OPTIMIZATION OF PROCESS PARAMETERS FOR MICROBIAL CELLULOSE PRODUCTION FROM RICE-WASHING WASTEWATER (*NATA-DE-LERI*) BY *Acetobacter xylinum*

Alwani Hamad[1*], Giswantara[2], Endar Puspawiningtyas[3], Regawa Bayu Pamungkas[4], Abdul Haris Mulyadi
Program Studi Teknik Kimia, Fakultas Teknik dan Sains, Universitas Muhammadiyah Purwokerto

Informasi Makalah

Dikirim, 24 Februari 2023
Direvisi, 31 Maret 2023
Diterima, 1 April 2023

Kata Kunci*:*

bacterial cellulose,
nata de leri,
Acetobacter xylinum,
limbah cucian beras
fermentasi

INTISARI

Nata adalah selulosa dari bakteri yang diperoleh dari fermentasi *Acetobacter xylinum*. Nata sering ditemukan dalam makanan penutup sebagai suplemen kesehatan karena kandungan seratnya yang tinggi. Nata tidak hanya dihasilkan dari air kelapa yang dikenal dengan *nata-de-coco*, tetapi juga dapat dihasilkan dari sumber lain seperti air limbah cucian beras yang disebut dengan *nata-de-leri*. Penelitian ini bertujuan untuk mengoptimalkan parameter dalam fermentasi *nata-de-leri* dengan metode statistik dalam rangka meningkatkan produksi nata. Hal yang dilakukan yaitu, pertama melakukan desain untuk menyeleksi parameter yang berpegaruh menggunakan respon yield menggunakan Design Placket-Burman. Ada delapan factor yang kaji untuk kemudian didapatkan faktor yang paling berpengaduh agar mudah dalam optimasi nantinya. Hal kedua yang dilakukan adalah optimasi empat parameter yang paling berpengaruh menggunakan Central Composite Design (CCD) dengan respon hasil yield. Hasil dari penelitian menunjukkan bahwa konsentrasi gula, konsentrasi $CO(NH_2)_2$, konsentrasi starter inokulum dan waktu fermentasi merupakan faktor yang penting dalam produksi *nata de leri*. Hasil parameter untuk menghasilkan yield optimal dalam produksi *nata de leri* ketika menggunakan 500 ml substrat limbah cucian beras adalah menggunakan gula sebanyak 17,5 g; $CO(NH_2)_2$ sebanyak 12 g; volume starter sebanyak 60 mL dan waktu fermentasi selama 12 hari. Dari parameter tersebut menghasilkan rendemen sebesar 97,20%. Dari optimasi dalam penelitian ini menghasilkan peningkatan produksi nata de leri sebesar 20% dari kondisi awal sebelum dioptimasi.

Keyword*:*

bacterial cellulose,
nata de leri,
Acetobacter xylinum,
rice washing wastewater,
fermentation

ABSTRACT

Nata is a bacterial cellulose obtained from the fermentation of *Acetobacter xylinum*. It is often found in desserts as a health supplement because of its high fiber content. It is not only produced from coconut water, known as *nata-de-coco* but can also be produced from other sources, such as wastewater from rice washing, called *nata-de-leri*. This study aimed to optimize the parameters of bacterial cellulose (*nata-de-leri*) fermentation by statistical methods to improve production. First, the Placket-Burman Screening Design was applied to address the essential parameter components affecting *nata-de-leri* production. Eight factors are continuously checked, and unimportant elements are removed to obtain a smaller, more manageable set of characteristics. Second, a feedback surface method using Central Composite Design (CCD) was used to determine the optimal level of fermentation parameters using yield response. The results of this study show that sucrose concentration, $CO(NH_2)_2$ concentration, inoculum concentration and fermentation time are believed to be the main factors for nata production. The estimated optimal values *nata-de-leri* production when using 500 ml of rince washing wastewater are as follows: sucrose is 17.5 g; $CO(NH_2)_2$ is 12 g; volume of starter is 60 ml; fermentation time for 12 days. This results in a yield of 97.20%. From this study, the optimum yield in *nata-de-leri* is 20% larger than the baseline parameters.

Korespondensi Penulis:

Alwani Hamad
Program Studi Teknik Kimia, Universitas Muhammadiyah Purwokerto
JL. Raya Dukuhwaluh Purwokerto, 53182
Email: alwanihamad@ump.ac.id

1. INTRODUCTION

Bacterial cellulose (BC) is produced by *Acetobacter xylinum* at the air-liquid interface of coconut water called nata-de-coco. Not only coconut water is used as a substrate like nata, but the rice washing wastewater can also be used as *nata-de-leri*. *A. xylinum* uses nutrients in coconut water, forming a thin, viscous, transparent cellulose layer on the surface of the medium, this layer thickens over time to form a thick sheet after 7-20 days [1]–[3]. Although microbial cellulose finds applications in a number of fields, cellulose production yields must be addressed to make it economically compatible. Therefore, it becomes necessary to optimize the yield of cellulose production through the use of process improvement strategies. Many scientist have optimized media compositions and process parameters to increase microbial cellulose (BC) production [4]–[6].

It is frequently asserted, although without compelling scientific support, that used rice wash water is an effective plant fertilizer. Prior to cooking, milled rice is frequently washed to get the bran, dust, and debris off. However, washing rice can also significantly reduce the quantity of nutrients in rice that are water-soluble, and the water that remains after washing rice is frequently just dumped into the environment. This wastewater is known as "washed rice water," and it has been discovered to contain a variety of vital plant nutrients, including vitamins and carbohydrates[7], [8]. Thus, this is a potential source of nutrition in bacterial cellulose fermentation as it is called *nata- de -leri.*

Optimization is an important step in the development of a synthesis protocol, and the evaluation of the interaction of reaction parameters influencing bacterial cellulose biosynthesis and their optimization is required to increase yield[9]–[11]. There is little information in the literature about optimizing the process of bacterial cellulose fermentation, particularly when using different types of substrates[2]. The response surface methodology (RSM) can predict the optimal levels of each parameter and their corresponding response values to eliminate the limitations of a single-factor optimization process, which can explain the combined effects of all the factors influencing a process[9], [12]. The Plackett-Burman design is an efficient and quick way to identify the relevant variables for optimization. In addition, the RSM employs an experimental design[11], [13], [14]. The objective of this study is the optimization of *nata-de- leri* production from *A. xylinum*. Media components and process parameters were screened by Plackett-Burman Design (PBD) and optimization of key factors was performed using Central Composite Design (CCD).

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2. METHODS

2.1 Microorganisms and preparation of starter of *nata-de-leri*

Acetobacter xylinum (wild-type) cultures obtained from the Food and Bioprocess Research Center, Universitas Muhammadiyah Purwokerto, Indonesia, were maintained in liquid medium. *A. xylinum* grown in 500 ml coconut water was inoculated into sterile medium containing 17.5 g fructose, 3 g $CO(NH_2)_2$ in 500 ml coconut water and adjusted to pH 4.5 with glacial acetic acid. The inoculated medium was statically cultured in a sterilized container at 30°C for 7 days culture[15]–[18]

2.2 Media and growth conditions

500 ml of culture medium (wastewater from rinsing rice) was placed in a 30 x 20 x 5 cm aseptic container, inoculated with bacteria, and cultured at room temperature for 8 to 16 days in liquid interface then sterilized media of fermentation. The culture were added sucrose as a carbon source, $CO(NH_2)_2$ as a nitrogen source, K_2HPO_4 as a phosphate source, and biotin[16], [18].

2.3 Screening of the key culture parameters using Placket Burman Design (PBD)

A Plackett-Burman design was used to identify the factors that significantly affect to the nata production. In this experiment using Plackett-Burman design was used to screen eight variables including the ratio of rice to rinsing water, sucrose concentration, $CO(NH_2)_2$ concentration, K_2HPO_4 concentration, pH, fermentation days, starter concentration, and volume of biotin (**Table 1)** at high (+1), medium (0), and low (-1) levels. The 27 experimental trials were conducted to determine the impact on nata yield production (**Table 2**). Yield is calculated from the percentage of the mass of nata was produced from fermentation per mass of liquid of culture media (wastewater from rinsing rice)[11], [19].

To determine the optimal levels of the critical variables identified by the Plackett-Burman design, the RSM design using Central Composite Design (CCD) method was used for experimental design for optimization of influence factors that resulted from the PB design. [12], [20]. The result of the Plackett-Burman design was a list of parameters significantly affecting the responses. The four highest influence factors were chosen for optimization, including sucrose concentration, urea concentration, fermentation time, and starter volume. Each significance variable was examined at five different levels (-α, -1, 0, +1, +α), with 0 representing the central value of each variable. **Table 3** shows the results of the four main factors that influenced the yield nata. **Table 5** shows the experimental conditions tested according to the CCD design using the response of the yield nata production taken as the answer (Y) and factors as the independent variable (X). A central composite design (CCD) consisting of 29 center point replicates was run to optimize the independent factors to get the optimum response (yield). The fermentation was conducted as the previous preparation using 500 mL of culture media wastewater from rinsing as the run was described in **Table 5**.

Table 1. Component of factors and their high (+), basal (0) and low level (-) of Placket Burman method

Symbol Designated	Variables	Low level (-1)	central level (0)	High Level (+1)
R	Rice to rinsing water ratio	1:1	1:2	1:3
C	Sucrose concentration (%w/v)	1	3.5	6
N	$CO(NH_2)_2$ concentration (%w/v)	0	0.6	1.2
P	K_2HPO_4 concentration (%w/v)	0	0.6	1.2
pH	pH	6	4,5	3
LF	Fermentation time (days)	8	12	16
V	Starter concentration (%v/v)	2	12	22
B	Biotin (drops)	0	3	6

Table 2 Plackett–Burman design for 8 variables for nata yield production

Run	Variables								Yield (%)
	R	C	N	LF	pH	B	P	V	
1	1:3	1	1.2	16	6	6	0	2	11.81
2	1:1	6	0	8	6	6	1.2	22	45.95
3	1:1	6	1.2	16	6	6	1.2	2	89.09
4	1:1	6	0	8	6	6	1.2	22	48.05
5	1:1	6	1.2	8	3	6	0.6	22	42.32
6	1:2	3.5	0	12	4.5	3	0.6	2	65.01
7	1:3	1	0.6	8	6	0	1.2	22	40.72
8	1:3	1	1.2	8	6	0	1.2	22	42.33
9	1:3	6	0	16	6	0	0	22	37.80
10	1:1	6	1.2	8	3	0	0	2	44.03
11	1:1	1	0	8	3	0	0	2	40.87
12	1:1	1	0	8	3	0	0	2	42.97
13	1:3	6	1.2	8	3	6	0	22	53.37
14	1:3	6	0	16	6	0	0	22	30.99
15	1:3	6	0	16	3	0	1.2	2	26.41
16	1:3	1	1.2	16	6	6	0	2	15.17
17	1:1	1	1.2	16	3	0	1.2	22	26.41
18	1:2	3.5	0.6	12	4.5	3	0.6	2	61.57
19	1:1	1	0	16	3	6	0	22	25.98
20	1:2	3.5	0.6	12	4.5	3	0.6	12	63.59
21	1:3	1	0	8	3	6	1.2	2	43.15
22	1:1	6	1.2	16	6	6	1.2	2	98.10
23	1:3	6	0	16	3	0	1.2	2	27.19
24	1:3	-1	0	8	3	6	1.2	2	58.88
25	1:1	1	1.2	8	3	0	0	2	44.79
26	1:1	-1	0	16	3	6	0	22	26.51
27	1:1	-1	1.2	16	3	0	1.2	22	28.76

Note: the symbols in code in variables are referred to **Table 1**

2.4 Optimization of the main independent variables using Response Surface Methodology (RSM)

Table 3. Variables and their levels for CCD

Variables	Range level in 500 ml culture of wastewater from rinsing rice				
	-Star point	Low level	Center level	High level	+Star point
	(-α)	(-1)	0	(+1)	(+α)
Sucrose concentration (g)	0.5	5	17.5	30	50
$CO(NH_2)_2$ (g)	0	0.5	3	6	12
Fermentation time (days)	6	8	12	16	20
Volume of Starter (ml)	1	10	60	110	150

2.5 Data analysis

Statistical experimental designs were developed and analyzed using Minitab 15 (Minitab Inc., USA). A three-dimensional surface plot was created to visualize the interaction between the variables of interest and their optimal values.

3. RESULTS AND DISCUSSION

3.1 Screening of the independent variables: Plackett-Burman (PB) design

Main effects plots (**Figure 1**) and analysis of yield using Plackett Burman Design show that carbon source using sucrose, nitrogen source using $CO(NH_2)_2$, fermentation time and amount of starter have a large impact to nata de léri production compared to other variables (ratio of rice to rinse water, phosphate source, pH, and biotin). This is due to the carbon and nitrogen sources that help *Acetobacter xylinum* grow and create its nata layers as known as nata-pelikes. The growth of nata-pelikes is also affected by the amount of the organism itself and the incubation period [1], [16], [17], [21]. Therefore, to optimize nata production, it is necessary to optimize the concentrations of key variables at various concentrations or levels while keeping other components of the medium constant. **Table 4** show the regression analysis for PB design. As it seen that the factor C (concentration of sucrose) was the most important parameter for increasing yield of nata de leri ($p<0.05$). The main effect as shown in **Figure 1** that the four parameters including sucrose concentration, $CO(NH_2)_2$ concentration, fermentation time and concentration of starter were the influence factors that increasing the yield of nata de leri. Thus these parameters was further developed for the optimization process using RSM.

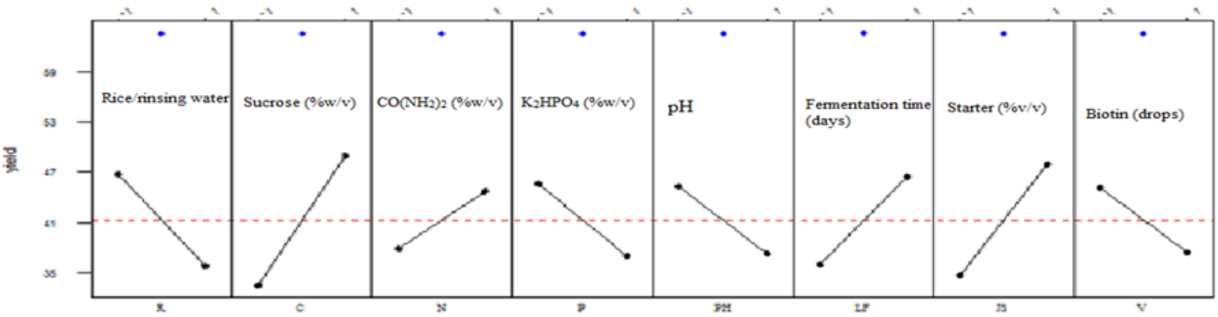

Figure 1. Main effect plot for the screening of medium components using PB design.

Table 4 Results of regression analysis for the Plackett–Burman design.

Term	Effect	Coef	StDev Coef	T	P
Constant		43.772	3.201	13.67	0.000
R	-10.941	-5.471	3.395	-1.61	0.125
C	15.372	7.686	3.395	2.26	0.036
N	6.840	3.420	3.395	1.01	0.327
P	-8.607	-4.303	3.395	-1.27	0.221
PH	-7.999	-3.999	3.395	-1.18	0.254
LF	10.419	5.209	3.395	1.53	0.142
JS	13.197	6.599	3.395	1.94	0.068
V	-7.769	-3.884	3.395	-1.14	0.268

Note: the symbol in terms is referred to Table 1

Table 5 CCD matrix for the four variables and experimental results.

Run	Sucroce (g)	$CO(NH_2)_2$ (g)	Fermentation time (days)	Volume of Starter (ml)	Yield (%)
1	5	6	16	10	NA
2	30	6	8	110	86.36
3	30	0.5	8	110	66.34
4	17.5	3	12	150	79.31
5	17.5	3	12	60	91.54
6	17.5	3	12	60	95.52
7	30	6	16	10	NA
8	30	0.5	16	110	66.08
9	50	3	12	60	79.58
10	17.5	0	12	60	85.93
11	5	6	16	110	73.57
12	17.5	3	6	60	87.49
13	30	0.5	16	10	42.39
14	30	6	8	10	39.20
15	5	6	8	10	45.16
16	30	0.5	8	10	41.02
17	30	6	16	110	90.70
18	17.5	12	12	60	88.25
19	5	6	8	110	75.75
20	17.5	3	12	1	NA
21	5	0.5	16	110	55.56
22	5	0.5	8	110	53.71
23	17.5	3	12	60	94.26
24	5	0.5	8	10	32.75
25	17.5	6	12	60	94.21
26	5	0.5	16	10	NA
27	17.5	3	12	60	93.57
28	0.5	3	12	60	NA
29	17.5	3	20	60	58.09

NA: not available/ there is no result of nata

3.2 Optimization of the independent variables: Response surface methodology (RSM)

Fermentation variables that were found to have a significant impact on the production of nata were tested at the values shown in **Table 5** for CCD including the level amount of the sucrose and urea, the fermentation time and the volume of the starter inoculum. To optimize the four independent variables, a CCD consisting of a series of 29 experiments with 4 independent experiments at a central point was performed. The results show that the yields of the nata from experiments had a value range from 0.00 to 95.53%. The experiment also shows the CCD variables and their levels. All experiments were performed in a sterile container containing 500 ml of medium (rince-rice water). After it was analyzed using Minitab software, the quadratic model expressed in the equation describes the amount of yield (Y) as a function of sucrose (X_1), $CO(NH_2)2$ (X2), fermentation time (X_3), and starter volume (X4). The equation was express as below:

$$Y = -4.330 + 2.570 (X1) + 4.370 (X_2) + 2.324 (X_3) + 0.929 (X_4) - 0.053 (X_1)^2 - 0.093 (X_2)^2 - 0.261 (X_3)^2 - 0.008 (X_4)^2 - 0.113 (X_1)(X_2) + 0.056 (X_1)(X_3) + 0.001 (X_1)(X_3) - 0.299 (X_2)(X_3) + 0.050 (X_2)(X_3) + 0.037 (X_3)(X_4)$$

Table 6. Analysis of Variances of CCD

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	25093.5	25093.48	1792.39	4.27	0.005
Linear	4	14858.7	2377.47	594.37	1.42	0.28
Square	4	8026.1	7996.08	1999.02	4.76	0.012
Interaction	6	2208.6	2208.64	368.11	0.88	0.536
Residual Error	14	5874.7	5874.65	419.62		
Lack-of-Fit	11	5866.3	5866.34	533.1	192.39	0.001
Pure Error	3	8.3	8.32	2.77		
Total	28	30968.1				

S = 20.48 R-Sq = 81.0% R-Sq(adj) = 62.1%

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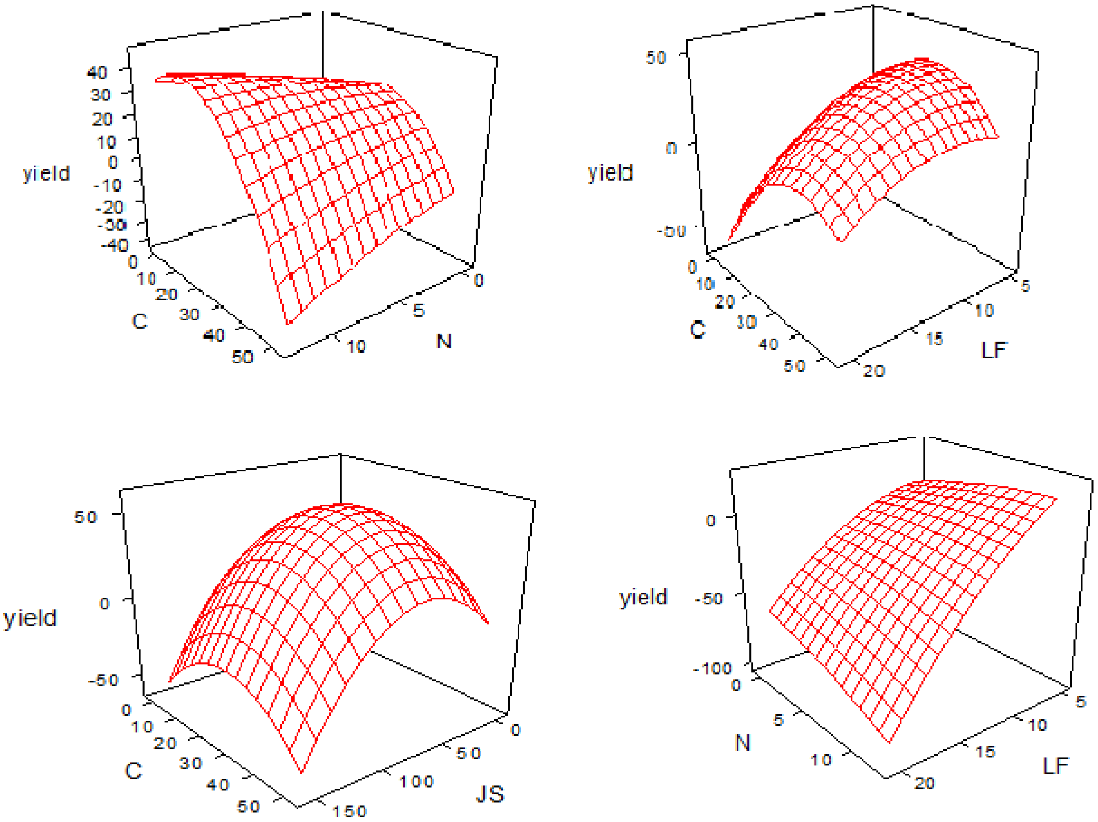

C: Sucrose (g); N ($CO(NH_2)_2$ (g); LF: Fermentation time (days); JS: Starter (ml)

Figure **2:** Surface plot of showing the interaction effects from CCD design

Estimation coefficients for response yield are shown in that equation. The significance of each coefficient was determined by t-test and P-value. The larger the T value and the smaller the P value, the larger the corresponding coefficient. Responses obtained under various combinations of variables and the experimental design defined in Table 6 were analyzed using analysis of variance (ANOVA) appropriate to the experimental design in Table 5 was found to be significant ($p<0.05$) and the lack of fit was not significant. The coefficient of determination value ($R^2 = 0.810$), although accurate, suggests that the model fits well.

A 3D plot (Fig. 2) visually interprets the interaction between the two factors. Response surface plots allowed us to see interactions between variables up to interaction effects. Estimated optimum values for the production of nata de léri from the model were: the amount of sucrose17.5 g, the amount of $CO(NH_2)_2$ was 12 g, the volume of starter was 60 ml, and the fermentation time for 12 days in 500 ml of rinse rice wastewater. This condition has resulted in a yield of 97.20% bacterial cellulose. This optimization result was consistent with the work that had been done previously in the production of nata de pina, which is bacterial cellulose made from pineapple juice. When the substrate of pineapple juice used in the process was a 500 ml volume, 17.5 grams of sucrose and 60 milliliters of starter volume were required. In spite of this, it fermented for a total of 12 days using only 3 grams of urea as a source of nitrogen. [10].

4. CONCLUSION

This optimization work for increasing the production of nata de leri using rice washing wastewater was investigated using the Placket Burman design and then followed by the RSM using Central Composite Design (CCD). The result demonstrated *using* CCD design can obtain optimal yields of Nata de leri production from *Acetobacter xylinum*. From this study, it can be concluded that the optimal nata de -leri yield has resulted in 20% greater than the baseline parameters.

ACKNOWLEDGEMENT

Financial support from the Annual Grant Competition from Lembaga Penelitian dan Pengabdian Masyarakat (LPPM), Universitas Muhammmadiyah Purwokerto under contract number A11-III/320-SPj/LPPM/XII/2014, is acknowledgment.

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