp. was half that of the amount of proteolytic activity of *Lactobacillus* sp., which indicated 6 U/mg to 11 U/mg. This shows lactic acid bacteria's highest extracellular proteolytic activity [68].

Based on analysis, Jack beans should be submerged in water for several hours to soften and hydrate them. This creates a moist environment that is favorable for the

growth of bacteria. Lactobacillaceae and Enterobacteriaceae can survive and thrive in this environment. The bacteria break down complex carbohydrates and proteins into small molecules.

The Lactobacillaceae and Enterobacteriaceae have synergistic interaction microflorae during the soaking process. Lactobacillaceae bacteria produce lactic acid, which helps lower the environment's pH, and create a more acidic environment, favorable for the growth of the fungi that will eventually form the tempeh. The Enterobacteriaceae bacteria produce enzymes that help to break down the complex carbohydrates and proteins in soybeans, making them more easily digestible and nutritious. Lactobacillaceae and Enterobacteriaceae interaction during tempeh fermentation is an important part of the process, and their synergistic interaction helps ensure the fermentation's success and the production of high-quality, nutritious tempeh.

## 4. Conclusions

Soaking Jack beans in distilled water for different periods during tempeh fermentation results in higher protein and amino acid content—sixteen essential and non-essential amino acids required for the human body. The soaked Jack beans' highest protein and amino acids were after 12 h, with 35% crude protein and L-glutamate amino acid; the lowest was L-proline. Proteolytic microbial species have been found in the soaking process, indicated by a clear zone around the colony on SMA agar. Biochemical analysis using the Vitek 2.0 system identified *Staphylococcus aureus*, *Enterococcus faecium*, *Enterococcus durans*, *Pediococcus pentosaceus*, *Stenorophomonas maltophilia*, *Cronobacter sakazakii*, and *Klebsiella pneumonia* ssp. All microbial species found in the soaking process, namely Lactobacillus and Enterobacteriaceae, thrived in this environment. These two types of bacteria can help create a favorable environment for the growth of the fungus *Rhizopus oligosporus*, the primary organism responsible for the fermentation process that transforms the soaked beans into tempeh. Optimizing the soaking process for Jack beans tempeh increases the crude protein, improves quality of tempeh, and promotes greater interest and investment in producing alternative protein sources.

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