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Optimization of antioxidant activity and characterization of probiotic cascara beverage fermented using water kefir grain

[Optimasi aktivitas antioksidan dan karakterisasi minuman probiotik cascara hasil fermentasi menggunakan bibit kefir air]

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ABSTRACT

Cascara is a beverage derived from dried coffee cherry pulp, known to have good functional value but with a short shelf life. Fermentation is a technique that can enhance cascara beverages' shelf life and quality and their functional value due to the presence of probiotics and increased antioxidant compounds. The study aimed to optimize the fermentation conditions of cascara tea with water kefir seeds to improve the microbiota content of Lactobacillus sp, Bifidobacterium sp, and Saccharomyces cerevisiae and the antioxidant activity of the beverage. The method in this study used Response Surface Methodology (RSM) with a design selection of Central Composite Design (CCD), using two independent variables, namely fermentation time (X1) and Grain Concentration (X2), with a response of antioxidant activity (%). The findings indicated that a fermentation period of 48 hours and a grain concentration of 8.58% resulted in a probiotic drink exhibiting the maximum antioxidant activity of 65.53%. This optimum treatment resulted in a drink with IC_{50} antioxidant activity of 173.66 ppm, total phenol 477.24 mg GAE/mL, brightness value (L) 30.69, redness (a*) 14.06, yellowness (b*) 29.57, pH 3.57, TPT 8.80 (ºBrix), viscosity 1.62 mPa.s, total LAB 8.54 log CFU/ml, and total yeast & mold 10.93 log CFU/ml.*

Keywords: Antioxidant activity; Cascara tea; Probiotic Beverage, Water kefir

ABSTRAK

Cascara adalah minuman yang berasal dari limbah kulit kopi kering dan diketahui memiliki nilai fungsional yang baik namun memiliki umur simpan yang singkat. Fermentasi merupakan teknik yang dapat ditawarkan untuk meningkatkan nilai fungsional, umur simpan dan kualitas produk minuman *cascara* dengan kandungan probiotik dan peningkatan senyawa antioksidan. Oleh sebab itu, teh *cascara* merupakan bahan baku potensial untuk pembuatan minuman probiotik tinggi antioksidan. Penelitian bertujuan untuk mengoptimalkan kondisi fermentasi teh *cascara* dengan bibit water kefir untuk meningkatkan kandungan microbiota yaitu golongan *Lactobacillus sp, Bifidobacterium sp, Saccaromyces cerevisiae* dan meningkatkan aktivitas antioksidan minuman. Metode yang digunakan yaitu *Response Surface Methodology (RSM)* dengan pemilihan desain yaitu *Central Composite Design (CCD),* menggunakan 2 independen variabel yaitu waktu fermentasi (X1) dan Konsentrasi Grain (X2) dengan respon aktivitas antioksidan (%). Hasil penelitian menunjukkan perlakuan optimum yang dapat dilakukan untuk produksi *cascara water kefir* yaitu waktu fermentasi 48 jam, dan konsentrasi grain 8.58% yang menghasilkan minuman probiotik dengan aktivitas antioksidan optimum yaitu 65.53%. Perlakuan optimum ini menghasilkan minuman dengan aktivitas antioksidan IC_{50} 173.66 ppm, total fenol 477.24 mg GAE/mL, Nilai kecerahan (L*) 30.69, kemerahan (a*) 14.06, kekuningan (b*) 29.57, pH 3.57, TPT 8.80 (ºBrix), viskositas 1.62 mPa.s, total BAL *8,54 log Cfu/ml*, dan total khamir& kapang *10,93 log Cfu/ml.*

Kata kunci: Aktivitas Antioksidan; *Cascara*; Kefir Air; Minuman Probiotik

Introduction

Cascara (the Spanish word for husk) is a tea beverage made from dried coffee cherry pulp (Arpi et al., 2021). Studies have indicated that cascara contains a high level of antioxidant activity because of phenolic components, particularly chlorogenic acid (Oktaviani et al., 2020). Cascara is consumed as a tea through brewing (Murlida et al., 2021). According to Heeger et al. (2017), coffee pulp has the potential to yield cascara, a beverage with total polyphenols of 4.9 and 9.2 mgGAE/g and antioxidant activity of 51 to 92

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μmol, or Trolox (TE)/g dry matter. Geremu et al. (2016) found that cascara has a high amount of total polyphenols (1809 mg GAE/g) and an IC_{50} antioxidant activity of 0.4 mg/mL. Due to its antioxidant activity and high bioactive compounds, cascara has been used as a functional beverage (Arpi et al., 2021; Murlida et al., 2021; Sales et al., 2023). Tea beverages in liquid form have a disadvantage in terms of shelf life. To increase the shelf life and quality of cascara beverage products and improve their functionality, they are processed by fermentation.

Fermentation is a processing technique that utilizes microorganisms' growth and metabolic processes to stabilize desired agricultural products in food products (Terefe, 2016). Fermentation enhances food quality and safety while extending shelf life and improving flavor. Fermented products are considered functional foods that provide health benefits due to the increase in bioactive components and changes in chemical composition with the help of microbes and enzymes (Singh & Verma, 2018). In addition, fermented products can increase the growth of probiotics that are beneficial to gastrointestinal health and improve microbial balance in the digestive tract (Koirala & Anal, 2021). In the development of fermented beverage products, cascara has been used as an ingredient to produce kombucha; the results show there is an increase in antioxidant activity and phenolic compound content in the resultant kombucha (Nurhayati et al., 2020; Rohaya et al., 2022). One of the fermented beverages that has the potential to be developed is water kefir made from cascara (Moretti et al., 2022).

Water kefir is a probiotic beverage fermented using a group of microorganisms in kefir grains containing proteins and polysaccharides (Cufaoglu & Erdinc, 2023). The microbiota contained in kefir grains usually consists of lactic acid bacteria (*Lactobacillus ssp., Streptococcus ssp.,* and *Leuconostoc ssp.*), khamir (*Saccharomyces ssp.,* and *Dekkera ssp.*), and acetic acid bacteria (Moretti et al., 2022; Wang & Wang, 2023), especially *Lactobacillus casei/paracasei*, *Lactobacillus harbinensis*, *Lactobacillus hilgardii*, *Bifidobacterium psychraerophilum/crudilactis*, *Saccharomyces cerevisiae*, *Dekkera bruxellensis, acetobacter lovaniensis/fabarum* (Laureys & De Vuyst, 2014). The microbiota is encapsulated in a dextran polysaccharide matrix that appears firm, small, translucent, and irregular shape of granules (Moretti et al., 2022).

The water kefir product has been developed from roselle flower tea (Hastuti & Kusnadi, 2016); this study observed the organoleptic and physical quality of water kefir from various brands of roselle flower tea. Another survey of the antibacterial benefits of water kefir from soursop leaf tea was conducted by Muizuddin and Zubaidah (2015), who found that water kefir from soursop leaf tea has antibacterial activity against *S. aureus* and *E. coli.* The utilization of water kefir *grain* in Butterfly pea flower tea is also carried out by Setiawati & Kusnadi (2021), who optimized the antioxidant activity of water kefir with the factors of grain concentration and fermentation time. Another study by Zamzami (2019) utilized cascara extract added to cow's milk kefir formulation as much as 0.5-1.5%. This study did not use pure cascara extract as the main ingredient for making kefir.

In the kefir fermentation process, fermentation time and grain concentration are the most critical parameters because they can affect the final quality of the kefir (Guzel-Seydim et al., 2021). Similar findings were obtained by Setiawati & Kusnadi (2021), which showed that fermentation with water kefir microorganisms was able to increase its antioxidant activity compared to tea before fermentation. The optimum conditions of the cascara water-kefir fermentation process for butterfly pea flower tea were 36 hours, 2 minutes for fermentation time, and 15% for kefir grains concentration. Fermentation of cascara with indigenous lactic acid bacteria also produces higher antioxidant activity than cascara extract without fermentation (Oktaviani et al., 2020). Based on several studies on water kefir development and the utilization of cascara described above (Moretti et al., 2022; Nurhayati et al., 2020; Oktaviani et al., 2020; Rohaya et al., 2022; Zamzami, 2019), it has never been done to develop fermented cascara tea using water kefir grains that contain a variety of microbiota, including yeast, lactic acid bacteria, and acetic acid bacteria. Studies on optimizing the cascara tea fermentation process using water kefir grains on its

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antioxidant activity and the characterization of fermented products need further investigation. Therefore, this research aims to provide a diversified fermented beverage, water kefir, made from cascara tea with a high functional value. The primary criteria that need to be investigated to produce cascara water kefir with the most antioxidant activity and assess the qualities of the finished product are the length of the fermentation process and the concentration of the grains.

Materials and Methods

Materials

The tools used were glass jars, plastic spoons, non-metal sieves, analytical scales, measuring cups (Pyrex), test tubes (Iwaki Pyrex), measuring pipettes, pipette pumps, dropper pipettes, Petri dishes (Iwaki Pyrex), colorimeter (CS-10), pH meter (Mettler Toledo), viscometer (Brookfield), UV-Vis spectrophotometer (Shimadzu), Refractometer (Atago).

The materials used were cascara arabica "Ijen," water kefir grain, sugar, mineral water, DPPH reagent(2,2-diphenyl-1-picrylhydrazyl) dissolved in methanol, distilled water, Folin Ciocalteu reagent (1:10), Na2CO3 7.5%, gallic acid standard, phosphate buffer, sterile MRSA (Man Ragosa Sharpe Agar) media, sterile PDA (Potato Dextrose Agar) media.

Methods

This study employed the Response Surface Methodology (RSM) with a Central Composite Design (CCD) and Design Expert 10.01 software. The inputted variables were the grain concentration (5% to 15%) and fermentation time (24 to 48 hours). The response observed in this study was antioxidant activity (%). Details of the independent variables and experimental levels are shown in Table 1.

Table 1. Determination of variables and levels in experimental design

VI	Level				
	-1.414	**-1**	**0**	**+1**	**+1414**
X1	2.93	5	10	15	17.07
X2	19.03	24	36	48	52.98

Description: VI= Independent Variable; X1= Seedling Concentration; X2= Fermentation Time

The test results for the antioxidant activity response were then entered into the Design Expert 10.01 program to get calculations and suggest a model for each response. Furthermore, the next step in the optimization process was determining the optimal peak point by identifying several expected criteria for each factor and response. The factor and response limits are defined according to Table 2.

Table 2. The setting criteria in determining the optimum point

Factor and Response	Criteria
Grain concentration (%)	*In Range*
Fermentation time (Hour)	*In Range*
Antioxidant activity (%)	*Maximize*

The optimization constraints were set to produce an optimum condition. The optimum treatment selection will be based on the highest desirability value (Montgomery, 2017). After obtaining the optimum points, the process continued the verification process based on the predictions set by the program. The verification process was an action to prove that a particular method produced optimum results (Montgomery, 2017). The program predictions and verification results based on laboratory experiments were compared with calculations in Design Expert 10.01 and the paired-t test. The expected result used a verification value above 5% (not significantly different). After the verification process for the optimum results, the process proceeded to the next stage, characterization.

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Water kefir grain adaptation

Water kefir seedlings were washed using mineral water two times. A 500 ml of water and 10% sugar mixture were prepared and pasteurized at 72℃ for 15 minutes. After the extracted tea had cooled, grains of water kefir were added. The grains were separated (filtered) every 48 hours, and the same process was used to add a fresh mixture of sugar and water. Before being put to use, this procedure was carried out seven times.

Preparation of cascara water kefir

Cascara arabica "Ijen" was obtained from coffee farms at the hillside of Mount Ijen, Bondowoso Regency, East Java. Cascara arabica was taken from the skin of ripe arabica coffee beans. Coffee beans and skin were separated in fresh condition to obtain pulp, and the coffee skin was dried. Cascara tea was prepared by heating 200 ml of water to a temperature of 100℃, then turning off the heat and putting 2 grams of cascara into the hot water, then brewing for 12 minutes. After brewing, sugar was added to 10% of the total solution. Then, it was cooled down to 25℃. After being cooled down, kefir grains were added with various concentrations suggested by Design Expert 10.01, and fermentation was carried out according to the time recommended by Design Expert 10.01. Ultimately, kefir grains were filtered from kefir water and used for further analysis.

Antioxidant activity measurement

Antioxidant activity was measured by reducing DPPH radicals in a methanol solution. The sample was reacted with DPPH solution. The absorbance of the sample was then measured at a wavelength of 517 nm using a UV-Vis spectrophotometer. The antioxidant activity of the sample was calculated by Equation (1) (Baliyan et al., 2022).

$$\%\ \text{Antioxidant Activity} = (A_0 - A_1)/A_0 \times 100 \quad (1)$$

A_0 = absorbance of control containing methanol
A_1 = absorbance of the tested sample

Determination of total phenolic content

Total phenolics were tested using the Folin-Denis method. The standard gallic acid solution was made by dissolving gallic acid into distilled water with several concentrations. The standard solution was used to make a standard curve. The mixture of standard solution and reagents measured absorbance with a wavelength of 756 nm. The results of the measurement of absorbance and concentration to obtain a standard curve of gallic acid resulted in Equation (2) (Kristanti et al., 2022): $y = ax + b$, where y = Absorbance of the sample, x = total phenol concentration of the sample.

Color (L, a, b) measurement

The color of the tea and water kefir samples was measured with a colorimeter. Color measurement is done by putting the device on the sample's surface covered with clear plastic. The color will be defined as brightness (L*), redness-greenness (a*), and yellowness-blueness (b*) value (Putri et al., 2021).

Determination of pH

Tea and cascara water kefir samples were taken as much as 30 ml and poured into a 50 ml beaker glass. The calibration process was carried out on the pH meter by inserting it into a buffer solution of pH 7 and 4. After each buffer solution was replaced, the probe was cleaned using distilled water. Sample measurement was done by inserting a clean probe into the sample placed into the beaker. For every sample displacement, the probe tip must always be cleaned with distilled water (Putri et al., 2023).

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Total dissolved solids measurement

Total dissolved tea solids and cascara water kefir were measured using a digital refractometer. The measurement procedure is done by dropping 1 to 2 drops of sample on the refractometer's glass surface and then leaving for 1 minute. Total dissolved solids were read digitally and appeared on the screen, stating the total dissolved solids in ^{o}Brix units (Nurhayati et al., 2020).

Determination of water kefir viscosity

The viscosity of kefir was tested using a viscometer (Brookfield RVT), and 50 ml of sample was put into a beaker glass. The sample's viscosity was measured using spindle no. 2 at 125 rpm with a 5-85% readability. The viscosity test was carried out for two minutes until it reached a stable condition, and then the sample was conditioned at room temperature. The result was recorded as water kerfir viscosity and expressed in mPa.s. (Setyawardani et al., 2020).

Total bacteria analysis

Sterile MRSA (Man Ragosa Sharpe Agar) as lactic acid bacteria growth media and sterile PDA (Potato Dextrose Agar) as mold and yeast growth media were prepared. A sterile phosphate buffer solution was prepared for sample dilution. Dilutions were carried out until obtaining a dilution result of 10^{-9}. From the last three dilution results, 10^{-7}, 10^{-8}, and 10^{-9}, 1 ml were taken and poured into a petri dish. The prepared sterile media was poured into a petri dish using the pour plate method. The number of colonies was counted using a colony counter after completing the 48-hour incubation period (Rohaya et al., 2022).

Statistical analysis

Statistical analysis for Response Surface Methodology uses the Design Expert 10.01 program, which will generate various data such as analysis of variance (ANOVA), Sequential Model of Sum Square, lack of fit, Summary Statistic Response, Contour plot, and Response Surface Curve. Meanwhile, the paired-t test was conducted using Minitab 16.

Result and discussion

Effect of grain concentration and fermentation time on antioxidant activity response

The antioxidant activity of cascara water kefir ranged from 41.72% to 72.11%. Based on the Design Expert 10.01 program, the suggested model in this study was the Quadratic vs 2FI model. Figure 1 shows the optimization results' contour plot surface and response surface curves with antioxidant activity response. The antioxidant activity equation obtained based on the calculation from Design Expert 10.01 is described in Equation (3).

$$\text{Antioxidant activity} = 62.82 + 3.78X_1 - 3.04X_2 - 1.15X_1X_2 - 1.02X_1^2 - 8.89X_2^2 \quad (3)$$

The Y coefficient (response) showed the amount of each unit increased in the X coefficient (factor). A positive amount of the X coefficient would cause an increase in the Y coefficient, while a negative amount of the X coefficient would cause a decrease in the Y coefficient (Mourabet et al., 2017). The coefficient value of fermentation time (X1) and grain concentration (X2) in the production of cascara water kefir had a positive value of 3.78 for X1 and a negative 3.04 for X2, indicating a positive and negative relationship that affects the antioxidant activity of cascara water kefir.

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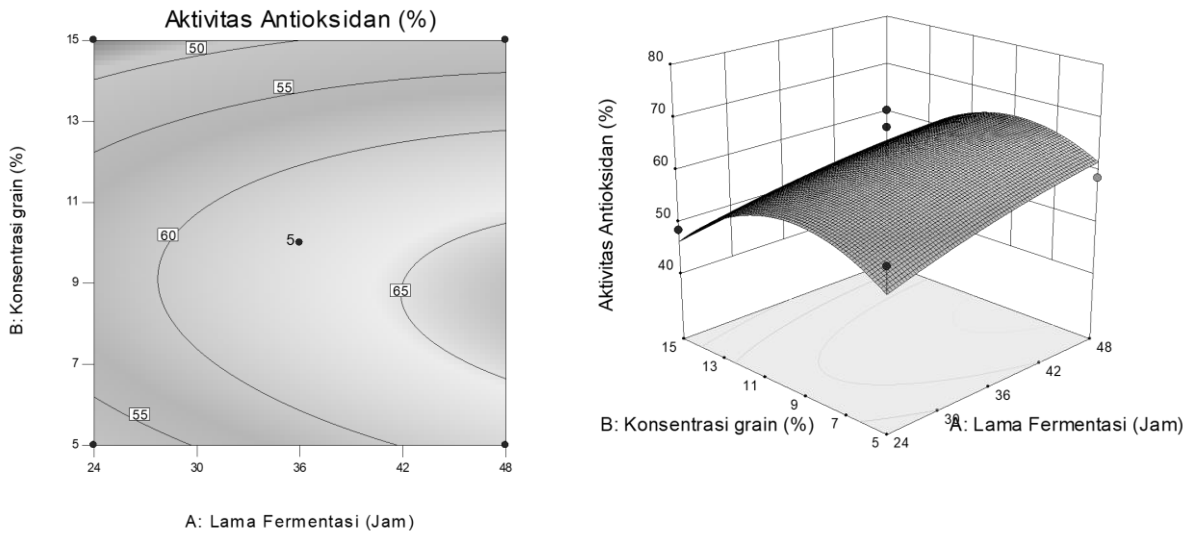

(a) (b

Figure 1. Contour Plot (a) dan Response Surface Curve (b) to antioxidant activity response

The mathematical equation model of the antioxidant activity response showed a relationship between the response and the fermentation time factor (X1); if the fermentation time were extended, the antioxidant activity of cascara water kefir produced would increase by 3.78. Furthermore, the antioxidant activity would decrease by 1.02 when the fermentation time (X1) was extended to its square value. The mathematical model of the relationship between the grain concentration factor (X2) and antioxidant activity showed a relationship whereby the grain concentration increased, and the antioxidant activity decreased by 3.04. Furthermore, if the grain concentration was multiplied by its square value, the antioxidant activity decreased by 8.89. In addition, the antioxidant activity decreased by 1.15 by the interaction between X1 and X2.

The increase in antioxidant activity during longer fermentation time resulted in the formation of organic acids by LAB for a more extended period. These organic acids work synergistically by transferring H^+ ions to free radicals, thus increasing antioxidant activity (Pratimasari, 2009). In addition, yeast can produce the enzyme vinyl phenol reductase. Combined with the enzyme ferulic acid reductase, it can convert cinnamic acid and ferulic acid into phenolic compounds through decarboxylation (Zubaidah et al., 2018). Lactobacillus bacteria are known to have the ability to break down ferulic acid into 4-vinyl phenol and cinnamic acid into 4-vinyl guaiacol due to the activity of the ferulic acid reductase and vinyl phenol reductase enzymes (Nurdyansyah & Hasbullah, 2018) which then increased the antioxidant activity in kefir. However, when the fermentation time was extended, more acid compounds formed, causing adverse effects on phenolic compounds acting as antioxidants (Budijanto et al., 2013).

The decrease in antioxidant activity with increasing kefir seed concentration is thought to be due to the formation of acidic compounds that cause damage to phenolic compounds, which play roles as antioxidants (Mahendra et al., 2019). One of the phenolic compounds that acids can damage is tannins, one of the various polyphenol compounds in the coffee pulp. (Pleissner et al., 2016). Fermentation can decrease by 42.65% the number of tannins in a product (Setiarto & Widhyastuti (2017), mainly influenced by LAB and yeast (Budijanto et al., 2013).

Lactic acid-producing bacteria, acetic acid, and yeast can hydrolyze sugar and carbohydrate components and convert them into organic acids. The more acidic compounds are formed, the lower pH can be performed. At the tannin molecule's center are carbohydrates such as d-glucose, which are also hydrolyzed due to tannin degradation (Izawa et al., 2010). Therefore, after a specific fermentation time, the organic acids formed can increase the antioxidant activity of a sample. However, at a certain peak point,

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the accumulation of these acids also contributes to the degradation of polyphenolic compounds, such as tannins, that have antioxidant properties.

Optimization of antioxidant activity of cascara water kefir

The production of cascara water kefir was optimized by confirming the optimum kefir grain concentration and fermentation period. Experimental trials were conducted by combining independent variables and observing the antioxidant activity response obtained from the combination. The experimental results data were based on Design Expert 10.01 design with antioxidant activity response (Table 3).

Table 3. Experimental design

STD	Run	Coded variable		Actual variable		Respons
		X1	X2	Time (Hour)	Concentration (%)	Antioxidant activity (%)
1	1	-1.000	-1.000	24	5	56.81
2	4	1.000	-1.000	48	5	58.76
3	6	-1.000	1.000	24	15	48.61
4	9	1.000	1.000	48	15	45.94
5	10	-1.414	0.000	19.03	10	50.21
6	8	1.414	0.000	52.97	10	72.11
7	5	0.000	-1.414	36	2.93	49.15
8	7	0.000	1.414	36	17.07	41.72
9	3	0.000	0.000	36	10	68.38
10	11	0.000	0.000	36	10	71.58
11	12	0.000	0.000	36	10	55.56
12	2	0.000	0.000	36	10	61.43
13	13	0.000	0.000	36	10	57.16

Summary Statistic Response shows the summary statistics of all responses. According to de Lima et al. (2022), the R-Square value indicates the adequacy of the model conformity to the actual data; when the data has an R-Square value that is close to 1, it indicates a high degree of conformity between the actual results and the predicted values. In the context of this study, the R-Square value of 0.7032 for the antioxidant activity response represents about 70.32% of the variation can be explained while using the suggested model.

The results of the variance analysis in Table 4 showed that the models were not significantly different and had a p-value of 0.0747. This result indicates that the model might have a negligible effect on the response. These results suggested that the combination of fermentation time and grain concentration resulted in relatively similar values of antioxidant activity. However, the analysis of the sequential model of sum square showed that the selected model used was a quadratic vs. 2FI model with a significant p-value (p-value of 0.0334) and no significant lack of fit value (p-value 0.4915). Montgomery (2017) explains that a selected model must have a p-value <0.05 for the sequential model of sum square and a p-value>0.05 for lack of fit. These results indicated that the model could predict low error-level data. A quadratic model was suggested based on the Design Expert 10.01 computerized system with these results.

The insignificant ANOVA results might be due to the formation of acidic compounds during the fermentation process. However, at a certain acid level, it can lead to a decrease in phenolic compounds, especially tannins in coffee pulp (Pleissner et al., 2016); therefore, the change in the antioxidant activity of cascara water kefir was not significant. Acidic compounds are released from the degradation of the groups of microorganisms found in water kefir grains (lactic acid bacteria, acetic acid bacteria, yeast), which form

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acetic acid, gluconic acid, and glucuronic acid (Jasman et al., 2015). The fermentation product by water kefir grain, the supernatant culture of water kefir, had antioxidant DPPH free radical inhibitory activity, especially from Acetobacter pasteurianus (Luang-In et al., 2018). The decrease in tannins caused by acidic compounds occurs due to the activity of LAB and yeast in the fermentation process, which degrades tannin compounds at low pH levels, thus reducing total phenols and resulting in a decrease in antioxidant activity (Nurhayati et al., 2020). Setiarto & Widhyastuti (2017) found that the tannin content decreased to 42.65% through fermentation.

Table 4. ANOVA result of antioxidant activity response

Source	*Sum of Squares*	*df*	*Mean Square*	*F Value*	*p-value*	
Quadratic vs 2FI	549.35	2	274.67	5.74	0.0334	*Suggested*
Model	793.32	5	158.66	3.32	0.0747	*not significant*
A-Fermentation time	114.39	1	114.39	2.39	0.1659	
B-Grain concentration	124.25	1	124.25	2.60	0.1511	
AB	5.34	1	5.34	0.11	0.7482	
A^2	7.28	1	7.28	0.15	0.7081	
B^2	549.22	1	549.22	11.48	0.0116	
Residual	334.86	7	47.84			
Lack of Fit	140.53	3	46.84	0.96	0.4915	*not significant*
Pure Error	194.33	4	48.58			

The insignificant ANOVA results might be due to the formation of acidic compounds during the fermentation process. However, at a certain acid level, it can lead to a decrease in phenolic compounds, especially tannins in coffee pulp (Pleissner et al., 2016); therefore, the change in the antioxidant activity of cascara water kefir was not significant. Acidic compounds are released from the degradation of the groups of microorganisms found in water kefir grains (lactic acid bacteria, acetic acid bacteria, yeast), which form acetic acid, gluconic acid, and glucuronic acid (Jasman et al., 2015). The fermentation product by water kefir grain, the supernatant culture of water kefir, had antioxidant DPPH free radical inhibitory activity, especially from *Acetobacter pasteurianus* (Luang-In et al., 2018). The decrease in tannins caused by acidic compounds occurs due to the activity of LAB and yeast in the fermentation process, which degrades tannin compounds at low pH levels, thus reducing total phenols and resulting in a decrease in antioxidant activity (Nurhayati et al., 2020). Setiarto & Widhyastuti (2017) found that the tannin content decreased to 42.65% through fermentation.

The results then proceed to the optimization stage. Design Expert 10.01 would predict the optimum result based on the existing data. Based on the optimization process, the prediction of the most optimal fermentation time was 48 hours, and the grain concentration was 8.58% in making cascara water kefir probiotic beverage, with the highest desirability value of 0.809 or 80.9%. According to Montgomery (2017), the desirability value is used to determine an optimal solution's accuracy level. Manohar et al. (2013) explained that when the desirability value is close to 1, the higher value of optimization accuracy predicted by the Design Expert 10.01 program. Thus, the optimal process conditions were predicted to produce a product with an antioxidant activity of 66.31%.

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Verification

Table 5. Comparison of Prediction and Verification Results

	Antioxidant activity (%)
Prediction	66,31
Verification	65.53±7.82
P-Value	0.878

The process optimization results obtained were then verified by conducting experiments three times. Based on the verification results (Table 5), the antioxidant activity of the experimental results was 65.53%. This value was similar to the prediction from Design Expert 10.01 of 66.31%. The statistical paired-T test showed that the antioxidant activity value compared to the prediction and verification had a $p>0.05$ value, which was not significantly different. It would be stated that the antioxidant activity between cascara water kefir prediction and verification was similar.

Characterization of the cascara tea and cascara water kefir

Table 6 shows the characterization results of cascara tea and cascara water kefir optimization: IC_{50} antioxidant activity using DPPH reagent, total phenol, color (L, a, b), pH, total soluble solids, total LAB, and total yeast and mold. The IC_{50} antioxidant activity of the tea raw material showed a lower value (124.77 ppm) than that of the cascara water kefir product (173.66), and both samples have moderate antioxidant activity. The smaller the IC50 value, the stronger the antioxidant activity against free radicals, which can be said to have high antioxidant activity (Munteanu & Apetrei, 2021). Antioxidant activity is highly strong when the value of $IC_{50}<50$ ppm, strong if between 51 to 100 ppm, moderate if between 101 to 250 ppm, and low if between 251 to 500 ppm (Fauzi et al., 2022).

The qualitative analysis showed that both cascara tea samples have a higher phenolic content than cascara water kefir. The degradation process of phenolic compounds such as tannins can cause a decrease in antioxidant activity and total phenol values. The presence of bacteria (LAB, BAA) and yeast during fermentation can produce external enzymes, namely tannase, which can break down the ester bonds in tannin compounds. This causes a decrease in total phenols (Nurhayati et al., 2020; Rohaya et al., 2022). Tannins are phenol compounds easily hydrolyzed by acids, bases, or enzymes (Mahendra et al., 2019; Nurhayati et al., 2020). Sugar groups in tannin molecules will be hydrolyzed during fermentation by LAB (Mahendra et al., 2019). Therefore, the diversity of microbiota contained in water kefir grains contributes to decreased phenol compounds and antioxidant activity.

Table 6. Comparison of characterization results of cascara tea and cascara water kefir

Parameters	Cascara tea	Cascara water kefir
IC_{50} (ppm)	124.77	173.66
Quantitative Phenol	+++	+++
Total phenolic content (mg GAE/mL)	490.06 ± 0.00	477.24 ± 0.00
L	24.08 ± 0.93	30.69 ± 2.94
A	15.56 ± 0.31	14.06 ± 2.68
B	20.37 ± 1.81	29.57 ± 0.88
pH	5.47 ± 0.04	3.57 ± 0.06
Total dissolved solid (^{o}Brix)	9.83 ± 0.32	8.80 ± 0.65
Viscosity (mPa.s)	1.59 ± 0.01	1.62 ± 0.01
Total LAB (log Cfu/ml)	0.00 ± 0.00	8.54 ± 0.10
Total Khamir & Mold (log Cfu/ml)	10.63 ± 0.59	10.93 ± 0.04

Acidic compounds formed during the fermentation process can also reduce the pH of cascara tea from 5.47 to 3.57. The changes in acidic conditions can degrade tannin compounds and increase the product's

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lightness. As seen in Table 6, the lightness increased from 24.08 to 30.69 after fermentation. The longer the fermentation time, the lighter the color will be due to the impact of the acidity level (Nurhayati et al., 2020). Hydrolysis of tannins also changed the redness value (a*), which became lower in cascara water kefir as the thearubigin's reddish-brown color faded. The results showed an increase in the yellowness (b*) of the cascara water kefir product after fermentation; a more positive value indicates a more yellow color. The increase in the yellow color (b+) level is related to the change in tannins, which produce a light color in an acidic condition, as well as the degradation of thearubigin compounds (Firdaus et al., 2020). This result aligns with a study conducted by Wistiana (2014), which showed increased brightness and yellowness and decreased redness values of fermented tea.

The total soluble solids decreased after fermentation. The value of total dissolved solids in cascara tea was 9.83 °Brix and decreased to 8.80 °Brix after fermentation. Microorganisms in water kefir break down soluble solids in water kefir cascara, such as sugar, into lactic acid and alcohol, reducing the total soluble solids in cascara tea (Mulyani et al., 2021). The results showed that cascara water kefir contained microorganisms such as LAB, yeast, and mold, as high as 8.54 and 10.93 log Cfu/ml, respectively. Cascara tea does not contain LAB.

Meanwhile, it contains a lower amount of yeast and mold compared to Cascara water kefir. The increase in the content of microorganisms in tea is due to the addition of water kefir grain containing LAB and yeast. During the fermentation process, the substrate in the form of sugar is sufficient as a nutrient for microorganisms to grow and multiply. The fermentation process will cause the breakdown of sugar by LAB for its growth and development, increasing the number of microorganisms after fermentation (Ayuni et al., 2020).

In this study, the fermentation process of cascara tea showed increased viscosity after the fermentation process into cascara water kefir. A complex microbiological system in a polysaccharide matrix of levan (glucose polymer) and dextran (fructose polymer), created by bacteria, is thought to be responsible for the increase in viscosity (Fels et al., 2018; Lynch et al., 2021). In cascara water kefir, lactic acid bacteria (LAB), acetic acid bacteria (BAA), and yeast have a symbiotic relationship. When the bacteria in water kefir thrive in the right conditions, glucan will be produced, increasing the grains' biomass and raising viscosity.

Conclusion

The optimum conditions for producing cascara water kefir probiotic beverage were 48 hours of fermentation time and 8.57% kefir seed concentration. The optimum combination of cascara water kefir produced antioxidant activity of 65.53%, IC50 173.66 ppm, total phenols 477.24 mg GAE/mL, Lightness value (L*) 30.69, redness (a*) 14.06, yellowness (b*) 29.57, pH 3.57, TPT 8.80 (°Brix), viscosity 1.62 mPa.s, total LAB 8.54 log Cfu/ml and total yeast & mold 10.93 log Cfu/ml. Cascara water kefir is an excellent source of antioxidant activity; it may be created with the optimal fermentation time and kefir grain concentration found in this study. The suggestions for further research were the characterization of phenolic compounds contained in cascara water kefir and comparing them with raw materials from cascara tea to determine the dynamic compounds during fermentation. Kefir grains from this study can be used to produce cascara water kefir, a good antioxidant activity source. The suggestions for further research were the characterization of phenolic compounds contained in cascara water kefir and comparing them with raw materials from cascara tea to determine the dynamic compounds during fermentation.

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