Effect of Physicochemical Process Variables on Natural Indigo Dye Production from *Strobilanthes cusia* Leaves by Response Surface Methodology

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Received: August 10, 2021
Accepted: February 21, 2022

DOI: 10.22146/ijc.68335

Abstract: *The recovery process of indigoid compounds involves enzymatic hydrolysis of indigo precursors continued by oxidation reaction to synthesize indigo pigment. The purpose of this research was to evaluate the effect of physicochemical process variables, i.e., temperature, time, and pH aeration, on indigo yield from* Strobilanthes cusia *leaves. Small leaf pieces were immersed in distilled water and heated at temperatures (40, 50, and 60 °C) and duration (1, 2, and 3 h). The extract was aerated at different pHs (8, 10, and 12) to form the indigo product. The indigo concentration was quantified through a visible spectrophotometer and high-performance liquid chromatography (HPLC). The optimized condition for indigo production was studied using response surface methodology (RSM). Temperature, time, and interaction between temperature and time significantly affected the indigo yield. The optimized conditions for extraction of indigo dyes were determined to be at 60 °C for 1 h and pH 8 for maximizing the indigo yield. On that condition, the indigo concentration quantified by HPLC was 1.15% (w/v) which was lower than that by the spectrophotometry. By spectrophotometric analysis, the actual indigo content of 1.68% (w/v) on that optimum condition was close to the predicted indigo content of 1.77% (w/v) using RSM.*

Keywords: *indigo;* Strobilanthes cusia*; temperature; time; pH; response surface methodology*

INTRODUCTION

Nowadays, one of the most polluting industrial sectors is the textile industry, producing large quantities of water pollutants contaminated with all types of chemicals. Indeed, the production of textile dyes is globally estimated at just over 10.000 tons annually, and around 100 tons annually of dyes are released into wastewater [1]. Approximately 7 × 10^7 tons of synthetic dyes are produced for the textile industry worldwide per year, of which nearly 10% of the dyestuffs are discharged to the environment as effluent after dying and processing [2]. Moreover, synthetic dye such as azo dyes that account for most textile dyestuffs is a cause of significant concerns. Most of them are highly toxic and carcinogenic [3].

To minimize the impact on health and the environment, natural dyes can be used as an alternative to reduce the use of synthetic dyes. Natural dyes can be derived from natural resources classified as insects or animals, minerals, fungi, and various plant parts, including roots, barks, leaves, flowers, and fruits [4-5]. As natural dyes are from renewable sources and biodegradable wastewater, they can sustain the environment [6]. In addition, natural dyes are generally less toxic and allergenic than synthetic dyes [7] and sometimes have a therapeutic effect on health [8].

Indigo, indigo-blue, or indigotin, is one of the most valuable sources of blue pigment and is extensively used [9-10]. Indigo dyes have been used for textiles coloration since prehistoric times, making them one of the oldest dyes used by human beings [11]. Recently, indigo has become the most produced dye worldwide [12]. The production of indigo dye has been increased more than doubled from 22,000 tons per year in 2001 to 50,000 tons per year in 2011 due to its high global market demand. The indigo demand of 50,000 tons in 2011, 95% of the total production, was used to dye over 4 billion denim garments manufactured yearly [13-14].

There are a variety of temperate, tropical, and subtropical plants from many different species, genera, and families that can be hydrolyzed to form indigo dyes. *Indigofera sp.* is the most famous indigo-bearing plant which is mainly native to tropical and subtropical regions (India, Southeast Asia, and the Middle East). In temperate climates, Indigo dyes can also be produced from other species such as *Isatis tinctoria* or dyer's woad (Mediterranean, Western Asia, North America, and Europe) and *Polygonum tinctorum* (Japan and China). *Strobilanthes cusia* is a common alternative used in subtropical locations that have potential commercial value in sustainable indigo production and medicinal use [11]. *Strobilanthes cusia* (Nees) Kuntze or *Baphicacanthus cusia* (Nees) Bremek., a member of the family Acanthaceae, is an herbaceous plant native to many regions of Asia such as northeast India, Myanmar, Thailand, and southern China. This plant has been widely used as traditional herbal medicine and dye in southwest China [15-17]. *Strobilanthes cusia* is an indigo-rich plant that the main indigoid pigments were mostly synthesized in the leaves and stems of *Strobilanthes cusia* [18].

Indigo pigment can be formed by extracting the leaves of indigo-yielding plants. By steeping underwater, some indigo precursors such as indican (indoxyl-β-D-glucoside) and isatan B (indoxyl-β-ketogluconate) accumulated in the cell vacuole of leaves are hydrolyzed by the action of endogenous β-glucosidase. The products are indoxyl and glucose. In aerobic conditions, two molecules of indoxyl combine spontaneously to form indigo. However, isatin can be generated from the hydroxylation of indoxyl and decomposition of indigo in atmospheric oxygen. Condensation of indoxyl with isatin leads to indirubin as a side reaction of indigo biosynthesis [19-20].

A previous study revealed that some physicochemical parameters during fermentation, such as fermentation temperature, fermentation duration, pH, dissolved oxygen (DO), and redox potential of the fermented solution, played an essential role in indigo dye formation from *Indigofera* plant biomass [21]. Furthermore, there was also a study that focused on some parameters like concentration of substrate-indole, pH, and temperature to optimize the production of indigo pigment [22]. The traditional method successfully produced indigo dyes from plants that were performed by steeping the leaves of the plant in water and adjusting the pH during this process to form indigo [23]. However, the drawbacks of extracting dyes from plant material are time-consuming processes and low color value produced [24]. This method is less efficient, and the quality and quantity of products are variables [23,25]. Therefore, it is necessary to carry out further studies for optimizing the indigo extraction process to produce high-quality and quantity products with an efficient and eco-friendly process.

This study aims to understand the effect of physicochemical process variables, i.e., temperature, time, and pH aeration, on the formation of natural indigo dyes. The optimal condition of three parameters was run by using the full factorial design on response surface methodology (RSM).

■ EXPERIMENTAL SECTION

Materials

Strobilanthes cusia plants were obtained from a farmer in Temanggung, a regency located in Central Java, Indonesia. Indigo standard ($C_{16}H_{10}N_2O_2$, 95% purity) was purchased from Aurora Silk LLC (USA). Calcium oxide or quicklime (CaO) used to synthesize calcium hydroxide solution ($Ca(OH)_2$). Dimethyl sulfoxide or DMSO ($(CH_3)_2SO$, ≥ 99.95% purity) was utilized for spectrophotometric analysis, which was purchased from Supelco Sigma-Aldrich Corporation. Acetonitrile (CH_3CN, 99.9% purity) utilized as a mobile phase for HPLC analysis was purchased from Merck (USA).

Procedure

Recovery of indigo from Strobilanthes cusia

The recovery process used in the experiment was based on the procedure conducted by Comlekcioglu et al. [26] with some modifications. The fresh leaves of *Strobilanthes cusia* were cleaned before being used. The leaves (5 g) were cut into smaller pieces, approximately 0.5 × 0.5 cm. Indigo precursors were hydrolyzed by putting the tiny leaves into the conical tube and adding 40 mL distilled water (leaves:distilled water = 1:8). This process was performed in anaerobic conditions so that the leaves were dipped underwater to exclude air. In this extraction process, there were several controlled parameters, namely temperature and time. The extraction process was carried out at 30, 60, and 90 °C for 1, 2, and 3 h, respectively. After the extraction process, the leaves were removed, and the extracts were quickly cooled to room temperature. The pH of the extract was adjusted to 8, 10, and 12, respectively, by adding a saturated solution of calcium hydroxide (0.1 g mL^{-1}), and the extract was aerated for 15 minutes using an aerator. The extract transforms its color from green-yellow into blue color after aeration is performed.

Quantification of indigo by visible spectrophotometry

The estimation of indigo yield in this experiment was quantified through a spectrophotometric method according to Puchalska et al. [27] and Qu et al. [28]. The indigo powder was dissolved in dimethyl sulfoxide (DMSO). The concentration of pure indigo was determined by the calibration curve method. The indigo concentration in an unknown sample was compared to a set of known concentrations of the standard sample. The standard indigo solutions were prepared in various concentrations (0, 25, 50, 100, and 200 ppm). Both standard indigo and unknown samples were dissolved in DMSO. The samples were added 5 mL DMSO and diluted 50-fold. The absorbance of sample and standard was measured using Vernier-SpectroVis plus SVISPL spectrophotometer assisted with the Logger Lite program.

Quantification of indigo by high-performance liquid chromatography (HPLC)

HPLC analysis was performed to quantify the yield of pure indigo pigment and identify the primary component. Samples and standards were dissolved in dimethyl sulfoxide and analyzed by the HPLC system. Chromatographic separation was carried out with a reversed-phase column (octadecylsilane, C18) at room temperature with a flow rate of 1.0 mL/min under an isocratic flow of acetonitrile in 0–20 min. Products were detected and quantified using a UV detector at 600 nm.

Optimization of indigo extraction conditions

Response surface methodology (RSM) was performed using Design Expert 10.0.3 software to determine the optimal condition that provided the highest indigo yield. Three independent parameters, i.e., extraction temperature (A), extraction time (B), and pH of the extract before oxidation (C), were investigated. Each parameter was coded in three levels, as shown in Table 1. Extraction temperature was coded as −1, 0, and 1 which the actual value was 40, 50, and 60 °C, respectively. With the same coded values as extraction temperature, the actual value of extraction time was 1, 2, and 3 h, respectively, and the actual value of pH was 8, 10, and 12, respectively. Statistical analysis was performed on the experimental results by regression and analysis of variance (ANOVA). Response surfaces and contour plots of the model runs were also reported in this work. Furthermore, the desirability function was predicted to optimize the indigo yield.

Table 1. Experimental design for optimizing indigo yield

Independent parameter	Symbol	Unit	Level		
			-1	0	1
Extraction temperature	A	°C	40	50	60
Extraction time	B	Hour	1	2	3
pH before oxidation	C	N/A	8	10	12

■ RESULTS AND DISCUSSION

Quantification of Crude Indigo Dye

It can be understood from the result above (Fig. 1) that the addition of calcium hydroxide solution before the aeration process, which was defined as the increase in pH of the extract, increased extraction yields considerably. According to a previous report published, adding a small number of alkalis such as carbonate or calcium hydroxide into the extract could accelerate the oxidation reaction of indoxyl forms indigo and increase the indigo yield. However, an excess of alkali used could decrease the yield of indigo [29]. Calcium hydroxide also allowed the indigo suspension to sediment more rapidly and resulted in larger indigo particles [30].

The results in Fig. 1(a) show that there is a significant increase in crude indigo yield with the extended extraction time at the lower temperature (40 °C). It seems that increasing time in the extraction process can accelerate enzyme activity to break down indican as a major indigo precursor to indoxyl. By contrast, increasing duration in hydrolysis reaction did not produce a considerable yield improvement at the higher temperature, as shown in Fig. 1(b) and 1(c). Even there is also a slight decline of indigo yield at a longer duration and higher temperature. It indicates that a lengthy extraction process at high temperatures may occur a loss of indigo [23].

Spectrophotometric Analysis

The concentration of pure indigo was examined using spectrophotometric analysis by dissolving in DMSO and the result is presented in Fig. 2. It is practically insoluble in water and many other common solvents, but some solvents will dissolve indigo to some extent. Indigo-blue was sufficiently unstable in DMSO, where the color of the solution could change over time from blue to purple or even red color. Gilbert 2007 also reported a red/brown color produced after 24 h when indigo-blue dissolved in DMSO [9]. The result (Fig. 2) reveals a maximum absorption wavelength of 616.9 nm,

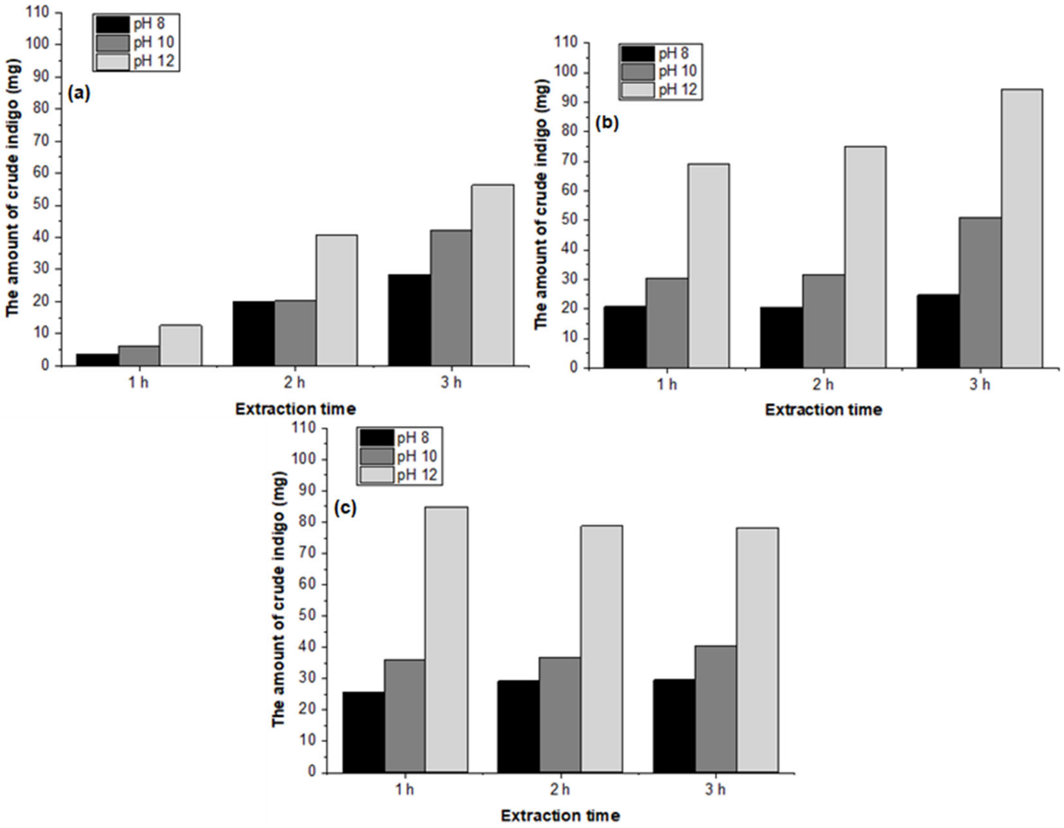

Fig 1. The amount of crude indigo yield at different pH (8, 10, and 12), extraction time (1, 2, and 3 h), and temperature: (a) 40 °C, (b) 50 °C, and (c) 60 °C

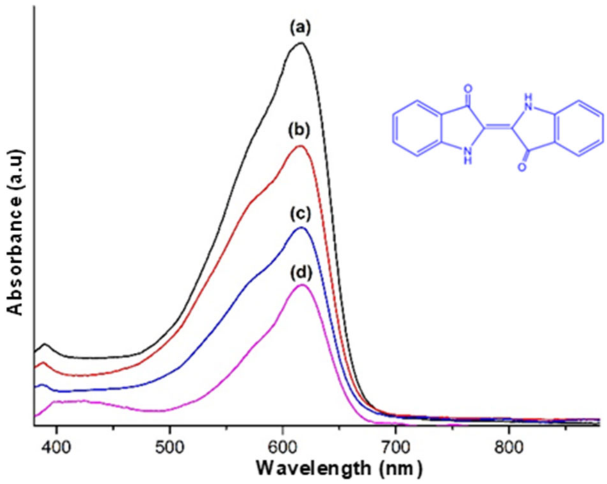

Fig 2. Visible absorption spectra of (a) extracted indigo-pH 8, (b) extracted indigo-pH 10, (c) extracted indigo-pH 12, and (d) indigo standard

indicating a maximum wavelength of indigo. In addition, the result shows that there is a broad spectrum of extracted indigo yield. The main factors that may cause the peak broadening are impurities such as calcium salts and various derivatives of indigo and indoxyl. The by-products of indigo synthesis, such as indirubin and isoindirubin that produce red pigment, have maximum absorption at 540 nm and 552 nm, respectively [27].

Characterization of Natural Indigo Dye

The vibrational spectra of extracted indigo and standard indigo is presented in Fig. 3. The strong band at 3426 cm^{-1} usually is due to the N–H stretching. The strong exhibited at 1628 cm^{-1} is attributed to C=O stretching vibration. Other peaks at 1458 and 1404 cm^{-1} are assigned to the stretching vibration of the C=C aromatic ring. The absorption bands which appear at the region below 1320 cm^{-1} correspond to the deformation of N–H and C–H vibration [31].

Optimization of Indigo Extraction

ANOVA for the quadratic regression model is used to describe the relationship between indigo yield and independent variables. This model analysis shows that the response variable is adequately fitted to the regression model, a second-order polynomial equation. The equation of yield can be expressed in the following equation:

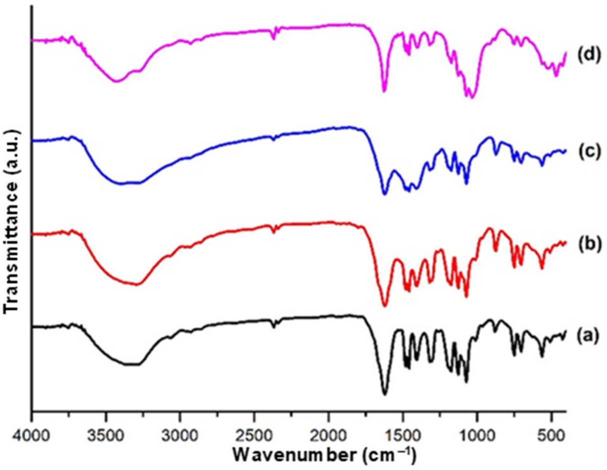

Fig 3. FT-IR spectra of a) extracted indigo-pH 8, (b) extracted indigo-pH 10, (c) extracted indigo-pH 12, and (d) indigo standard

$$Y = 1.19 + 0.44A + 0.15B - 0.07C - 0.24AB - 0.11AC - 0.054BC - 0.053A^2 - 0.014B^2 - 0.008406C^2$$

where Y represents the pure indigo yield as a function of studied independent variables, which are extraction temperature (A), extraction time (B), and pH before oxidation reaction (C).

In general, Table 2 is used to evaluate the significance level of each parameter toward the response variable, which is indigo concentration. A lower p-value ($p < 0.05$) and a higher F-value of each factor mean significantly affect the model. The results reveal that temperature and extraction time substantially influence the indigo yield, while pH is insignificant for this model. It indicates that pH has less influence on the increase of indigo yield. The highest F-value is for extraction temperature, indicating the most influential parameter to increase pure indigo yield. In addition, the interaction parameter between temperature and time (AB) provides statistical significance with a p-value equal to 0.25×10^{-2}. Conversely, the interaction between temperature and pH (AC) as well as time and pH (BC) are insignificant to the pure indigo yield.

In this study, the R^2 value of 0.8435 implies that 84.35% of the total variance is attributed to the response variable studied, which is the pure indigo yield. The predicted R^2 value reasonably agrees with the adjusted R^2 value because the difference between those data is less than 0.2. According to this regression analysis, the model

Table 2. Analysis of variance and regression of RSM

Source	Sum of squares	Df	Mean square	F value	p-value
Model	4.81	9	0.53	10.18	$< 1.00 \times 10^{-4}$
A-Extraction Temp.	3.45	1	3.45	65.70	$< 1.00 \times 10^{-4}$
B-Extraction Time	0.40	1	0.40	7.58	1.36×10^{-2}
C-pH	0.09	1	0.09	1.69	0.21
AB	0.66	1	0.66	12.62	0.25×10^{-2}
AC	0.16	1	0.16	3.02	0.10
BC	0.03	1	0.03	0.68	0.42
A^2	0.02	1	0.02	0.32	0.58
B^2	1.14×10^{-3}	1	1.14×10^{-3}	0.022	0.88
C^2	4.24×10^{-4}	1	4.24×10^{-4}	8.07×10^{-3}	0.93
Residual	0.89	17	0.05		
Corrected Total	5.71	26			
R^2					0.8435
Adjusted R^2					0.7606
Predicted R^2					0.5913
Adequate Precision					11.296

has a good precision value of 11.296. This value is greater than 4 so that the model meets the requirements for the optimization process. This good precision value measures the signal-to-noise ratio that compares the range of values predicted at the design point with the average prediction error. If it is greater than 4, the model has a signal that is strong enough to be used for optimization. In general, the results of the analysis show that most of the experimental results obtained are good enough to predict the concentration of extracted pure indigo (Fig. 4).

The interaction between temperature and time on the extraction process significantly affects the pure indigo yield, as shown in Table 2. At the lower temperature (40 °C), the higher extracted pure indigo yield was obtained at a longer extraction process. However, this process can be shortened by increasing the temperature, as presented in Fig. 5(a).

Fig. 5(b) and 5(c) show that the highest indigo yield is obtained at pH 8. At a relatively higher volume of the base added, indigo content decreased gradually. Russell and Kaupp [32] reported that indigo synthesized by the oxidation of indigo in basic conditions can involve the indoxyl radical as an intermediate. The effect of the excess base in a decline of the indigo yield can be rationalized by indolone formation in which the presence of base and oxygen may be converted to non-indigo products. Furthermore, adding the excess base can also reduce indigo purity by impurities such as calcium salts within the indigo product [30]. Some researchers also reported that the quantity of crude dye increased along with the increasing concentration of calcium hydroxide while

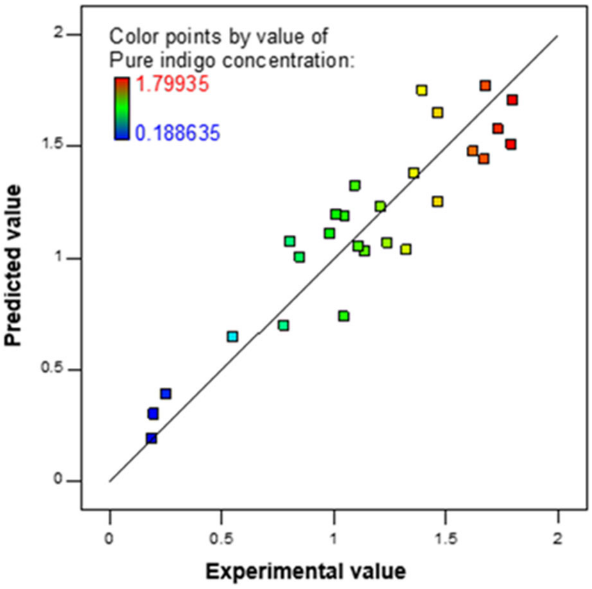

Fig 4. Predicted vs. experimental value for indigo yield (the straight line represents the predicted yield, and the points represent the experimental measurement)

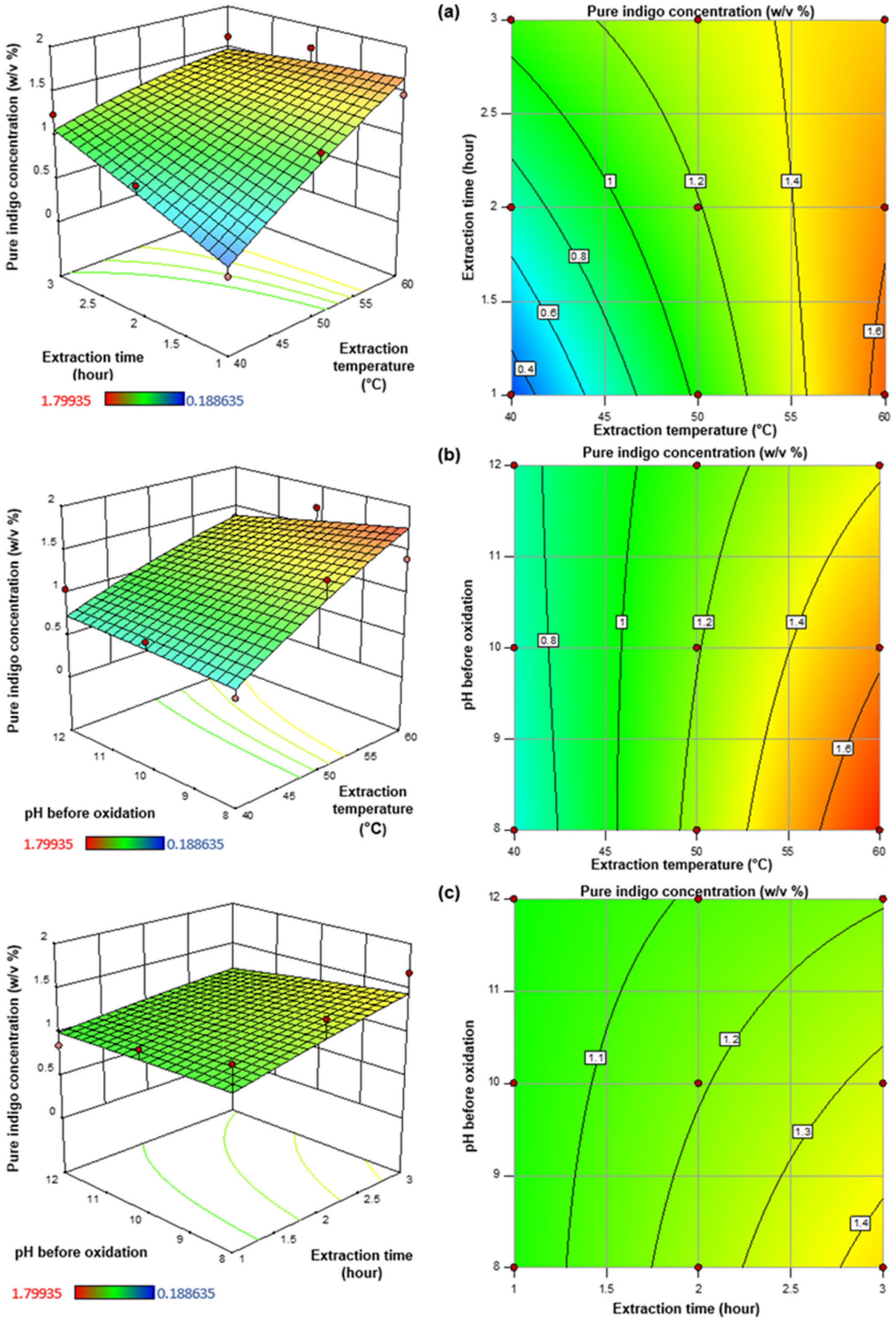

Fig 5. Response surfaces and contour plots of the pure indigo yield (w/v %) as a function of extraction temperature and time (a), extraction temperature and pH before oxidation reaction (b), extraction time and pH before oxidation reaction (c)

indigo content in the crude dye (% indigo per gram of crude dye) decreased [33]. A higher concentration of blue color could be achieved by declining the amount of lime [34].

Based on numerical optimization, the optimized process conditions for maximization of pure indigo concentration were achieved at 60 °C for one hour to break down indican to indoxyl and pH 8 to accelerate the reaction of indoxyl into indigo. An optimum indigo yield of 1.68% (w/v) was produced, which was adequately close to the predicted yield of 1.77% (w/v).

Determination of Natural Indigo Pigment by HPLC Analysis

Based on chromatogram data, there are a few impurities on the indigo sample compared to the indigo standard (Fig. 6). As shown in Table 3, the quantification of pure indigo shows some different results between the measurements using visible spectrophotometer and HPLC-UV. However, these findings provide a similar pattern. There is evidence to suggest that both Visible-spectrophotometry and HPLC-UV methods can accurately be used to determine the optimal variables that produce the highest pure indigo yield as long as both methods have the same tendency. The spectrophotometry has advantages over the HPLC method regarding analysis cost and instrumental operation, which is relatively inexpensive and easy to operate. However, compared to the spectrophotometric method, the HPLC method has greater sensitivity and higher precision. There are also some disadvantages of spectrophotometry. Impurities in the sample cannot be eliminated, increasing the calculated concentration. As shown in Table 3, the estimation of indigo concentration by HPLC is lower than that by the spectrophotometric analysis.

■ CONCLUSION

In this study, natural indigo dyes have been successfully extracted from leaves of *Strobilanthes cusia*. In addition, the response surface methodology (RSM) based on the full factorial design was used to examine the optimal parameters from three independent factors on the indigo extraction. The results reveal that temperature and time are the most important factors influencing the amount of extracted pure indigo yield.

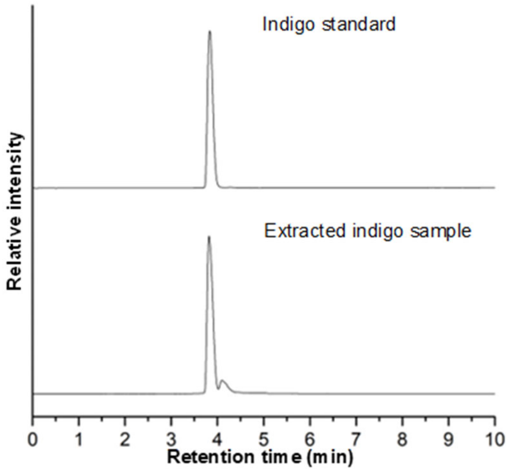

Fig 6. Chromatogram of the extracted indigo sample and standard

Table 3. Comparison of pure indigo concentration measured using Visible spectrophotometry and HPLC method

Sample	Control variables			Pure indigo yield (% w/v)	
	Extraction temp. (°C)	Extraction time (h)	pH before aeration	Visible-spectrophotometer	HPLC-UV
41A	40	1	8	0.19	0.22
42A	40	2	8	0.55	0.50
43A	40	3	8	0.80	0.64
51A	50	1	8	1.33	1.03
53A	50	3	8	1.67	1.09
61A	60	1	8	1.68	1.15
63A	60	3	8	1.80	1.18
63B	60	3	10	1.62	1.17
63C	60	3	12	1.21	0.95

Furthermore, an optimum of the pure indigo yield was achieved on conditions as follows: extraction temperature at 60 °C, extraction time of 1 h, pH before oxidation reaction of 8. Under that condition, the pure indigo yield of 1.68% (w/v) was experimentally obtained, which was a well fit for a predicted yield of 1.77% (w/v) by the regression model. This optimum condition could be used as a standard condition for natural dye extraction from *Strobilanthes cusia* leaves to provide the highest content of indigo dyes.

■ ACKNOWLEDGMENTS

This research was financially supported by Chemical Engineering Department, Faculty of Engineering, Universitas Gadjah Mada, and Riset Inovatif-Produktif (RISPRO) under contract number PRJ-103/LPDP/2019 between LPDP Ministry of Finance Indonesia and Directorate of Research Universitas Gadjah Mada.

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