OptimizationofC-phycocyaninproductionfrom S. platensis cultivated on mixotrophicconditionbyusingresponsesurfacemethodology

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Original Research Paper

Optimization of C-phycocyanin production from *S. platensis* cultivated on mixotrophic condition by using response surface methodology

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ABSTRACT

Production of phycocyanin from *Spirulina platensis* by mixotrophic condition was favored due to high growth rate result. The objective of this research was to obtain the statistical interaction models of molasses and urea to crude phycocyanin (CPC) production by applying response surface methology (RSM)associated central composite design approach. *S. platensis* was cultivated under mixotrophic condition by adding varied molasses and urea under continous illumination at 45.5 μmol m^{-2} s^{-1} for 6 days. By using 2nd order statistical models, optimum phycocyanin production was recorded on 114.74 mg L^{-1} and 0.196 L^{-1} of urea and molasses, respectively. Molasses could be the promising substrate for phycocyanin production of the microalgae.

1. Introduction

Spirulina platensis is one of the favored microalgae in the market. Its phycocyanin pigment is utilized as natural blue colorant on health food, drink, pharmacy, and cosmetics (Silveira et al., 2007; Kuddus et al., 2013). However high cost of phycocyanin production on *S. platensis* is the problem in the commercial production.

Chen et al. (1996) succesfully enhanced cell growth and phycocyanin production of *S. platensis* in batch culture under continous illumination. Later, Chen and Zhang (1997) found lower phycocyanin content when *S. platensis* was cultivated on batch-mixotrophic compared to autotrophic condition. Engineering strategies by fed batch cultivation were employed on its condition to optimize the phycocyanin production rate.

However Chainapong et al. (2012) reported the opposite result from previous research by using only batch culture, and found higher phycocyanin content during mixotrophic compared to autotrophic condition. It indicates the other parameter should be included in the research.

Nitrogen could be the most influencing parameter in the phycocyanin formation (Boussiba and Richmond, 1980). One of the promising nitrogen sources is urea that has low cost. However the previous research was not clearly reported on the optimum urea addition during mixotrophic cultivation of *S. platensis* to obtain the phycocyanin production.

Previous work informed that molasses has potential for use as substrate for cultivation of microalgae on hetero- and mixotrophic condition (Schmidt et al., 2005; Borsari et al., 2007). This substrate was easily collected, has lower cost compared to glucose, and potential to be applied on commercial scale. Andrade and Costa (2007) cultivated *S. platensis* under mixotrophic condition to study the growth rate and biomass production. However, previous research was not well done to optimize the result to obtain phycocyanin production. This research objective was to obtain the models by optimizing the interaction of urea and molasses on crude phycocyanin content, and production rate of *S. platensis* by using response surface methodology.

2. Material and methods

2.1. Strain and growth medium

The *S. platensis* strain was purchased from BBPAP Jepara. The culture media was distilled water containing 100% (v/v) modified Bangladesh medium (Azimatun Nur and Hadiyanto, 2014). Each medium was supplemented with 0.05–0.3 g $L^{-1}$of sugarcane molasses obtained from Madukismo Yogyakarta sugar industry. Nitrogen content in the medium was varied with 0–160 mg L^{-1} of urea.

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2.2. Culture condition

Cultivation was carried out in sterilized Erlenmeyer flask glass 2 L equipped with an Illumination lamp 20 W type flourescent to give light intensity of 45.5 μmol m^{-2} s^{-1} for 24 hour and diaphragm pumps providing sterilized air to agitate the medium. Each culture was inoculated with initial *S. platensis* biomass concentration of 0.200D_{670}. The *S. platensis* cultivation growth was determined daily by measuring the optical density (OD) at 670 nm wavelength and comparing the OD values with previously standard calibration curves of optical density versus *S. platensis* biomass dry weight (W_1) Eq. (1) by Olaizola and Duerr (1990).

$$W_1 = 0.5273 \times \mathrm{OD680\ nm} - 0.0138 \left(R^2 = 0.9982\right)$$

The culture was incubated for 6 days. Temperature of medium was maintained at 29–30 °C and the pH of the cultures was maintained at 8.5–9. Growth rate of *S. platensis* was calculated by exponential of the logarithmic phase in Eq. (1).

$$\mu = \frac{(\ln \mathrm{OD}_x - \ln \mathrm{OD}_0)}{t_x - t_0} \tag{1}$$

where μ is the specific growth rate, OD_x is the maximum optical density, OD_0 is the initial optical density at $t_0 = 0$, and t_x is the time of cultivation at maximum OD_x.

2.3. Phycocyanin extraction

The biomass was harvested using filter cloth 80 μm size. Wet extraction was performed by modifying from Boussiba and Richmond (1979). Each of the 40 mg wet biomass (W_2) 70% dw was added into 10 ml centrifugal tube. Phosphate buffer pH 7.2, 100 mM, was used as a solvent of wet extraction. The buffer consists of 10.64 K_2HPO_4 and 5.29 g L^{-1} KH_2PO_4. The suspension was sonicated for 1 h under 48 kHz, then stored in the refrigerator at 15 °C overnight and centrifuged at 6000 rpm. Filtrate containing phycocyanin and the residue was separated using filter paper of 80 μm. Each of the crude phycocyanin (CPC) supernatant was measured by spectrophotometer SP-300 at 620 nm to determine CPC content as Eq. (2).

$$\mathrm{CPC} = \frac{A_{620} \times V \times 100}{3.39 \times W_2 \times dw} \tag{2}$$

where CPC is the crude phycocyanin in %, A_{620} represents the absorbance of phycocyanin at 620 nm, 7.3 is the extinction coefficient of CPC at 620 nm, V is the volume of solvent, 100 represents 100%, W_2 is the weight of wet biomass and dw represents the percentage of dry weight.

Crude phycocyanin production rate was calculated using Eq. (3) as

$$\mu_{CPC} = \mu \times W_1 \times \mathrm{CPC} \tag{3}$$

where μ_{PPC} is the crude phycocyanin production rate (mg L^{-1} day^{-1}), μ is the growth rate of *S. platensis* (day^{-1}), W_1 is the dry weight of biomass (mg L^{-1}), and CPC represents crude phycocyanin content (%).

2.4. Statistic analysis

The effect of the two factors (urea and molasses addition) and their interactions were studied using uncoded response surface methodology (RSM) as shown in Table 1. The total number of experimental runs was 13 including replication. Central composite design (CCD) was employed for two factors. For urea (X_1), concentration was varied in the range from 0, 23, 80,137, and 160 mg L^{-1} and for molasses (X_2), concentration was varied in the range from 0.05, 0.087, 0.15, 0.263 to 0.3 g L^{-1}.

The 2nd order general polynomial model for RSM analysis was employed. The quadratic model for predicting the optimal CPC content and CPC production rate was according to Eq. (4), where y_i, and b_0 represent response variable and interception coefficient, respectively, and b_i, b_{ii}, and b_{ij} are regression coefficients. While n is the number of studied variables, x_i and x_j represent independent variables.

$$y_i = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=2}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j \tag{4}$$

Minitab 16 software was employed to analyze the statistical model and result. The model was expressed by cofficient of determination R^2, and significance of variable was checked by the F-test ($p < 0.05$)ANOVA.

3. Result and discussion

3.1. Phycocyanin accumulation

Phycocyanin content of *S. platensis* was influenced by urea and molasses addition as described in Table 2 with all of the parameter significances $P < 0.05$. However CPC was not significantly influenced by interaction between urea and molasses. Optimum CPC was found in 105 mg L^{-1} urea and 0.125 g L^{-1} molasses with 17.2% CPC (Fig. 1a).

In run 11, lowest value of CPC content of all the runs was found (6.92%). Urea was starvated and molasses was added at the middle

Table 1
Summary of the result response surface methodology central composite design.

Run order	Level type	Urea (mg L^{-1})	Molasses (g L^{-1})	CPC (%)	Growth Rate (day^{-1})	Biomass (g L^{-1})	CPC Rate (mg L^{-1} day^{-1})
1	1	137	0.263	12.53	0.354	0.393	17.432
2	−1	80	0.050	15.41	0.293	0.234	10.565
3	1	23	0.263	10.359	0.098	0.255	2.589
4	1	23	0.087	9.4	0.173	0.198	3.220
5	0	80	0.150	16.8	0.252	0.458	19.390
6	−1	160	0.150	13.9	0.394	0.314	17.197
7	0	80	0.150	16.84	0.259	0.405	17.664
8	0	80	0.150	16.41	0.254	0.452	18.840
9	1	137	0.087	16.24	0.345	0.223	12.494
10	−1	80	0.300	14.3	0.211	0.465	14.022
11	−1	0	0.150	6.92	0.051	0.181	0.639
12	0	80	0.150	16.68	0.249	0.445	18.475
13	0	80	0.150	16.57	0.257	0.447	19.043
14	Control	80	0	15.7	0.354	0.227	8.696

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Table 2
Interaction coefficient and estimation of the models.

Term	CPC (%)		μ (mg/l/d)		X_1 (g/l)		μ_{CPC} (mg/l/d)	
	Coeficient	P	Coeficient	P	Coeficient	P	Coeficient	P
Constant	−0.941	0.115	0.1671	0.002	−0.184	0.002	−14.043	0.001
X_1	0.239	0.000	0.0023	0.001	0.005	0.000	0.33	0.000
X_2	58.529	0.000	−0.737	0.036	4.04	0.000	168.77	0.005
X_1^2	−0.001	0.000	0.000	0.034	0	0.000	−0.002	0.003
X_2^2	−135.461	0.000	0.545	0.466	−10.89	0.000	−509.92	0.004
$X_1 \cdot X_2$	−0.230	0.001	0.003	0.030	0.0017	0.020	0.272	0.051
R^2 (%)	99.33		98.71		98.5		98.98	

level of 0.15 g L^{-1}. When urea was added to the medium with the same molasses concentration, CPC content also increased. This result is in agreement with Gagnon and Pick (2012), followed by Abd El-Baky (2003) who found the decrease of phycocyanin during nitrogen starvation.Urea as the source of nitrogen could be stored in the cell formed in phycocyanin pigment (Boussiba and Richmond, 1980). However, Chen et al. (1977) reported that phycocyanin was denatured in the presence of urea up to 7 M.

In run 1, 137 mg $L^{-1}$of urea and 0.263 g $L^{-1}$of molasses gave phycocyanin content 12.53%. When the lower level 0.087 g L^{-1}molasses was added on run 9, the phycocyanin increased up to 0.83 fold. Phycocyanin was totally inhibited at higher 0.05 g L^{-1}molasses on the same urea addition (runs 2, 10, 11). Borsari et al. (2007) reported the cultivation of Nostoc sp. that cultivated in mixotrophic condition and found the optimum phycocyanin absorbance at 1 g L^{-1} sugarcane molasses, while at 0.05 and 1.5 g L^{-1}, the value was inhibited.

Addition of molasses into medium seems inhibiting the light penetration, and medium was favored to form mixotrophic condition (Andrade and Costa, 2007). Chen and Zhang (1997) reported that phycocyanin in mixotrophic condition was lower than autotrophic condition. It indicated that nitrogen was not successfully stored as phycocyanin due to limitation of light intensity that important in photosynthetic reaction.

However, molasses as organic carbon source was favored influencing phycocyanin content due to its ion content. The phycocyanin content was higher when compared to autotrophic condition (Table 1). Younis et al. (2010) informed the Na^+ ion in the molasses up to 6% (w/v). While Abd El-Baky (2003) reported that increase in the salt concentration caused significant phycocyanin value in Spirulina maxima due to stress condition of the medium.

3.2. Growth rate profile

S. platensis cultivated in high urea concentration resulting high growth rate. In Table 1, highest growth rate was found in

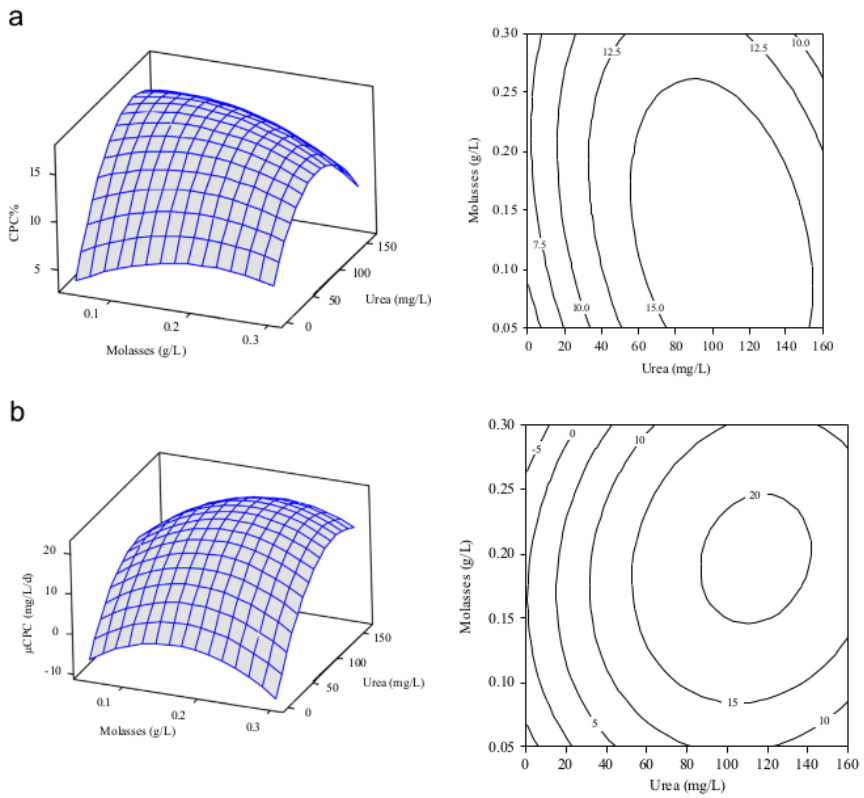

Fig. 1. Surface plot of *S. platensis* cultivated under mixotrophic condition in different molasses and urea concentration (a) CPC content and (b) CPC rate.

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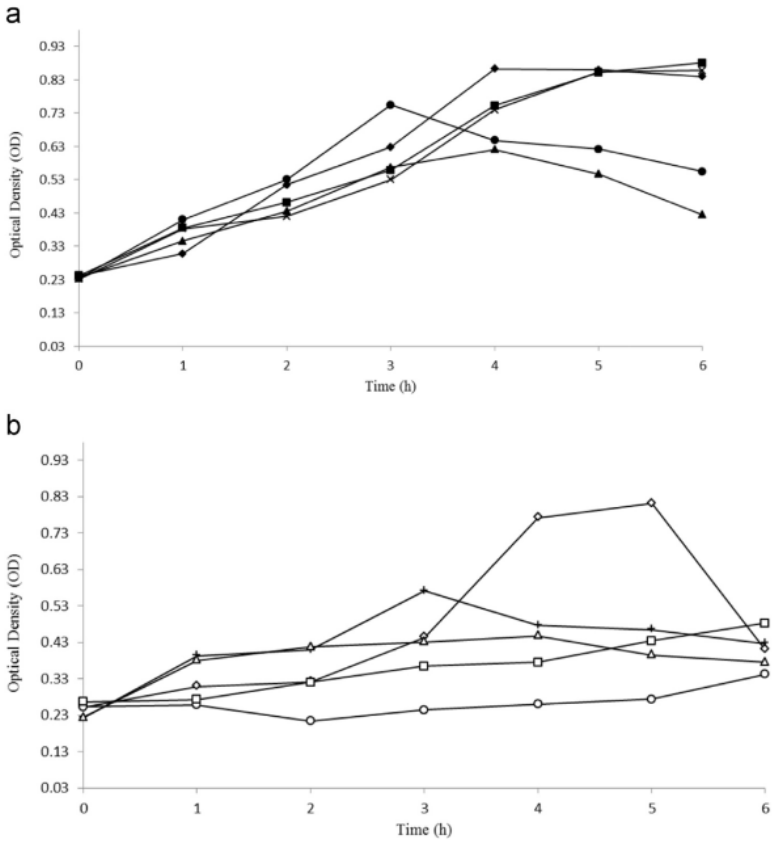

Fig. 2. Growth profile of *S. platensis* under mixotropic condition at concentration ■=0.087 g L^{-1} molasses, 80 mg L^{-1} urea, ▲=0 g L^{-1} molasses, 80 mg L^{-1} urea, ×=1.5 g L^{-1} molasses, 80 mg L^{-1} urea, ●=1.5 g L^{-1} molasses, 160 mg L^{-1} urea, ◆=0.263 g L^{-1} molasses, 137 mg L^{-1} urea, □=2.63 g L^{-1} molasses, 23 mg L^{-1} urea, ○=1.5 g L^{-1} molasses, 0 mg L^{-1} urea, ◇=0.263 g L^{-1} molasses, 80 mg L^{-1} urea, Δ=0.087 g L^{-1} molasses, 23 mg L^{-1} urea, +=0.087 g L^{-1} molasses, 137 mg L^{-1} urea.

160 mg L^{-1} of urea and 0.15 g L^{-1} of molasses. The value was decreased due to lower urea concentration (runs 5, 6, 11). Lowest growth rate was recorded in run 11 that contains urea starvation. Nitrogen was an important parameter in the growth rate of microalgae (Gagnon and Pick, 2012).

In Run 2, when 80 mg $L^{-1}$of urea was added in low molasses concentration (0.05 g L^{-1}), growth rate slightly increased. Compared to run 5 and 10, growth rate decreased when high molasses concentration was added. Molasses inhibited with constant −0.7 (P=0.036). This result was corresponded with Andrade and Costa (2007) who found inhibition of growth rate caused by molasses, for *S. platensis* cultivation in mixotrophic condition.

In Fig. 2, growth profile of *S. platensis* was described. Overall, the addition of urea gave shorter lag phase. It is the most influencing parameter on the modeled RSM with $P<0.05$ (Table 2). However when molasses dominate the medium, growth rate was lower. It could be the toxic content of the molasses that inhibited the growth rate as mentioned in previous research (Setyoningrum et al. 2014). Chojnacka and Zielinska (2012) also reported that addition of glucose at higher concentration on mixotrophic condition could also lower the biomass of Spirulina Sp.

On this research, the growth rate was higher when compared to autotrophic condition (Table 1). This result was in agreement with Chojnacka and Noworyta (2004) who found highest growth rate on mixotrophic compared to autotrophic and heterotrophic condition, and Smith et al. (2015) who demonstrated the influences of dissolved inorganic concentration, photosynthesis rate, dissolved oxygen, and respiration on the growth rate. In this research, mixotrophic condition was generated from the addition of molasses. Nevertheless, in this research, bicarbonate was added in to medium as inorganic carbon source. Organic and inorganic carbon supplied the *S. platensis* to grow on mixotrophic as the sum of autotroph and heterotrophic condition.

3.3. Phycocyanin production rate

The model resulted that molasses and urea gave positive interaction with $P<0.05$ (Table 1). Fig. 1b describes the optimum result of phycocyanin production rate. Highest phycoyanin production rate was found on 0.196 g L^{-1} of molasses and 114.74 mg L^{-1} of urea with result 21.25 mg $L^{-1}$$day^{-1}$.

Several researchers reported that condition of cultivation influenced the biomass production. Chojnacka and Zielinska (2012) informed that culture condition influenced biomass accumulation. Higher light irradiation exhibits uptake of glucose and nitrate-nitrogen as the substrate to be converted as biomass. The effect was opposite when higher nitrogen was added. In this research, highest biomass was found on 0.20 g L^{-1}molasses and urea concentration on 95.35 mg L^{-1}. It indicates that excess urea was generated when higher molasses was added. In runs 5 and 6, the results follow this condition.

Thus, in runs 3 and 4, when low urea was added (23 mg L^{-1}), followed by molasses (0.26–0.08 g L^{-1}), it gave higher CPC content, lower biomass, higher growth rate, and resulted in higher CPC rate. The biomass and phycocyanin contents were in contrast result. It proved that phycocyanin rate not only depended on the

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biomass, but also on the growth rate and phycocyanin content.

4. Conclusion

In summary, urea and molasses fed of mixotrophic *S. platensis* cultures were found to have optimum CPC production rate up to 21.25 mg L^{-1} d^{-1} on 114.74 mg L^{-1}, 0.196 g L^{-1} of urea and molasses respectively. Excess of urea was generated from mixotrophic condition that *S. platensis* unable to utilize for phycocyanin pigment. Overall, molasses is the potential substrate for phycocyanin production of *S. platensis*.

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