Optimization of fermentation time and grain concentration for water kefir production from butterfly pea flower (Clitoria ternatea)

A E Setiawati¹ and J Kusnadi²

¹Master of Agricultural Product Technology, Department of Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Brawijaya
²Department of Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia

E-mail: azizah_es@yahoo.co.id

Abstract. Herbal tea is known rich in bioactive compounds and pigments. Herbal tea can be derived from Butterfly pea flowers. Butterfly pea has a high level of anthocyanins and flavonoid levels. However, butterfly pea has not been used optimally in fermentation products such as water kefir. Water kefir has many benefits and potential antioxidant sources. This research aimed to optimize fermentation time (24-48 hours) and concentration grain (5-15%) for the production of butterfly pea water kefir with a good antioxidant activity using response surface methodology. Central Composite Design was used to develop models of the response. The response used in this research is antioxidant activity, flavonoid, anthocyanin and brightness. The optimum condition was obtained at 36 hours 2 minutes of fermentation time, and 15% of grain concentration using Design Expert 10.01. The optimum combination for butterfly pea water kefir resulted in 58 ppm of antioxidant activity, 39 of brightness, 81 mg/g QE of flavonoid and 4.2 ppm of anthocyanin. A validation experiment showed that no significant difference between actual and predicted values based on paired t-test (p>0.05) using Minitab 17.0.

1. Introduction
Herbal tea is usually made from dried leaves, seeds, wood, fruit or other plants that provide benefits [1]. Butterfly peas are one of the local raw materials that has not been used optimally. Butterfly pea flowers contain anthocyanins, flavonoids and high antioxidant activity which are beneficial for health [2]. Butterfly peas are widely used in food product research. Even though it has the potential as a fermented beverage product, butterfly peas are mostly used as a colourant. One of the potential fermentation products to be developed is water kefir [3].

Water kefir has a distinctive characteristic, especially taste like sparkling soda. This unique taste is produced due to the activity of yeast or khamir breaking down the sugar into alcohol and carbon dioxide. Water kefir can be used as an alternative to lactose-intolerant [6]. Water kefir is a natural source of antioxidants. The quality of water kefir is influenced by grain concentration [4], and fermentation duration [5].
This research aims to optimize fermentation time (24-48 hours) and grain concentration (5-15%) for the production of butterfly pea water kefir using Response Surface Methodology (RSM). Central Composite Design (CCD) was used to develop models of the response. Fermentation time and grain concentration used in this research were based on preliminary research and literature. The responses used in this research are antioxidant activity, flavonoid, anthocyanin and brightness. The optimum fermentation time and grain concentration from this study can be used for producing *Clitoria ternatea* water kefir that contains a good source of antioxidants.

2. Materials and methods

2.1. Preparation of butterfly pea water kefir
Butterfly pea was obtained from Panggreh village, Sidoarjo – East Java. Firstly, 250 ml of water was boiled, then cooled down to 80°C. Following that, 2 grams of butterfly pea and 5 ml of honey were added, then brewed for 10 minutes. The butterfly peas were then drained and the water was cooled to 25°C then the kefir grain was added. The concentrations of kefir added and fermentation time was based on the design suggestion from the Design Expert. Lastly, kefir grains were filtered from the water kefir and used for the next analysis.

2.2. Experimental design
This experiment used Responses Surface Methodology (RSM) with Central Composite Design (CCD) with Design Expert 10 software. The variables are grain concentration and fermentation time. Fermentation time with a lower limit of 12 hours and an upper limit of 48 hours. The grain concentration with a lower limit of 5% and an upper limit of 15%. The response analyzed consisted of antioxidant activity, flavonoid, anthocyanin and brightness. Independent variable use in this research and the level of experimental design are shown in Table 1.

| Independent variable | Levels          |
|----------------------|----------------|
|                      | -1.414         |
| Grain concentration  | -1             |
| Fermentation time    | 0              |
|                      | +1             |
|                      | +1.414         |

2.3. Determination of antioxidant activity
The test was conducted in a 96-well plate using Costar Plate Ref 3596. About 20 µL stock solution of extracts were put in various concentrations (100, 500, 1000, 1500, 2000 ppm) then, 180 µL of DPPH solution 0.147 mM were added to each well. The mixture was then incubated for 30 minutes at room temperature and darkroom. The absorbance was read at a wavelength of 517 nm using microplate reader spectrostar nano BMG labtech. Methanol was used as blank [7] and the quantity was expressed as ppm.

2.4. Determination of flavonoid
The test was conducted in a 96-well plate using Costar Plate Ref 3596. Into each well 10 µL of extract, 60 µL ethanol, 10 µL of 10% AlCl3, 10 µL of 1 M potassium acetate, and 120 µL distilled water were added. The mixture was homogenized and incubated for 30 minutes. The absorbance was measured at a wavelength of 415 nm in a microplate plate reader spectrostar nano BMG labtech. Quercetin was used as a standard solution, and the quantity was expressed as quercetin equivalent (QE) in an (mg QE/g) [8].
2.5. Determination of anthocyanin

The test was conducted in a 96-well plate using Costar Plate Ref 3596 and determined by pH difference. 3 mL of samples was mixed with 5 mL of two buffers, pH 1.0 (0.2 M potassium chloride adjusted with chloric acid) and pH 4.5 (0.2 M acetate of sodium adjusted with acetic acid), individually. About 0.2 ml of each mixture was placed into each well, followed by 30 minutes of incubation at room temperature. The absorbance was measured at 530 nm and 700 nm in a microplate plate reader using spectrostar nano BMG labtech [9].

2.6. Determination of brightness

A colour reader was used to observe the brightness. Colour brightness (L) indicates dark to bright white colours with values ranging from 0 – 100. The sample was placed into transparent plastic, then press the target button. Record the brightness value display on the colour reader monitor [10].

3. Results and discussion

3.1. Effect of fermentation time and grain concentration on responses

3.1.1. Antioxidant activity. The range of the antioxidant activity was 46.34 ppm until 291.71 ppm. The best value of the treatment combination is by using the minimum (lowest) value of the antioxidant activity value. Based on the Design Expert system, the recommended model is the quadratic vs. 2FI model (R=86.28%). Figure 1a showed the surface curve results of the optimization of the antioxidant activity response. There are 3 axes in the graph, the X-axis represents the length of fermentation, the Y-axis represents the concentration of grain and the Z-axis represents the high or low value of the antioxidant activity response. The equation of antioxidant activity is as follow (1):

\[ IC50 = 126.76 - 23.17 \times X - 42.84 \times X \times X + 13.37 \times X \times Y + 93.08 \times Y - 27.21 \times Y^2 \]  

(1)

The constant value of 126.76 indicates the response value of antioxidant activity predicted by the Design Expert. If there is no effect, then the value of the antioxidant activity is 126.76 ppm. The coefficient values of X(Fermentation time) and X(Grain concentration) show a relationship if the greater grain concentration used will increase antioxidant activity and the longer the fermentation time will decrease antioxidant activity. Antioxidant activity is increased by the fermentation process. Where probiotic lactobacilli from a non-dairy product can be useful for providing antioxidants [13]. The longer the microbial fermentation time will proliferate and cause the ability to break down glucose substrates [14].

3.1.2. Brightness. Brightness was ranging from 32.93 until 51.40. The best value of the treatment combination is by using the in-range value of the brightness. Based on the Design Expert system, the recommended model is the quadratic vs. 2FI model (R=79%). Figure 1b showed surface curve results of the optimization of brightness response. There are 3 axes in the graph, the X-axis represents the length of fermentation, the Y-axis represents the concentration of grain and the Z-axis represents the high or low value of the brightness response. The equation of brightness is as seen in equation (2):

\[ \text{Brightness} = 36.35 + 2.09 \times X_1 - 0.33 \times X_1^2 + 3.35 \times X_2 + 5.04 \times X_1^2 + 3.55 \times X_2^2 \]  

(2)

The constant value of 36.35 indicates the response value of brightness predicted by the Design Expert. If there is no effect, then the value of brightness is 35.35. The coefficient values of X(Fermentation time) and X(Grain concentration) show a relationship if longer fermentation time will increase brightness and greater grain concentration will decrease brightness. During the fermentation process, the brightness decreased due to changes in the pH value. The lower the pH, the anthocyanins will be stable [15]. Long fermentation time will make colour fade which is caused by tannins being damaged due to the presence of acids [16].
3.1.3. Flavonoid. Flavonoid was shown in the range of 63.00 mg/g QE until 84.67 mg/g QE. The best value of the treatment combination is by using the maximum (highest) value of the flavonoid content. Based on the Design Expert system, the recommended model is the quadratic model (R=98.85%). Figure 1c shows surface curve results of the optimization of the flavonoid response. There are 3 axes in the graph, the X-axis illustrates the length of fermentation, the Y-axis illustrates the concentration of grain and the Z-axis illustrates the high or low value of the flavonoid response. The equation of flavonoid content is as follow (3):

\[
\text{Flavonoid} = 77.27 + 4.93 X + 5.67 X - 3.42 XX - 3.53 X - 2.53 X^3
\]  

The constant value of 77.27 indicates the response value of flavonoids predicted by the Design Expert. If there is no effect, then the value of flavonoid is 77.27 mg/g QE. The coefficient values of X (Fermentation time) and X (Grain concentration) show a relationship if greater grain concentration and longer fermentation time will increase flavonoid. Flavonoid levels increase during the fermentation process due to the increase in phenol content during fermentation. In addition, fermentation also causes enzymatic reactions and stimulates the formation of flavonoids [17].

3.1.4 Anthocyanin. Anthocyanin was shown in the range of 1.78 mg/L until 4.79 mg/L. The best value of the treatment combination is by using the in-range value of anthocyanin. Based on the Design Expert system, the recommended model is the quadratic vs. 2FI model (R=91.84%). Figure 1d showed surface curve results of the optimization of the anthocyanin response. There are 3 axes in the graph, the X-axis demonstrates the length of fermentation, the Y-axis demonstrates the concentration of grain, and the Z-axis demonstrates the high or low value of the anthocyanin response. The equation of antioxidant activity is (4):

\[
\text{Anthocyanin} = 3.47 + 0.41 X + 0.45 X + 0.33 XX - 0.55 X - 0.33 X^3
\]  

The constant value of 3.47 indicates the response value of anthocyanin predicted by the Design Expert. If there is no effect, then the value of the antioxidant activity is 3.47. The coefficient values of X (Fermentation time) and X (Grain concentration) show a relationship if greater grain concentration and longer fermentation time can increase the anthocyanin. During the fermentation process, a colour change occurs due to a decrease in pH. Anthocyanins are stable under acidic conditions and the colour produced tend to be red [18].

3.2 Optimization

Optimizing the production of butterfly pea kefir is conducted by verifying the optimum fermentation time and the grain concentration. Optimization was carried out using Central Composite Design with Response Surface Methodology (RSM) method. Data from four responses obtained from the optimization results with the combined results analyzed using a Design Expert is shown in Table 2.

| No | Coded variable | Actual variable | Response |
|----|----------------|-----------------|----------|
|    | X | X | Fermentation time (hour) | Grain concentration (%) | Antioxidant activity-IC50 (ppm) | Flavonoid (mg/g QE) | Anthocyanin (ppm) | Brightness (L) |
| 1  | -1 | -1 | 24 | 5 | 291.71 | 66.00 | 2.82 | 51.40 |
| 2  | 1  | -1 | 48 | 5 | 257.67 | 63.00 | 3.11 | 45.40 |
| 3  | -1 | 1  | 24 | 15 | 85.13 | 84.67 | 2.91 | 41.43 |
| 4  | 1  | 1  | 48 | 15 | 104.56 | 68.00 | 4.52 | 48.83 |
The model used shows that a p-value of less than 0.05 with a lack of fit value of more than 0.05 because it will appropriate model to be used in the study. Lack of fit is the failure of the model shown to represent the data in the experiment. If the model has a significant effect on the p-value <0.05, then it will be inconsistent with the experimental design. Based on the expert design system, the recommended model is a quadratic vs 2FI model. This model is recommended because the p-value is very small compared to other models. If the p-value is greater or equal 0.05 (5%) it indicates that the probability of model error will be more than 5%, so that model becomes an insignificant effect. Annova data is shown in Table 3.

### Table 3. ANOVA for each response.

| Source   | Antioxidant activity | Flavonoid | Anthocyanin | Brightness |
|----------|----------------------|-----------|-------------|------------|
| Linier   | 0.0492               | 0.0006    | 0.0597      | 0.0159     |
| 2FI      | 0.0602               | 0.0008    | 0.0551      | 0.0152     |
| Quadratic| 0.6330               | 0.0918    | 0.7722      | 0.0922     |
| Cubic    | -                    | -         | -           | -          |
| Pure Error| -                   | -         | -           | -          |

The value of the quadratic coefficient of each factor will affect the shape of the curve that will be generated. The stationary point was determined based on the obtained regression equation and the resulting curve can be seen before knowing the surface characteristics of the brightness response. Calculating the second-order model will later be used to determine the optimum point of the response. The optimum point is known by determining the value of the stationary point. The results of the curve plot and the relationship between fermentation time and grain concentration are on the response [11]. The surface curve is shown in Figure 1. The model shows a p-value of less than 5% or P<0.05, so the model has a significant effect on the brightness response. If the lack of fit test is greater than p-value 5%, then the model used is appropriate [12].
3.3. Validation
Butterfly pea water kefir was carried out for the validation step. Optimum product triplicate test and the result concluded that there were no significant differences between prediction data and actual data by Design Expert 10.0. Validation data based on pair t-test (p>0.005) using Minitab 17.0. This research has optimization criteria. Length of fermentation, grain concentration and brightness are in range. Antioxidant activity is minimized. Flavonoid and anthocyanin are maximized. Optimization criteria that have been set will produce optimum conditions according to Design Expert 10.01. It will be selected based on the highest desirability value. Desirability has a scale of 0-1, if the value is close to 1, it will be the optimal solution, and if the desirability is close to 0 then there will be a response that is out of bounds [19]

4. Conclusions
This research concludes that the optimum condition was obtained at 36 hours 02 minutes of fermentation time, and 15% of grain concentration using Design Expert 10.01. The optimum combination for butterfly pea water kefir resulted in 58 ppm of antioxidant activity, 39 of brightness, 81 mg/g QE of flavonoid and 4.1 ppm of anthocyanin. The optimum fermentation time and grain concentration from this study can be used for producing Clitoria ternatea water kefir that contains a good source of antioxidant activity.

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