JBAT 12(2) (2023) 129 – 136

Jurnal Bahan Alam Terbarukan

Accredited SINTA 2 by Ministry of Research and Technology/National Research and Innovation Agency
SK No. 200/M/KPT/2020
http://journal.unnes.ac.id/nju/index.php/jbat

p-ISSN 2303 0623
e-ISSN 2407 2370

Process of Bacterial Cellulose Production from Tofu Wastewater Without Pretreatment Using *Acetobacter xylinum*

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DOI: https://doi.org/10.15294/jbat.v12i2.47510

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Article Info

Article history:
Received
9 September 2023
Revised
28 November 2023
Accepted
4 December 2023
Online
5 December 2023

Keywords:
Acetobacter xylinum;
Bacterial cellulose;
Nata de soya;
Tofu wastewater;
Static batch

Abstract

Bacterial cellulose (BC) is an alternative cellulose source that warrants more investigation due to its limited efficiency and high production costs. This research aims to investigate the generation of bacterial cellulose from tofu wastewater using *Acetobacter xylinum* without pretreatment. The experiment was run on two media: tofu wastewater with and without additional sugar. The fermentation was run in a dark cabinet using a static batch method in numerous fermenter trays, each with a working volume of 1000 mL. Each experiment used 900 mL of tofu wastewater medium plus an extra 100 mL inoculum (equal to 10% v/v), which was then cultured at room temperature and harvested on days 6, 12, 18, 24, and 30. The weight of nata de soya, BC, and residual sugar were all measured from the samples. The results of the experiments revealed that the best incubation time was 18 days. The fermentation employing tofu wastewater medium with added sugar yielded nata de soya of 227.3 g/L and BC of 32.2 g/L, while the medium without added sugar yielded 103.9 g/L and BC of 9.3 g/L. The medium with added sugar yielded higher BC productivity, 1.79 g/L.day, compared to 0.57 g/L.day in the medium without added sugar. On the other hand, the BC results per sugar consumption were 0.62 g BC/g sugar and 0.36 g BC/g sugar, respectively, for the medium with and without added sugar.

INTRODUCTION

Cellulose is a natural polymer that is environmentally friendly and widely used. The primary source of cellulose is lignocellulosic material through a delignification process (Mulyadi, 2019). The delignification process requires high mechanical and thermal energy, potentially impacting environmental pollution. In addition, developing plants for cellulose production requires a long time and a large area of land. Therefore, a study to find alternative sources of cellulose is a fascinating study to do.

Bacterial cellulose (BC) is an alternative source of cellulose that has been extensively studied for various purposes. Among other things, the development of bacterial cellulose aims to be utilized as composite materials (Almeida et al., 2022; Luz et al., 2020), bioelectronic materials (Zhang et al., 2022), absorbents (Guimarães et al., 2023; Madeira et al., 2020; Nguyen Ngo et al., 2023), water treatment (Yaqoob et al., 2020), and bacterial filter (Suryanto et al., 2023). Thus, bacterial cellulose is an industrial commodity with a broad market and is continually growing.

The advantage of bacterial cellulose is that it is pure cellulose, which does not require a complex separation process as in the process of delignification of wood (Seto & Sari, 2013). However, low efficiency and high production costs are still obstacles to the development of bacterial cellulose (Li et al., 2023). One of the factors related

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to efficiency and production costs is how to get cheap sources of raw materials or substrates so that bacterial cellulose can become technically and commercially feasible. Various substrates have been investigated for the production of bacterial cellulose, including palm frond juice (Azmi et al., 2023), lotus rhizome (Nie et al., 2022), paper waste sludge (Nguyen Ngo et al., 2023), cashew apple juice (Guimarães et al., 2023), orange peel (Tsouko et al., 2020), and cassava wastewater (Sari et al., 2022).

Tofu wastewater is an alternative potential raw material for bacterial cellulose production in Indonesia. It is important to study because of its abundant availability and relatively high organic matter content, namely the BOD value of 8852 mg/L (Rajagukguk, 2020). The tofu production produces wastewater of as much as 20.4 L/kg of soybean (Sjafruddin et al., 2022). It has the potential to pollute the environment because its organic load can disrupt the biotic ecosystem (Aris et al., 2021). Unfortunately, the carbohydrate content in tofu wastewater is a complex sugar that must be converted to simple sugars to be utilized by BC producers, which typically only use simple sugars.

Some bacteria have been widely studied as a monoculture in bacterial cellulose production. Among them are *Acetobacter* sp., *Gluconobacter* sp., and *Komagataeibacter* sp. However, *Acetobacter xylinum* is a species that is easy to find and commonly used to produce bacterial cellulose using the simple sugar of sucrose. Therefore, monoculture fermentation of *A. xylinum* in tofu wastewater substrate requires a pretreatment process to convert complex sugar into simple sugar. Nonetheless, the acidic nature of tofu wastewater is expected to cause the hydrolysis to run naturally. The other fermentation method is co-culture between two bacteria, such as *Bacillus cereus* co-culture with *Komagataeibacter xylinus* (Li et al., 2023) or *Komagataeibacter nataicola with Lactobacillus* (Jiang et al., 2023). The co-culture method is intended to realize cooperation among the bacteria.

Several factors, including sugar content, nitrogen content, and incubation time, influence the productivity of bacterial cellulose. An incubation time that is too long has the potential for medium contamination, the nata layer being thick, and the texture becoming hard (Marliyana et al., 2021). The length of incubation affects the physical quality, fiber content, and organoleptic properties of nata de cassava. The best nata de cassava product was obtained on the 13th day of fermentation with a thickness of 1.37 cm (Putriana & Aminah, 2013). In contrast to nata de cassava, the best nata de coco was obtained on the 10th day of fermentation, which was 600 g/L medium (Gresinta et al., 2019). Fatima et al. (2023) reported that fermentation and drying conditions affected the properties of the resulting cellulose bacterial membrane. Meanwhile, acid treatments affect the bacterial cellulose characteristics from cassava wastewater (Sari et al., 2022).

This study aims to investigate the use of tofu wastewater without pretreatment to produce bacterial cellulose using *A. xylinum* in a fermentation process. Tofu wastewater with and without added sugar was used as the experimental media. The weight of nata de soya and bacterial cellulose were recorded at a regular incubation time. Its residual sugar in the broth was also examined in the incubation period. The performance of the fermentation process was evaluated in the parameters of productivity of nata de soya and bacterial cellulose, bacterial cellulose content, and yield of bacterial cellulose to sugar consumption. Additionally, the kinetic sugar consumption rate was also investigated.

EXPERIMENTAL METHOD

Microorganism and Medium

Acetobacter xylinum was obtained from The Laboratory of Soil Biology and Biotechnology, Faculty of Agriculture, Universitas Sebelas Maret. The medium materials consisted of tofu wastewater (obtained from a small scale of tofu in Jebres, Surakarta, Indonesia), sugar (cane sugar obtained from the commercial market in Surakarta), yeast extract (obtained from Sigma Aldrich), and other chemicals from Merck Chemicals (K_2HPO_4, $MgSO_4 7H_2O$, $(NH_4)_2SO_4$, and glacial acetic acid). The bacteria was grown and stored in a stock culture medium at room temperature. The stock culture and inoculum used the same medium, i.e., tofu wastewater containing (g/L): sugar, 100; yeast extract, 2; K_2HPO_4, 4; $MgSO_4$, 0.5; and $(NH_4)_2SO_4$, 13. The production medium was prepared using tofu wastewater containing (g/L): sugar, 100 g/L; and $(NH_4)_2SO_4$, 26. The pH of the medium was adjusted to around 3.95 with glacial acetic acid.

Inoculum Development

One hundred milliliter medium was sterilized at 121°C for 20 minutes, cooled, and filtered. It was inoculated with a stock culture of 10% v/v and incubated in an incubator shaker at room temperature and 75 rpm for seven days (the target cell population was about 6.0×10^8 cells/mL). The inoculum is ready to use.

Bacterial Cellulose Production

Nine hundred milliliters of the sterilized medium were inoculated with 100 mL of inoculum. It was filled in a tray bioreactor, covered with paper, and placed in a dark cabinet. It was incubated at room temperature until specific days to harvest, i.e., 6, 12, 18, 24, and 30 days. The sample was chosen to analyze nata de soya (wet weight), bacterial cellulose (dry weight), and total sugar. The experiments were carried out as triplicates, and the data were presented at the average value.

Chemical Analysis

Residual sugar was analyzed as total sugar by the modified Dubois method (Margono et al., 2018). Nata de soya was harvested from the fermentation broth and weighed as the weight of nata de soya (wet weight) expressed in units of g/L. Meanwhile, bacterial cellulose was obtained from wet nata de soya, which was pressed and dried at 50°C to constant weight (g/L).

RESULTS AND DISCUSSION

Sugar Consumption Rate

These experiments of bacterial cellulose production used two types of media, i.e., tofu wastewater with and without added sugar. The residual sugar profiles during fermentation of each medium are shown in Figure 1.

The end residual sugar in the wastewater with added sugar is 38.9 g/L (Figure 1), higher than the original sugar in the tofu wastewater of 30 g/L. It shows that during the 30-day incubation period, *A. xylinum* utilized the extra sugar (simple sugar) rather than the wastewater's original sugar (complex sugar). Meanwhile, the wastewater without added sugar resulted in a final residual sugar of 12.9 g/L, much less than the original sugar in the wastewater. It suggests that a hydrolysis event occurred on the complex sugar once the simple sugar was used up.

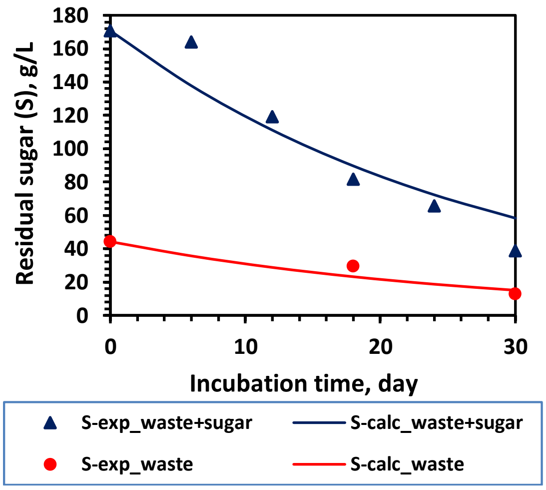


Figure 1. Residual sugar profiles of experimental and calculation results.

This profile of residual sugar shows that the sugar consumption rate by *A. xylinum* can be approximated by the first order of sugar consumption rate. The mathematical model for the sugar consumption rate is shown in Equation (1).

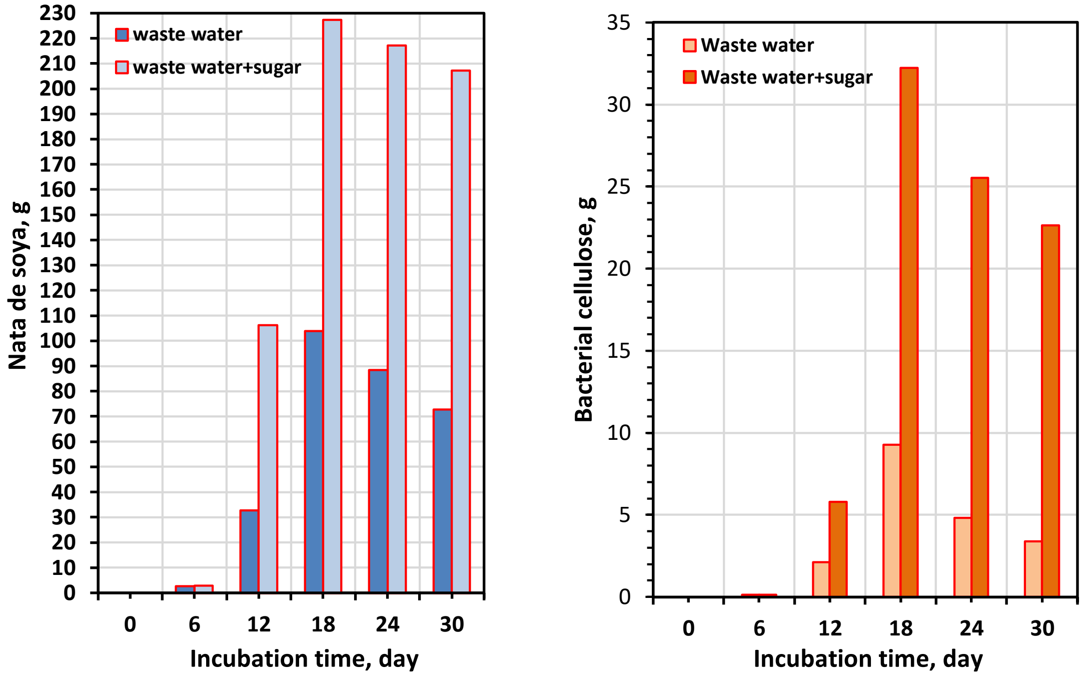
$$\frac{dS}{dt} = -k_d S \qquad (1)$$

S is the residual sugar (g/L), t is the incubation time (day), and k_d is the coefficient of sugar consumption rate (day^{-1}). Based on the experimental data, the k_d value is 0.036 day^{-1} with the root mean square error (RMSE) of 14.4, referring to the RMSE formula by Salakkam et al. (2023). This RMSE value is quite worthy compared to another previous result, i.e., the RMSE 29.8 for the mathematical model of substrate consumption in bioethanol production (Salakkam et al., 2023). Implementing Equation (1) and the value of k_d on fermentation data using medium without added sugar shows that Equation (1) matches the experimental data of medium without added sugar with the RMSE of 3.8.

Profiles of Nata De Soya and Bacterial Cellulose

Nata de soya and bacterial cellulose increased over the first 18 days of incubation. However, after the incubation period of 18 days, nata de soya and bacterial cellulose declined until the fermentation was terminated. The growth of nata de soya and bacterial cellulose in the two media are presented in Figure 2.

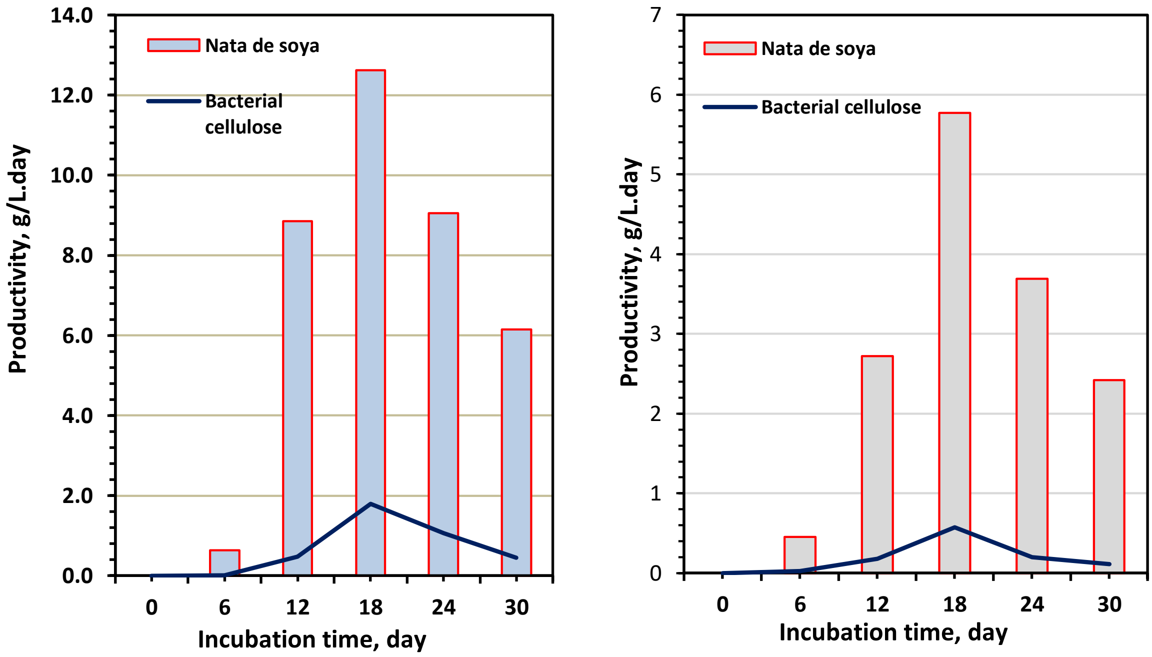
The weight of nata de soya achieves its maximum weight, which is 227.3 g/L for the medium with added sugar and 103.9 g/L for the



(a) (b)

Figure 2. The profiles of nata de soya and bacterial cellulose throughout fermentation.

medium without added sugar, respectively, at 18 days incubation. The maximal weights of bacterial cellulose for the medium with and without added sugar are 32.2 g/L and 9.3 g/L, following the same pattern of nata de soya (Figure 2b). Nata de soya and bacterial cellulose have lost weight after 18 days of incubation due to decreased sugar and increased acid acting as a product inhibitor metabolite (Putriana & Aminah, 2013). Additionally, cells will lose their reserve energy and die due to dietary inadequacies. Bacterial cells that have expired will be lysed and decomposed. Other results in nata de



(a) (b)

Figure 3. Productivity of nata de soya and bacterial cellulose (a. wastewater+sugar; b. wastewater).

soya growth during the incubation period were reported by Nurhayati (2006); it reached 418.1 g/L on the seventh day of incubation and fell to 247.6 g/L on the fourteenth day of incubation.

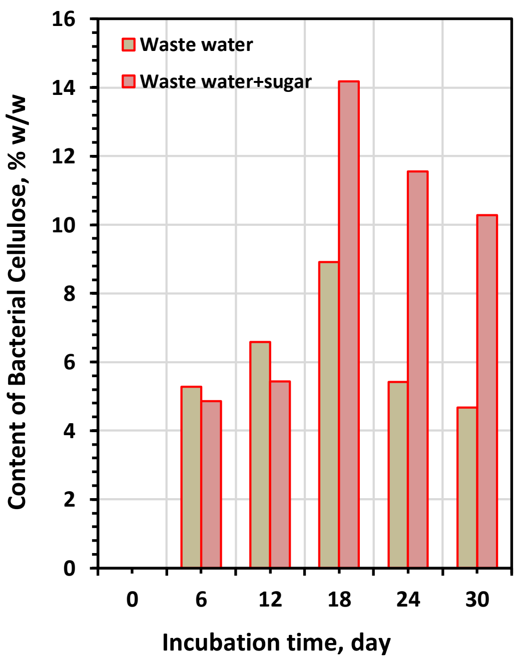
One of the evaluating elements for the production fermentation process is the productivity of nata de soya and bacterial cellulose. They indicate the production rate per medium volume per incubation period represented in units of g/L.day. Figure 3 displays the productivity of bacterial cellulose and nata de soya throughout fermentation.

Nata de soya fermentation using a medium with added sugar resulted in the highest productivity of 12.6 g/L.day, while the highest productivity of bacterial cellulose is 1.79 g/L.day (Figure 3a). The maximum productivity of nata de soya on a medium without additional sugar is recorded in Figure 3b, reaching 5.77 g/L.day and 0.57 g/L.day for its bacterial cellulose productivity. Most of the greater productivity of nata de soya at the same incubation time is produced by fermentation utilizing a tofu wastewater medium with added sugar. Previous research employing tofu wastewater medium with 100 g/L of additional sugar and 14 days of incubation resulted in cellulose productivity of 1.58 g/L/day (Budiarti, 2008).

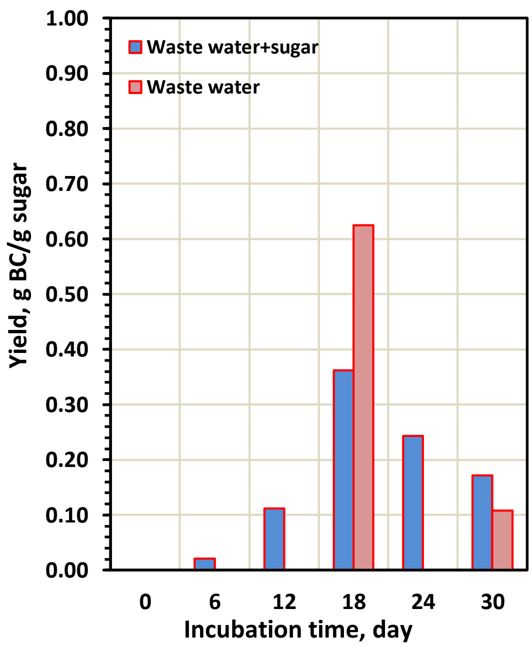
The content of bacterial cellulose in nata de soya (% w/w) fluctuates with incubation time. Figure 4a depicts the profile of bacterial cellulose content in nata de soya during fermentation. On the other hand, yield—the amount of bacterial cellulose generated relative to the amount of sugar consumed—also varies with incubation duration. Figure 4b displays the yield of bacterial cellulose production to the amount of sugar consumed (g BC/g sugar).

Figure 4a demonstrates that the bacterial cellulose content in nata de soya peaks after an 18-day incubation period and then declines. Tofu wastewater with and without added sugar resulted in maximum percentages of 14.18% w/w and 8.91% w/w, respectively. A similar phenomenon happens in the pattern of yield-bacterial cellulose to sugar consumption. It reaches maximum values of 0.36 g BC/g sugar and 0.62 g BC/g sugar, respectively, for the medium with and without added sugar (Figure 4b). It takes 18 days for this maximum value to be reached. However, the yield produced on the medium without added sugar is greater than the other medium. This oddity demonstrates the necessity for more research into the best medium for maximum yield.

Several production parameters that have been previously evaluated show that the optimal fermentation time for the production of nata de soya and bacterial cellulose is 18 days. The weight of nata and bacterial cellulose achieved their highest values at 18 days of incubation, along with productivity, percentage of cellulose in nata, and



(a)



(b)

Figure 4. Bacterial cellulose content in nata de soya (% w/w) and yield of bacterial cellulose (BC) to sugar consumption

Table 1. Various performances of bacterial cellulose production using various substrates

No	Substrate	Species	Productivity, g BC/L.day	Yield, g BC/g sugar	BC Fraction, % w/w	References
1.	Coconut water with added sugar	*Acetobacter xylinum*	-	-	0.0015	Local market
2.	Soya whey with added sugar	*Acetobacter xylinum*	1.58	-	-	(Budiarti, 2008)
3.	Oil palm frond juice	*Acetobacter xylinum*	-	0.195	-	(Azmi et al., 2023)
4.	Hydrolyzed Paper waste sludge	*Acetobacter xylinum*	0.8	-	-	(Ngo et al., 2023)
5.	Hydrolyzed Lotus rhizome	*Acetobacter pasteurianus*	0.26	0.56	-	(Nie et al., 2022)
6.	Hydrolyzed Orange peel waste	*Komagataeibacter sucrofermentans*	1.55	0.45	-	(Tsouko et al., 2020)
7.	Aerola byproduct	*Kombucha culture*	0.40	-	-	(Leonarski et al., 2021)
8.	Confectionery wastes	*Komagataeibacter sucrofermentans*	0.63	0.36	-	(Efthymiou et al., 2022)
9.	Tofu wastewater with added sugar	*Acetobacter xylinum*	1.79	0.36	14.18	Recent work
10.	Tofu wastewater	*Acetobacter xylinum*	0.57	0.62	8.91	Recent work

yield of bacterial cellulose to sugar consumption. The value of the measured parameters will decrease with an incubation period longer than 18 days. All experimental results might be competitive, but comparing them to findings from earlier research would be preferable. The findings of some earlier investigations that can be compared are presented in Table 1.

CONCLUSION

The consumption rate of sugar in the fermentation of tofu wastewater using *Acetobacter xylinum* followed the first-order consumption rate with a consumption rate coefficient of 0.036 day^{-1}. At the optimal incubation time of 18 days, tofu wastewater with added sugar resulted in nata de soya of 227.3 g/L and bacterial cellulose of 32.2 g/L. This result is comparable to the productivity of nata de soya of 12.60 g/L.day and bacterial cellulose of 1.79 g/L.day. In comparison, the medium without added sugar resulted in nata de soya of 103.9 g/L and bacterial cellulose of 9.3 g/L or equivalent to nata de soya productivity of 5.77 g/L.day and bacterial cellulose of 0.57 g/L.day. The highest yields of bacterial cellulose to sugar consumption were 0.62 g BC/g sugar and 0.36 g BC/g sugar for the medium with and without added sugar, respectively. The maximum content of bacterial cellulose is 14.18% w/w and 8.91% w/w for fermentation using wastewater medium with and without added sugar, respectively.

ACKNOWLEDGEMENTS

The authors would like to thank Universitas Sebelas Maret for supporting this research through the scheme of Penelitian HGR-UNS with contract number 228/UN27.22/PT.01.03/2023.

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