

Determining the best composition of bacterial cellulose medium made from glycerol and soybean-soaked wastewater for nata de coco production using Taguchi method

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Abstract. The demand for cellulose is increasing every year. Therefore, developing bacterial cellulose (BC) production in the form of nata de coco in Indonesia needs to be done. One of the ways that can be done to support it is by modifying the carbon and nitrogen sources used. In this study, we use soybean-soaked wastewater as the nitrogen source alternative and glycerol as the carbon source alternative. Taguchi methods are used to obtain the best composition of glycerol, soybean-soaked wastewater, and ZA to make the best bacterial cellulose based on its thickness. To verify the best composition obtained by using the Taguchi Method, a confirmatory experiment was carried out. The best composition is soybean-soaked wastewater concentration 20%, glycerol concentration 1%, and ZA concentration 0,2%.

1 Introduction

Cellulose is an organic compound in the form of a polysaccharide that can be produced by several living organisms such as plants, algae, and bacteria. The annual demand for cellulose is expected to increase every year with the growing use of cellulose by the pharmaceutical and technical industries [1]. Therefore, the cellulose demand needs to be met.

BC is a cellulose produced by bacteria. Many bacteria can produce BC, such as genus *Achromobacter*, *Alcaligenes*, *Aerobacter*, *Agrobacterium*, *Azotobacter*, *Komagataeibacter*, *Acetobacter*, *Pseudomonas*, *Rhizobium*, *Sarcina*, and *Rhodobacter* [2]. One of the most commonly used bacteria for producing BC is *Komagataeibacter xylinus* (formerly known as *Gluconacetobacter xylinus* or *Acetobacter xylinum*) because it is classified as an acetic acid bacteria that synthesizes cellulose as its primary metabolite [3]. In Indonesia, BC is usually produced as nata de coco, which is made from coconut water fermentation using *Komagataeibacter xylinus*.

To fulfil the cellulose demand, the production can be done in the form of BC. One of the advantages of using BC to replace cellulose is that BC can be made according to the thickness required, such as for food around 1 cm and thin thickness for pharmaceutical needs [4]. However, one of the problems with BC industrialization, especially nata de coco industry in Indonesia is the expensive production costs [5]. Therefore, the BC industry can be developed by saving production costs.

There are several factors that influence the production of nata de coco such as carbon source, nitrogen source, pH, oxygen availability, other nutrients, temperature, and fermentation time [6]. The development of BC production

can be done by changing the carbon source and nitrogen source using better alternative with lower cost. Both factors are important because nitrogen can stimulate the growth of *Komagataeibacter xylinus* and is closely related to the volume of cellulose formed [7]. Carbon serves as an energy provider for bacterial growth.

There are some cheaper alternatives that can be used as a nitrogen source, such as soybean oil production wastewater, tofu workshops wastewater, and the wastewater from breweries and wineries [8]. In this study, soybean-soaked wastewater was used as a nitrogen source because it has organic nutrition and protein extracted from soybean that has the potential for fermentation of BC. For carbon sources, there are some alternatives that can be used such as sucrose, lactose, glucose, fructose, and glycerol. Glycerol is selected in this study as the carbon source because it produces a better yield than glucose, the usual carbon source for the fermentation of nata de coco [9-10].

Research is needed to use those materials for BC production to get the best composition that can yield the best BC based on its thickness. The method that can be used to obtain the best composition is full factorial design. However, this method requires a lot of time and costs. Therefore, Taguchi method was used for this study. Taguchi method is a high-efficiency design method that uses mathematical statistics in which a small number of experiments can discover significant factors quickly with the principle of orthogonality using the orthogonal table [11]. The benefit of using this method is that it requires less computational time, less cost, and is easy to use. The orthogonal table was designed using 3-factors with 2-levels, with the factors being soybean-soaked wastewater, ammonium (ZA), and glycerol. ZA is usually used as a nitrogen source in nata de coco production. In this study,

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ZA was used as a factor to determine whether a combination of ZA and soybean-soaked wastewater could optimize the production of BC. The formation of BC in nata de coco fermentation in this study was monitored using real-time image processing to reduce the chance of contamination during observation.

This study aimed to find the best composition of glycerol, soybean-soaked wastewater, and ZA to produce the best yield of BC based on its thickness with the help of real-time image processing during observation. The selection of BC with the best thickness aims to make production costs more efficient by producing BC with the best thickness using as little time as possible.

2 Experimental design

2.1 Materials and equipment

This study used some materials, such as *Komagataebacter xylinus* starter, soybean-soaked wastewater, glycerol, coconut water, ZA, vinegar, and glucose. The equipment used was a fermenter, camera, lamp, Raspberry Pi 4, and monitor.

Soybean-soaked wastewater used in this study was from Tempe Londho. Based on laboratory test results, nitrogen content in the wastewater was 0.0172%. The starter solution was made using 1.5 l of filtered coconut water added with 5% technical glucose (75 g), 0.134% vinegar (2 ml), and 0.2% ZA (3 g). The solution was heated until it boiled ($T = 100^{\circ}\text{C}$) for 30 seconds and then lowered the heating temperature ($T = 60^{\circ}\text{C} \pm 5$) for 10 minutes. Then, let it stand at room temperature until it reaches average temperature ($T \leq 30^{\circ}\text{C}$). Put the solution into a fermenter bottle aseptically and 20% *Komagataebacter xylinus* was added. The solution was fermented for 8 days to be used.

The fermenter used for medium production refers to Nugroho et al. [12], which measures 35 cm in length, 25 cm in width, and 6 cm in height. It is made from acrylic with the exterior colored non-reflective black. There is a transparent area with a width of 6 cm and height of 4 cm on one side with a USB camera (MEX121 Web Cam M-Tech WB 100 5MP 480p) placed in front of it to capture images depicting the thickness of the BC. A 145 lux LED light is placed above the camera to provide lighting. The fermenter arrangement is shown in Figure 1.



Fig. 1. Fermenter design [12].

2.2 Identification of factors and its levels

The control factors used in this study were glycerol, soybean-soaked wastewater, and ZA. The response in this

study is the thickness of BC. For glycerol, the concentrations used were 2% [9] and 1% [13]. For soybean wastewater, the concentrations used are 20% and 30% [14]. For ZA, the concentrations used are 0.2% and 0% to determine if soybean-soaked wastewater can replace ZA. The following table shows the factors and levels used.

Table 1. Control factors and levels.

Factor	Code	Level	
		1	2
soybean-soaked wastewater concentration	A	20%	30%
glycerol concentration	B	2%	1%
ZA concentration	C	0%	0,2%

2.3 Bacterial cellulose fermentation

Fermentation of BC started with the production of medium. Design of experiment of the medium production was done by using Taguchi orthogonal array. Since there are 3 factors and 2 levels for each factor, the orthogonal array used is L_4 . The orthogonal array table used is shown in Table 2.

Table 2. Medium production orthogonal array.

Experiment	Factor		
	A	B	C
Experiment 1	20%	2%	0%
Experiment 2	20%	1%	0,2%
Experiment 3	30%	2%	0,2%
Experiment 4	30%	1%	0%

This combination was added to filtered coconut water whose volume depends on soybean-soaked wastewater. For 30% soybean-soaked wastewater needs 70% coconut water and for 20% soybean-soaked wastewater needs 80% coconut water. Then, 0.16% acetic acid was added. The medium was boiled ($T = 100^{\circ}\text{C}$) for 30 seconds and then the heating temperature was lowered ($T = 60^{\circ}\text{C} \pm 5$) for 10 minutes. Then, let it stand at room temperature until it reaches average temperature ($T \leq 30^{\circ}\text{C}$). The medium was put into a fermenter aseptically. 20% *Komagataebacter xylinus* was added to start the fermentation of bacteria cellulose. The fermenter was closed using newspaper. The thickness measurement of BC was done on day 8 because it has reached the

maximum thickness or has entered the stagnant phase [15-16]

2.4 Image processing preparation

In this study, image processing is used to capture the thickness of the BC. Preparation of image processing was done by using Raspberry Pi 4 preinstalled with Raspbian OS, Python, OpenCV, and MySQL database. The database contains some information such as date, time, fermentation code, and bacterial cellulose thickness. Date and time are filled automatically to the current time and date on the Raspberry Pi. The fermentation code is typed manually when starting the fermentation program file command. Outcome of the programming script measurement and calculation is the condition of the bacterial cellulose.

2.5 Data collection

The data is in the form of an image that describes the thickness of the BC and is taken using a USB camera. The images are then converted into information, stored in a MySQL database, and sent to the user's Telegram using the Telegram Bot API when connected to the internet.

2.6 Data analysis

Data analyzed using programming language R 4.4.0 to measure S/N ratio and analysis of variance (ANOVA). S/N refers to the term 'signal' which is the desirable value (mean) for the response and 'noise' which is the undesirable value for the response. Analyses of the effects of each factor were performed with an S/N response table. It shows the calculated S/N ratios of control factors at each level. ANOVA is a part of statistical analysis methods commonly used to test the comparison of two or more means of a population or sample data. ANOVA method was used to calculate the percentage of contribution of each factor.

2.7 Confirmatory experiment

Confirmatory experiment is the validation of the optimal factors and level that has been obtained in the previous analysis [17]. It is done by comparing the predicted means of BC thickness at the optimum level and its confidence interval with the results from the confirmatory experiment and its confidence interval. When both of the intervals are intersected, the optimal factors and level is validated.

3 Results and discussion

3.1 BC thickness

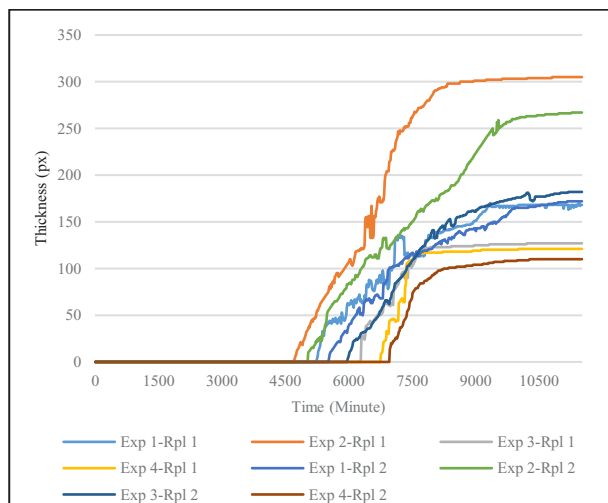


Fig. 2. BC thickness during fermentation.

Figure 2 shows the thickness of BC during fermentation for each experiment. Experiment 2 had the thickest BC for both replications while experiment 4 had the thinnest BC for both replications. These results are different from the results of research conducted by Maloringan and Nugroho [14] which soybean-soaked wastewater that produces the best BC thickness is 30%. There was difference in the carbon source used, where in Maloringan and Nugroho's research, glucose was used. Therefore, further research regarding the comparison of the relationship between several carbon sources and several nitrogen sources is needed.

The data of BC thickness on day 8 for each experiment and its replication is shown in Table 3.

Table 3. BC thickness on day 8.

Experiment	BC thickness (px)	
	Replication 1	Replication 2
Experiment 1	168	172
Experiment 2	305	267
Experiment 3	127	182
Experiment 4	121	110

3.2 BC thickness mean and S/N ratio

Based on the obtained data on BC thickness, the thickness mean and its S/N Ratio were calculated for each experiment. The equations for thickness mean and S/N ratio shown below

$$\bar{y} = \frac{\sum_{i=1}^n y_i}{n} \tag{1}$$

$$S/N \text{ ratio} = -10 \log \left(\frac{1}{n} \sum_{i=1}^n \frac{1}{y_i^2} \right) \tag{2}$$

The calculated results of the thickness mean and S/N ratio are summarized in Table 4.

Table 4. Mean and S/N ratio.

Experiment	Means (px)	S/N ratio
Experiment 1	170	44,61
Experiment 2	286	49,07
Experiment 3	154,5	43,36
Experiment 4	115,5	41,22

3.3 Effect for S/N ratio yield approach

Quality characteristic of the S/N Ratio used in this study is the larger the better because the larger the thickness of the BC is the better. The optimum conditions on this approach obtained by sorting the difference value of the effect for S/N ratio yield. The greater the difference value of a factor means the factor contributes more to the thickness of the BC.

Table 5. Effect for S/N ratio yield.

	Factor A	Factor B	Factor C
Level 1	46,84	43,985	42,915
Level 2	42,29	45,145	46,215
Difference	4,55	1,16	3,3
Rank	1	3	2

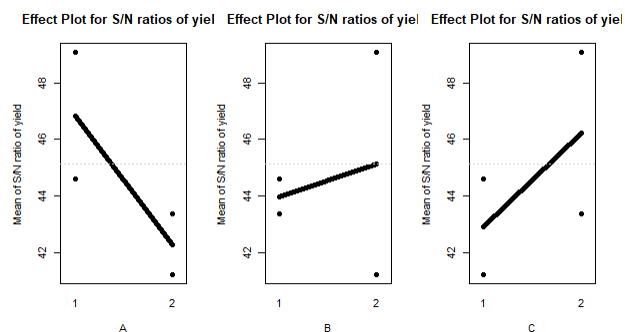


Fig. 3. Effect plot for S/N ratio yield.

Based on Table 5, the greater difference value is soybean-soaked wastewater concentration (A), followed by ZA concentration (C), and glycerol concentration (B).

Each factor effect was plotted in a graph that shown in Figure 3. The highest plots of each factor were chosen as the best composition for making BC with larger thickness. The best composition obtained by this approach are A1, B2, and C2.

3.4 Two-way ANOVA approach

The ANOVA used in this study was two-way ANOVA because it uses 3 independent variables (3 controlled factors) with 1 dependent variable (BC thickness). The hypothesis null is rejected if F-ratio is greater than F-table. Result of two-way ANOVA is shown in Table 6.

Table 6. Two-way ANOVA.

Factor	Sum of squares	Degree of freedom	Mean squares	F _{ratio}	Contribution
A	17298	1	17298	29,99	50,02 %
B	2964,5	1	2964,5	5,14	8,57%
C	12012,5	1	12012,5	20,83	34,74 %
Error	2307	4	576,75		
Total	34578	7			

From Table 6, F-table ($F_{(0,05;1;4)} = 7.709$) was compared to F ratio for each factor with the level of significance $\alpha = 5\%$. It was found that glycerol F ratio was lower than F-table. Therefore, glycerol concentration (B) did not affect the response significantly. It can be seen from experiment 2 and experiment 4 that has similar glycerol concentrations but very different results.

Soybean-soaked wastewater concentration (A) has the biggest contribution to the thickness of BC (50,02%) followed by ZA concentration (C) (34,74%). Based on the experiment, using 20% soybean-soaked wastewater concentration produced BC with better thickness than 30%.

3.5 Confirmatory experiment

To carry out a confirmatory experiment, a predicted value (μ_{pred}) and confidence interval (CI) for predicted values was calculated based on the means of the best composition that has been obtained. The equations of CI for predicted values shown below

$$CI = \pm \sqrt{\frac{F_{(\alpha;v_i;v_e)} \times MS_e}{n_{eff}}} \tag{3}$$

$$n_{eff} = \frac{\text{number of experiment}}{1 + \text{degree of freedom total}} \tag{4}$$

The predicted value of the best composition is 286 px with the calculated result of CI for predicted values is $\pm 66,68$. Therefore, the prediction confidence interval is

$$\begin{aligned} \mu_{pred} - CI &\leq \mu_{pred} \leq \mu_{pred} + CI \\ 286 - 66,68 &\leq 286 \leq 286 + 66,68 \\ 219,32 &\leq 286 \leq 352,68 \end{aligned}$$

Confirmatory experiment was done by fermenting BC using the best composition. The following figure is BC which is fermented using the best composition.

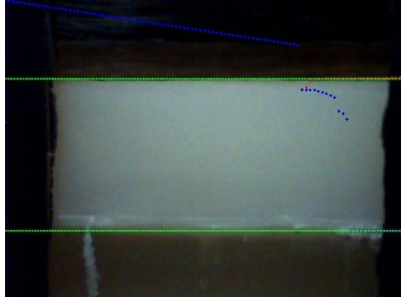


Fig. 4. Confirmatory experiment result.

The thickness of the BC as the confirmatory experiment result was 242 px. The CI for experiment value was calculated with equation below

$$CI = \pm \sqrt{F_{(\alpha, v_i; v_e)} \times MS_e \times \left(\frac{1}{n_{eff}} + \frac{1}{r}\right)} \quad (5)$$

$$r = \text{number of confirmatory experiment} \quad (6)$$

The calculated result of CI for experiment value is $\pm 94,30$. Therefore, the experiment confidence interval is

$$\begin{aligned} \mu - CI &\leq \mu \leq \mu + CI \\ 242 - 94,30 &\leq 242 \leq 242 + 94,30 \\ 147.7 &\leq 242 \leq 336.3 \end{aligned}$$

From the calculation results, the prediction confidence interval intersects with the experiment confidence interval. The intersection between the intervals means the best composition was validated.

4 Conclusion

By using Taguchi method and ANOVA, the best composition of BC medium to produce the best yield of BC based on its thickness are soybean-soaked wastewater concentration (20%), ZA concentration (0,2%), and glycerol concentration (1%). The greatest contribution is given by soybean-soaked wastewater concentration (50.02%) followed by ZA concentration (34.74%). Glycerol concentration didn't affect the response significantly. The predicted result compared to the confirmatory experiments is validated. Therefore, the obtained composition is proven to be effective. However, further research needs to be done regarding its industrial production implementation in real environment.

We express our deepest gratitude to Universitas Gadjah Mada for providing the research grant for our final project through the Final Project Recognition Grant Universitas Gadjah Mada Number 5075/UN1.P.II/Dit-Lit/PT.01.01/2023.

References

1. R. Naomi, R. Bt Hj Idrus, M.B. Fauzi, Plant- vs. Bacterial-Derived Cellulose for Wound Healing: A Review. *IJERPH* **17**, 6803 (2020). <https://doi.org/10.3390/ijerph17186803>
2. S.A. Liany, W. Syafira, A. Putri, A.U. Khasanah, Effect of Bacterial Cellulose (BC) Formation on Various Substrate Variations and Combinations. *BIB* **13**, 13–20 (2022). <https://doi.org/10.22146/bib.v13i2.4396>
3. I.A.A. Fernandes, A.C. Pedro, V.R. Ribeiro, D.G. Bortolini, M.S.C. Ozaki, G.M. Maciel, C.W.I. Haminiuk, Bacterial cellulose: From production optimization to new applications. *Int. J. Biol. Macromol* **164**, 2598-2611 (2020). <https://doi.org/10.1016/j.ijbiomac.2020.07.255>
4. S.M. Choi, K.M. Rao, S.M. Zo, E.J. Shin, S.S. Han, Bacterial Cellulose and Its Applications. *Polymers* **14**(6), 1080 (2022). <https://doi.org/10.3390/polym14061080>
5. C. Zhong, Industrial-Scale Production and Applications of Bacterial Cellulose. *Front. Bioeng. Biotechnol* **8**, 605374 (2020). <https://doi.org/10.3389/fbioe.2020.605374>
6. S.S. Muthu and R. Rathinamoorthy, Bacterial Cellulose Sustainable Material for Textiles, (Springer, Singapore, 2021)
7. H. Mutmainnah, F. Renhoat, Pengaruh Jenis Sumber Nitrogen Terhadap Karakteristik Nata De Sago. *Bionature* **23**(2), 84-90 (2022). <https://doi.org/10.35580/bionature.v23i2.37729>
8. H. Zhou, J. Wang, H. Yin, C. Wang, L. Zhang, X. Zhang, N. Qiao, D. Yu, Bacterial cellulose production from wastewater and the influence of its porosity on the fluorescence intensity of prepared carbon dots. *Biochemical Engineering Journal* **187**, 108633 (2022). <https://doi.org/10.1016/j.bej.2022.108633>
9. D. Mikkelsen, B.M. Flanagan, G.A. Dykes, M.J. Gidley, Influence of different carbon sources on bacterial cellulose production by *Gluconacetobacter xylinus* strain ATCC 53524. *J. Appl. Microbiol.* **107**(2), 576–583 (2009). <https://doi.org/10.1111/j.1365-2672.2009.04226.x>
10. A. Adnan, G.R. Nair, M.C. Lay, J.E. Swan, R. Umar, Glycerol as A Cheaper Carbon Source In Bacterial Cellulose (BC) Production By *Gluconacetobacter Xylinus* DSM46604 In Batch Fermentation System. *MJAS* **19**(5), 1131–1136 (2015)
11. I.O. Fagbolagun, S.A. Oke, The Optimization of Packaging System Process Parameters Using Taguchi Method. *IJIEEM* **2**, 1-13 (2020). <https://doi.org/10.24002/ijeem.v2i1.3694>

12. D.A. Nugroho, L. Sutiarmo, E.S. Rahayu, R.E. Mashitoh, Kinetic observations of bacterial cellulose thickness formation using image processing approach during the fermentation process. *Food Research* **6**(1), 210-214 (2022).
[https://doi.org/10.26656/fr.2017.6\(1\).093](https://doi.org/10.26656/fr.2017.6(1).093)
13. W. Soemphol, P. Hongsachart, V. Tanamool, Production and characterization of bacterial cellulose produced from agricultural by-product by *Gluconacetobacter* strains. *Materials Today: Proceedings* **5**(5), 11159–11168 (2018).
<https://doi.org/10.1016/j.matpr.2018.01.036>
14. Y.G. Maloringan, D.A. Nugroho, Production of Nata de Coco Using Soaked Soybean Wastewater as the Alternative Usage of Zwavelzuur Ammoniak (ZA). *KnE Life Sciences*, 43-50 (2017).
<https://doi.org/10.18502/cls.v4i2.1656>
15. E.E. Al-Shamary, A. K. Al-Darwash, Influence of Fermentation Condition and Alkali Treatment on the Porosity and Thickness of Bacterial Cellulose Membranes. *TOJSAT* **3**(2), 194-203 (2013)
16. A. Khusna, A. Prastujati, S. Setiadevi, M. Hilmi, Effect of starter sources and old fermentation on making nata de whey towards chemical quality. *E3S Web Conf.* **142**, 04001 (2020).
<https://doi.org/10.1051/e3sconf/202014204001>
17. O. Novareza, I.P. Tama, N.W. Setyanto, L.T.W.N. Kusuma, P.C. Nirmalasari, Application Of Taguchi Experiment For The Composition Optimization Of Raw Material In Making Of Terrazo Chair. *JEEST* **4**(2), 103-110 (2017).
<http://doi.org/10.21776/ub.jeest.2017.004.02.6>