Research Article

The Role of *Bacillus subtilis* in Increasing the Quality and Nutritional Content of Sago Pith

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Abstract | This study aimed to determine the optimal substrate type and fermentation time for improving the quality and nutritional value of fermented sago pith (FSP). The materials used in this study were sago pith (SP), *Bacillus subtilis*, cassava leaves (CL), Indigofera leaves (IL), tofu dregs (TD), chemicals, and laboratory equipment. The substrates consisted of mixtures of sago pith with cassava leaves, Indigofera leaves, and tofu dregs. The experiment was conducted using a completely randomized design (CRD) with a 3 × 3 factorial arrangement and three replications. Factor A was the substrate type: A1 (80% SP + 20% CL), A2 (80% SP + 20% IL), and A3 (80% SP + 20% TD). Factor B was the fermentation time: B1 (2 days), B2 (4 days), and B3 (6 days). The observed variables were cellulase activity, crude fiber reduction, protease activity, and crude protein. The results showed a highly significant interaction ($P < 0.01$) between substrate type and fermentation time on cellulase activity, crude fiber reduction, protease activity, and crude protein. It can be concluded that the combination of 80% sago pith and 20% cassava leaves fermented for 4 days produced the best improvement in the quality of sago pith.

Keywords | *B. subtilis,* Fermentation time, Sago pith, Substrate composition, Enzymatic activity, Nutritional enhancement

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INTRODUCTION

Sago pith is the inner part of the sago trunk obtained after the removal of the outer fibrous layer. Indonesia is one of the largest producers of sago, with plantation areas reaching 206,150 ha and total production of 381,065 tons. In West Sumatra alone, sago plantations cover approximately 960 ha with a production of 1,725 tons (Directorate General of Plantations, 2019). However, only about 15–20% of this substantial potential has been utilized for human consumption (Moshawih *et al.*, 2025; Djulardi *et al.*, 2023). The utilization of sago pith as poultry feed remains limited, mainly due to its low nutritional quality. Sago pith contains 18.25% crude fiber, 5.31% crude protein, 1.83% crude fat, 0.24% calcium, and 0.65% phosphorus (Djulardi *et al.*, 2023). The high crude fiber

and low protein contents indicate that sago pith requires processing to improve its nutritional value, particularly for poultry feed.

Fermentation is a biological process that converts complex organic compounds into simpler forms through the activity of microorganisms. This process has been widely reported to improve the nutritional quality of feed ingredients (Mirnawati *et al*., 2022). According to Al-Maqtari *et al.* (2019) and Ciptaan *et al.* (2022), fermentation can increase protein content, enhance digestibility, and stimulate the formation of amino acids and enzymes. Previous research showed that fermentation of sago pith using Neurospora sp. mold at a ratio of 80% sago pith and 20% tofu residue for 9 days increased crude protein to 18.26% and reduced crude fiber to 7.31% (Djulardi *et al*., 2023; Nensih, 2006). However, the major limitation of using *Neurospora* sp. is the long fermentation period required. Therefore, alternative microorganisms with faster fermentation capability are needed, such as *Bacillus subtilis*.

Ciptaan *et al.* (2024) reported that fermentation of a mixture of 80% soybean milk residue and 20% Indigofera leaves using *Bacillus subtilis* for 6 days produced phytase activity of 6.71 U/mL, crude protein content of 41.82%, nitrogen retention of 61.41%, crude fiber of 10.39%, crude fiber digestibility of 56.51%, and metabolizable energy of 2,199.80 kcal/kg. In addition, *Bacillus subtilis* has probiotic properties (Vananda *et al*., 2024; Hai *et al*., 2025; Mirnawati *et al*., 2025). Supplementation of *Bacillus subtilis* up to 500 g/ton of feed was reported to maintain carcass and meat production in male broiler chickens while reducing feed intake (Hananto, 2014; Tang *et al*., 2021; Cai *et al*., 2024). These findings indicate that *Bacillus subtilis* is a promising microorganism for improving feed quality through fermentation.

Several factors influence the success of the fermentation process, including substrate composition and fermentation time. The substrate serves as a growth medium that provides essential nutrients for microbial activity. Ideal fermentation substrate must contain adequate sources of carbon (C), nitrogen (N), and other essential elements in an appropriate C:N ratio (Ginesy *et al*., 2017; Ciptaan *et al*., 2025). Sago pith has a very low crude protein content (5.31%), indicating a deficiency of nitrogen. Therefore, nitrogen-rich materials must be added to the substrate to support microbial growth. Potential nitrogen sources include cassava leaves (CL) with a crude protein content of 31.75% (dry matter basis), Indigofera leaves (IL) with 28.89% crude protein, and tofu dregs (TD) with 27.69% crude protein (Diarra and Anand, 2020; Bhavna *et al*., 2024).

Fermentation time is another critical factor affecting microbial growth and enzyme production. A longer fermentation period generally allows greater microbial proliferation and enzyme synthesis (Mirnawati *et al*., 2019a, 2024). Increased microbial populations result in higher enzyme activity, which enhances the degradation of complex nutrients into simpler, more digestible forms. *Bacillus subtilis* is known to produce various enzymes, particularly cellulase and protease. Mirnawati *et al.* (2019b) reported that palm kernel meal fermented with *Bacillus subtilis* for 6 days produced mannanase activity of 24.27 U/mL, protease activity of 10.27 U/mL, and cellulase activity of 17.13 U/mL.

Fermentation of sago pith using *Bacillus subtilis* is therefore expected to yield high cellulase activity, leading to the degradation of cellulose and a subsequent reduction in crude fiber content. Increased protease activity facilitates the breakdown of protein into amino acids, which are more readily utilized by *Bacillus subtilis* for growth. Enhanced microbial growth ultimately contributes to an increase in the crude protein content of the fermented product. The nutritional quality of crude protein can be evaluated through nitrogen retention, whereas a reduction in crude fiber is generally associated with increased crude fiber digestibility.

Although previous studies have demonstrated the potential of microbial fermentation to improve the nutritional quality of sago pith and other agro-industrial by-products, information on the interaction between substrate composition and fermentation time using *Bacillus subtilis* remains limited. In particular, systematic evaluations of the combined effects of nitrogen-rich substrates and fermentation time on enzymatic activity, fiber degradation, and protein enrichment of sago pith are still lacking. Therefore, this study aimed to determine the optimal substrate type and fermentation time for improving enzymatic activity, crude fiber reduction, and crude protein content of sago pith fermented with *Bacillus subtilis*.

MATERIALS AND METHODS

Study site

This research was conducted at the Animal Biotechnology Laboratory, Non-Ruminant Nutrition Laboratory, and the Experimental Animal Facility of the Faculty of Animal Husbandry, Andalas University, from May 7th to August 31th 2024.

Preparation of inoculum and fermentation procedure

Bacillus subtilis was obtained from the National Research and Innovation Agency (BRIN). The inoculum was prepared by culturing the isolate on Nutrient Agar and

subsequently propagated in Nutrient Broth at 37°C for 24 h. The bacterial suspension was adjusted to approximately 1 × 10^8 CFU/mL before use. Sago pith, cassava leaves, Indigofera leaves, and tofu dregs were oven-dried at 60°C and ground to pass a 1-mm sieve. Substrates were prepared according to the experimental design (80% sago pith + 20% supplement). Moisture content was adjusted to approximately 60% using sterile distilled water. Each substrate mixture was inoculated with 5% (v/w) *Bacillus subtilis* suspension, mixed thoroughly, packed into sterile polypropylene bags, and incubated under aerobic conditions at 37°C for 2, 4, or 6 days.

Research materials

The materials used in this study included cassava leaves (CL), sago pith (SP), tofu dregs (TD), Indigofera leaves (IL), and *Bacillus subtilis* obtained from National Research and Innovation Agency (BRIN). Other materials consisted of Nutrient Agar (Merck.), distilled water, alcohol 70% (Onemed), Brooks *et al*. solution, casein, phosphate buffer (pH 7), NaOH 0.5 N (Himedia), H_2SO_4 (Merck), and chemicals used for proximate analysis. A total of 30 broiler chickens aged 6 weeks with an average body weight of 1.5 kg were used in the feeding trial. The equipment used in this study included an incubator , autoclave, plastic containers, beakers, measuring cylinders, test tubes, test tube racks, hot plate, micropipettes and tips, spectrophotometer UV-VIS 1800 (Shimadzu USA MFG inc.), Eppendorf tubes, centrifuge, Erlenmeyer flasks (250 mL), analytical balance, polypropylene plastic bags (15 × 25 cm), oven, filter paper, aluminum foil, grinder, tissue paper, and metabolic cages equipped with individual drinkers.

Research design

This study employed a laboratory experimental method using a Completely Randomized Design (CRD) with a 3 × 3 factorial arrangement and three replications. Factor A was the substrate composition, consisting of A1 (80% sago pith + 20% cassava leaves), A2 (80% sago pith + 20% Indigofera leaves), and A3 (80% sago pith + 20% tofu dregs). Factor B was the fermentation time, consisting of B1 (2 days), B2 (4 days), and B3 (6 days).

Total bacterial count

Total bacterial population was determined using the total plate count method. Ten grams of fermented sample were homogenized in 90 mL sterile physiological saline and serially diluted up to 10^{-9}. Aliquots (0.1 mL) were spread on Nutrient Agar plates and incubated at 37°C for 24 h. Colonies were counted and expressed as CFU/g dry matter.

Measured parameters

Cellulase activity

Enzyme activity begins with crude enzyme extraction. Ten grams of fermented sample were homogenized in 90 mL of 0.05 M phosphate buffer (pH 7) and shaken for 2 h. The mixture was filtered and centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was collected as crude enzyme extract for cellulase activity analysis. One mL of crude enzyme was mixed with 1 mL of extract (0.5 mL carboxymethyl cellulose + 10 mL acetate buffer), and incubated for 30 minutes at 40°C in a water bath shaker. The reaction was stopped by adding 1 mL of acetone reagent and heating in boiling water for 20 min. After cooling, 1 mL of phosphomolybdate reagent and 7 mL of distilled water were added. Absorbance was then measured at a wavelength of 575 nm. To determine the amount of cellulase activity (Miller, 1959), the following formula is used:

$$Cellulase\ Activity\ (U/ml) = \frac{X \times P \times 1000}{T \times BM}$$

Description: X= Standard curve convention result, P= Dilution, T= Time, BM= glucose molecular weight.

Crude fiber reduction

Crude fiber content of fermented sago pith was determined using the standard acid–alkali digestion method. One gram of sample was placed in a 250 mL beaker and treated with 50 mL of preheated 0.3 N H_2SO_4. The mixture was gently heated and filtered, after which the residue and filter paper were returned to the beaker. Subsequently, 25 mL of 0.3 N NaOH were added, and the mixture was reheated for 30 min and filtered again using pre-weighed filter paper (A g). The residue was thoroughly washed with hot distilled water and rinsed with 25 mL of acetone. The filter paper containing the residue was transferred to a porcelain crucible and dried at 105–110 °C for 1 h. After cooling in a desiccator, the sample was weighed (B g) and then ashed in a muffle furnace at 400–600 °C until a constant weight was obtained. The crucible was cooled again in a desiccator and weighed (C g). Crude fiber content was calculated from the difference in weight before and after ashing.

Crude fiber content can be calculated using the following formula:

$$\text{Crude fiber (\%)} = \frac{B - C - A}{x} \times 100\%$$

Description: B= Weight of dish + sieve residue, C= Weight of dish + ash, A= Weight of filter paper, X= Weight of sample.

To determine the percentage decrease in crude fiber, use the following formula:

$$Percentage\ decrease\ in\ CF = \frac{Initial\ CF - Final\ CF}{Initial\ CF} \times 100\%$$

Description: Initial CF= Crude fiber before treatment, Final CF= Crude fiber after treatment

Protease activity

Enzyme activity begins with crude enzyme extraction. Ten grams of fermented sample were homogenized in 90 mL of 0.05 M phosphate buffer (pH 7) and shaken for 2 h. The mixture was filtered and centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was collected as crude enzyme extract for protease activity. Protease activity was measured using the Cupp and Enyard (2008) method. The procedure for measuring protease activity is as follows: First, take 2.5 mL of 1% casein solution using a pipette and added phosphate buffer (0.1 M pH 7). Then, place it in a reaction tube and mix with a vortex mixer. Next, incubated it in a water bath (37°C for 10 minutes) and added 1 mL of enzyme extract. Then, incubated it in a 50°C water bath for 10 minutes. Centrifuge (5000 rpm for 15 minutes at 4°C), filter, and take the supernatant. Take 2 mL of the supernatant using a pipette and place it in a test tube, then added 5 mL of 0.5 N NaOH and 0.5 mL of Folin Ciocalteau reagent and leave for 10 minutes. After that, measure the absorbance with a spectrophotometer at a wavelength of 650 nm. Protease activity is calculated using the formula:

$$Protease\ activity\ \left(\frac{U}{mL}\right) = \frac{Y \times a}{b} \times \frac{1}{t}$$

Description: Y= Sample absorbance, a= Value of a from the regression curve Y = a + bx, b= Value of b from the regression curve Y = a + bx, t= Incubation time.

Crude protein

Crude protein (CP) can be calculated using the Kjeldahl method (1883), which consists of three stages: destruction, distillation, and titration. Weigh 1 g of the sample, place it in a Kjeldahl flask, and added 1 g each of catalyst (selenium) and 25 mL of concentrated H2SO4, then destroy until clear, then cool. Added 500 mL of distilled water. Take 10 mL of the filtrate and place it in a distillation flask, added 25 mL of 0.3 N NaOH, 75 mL of distilled water, and a boiling stone, then distill until a pop occurs. The distillate is collected in 25 mL of 0.3 N H_2SO_4 containing 3 drops of methyl red indicator. After boiling (the distillation process is complete), titrate the distillate with 0.1 N until the color changes. Also perform a blank titration. The crude protein content is calculated using the formula:

$$CP\ (\%) = \frac{(Y - Z) \times N \times 0{,}014 \times C \times 6.25}{X} \times 100\%$$

Description: Y= Blank titration (mL), Z= Sample titration (mL), N= NaOH normality used, C= Dilution, X= Sample weight, 0.014= Atomic weight of N, 6.25= N in protein is only 16%.

The calculation of crude protein increase is done using the following formula:

$$Improving\ crude\ protein\ (\%) = \frac{(Y - X)}{Y} \times 100\%$$

Description: X= Initial CP (%BK), Y= Final CP (%BK).

Statistical analysis

All data were analyzed using analysis of variance (ANOVA). When significant differences were detected, treatment means were compared using Duncan's Multiple Range Test (DMRT) according to Steel and Torrie (1991) at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of fermented sago pith with *Bacillus subtilis* on cellulase activity

The average activity of sago pith cellulase and various substrate mixtures fermented with *Bacillus subtilis* can be seen in Table 1.

Table 1: Average cellulase activity (U/mL) of fermented sago pith with *Bacillus subtilis* in each treatment.

Factor A (substrate composition)	Factor B (fermentation time)			SEM
	B1	B2	B3	
A1	12.96^{cA}	15.81^{aA}	13.06^{bA}	0.02
A2	12.02^{cB}	14.78^{aB}	12.33^{bB}	
A3	11.63^{cC}	13.80^{aC}	12.05^{bC}	

Note: Lowercase letters in rows and uppercase letters in columns indicate significant differences ($P < 0.01$). A1 (80% sago pith + 20% cassava leaves), A2 (80% sago pith + 20% Indigofera leaves), and A3 (80% sago pith + 20% tofu dregs), B1 (2 days), B2 (4 days), and B3 (6 days).

The results of the diversity analysis indicated a very significant interaction ($P < 0.01$) between factor A and factor B on cellulase activity. In addition, each individual factor A and factor B also had a very significant effect ($P < 0.01$) on cellulase activity. Table 1 shows that the addition of cassava leaves (A1) to the substrate produced the highest cellulase activity at all fermentation periods, namely B1 (2 days), B2 (4 days), and B3 (6 days). The optimal cellulase activity was achieved in treatment A1B2. The high cellulase activity observed in A1B2 was associated with the large microbial population, as indicated by the total colony count of 30.8×10^9 CFU/g. This high microbial abundance resulted from the addition of 20% cassava leaves, which contributed a higher crude protein content than the addition of 20% indigofera leaves or 20% tofu dregs. Crude protein serves as a nitrogen source, which

is essential for microbial cell growth. Similar trends were reported by Spohn *et al.* (2016), who stated that nitrogen is utilized by microorganisms for cell formation. The greater the protein content in the substrate, the higher the availability of nitrogen for bacterial growth, leading to an increase in enzyme production. This is further supported by Fitriana and Asri (2022), who reported that bacterial enzyme secretion increases with enhanced bacterial cell growth. Consequently, an increase in bacterial population leads to greater enzyme production, particularly cellulase. *Bacillus subtilis* is widely recognized as an efficient cellulase-producing bacterium involved in lignocellulosic degradation. The present findings support recent reports showing that *Bacillus subtilis* enhances cellulase production and fiber breakdown in agro-industrial by-products, thereby improving their potential as feed resources (Devi *et al.*, 2023; Mushtaq *et al.*, 2024; Ciptaan *et al.*, 2025).

A fermentation period of 4 days resulted in the highest cellulase activity compared with fermentation periods of 2 days and 6 days in treatments A1, A2, and A3. The low cellulase activity on the second day was attributed to the adaptation phase of the microorganisms, during which microbial growth was still limited. In contrast, the lower cellulase activity on the sixth day was caused by the excessive fermentation period, which led to the microorganisms entering the death phase. Fermentation time determines the opportunity for microbes to grow and utilize the nutrients in the substrate; however, an excessively long fermentation period reduces nutrient availability and consequently decreases microbial growth. Thus, enzyme production during fermentation reaches a maximum at an optimum time and then declines either rapidly or gradually after this point (Mushtaq *et al.*, 2024; Xiang *et al.*, 2025). The decrease in cellulase activity on the sixth day was also influenced by the accumulation of cellulose hydrolysis products, which can inhibit cellulase activity. Glucose and cellobiose act as enzyme inhibitors in cellulose hydrolysis. In particular, cellobiose inhibits the activity of exoglucanase or cellobiohydrolase, key components of the cellulase enzyme complex.

Table 1 further shows that the highest cellulase activity was obtained in treatment A1B2, which consisted of a substrate mixture of 80% sago pith (SP) + 20% cassava leaves (CL) and a 4-day fermentation period, producing a cellulase activity of 15.81 U/mL. This value is higher than that reported by Ciptaan *et al.* (2025), who obtained a cellulase activity of 14.61 U/mL using *Bacillus subtilis* with a palm kernel meal substrate and a fermentation period of 4 days.

Effect of fermented sago pith with *Bacillus subtilis* on decrease in crude fiber

The average decrease in crude fiber content of sago pith and various fermented substrate mixtures with *Bacillus subtilis* can be seen in Table 2.

Table 2: Average reduction in crude fiber (%CF) of sago pith fermented with *Bacillus subtilis* in each treatment.

Factor A (substrate composition)	Factor B (fermentation time)			SEM
	B1	B2	B3	
A1	55.73^{cA}	65.72^{aA}	60.04^{bA}	0.27
A2	41.72^{cB}	58.62^{aB}	47.77^{bB}	
A3	41.35^{cB}	51.58^{aC}	45.84^{bC}	

Note: Lowercase letters in rows and uppercase letters in columns indicate significant differences ($P < 0.01$). A1 (80% sago pith + 20% cassava leaves), A2 (80% sago pith + 20% Indigofera leaves), and A3 (80% sago pith + 20% tofu dregs), B1 (2 days), B2 (4 days), and B3 (6 days).

The results of the diversity analysis indicated a very significant interaction ($P < 0.01$) between factor A and factor B on crude fiber reduction. In addition, factor A and factor B individually also had a very significant effect ($P < 0.01$) on crude fiber reduction. Table 2 shows that the addition of cassava leaves (A1) to the substrate resulted in the highest reduction in crude fiber at all fermentation periods, namely B1 (2 days), B2 (4 days), and B3 (6 days). The greatest crude fiber reduction and the lowest crude fiber content were observed in treatment A1B2. This result was associated with the high microbial population, as indicated by the total colony count of 30.8×10^9 CFU/g. The high microbial growth was attributed to the addition of 20% cassava leaves, which contributed the highest crude protein content compared with 20% indigofera leaves and 20% tofu dregs. Crude protein serves as a source of nitrogen, which is essential for microbial cell growth. Similar trends were reported by Spohn *et al.* (2016), who reported that nitrogen is utilized by microorganisms for cell formation. The higher the protein content in the substrate, the greater the nitrogen availability for bacterial growth, which in turn increases enzyme production. Enhanced enzyme production, particularly cellulase, accelerates the hydrolysis of cellulose into glucose, thereby increasing crude fiber reduction and decreasing crude fiber content at the end of fermentation (Demissie *et al.*, 2024; Ciptaan *et al.*, 2025).

A fermentation period of 4 days produced the highest crude fiber reduction and the lowest crude fiber content compared with fermentation periods of 2 days and 6 days in treatments A1, A2, and A3. The relatively high crude fiber content on day 2 was caused by the microorganisms being in the adaptation phase, resulting in limited microbial growth and low cellulase production. Consequently, only a small proportion of crude fiber was degraded. In contrast, the increase in crude fiber observed on day 6 was attributed to the excessive fermentation time, which led the microorganisms to enter the stationary phase

followed by the death phase, thereby reducing enzyme activity. Fermentation time determines the opportunity for microorganisms to grow and utilize nutrients in the substrate; however, excessively long fermentation reduces nutrient availability, leading to decreased microbial growth and enzyme production, which ultimately limits crude fiber degradation (Zhu *et al*., 2024; Ciptaan *et al*., 2024; Mirnawati *et al*., 2024).

Based on Table 2, the highest crude fiber reduction was obtained in treatment A1B2, with the lowest crude fiber content in the substrate mixture of 80% SP + 20% cassava leaves (CL) at a 4-day fermentation period, resulting in a crude fiber reduction of 65.72%. This result is higher than that reported by Ciptaan *et al*. (2025), who used *Bacillus subtilis* with a substrate mixture of soybean milk residue and cassava leaves and obtained a crude fiber content of 7.04% after 4 days of fermentation.

Effect of fermented sago pith with *Bacillus subtilis* on protease activity

The average activity of fermented sago pith protease (FSP) with *Bacillus subtilis* can be seen in Table 3.

Table 3: Average protease activity (U/mL) of FSP with *Bacillus subtilis* for each treatment.

Factor A (substrate composition)	Factor B (fermentation time)			SEM
	B1	B2	B3	
A1	2.21^{cA}	8.36^{aA}	5.22^{bA}	0.05
A2	1.72^{cB}	6.36^{aB}	4.34^{bB}	
A3	1.32^{cC}	5.45^{aC}	3.89^{bC}	

Note: Lowercase letters in rows and uppercase letters in columns indicate significant differences (P < 0.01). A1 (80% sago pith + 20% cassava leaves), A2 (80% sago pith + 20% Indigofera leaves), and A3 (80% sago pith + 20% tofu dregs), B1 (2 days), B2 (4 days), and B3 (6 days)

The results of the diversity analysis demonstrated a very significant interaction (P < 0.01) between factor A (substrate mixture) and factor B (fermentation time) on protease activity. Both factor A and factor B individually also had a very significant effect (P < 0.01) on protease activity. Based on the data presented in Table 3, treatment A1 (80% SP + 20% cassava leaves/CL) consistently produced higher protease activity than treatments A2 (80% SP + 20% indigofera leaves/IL) and A3 (80% SP + 20% tofu dregs/TD) at all fermentation periods, namely B1 (2 days), B2 (4 days), and B3 (6 days). The optimal protease activity was achieved in treatment A1B2, consisting of 80% SP + 20% CL with a fermentation period of 4 days.

The high protease activity observed in treatment A1B2 was closely related to the high microbial population, as indicated by a total colony count of 30.8×10^9 CFU/g. This high microbial growth was attributed to the higher crude protein content of cassava leaves compared with indigofera leaf flour and tofu dregs. Protein in the substrate serves as a nitrogen source for microbial cell synthesis; therefore, a higher protein content promotes more intensive microbial growth, which subsequently enhances enzyme production. These observations support Fitriana and Asri (2022), who reported that bacterial enzyme secretion increases in line with bacterial cell growth. As microbial populations increase, enzyme activity also rises, particularly protease activity, since *Bacillus subtilis* is a well-known protease-producing bacterium. This is supported by Efendi *et al*. (2017), who stated that *Bacillus subtilis* is a proteolytic bacterium capable of producing high protease activity, reaching 2.162 U/mL at 46 hours of incubation.

The lower protease activity observed on day 2 was due to the microorganisms still being in the adaptation phase, resulting in limited microbial growth. In contrast, the decrease in protease activity on day 6 was caused by the excessive fermentation time, which led the microbes to enter the death phase. Fermentation time determines the opportunity for microbes to grow and utilize nutrients in the substrate; however, overly long fermentation reduces nutrient availability and consequently decreases microbial growth. This condition was reflected by the decline in total microbial colonies during the 6-day fermentation period. A reduction in microbial population inevitably leads to a decrease in enzyme activity, including protease. According to Efendi *et al*. (2017), protease activity is directly proportional to bacterial growth; the greater the number of bacterial cells, the higher the protease activity produced, and vice versa. Based on Table 3, treatment A1B2 produced the optimal protease activity of 8.36 U/mL. This value is lower than that reported by Devi *et al*. (2023), who found that the fermentation of palm kernel meal using *Bacillus subtilis* and *Lactobacillus fermentum* resulted in a protease activity of 10.95 U/mL.

Effect of fermented sago pith with *Bacillus subtilis* on increase in crude protein

The average increase in crude protein content of fermented sago pith (FSP) with *Bacillus subtilis* can be seen in Table 4.

Table 4: Average increase in crude protein (%CP) of FSP with *Bacillus subtilis* for each treatment.

Factor A (substrate composition)	Factor B (fermentation time)			SEM
	B1	B2	B3	
A1	94.20^{cA}	129.29^{aA}	109.29^{bA}	1.09
A2	92.35^{cA}	112.16^{aB}	99.79^{bB}	
A3	78.08^{cB}	110.36^{aB}	95.45^{bC}	

Note: Lowercase letters in rows and uppercase letters in columns indicate significant differences (P < 0.01). A1 (80% sago pith + 20% cassava leaves), A2 (80% sago pith + 20% Indigofera leaves), and A3 (80% sago pith + 20% tofu dregs), B1 (2 days), B2 (4

days), and B3 (6 days).

The results of the diversity analysis demonstrated a very significant interaction ($P < 0.01$) between factor A (substrate mixture) and factor B (fermentation time) on the increase in crude protein. In addition, both factor A and factor B individually exhibited a very significant effect ($P < 0.01$) on crude protein enhancement. Based on the data presented in Table 4, treatment A1 (80% SP + 20% cassava leaves/CL) consistently resulted in a higher increase in crude protein than treatments A2 (80% SP + 20% indigofera leaves/IL) and A3 (80% SP + 20% tofu dregs/TD) at all fermentation periods, namely B1 (2 days), B2 (4 days), and B3 (6 days). The highest increase in crude protein was observed in treatment A1B2, which consisted of a substrate mixture of 80% SP + 20% CL with a fermentation period of 4 days.

The substantial increase in crude protein in treatment A1B2, reaching 129.29% (from 10.60% DM before fermentation to 24.29% DM after fermentation), was closely associated with the vigorous growth of *Bacillus subtilis* in this treatment. This was evidenced by the total colony count of 30.8×10^9 CFU/g. A high microbial population contributes directly to an increase in crude protein content in the fermented product. Similar trends were reported by Iyayi (2004), who reported that microorganisms function as single-cell proteins containing approximately 40–60% protein. The pronounced increase in crude protein was also related to the enzymes produced by the microorganisms; greater microbial growth leads to higher enzyme production, and enzymes themselves are proteinaceous compounds that contribute to the crude protein content of fermentation products. This is in accordance with Shakilanishi and Shanthi (2024), who stated that enzymes are proteins that act as specific biological catalysts for particular substrates. As shown in Table 4, treatment A1B2 exhibited the highest crude protein increase of 129.29% (from 10.60% DM before fermentation to 24.29% DM after fermentation). This value is substantially higher than that reported by Gabrella *et al.* (2022), who found that broiler finisher feed fermented with Bacillus subtilis inoculum at 5% of total feed resulted in only a 9.31% increase in crude protein (from 19.85% before fermentation to 21.70% after fermentation).

CONCLUSION

This study demonstrated a significant interaction between substrate composition and fermentation time in influencing enzymatic activity and the nutritional quality of sago pith fermented with *Bacillus subtilis*. The combination of 80% sago pith and 20% cassava leaves fermented for four days produced the most favorable results, as indicated by the highest cellulase and protease activities, the greatest reduction in crude fiber, and the highest increase in crude protein. These findings suggest that *Bacillus subtilis* fermentation is a promising biological approach for upgrading low-quality sago pith into a higher-value feed ingredient, particularly for poultry nutrition. Moreover, this fermentation strategy shows strong potential for practical application in the development of sustainable local feed resources.

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NOVELTY STATEMENT

This study presents a novel bacterial fermentation framework based on a fixed substrate ratio of 80% sago pith to 20% cassava leaves with a 4-day fermentation period using *Bacillus subtilis* for the bioconversion of low-quality feed resources. This experimental framework offers a new scientific basis for developing efficient bacterial fermentation systems to upgrade low-quality, fiber-rich local feed resources.

AUTHOR'S CONTRIBUTION

M contributed to research concepts, technical and logistic support, and supervised the research. CG, RKR and GY contributed to experimental design, data collection and execution. AS and HA contributed to data collection, analyses and write up of the manuscript. ARI contributed to writing the final drafted manuscript.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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