Utilization of Azolla microphylla as Nitrogen Source in Nata de Coco Production Affected by Various Fermentation Conditions

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Received: 15 – 10 – 2025 , Accepted: 31 – 12 – 2025

ABSTRACT

Nata de coco is a bacterial cellulose produced by *Acetobacter xylinum* during fermentation. This study investigated the use of *Azolla microphylla* as a natural nitrogen source and evaluated the influence of sucrose and starter concentration, temperature and carbon source on nata quality. Treatments included combinations of sucrose levels (2–8%) and starter inoculum (5–10%), as well as combination of temperatures (room temperature, 30 °C) and carbon sources (glucose, fructose, sucrose, lactose). Results showed that combination of sucrose and starter significantly affected nata thickness (0.47–1.20 cm), hardness (7.03–19.94 N), pH (3.60–4.07), and crude fiber (6.44–8.69%) ($P < 0.05$). Higher starter enhanced thickness and fiber, while excess sucrose reduced both due to osmotic stress. Combination of fermentation temperature and carbon source also influenced nata characteristics, with thickness ranging 0.43–1.15 cm, hardness 7.03–19.94 N, pH 3.63–4.02, and crude fiber 6.44–8.87%. Fructose combined with 30 °C incubation supported greater cellulose formation, while lactose at room temperature showed lower performance. These findings confirm that *A. microphylla* can effectively serve as a nitrogen source and that fermentation efficiency is strongly regulated by carbon type and temperature conditions.

Keywords: Acetobacter xylinum, ammonium sulfate, edible cellulose, floating plants, optimization

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Husna, A., Reviana, N. T., Pradita, S., Saati, E. A., Siskawardani, D. D., Fahmi, I. Z. (2025). Utilization of Azolla microphylla as Nitrogen Source in Nata de Coco Production Affected by Various Fermentation Conditions. *Jurnal Agroindustri Halal,* 11(3), 341 – 350

INTRODUCTION

Nata de coco is a fermented food product made from coconut water, produced through the action of the bacterium *Acetobacter xylinum*, which synthesizes edible cellulose in the form of a white, chewy, and high-fiber gel. In commercial production, ammonium sulfate, commonly referred to as ZA (zwavelzure ammoniak), is widely used as a nitrogen source to support the growth and metabolic activity of *A. xylinum*. Although effective, ZA is a synthetic compound primarily used as an agricultural fertilizer to enhance soil nitrogen content. Its application in food fermentation raises significant safety concerns. Residual ammonium compounds that are not fully metabolized during the fermentation process may remain in the final product, posing potential health risks with long-term consumption. These risks may include gastrointestinal irritation, nausea, and metabolic imbalances. Furthermore, chronic intake of inorganic nitrogen compounds has been associated with systemic toxicity and adverse effects on liver and kidney function (Ota et al., 2006). These issues highlight the need for safer, natural alternatives to synthetic nitrogen sources in food-based fermentation systems.

One promising candidate is *Azolla microphylla*, a free-floating aquatic plant known for its relatively high nitrogen content, ranging from 2 to 5 g per 100 g of biomass (Santosa et al., 2021). As an organic alternative, *A. microphylla* could potentially replace synthetic nitrogen in nata de coco production. However, the use of organic nitrogen sources may influence fermentation dynamics, potentially affecting *A. xylinum*'s ability to grow and synthesize bacterial cellulose. The successful formation of nata depends on the balance and availability of both nitrogen and carbon. These macronutrients are essential for driving microbial metabolism, particularly sugar breakdown and cellulose biosynthesis.

The use of *A. microphylla* as a nitrogen source has been reported to result in nata with slightly lower thickness, yield, and crude fiber content compared to conventional sources such as mung bean sprouts (Santosa et al., 2021). Nevertheless, the quality of nata can be improved by optimizing fermentation parameters. Sugar concentration plays a pivotal role, as it serves as the main carbon source for bacterial activity. Studies suggest that around 5–6% sugar concentration yields optimal cellulose production, while levels that are too low or too high may lead to poor bacterial growth or osmotic inhibition (Yanti et al., 2017). Similarly, starter concentration within the range of 5–10% ensures uniform fermentation and efficient bacterial activity (Hendrawati et al., 2019). Additionally, fermentation temperature and the type of carbon source significantly influence the final product characteristics. Incubation at 30 °C using simple sugars such as fructose or glucose has been shown to enhance cellulose synthesis.

Despite previous studies highlighting the importance of individual factors such as sugar concentration, starter inoculum, or temperature, comprehensive investigations on the combined effects of fermentation conditions remain limited. In particular, the interaction between temperature and carbon source, as well as the balance between sugar and starter concentrations, plays a crucial role in determining the efficiency of cellulose synthesis and the final quality of nata de coco. Temperature directly affects the metabolic rate of *A. xylinum*, while the type of carbon source determines the accessibility and speed of sugar utilization during fermentation (Talawar et al., 2015). Similarly, the interplay between sugar concentration and inoculum size influences both substrate availability and microbial density, which together shape the cellulose yield, thickness, and structural properties of the nata gel (Lahiri et al., 2021). However, only a few studies have specifically addressed these combined treatments, especially when organic nitrogen sources such as *Azolla microphylla* are used. Therefore, exploring these fermentation parameter combinations is essential to better understand their synergistic impact and to optimize nata de coco production under more sustainable and natural conditions.

The aim of this research was to evaluate the effects of fermentation parameters, including combinations of temperature and carbon source, as well as sugar and starter concentrations, on nata de coco production using *Azolla microphylla* as an alternative nitrogen source.

MATERIAL AND METHODS

Nata Starter preparation

The nata starter was prepared by inoculating pasteurized coconut water with *A. xylinum* (Edupark University of Muhammadiyah Malang collection) and incubated for 7 days at room temperature (26.3 °C).

Nata production influenced by combination of carbon source and temperature

The production of nata de coco refers to Sihmawati et al. (2016) with modification. The coconut water was kept at room temperature for 3 days then was filtered to remove debris. Meanwhile, *A. microphylla* was cultivated at Edupark University of Muhammadiyah Malang and harvested at 8 days old. 20 g of *A. microphylla* were washed, drained, crushed, and blended with 1 L of coconut water. The mixture was then filtered to obtain the filtrate, boiled, and added with various carbon sources namely sucrose (P1), glucose (P2), fructose (P3), and lactose (P4) at concentration of 2 % (w/v). Subsequently, the mixture was poured into a sterile jar and cooled down until room temperature before inoculated with a 50 mL nata starter consisting of *A. xylinum*. The fermentation process was carried out for 10 days in two different temperatures, i.e. room temperature (26,3 °C/Q1) and 30 °C in an incubator (Q2). The two variables (carbon source ; temperature) were combined to obtain 8 combinations, namely P1Q1 (sucrose ; room temperature), P1Q2 (sucrose ; 30 °C), P2Q1 (glucose ; room temperature), P2Q2 (glucose ; 30 °C), P3Q1 (fructose ; room temperature), P3Q2 (fructose ; 30 °C), P4Q1 (lactose ; room temperature), and P4Q2 (lactose ; 30 °C). The thick white solid formed on the surface of the mixture called nata was harvested and kept in the refrigerator before being subjected to physical and chemical analysis. The experiment was carried out using triplicate.

Nata production influenced by combination of starter and carbon concentration

Nata was produced using coconut water, bacteria, and procedure as explained above. The carbon concentrations were 2% (A1), 4% (A2), 6% (A3), and 8% (A4) while the starter concentrations were 5% (B1) and 10% (B2). Both variables (carbon concentration ; starter concentration) were combined to obtain 8 combinations, namely A1B1 (2% ; 5%), A1B2 (2% ; 10%), A2B1 (4% ; 5%), A2B2 (4% ; 10%), A3B1 (6% ; 5%), A3B2 (6% ; 10%), A4B1 (8% ; 5%), and A4B2 (8% ; 10%). The nata were harvested and kept in the refrigerator before being analyzed for its yield, as well as physical and chemical characteristics. The experiment was carried out using triplicate.

Physical and chemical properties determination of nata

Nata de coco from each combination was evaluated for its yield, physical properties including thickness and hardness, as well as chemical characteristics particularly pH and crude fiber. The yield of nata was calculated using the following formula (Jagannath et al., 2008):

$$Yield\ (\%) = \frac{Nata's\ weight\ (g)}{Media's\ weight\ (g)} \times 100\% \qquad (1)$$

The thickness of nata was measured using a Vernier caliper, while the hardness value was determined using texture profile analyzer (TPA) by Bourne (2002) method. The nata was cut into 3x3 cm and placed in the TPA plate, then the sample was pressed using the probe with 5 mm/s of speed until penetration distance reached 50 % of its thickness.

For pH, 5 g of each sample were chopped into small pieces and added with aquadest, then the calibrated pH meter probe was dipped into the mixture and the pH values were recorded (AOAC, 2005). The crude fiber was analyzed using SNI 01-2891-1992 method. The nata was crushed and weighed for about 2 g, placed in an erlenmeyer, then 50 ml of 1.25% $H2SO4$ was added. After the mixture was boiled for 30 minutes, 50 ml of 3.25% NaOH was added and reboiled for 30 minutes more. The aliquot was then filtered and rinsed with hot 25 ml 1.25% $H2SO4$, hot 25 ml aquadest, and hot 25 ml 96% ethanol, successively. The residue that was left on the filter paper was dried and weighed. Crude fiber content were determined using the following formula:

$$Crude\ fiber\ (\%) = \frac{a-b}{c} \times 100\% \qquad (2)$$

where a is final filter paper's weight (g), b is initial filter paper's weight (g), and c is sample's weight (g).

Statistical analysis

The data were analyzed using Analysis of Variance (ANOVA) to see the effect of each combination. The treatment combinations that had significant effect were further evaluated using DMRT (Duncan's Multiple Range Test) at α level = 5%.

RESULT AND DISCUSSION

Nata Yield

Nata is a thick white solid produced by *A. xylinum* and formed on the surface of the media fermentation. High thickness and yield are key physical indicators of high-quality nata de coco. Based on the result, the utilization of *A. microphylla* as the nitrogen source in nata production generated up to 33.05 % and 39% yield from raw material, as affected by carbon source and temperature (Fig. 1a), as well as carbon and starter concentration (Fig 1b), respectively. However, the variation of carbon source, temperature, as well as sucrose, and starter concentration did not give significant effect on the yield of nata. Santosa et al. (2021) reported that *A. microphylla* has 2-5 % nitrogen content and can be used as growth support of *A. xylinum* in nata production. In this research, the combination of 6% sucrose with 10 % nata starter produced the highest yield (33,5%). While for carbon source and temperature, the highest nata yield was obtained from fructose as carbon source at 30 °C. According to Shudeerkumar et al. (2015), high yield is achieved when the carbon-to-nitrogen (C/N) ratio of the media during fermentation is well controlled. This condition enables *A. xylinum* to convert all of the coconut water into nata without leaving any residue. The ability of *A. xylinum* to produce cellulose, along with substrate diversity, material composition, and environmental conditions, are among the factors that influence production outcomes (Andasuryani et al., 2021).

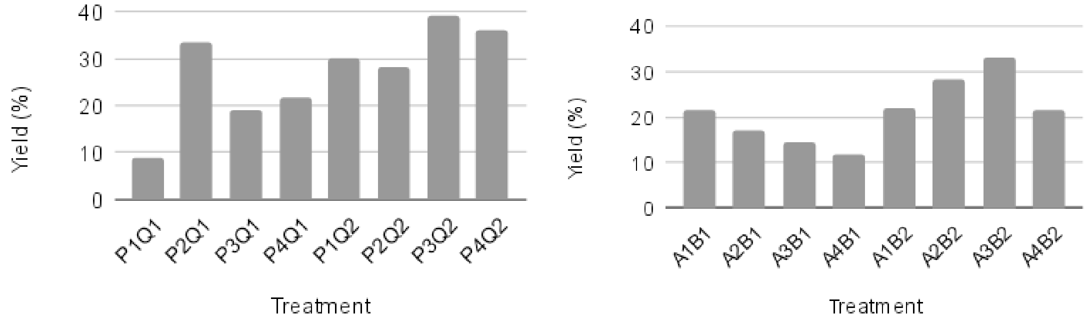

Figure 1. Nata yield affected by combination of carbon and temperature (left), as well as by combination of sucrose and starter concentration (right). P1Q1 (sucrose ; room temperature), P1Q2 (sucrose ; 30 °C), P2Q1 (glucose ; room temperature), P2Q2 (glucose ;

30 °C), P3Q1 (fructose ; room temperature), P3Q2 (fructose ; 30 °C), P4Q1 (lactose ; room temperature), P4Q2 (lactose ; 30 °C), A1B1 (2% carbon; 5% starter), A1B2 (2% carbon; 10% starter), A2B1 (4% carbon; 5% starter), A2B2 (4% carbon; 10% starter), A3B1 (6% carbon; 5% starter), A3B2 (6% carbon; 10% starter), A4B1 (8% carbon; 5% starter), and A4B2 (8% carbon; 10% starter).

Characteristics of nata affected by temperature and carbon source

The results showed that both fermentation temperature and the type of carbon source significantly influenced ($P < 0.05$) the thickness, hardness, and pH of nata de coco, while their interaction did not significantly affect crude fiber content (Table 1). Variations in thickness indicated that certain carbon sources responded differently depending on the incubation temperature, suggesting that both factors jointly modulate cellulose formation by *A. xylinum*. Hardness values also shifted according to the treatment combination, where changes in temperature altered the texture properties associated with different carbon sources. These patterns imply that temperature regulates the structural arrangement of bacterial cellulose, which in turn influences nata texture. Since nata quality is closely linked to chewiness rather than excessive hardness, these differences highlight the importance of controlling fermentation conditions to balance fiber composition and gel compactness (Pa'e et al., 2014).

Fructose, as a simple monosaccharide, tended to promote greater cellulose synthesis compared to more complex sugars, reflecting its higher metabolic accessibility for *A. xylinum*. The effect of incubation temperature was also evident, with 30 °C generally supporting more active sugar conversion into cellulose compared to lower temperatures. This aligns with the understanding that moderate temperatures enhance bacterial enzymatic activity, while deviations beyond the optimal range may reduce microbial performance (Nurdin, 2023). The interaction between carbon source and fermentation temperature further demonstrated that readily metabolized sugars were more effective at supporting cellulose production under favorable conditions, whereas less accessible sugars combined with suboptimal temperatures resulted in reduced nata yield. These outcomes emphasize that the efficiency of nata de coco formation depends not only on the type of carbon provided but also on how temperature conditions regulate bacterial metabolism (Attaqy et al., 2023).

Table 1. The physical and chemical characteristics of nata influenced by carbon source and temperature (mean±SD).

Treatments	Thickness (cm)	Hardness (N)	pH	Crude Fiber (%)
P1Q1	0.43^{a} ± 0.50	18.25^{b} ± 1.13	4,12^{b} ± 0.04	4.92^{a} ± 0.29
P2Q1	0.93^{a} ± 2.65	19.94^{bc} ± 5.23	3,51^{a} ± 0.08	8.87^{a} ± 1.16
P3Q1	0.64^{a} ± 1.77	18.72^{c} ± 2.14	4,44^{bc} ± 0.13	7.21^{a} ± 2.03
P4Q1	0.78^{a} ± 1.42	14.51^{bc} ± 4.41	4,66^{c} ± 0.31	6.43^{a} ± 0.20
P1Q2	0.79^{b} ± 4.19	16.84^{b} ± 2.62	4,17^{b} ± 0.05	6.20^{a} ± 2.39
P2Q2	0.64^{a} ± 3.73	7.03^{a} ± 1.52	3,92^{a} ± 0.55	5.98^{a} ± 2.38
P3Q2	1.15^{b} ± 1.49	15.63^{b} ± 2.16	4,42^{bc} ± 0.04	8.59^{a} ± 1.62
P4Q2	1.03^{b} ± 0.41	19.35^{c} ± 2.66	4,79^{c} ± 0.15	8.05^{a} ± 1.32

Numbers followed by the same letter in the same column indicate no significant difference based on the DMRT test at a 5% significance level ($\alpha = 0.05$). P1Q1 (sucrose ; room temperature), P1Q2 (sucrose ; 30 °C), P2Q1 (glucose ; room temperature), P2Q2 (glucose ; 30 °C), P3Q1 (fructose ; room temperature), P3Q2 (fructose ; 30 °C), P4Q1 (lactose ; room temperature), P4Q2 (lactose ; 30 °C).

Although fructose is also a monosaccharide, differences in bacterial metabolism may lead to less optimal texture (Sari et al., 2020). Disaccharides like lactose and sucrose require hydrolysis before being utilized, potentially reducing texture quality if hydrolysis is incomplete (Nurdin, 2023). Fermentation temperature also plays a key role; although 30°C supports optimal bacterial activity and cellulose formation, the highest hardness at room temperature (26.3 °C) may indicate denser cellulose layers due to slower metabolic rates, resulting in firmer texture (Hamad et al., 2011).

Analysis of variance showed a significant effect ($P < 0.05$) of carbon source on the pH of nata de coco. Fructose, as a monosaccharide, is rapidly metabolized by *A. xylinum* into organic acids, lowering the pH more effectively than glucose or lactose (Hamad et al., 2021). Although fructose supports fermentation, its metabolism is less efficient than glucose, resulting in a slower pH drop. Lactose-treated samples showed higher pH values, as *A. xylinum* lacks β-galactosidase to hydrolyze lactose into usable monosaccharides, leading to limited bacterial growth and minimal acid production. In contrast, sucrose, composed of glucose and fructose, can be hydrolyzed by microbial invertase into fermentable sugars. *A. xylinum* preferentially utilizes glucose from this hydrolysis for cellulose biosynthesis, contributing to better fermentation outcomes (Lasagni et al., 2024).

Analysis of variance showed that variations in temperature and carbon source, with the addition of *A. microphylla*, had no significant effect ($P \geq 0.05$) on the crude fiber content of nata de coco. The highest fiber content (8.87%) was observed with glucose at room temperature (26.3 °C), while the lowest was found with sucrose at the same temperature. All treatments exceeded the minimum crude fiber requirement (4%) based on SNI 01-4317-1996. Glucose and fructose resulted in higher crude fiber content due to their rapid bacterial metabolism and the absence of a need for enzymatic hydrolysis (Hamad et al., 2011). In contrast, sucrose requires hydrolysis before utilization, slowing cellulose production. While specific studies on temperature's impact on fiber content are limited, previous findings suggest that optimal fermentation conditions, including temperature and sugar concentration, influence crude fiber levels in cellulose-based fermented products (Maryam, 2024).

Characteristics of nata affected by sucrose and starter concentration

Analysis of variance revealed that both sucrose and starter concentration had a significant effect ($P < 0.05$) on the thickness, hardness, and pH of nata de coco. However, the combination had no significant effect on the crude fiber content of the nata (Table 2).

Table 2. The physico-chemical characteristics of nata influenced by sugar and starter concentration (mean±SD)

Treatment	Thickness (cm)	Hardness (N)	pH	Crude fiber (%)
A1B1	$0.73^{ab} \pm 0.06$	$12.27^{a} \pm 2.42$	$4.03^{c} \pm 0.05$	$7.75^{a} \pm 1.42$
A2B1	$0.69^{b} \pm 0.10$	$15.94^{a} \pm 8.01$	$3.87^{b} \pm 0.07$	$7.57^{a} \pm 1.58$
A3B1	$0.60^{b} \pm 0.10$	$15.59^{a} \pm 2.82$	$3.72^{a} \pm 0.12$	$7.39^{a} \pm 0.87$
A4B1	$0.47^{a} \pm 0.10$	$12.97^{a} \pm 5.47$	$3.60^{a} \pm 0.11$	$6.44^{a} \pm 1.52$
A1B2	$0.91^{ab} \pm 0.12$	$16.49^{a} \pm 4.87$	$4.07^{c} \pm 0.04$	$7.93^{a} \pm 1.01$
A2B2	$1.07^{b} \pm 0.20$	$10.46^{a} \pm 1.37$	$3.92^{b} \pm 0.16$	$8.17^{a} \pm 0.85$
A3B2	$1.20^{b} \pm 0.03$	$8.34^{a} \pm 0.38$	$3.87^{a} \pm 0.15$	$8.69^{a} \pm 1.71$
A4B2	$0.80^{a} \pm 0.27$	$7.91^{a} \pm 3.79$	$3.70^{a} \pm 0.15$	$7.48^{a} \pm 0.07$

Numbers followed by the same letter in the same column indicate no significant difference based on the DMRT test at a 5% significance level ($\alpha = 0.05$). A1B1 (2% carbon; 5% starter), A1B2 (2% carbon; 10% starter), A2B1 (4% carbon; 5% starter), A2B2 (4% carbon; 10% starter), A3B1 (6% carbon; 5% starter), A3B2 (6% carbon; 10% starter), A4B1 (8% carbon; 5% starter), and A4B2 (8% carbon; 10% starter)

The results indicated that sucrose concentration and starter inoculum significantly affected nata de coco thickness. At 5% starter concentration, increasing sucrose levels from 2% to 8% consistently reduced thickness, whereas at 10% starter, thickness increased up to 6% sucrose before declining at higher levels. These patterns suggest that substrate availability and inoculum size interact to influence cellulose production by *A. xylinum*. Excess sugar concentration may exert osmotic pressure that suppresses bacterial metabolism and cellulose synthesis, leading to thinner nata layers. Conversely, higher starter concentrations tended to support greater thickness, as larger bacterial populations accelerate sugar conversion into cellulose. This finding is consistent with previous studies that reported optimal cellulose formation at moderate sugar levels and sufficient starter inoculum (Yanti et al., 2017; Melindasari et al., 2025). Thus, the balance between microbial population and substrate availability is a key determinant of fermentation efficiency and nata formation.

Sucrose and starter concentration also affected the hardness of nata de coco. Increasing sugar concentration generally reduced hardness, indicating that osmotic stress at higher sugar levels can disrupt cellulose assembly and weaken the gel matrix. At lower sugar levels, higher starter concentrations tended to produce firmer textures, reflecting more active cellulose synthesis and denser gel formation. These results are in line with earlier findings that increasing sugar concentration beyond an optimal range decreases nata hardness (Manurung et al., 2024). While thickness contributes to texture, structural properties such as fiber compactness and porosity also play an important role. For instance, nata with a loose or porous cellulose network may remain soft despite being relatively thick, whereas denser cellulose matrices produce firmer textures (Halib et al., 2012).

The results showed that sucrose and starter concentrations influenced the final pH of nata de coco. Across treatments, pH values ranged between 3.60 and 4.07, indicating significant variation due to the interaction of sugar and inoculum levels. Increasing sugar concentration from 2% to 8% consistently reduced pH at both starter levels, suggesting that excess sugar is metabolized not only into cellulose but also into acetic acid, which accumulates in the medium and lowers the pH (Sun et al., 2018). Meanwhile, higher starter concentration (10%) tended to maintain a higher pH, likely because more rapid cellulose formation reduced the extent of acid accumulation. These findings align with Chairunnissa and Baila (2018), who also reported a final pH close to 4.0 in nata fermentation. In general, the pH after fermentation was lower than the initial coconut water (3.96), reflecting acid production during microbial metabolism, although at lower sugar concentrations (2%) the pH was slightly higher than the starting medium regardless of starter level.

Sucrose and starter concentration also affected the crude fiber content of nata de coco. Treatments with higher starter levels (10%) generally produced higher fiber values, indicating that larger microbial populations contribute more actively to cellulose biosynthesis (Purwani et al., 2022). At lower starter levels (5%), however, increasing sucrose concentration tended to decrease fiber yield, possibly due to osmotic pressure inhibiting bacterial activity. This pattern suggests that sufficient inoculum is required to effectively utilize the available sugar for cellulose formation. Previous studies reported similar trends, such as Rose et al. (2018), who found that higher sugar levels increased fiber content in nata de jackfruit, though the effect depended on microbial activity. The fiber content obtained in this study consistently exceeded the SNI 01-4317-1996 minimum standard of 4.5%, which may be attributed to the nutrient-rich substrate and favorable fermentation conditions. Fiber yield was also related to nata thickness, though differences in cellulose density and water content meant that thicker nata did not always correspond to higher fiber content (Maulani et al., 2018).

CONCLUSION

This study demonstrated that the utilization of *Azolla microphylla* as an alternative nitrogen source in nata de coco production can support cellulose synthesis by *Acetobacter xylinum* under various fermentation conditions. The results showed that both fermentation temperature and carbon source significantly affected nata thickness, hardness, and pH, while sucrose and starter concentration influenced all measured characteristics except crude fiber.

Variations in sugar type and temperature highlighted the importance of substrate accessibility and microbial metabolism, where simple sugars facilitated faster cellulose formation and temperature shifts regulated texture development. Changes in sucrose and starter concentration further revealed interactive effects, with sugar availability and inoculum size jointly determining thickness, hardness, and pH outcomes. Although crude fiber content was not significantly influenced by most interactions, treatments generally exceeded the minimum standard, indicating sufficient cellulose biosynthesis. Overall, fermentation parameters including temperature, type and concentration of carbon source, and inoculum level played a decisive role in shaping nata yield and quality when *A. microphylla* was used as a nitrogen source.

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