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Optimization of Pancreatic Lipase Inhibition in Fermented Beverages Brewed from Katuk Leaves Using RSM

[Optimasi Penghambatan Lipase Pankreas pada Fermentasi Seduhan Daun Katuk Menggunakan RSM]

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ABSTRACT

The inhibition of pancreatic lipase and the associated reduction of lipid absorption has become the most appropriate approach for treating obesity. Meanwhile, katuk (*Sauropus androgynus*) leaves are rich in polyphenols that act as natural bioactive compounds and are also responsible for the potential effect on metabolic diseases, including inhibition of pancreatic lipase activity. Naturally occurring polyphenols can inhibit pancreatic lipase and consequently affect fat digestion as well as energy intake. Therefore, this study aimed to optimize the inhibitory activity of pancreatic lipase, which plays an essential role in lipid absorption. In fermented katuk (*Sauropus androgynus*) leaves were brewed following RSM using a *Box Behnken* design. Data analysis was used to optimize the formulation with a response surface model consisting of three factors namely sucrose concentration ranging from 10-20% b/v, culture 10-20 b/v, as well as fermentation time of 1-5 days. The parameters tested were the percentage inhibition of pancreatic lipase, antioxidant activity, polyphenol, and total acid. Based on the RSM optimization results obtained from the three influencing factors, the optimum conditions were established namely 10.43% w/v sucrose, 10% v/v kombucha culture, and fermentation for 5 days. These conditions yielded the most optimal responses, with the percentage inhibition of pancreatic lipase, antioxidant activity, polyphenol, and total acid being 86.13%, 96.27%, 0.97 mg GAE/mL, and 1.11%, respectively. In general, the results demonstrated that the RSM method of the *Box Behnken* design and the parameter prediction values obtained using the model equation are in good agreement with the experimental values with at least R2 ≥ 0.8.

Keywords: antioxidant, katuk leaves, optimization, pancreatic lipase inhibitory activity, RSM

ABSTRAK

Penghambatan lipase pankreas dan kaitannya dengan reduksi penyerapan lipid telah menjadi strategi yang paling menguntungkan dalam membantu mengatasi obesitas. Daun katuk (Sauropus androgynus) merupakan salah satu makanan kesehatan sumber polifenol sebagai senyawa bioaktif alami yang berpengaruh terhadap penyakit metabolik salah satunya aktivitas dalam menghambat lipase pankreas. Polifenol yang terjadi secara alami dapat menghambat lipase pankreas dan dengan demikian memengaruhi pencernaan lemak dan memengaruhi asupan energi. Tujuan dari penelitian ini untuk mengoptimalkan aktivitas penghambatan lipase pankreas yang memainkan peran penting dalam penyerapan lipid melalui optimasi fermentasi seduhan daun katuk (Sauropus androgynus) sebagai minuman kesehatan menggunakan respon surface methodology (RSM) menggunakan desain Box Behnken. Dalam penelitian ini, analisis data digunakan untuk mengoptimalkan formulasi minuman fermentasi seduhan daun katuk (Sauropus androgynus) dari model permukaan respon yang terdiri dari tiga (3) faktor yaitu konsentrasi sukrosa (10-20% b/v) Konsentrasi kultur (10-20 b/v) dan waktu fermentasi dan (1-5 hari). Parameter respon yang akan diuji adalah prosentase penghambatan lipase pankreas, aktivitas antioksidan, polifenol dan total asam. Berdasarkan hasil optimasi RSM yang diperoleh dari ketiga faktor yang berpengaruh yaitu konsentrasi sukrosa (10,43% b/v), konsentrasi kultur kombucha katuk daun (10% v/v), dan waktu fermentasi (5 hari) dengan persentase penghambatan lipase pankreas, aktivitas antioksidan dalam menangkal radikal bebas 2-difenil-1-picrylhydrazyl (DPPH), polifenol dan total asam masing-masing sebesar 86,13%; 96,27%; 0,97 mg GAE/mL; 1,11%. Secara umum hasil membuktikan bahwa dengan menggunakan metode RSM desain Box Behnken nilai prediksi parameter menggunakan persamaan model berada dalam kesesuaian yang baik dengan nilai eksperimen dengan sedikitnya R^2 ≥ 0,8, seperti pada respon penghambatan lipase pankreas, aktivitas antioksidan dalam menangkal radikal bebas (DPPH), dan total asam.

Kata kunci: *aktivitas penghambatan lipase pankreas, antioksidan, daun katuk, optimalisasi, RSM*

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INTRODUCTION

High levels of fat or lipid in the human bloodstream potentially cause hyperlipidemia, which includes high cholesterol and triglycerides. Poor diet and lifestyle, together with lack of exercise can increase the risk of hyperlipidemia, which initially shows no symptoms, followed by an increase in total cholesterol and LDL as well as a decrease in HDL (Sukandar *et al.,* 2010). Meanwhile, herbal supplements are widely used to tackle blood cholesterol levels, an example of the products include red spinach leaf extract with antioxidant properties which helps relieve hyperlipidemia (Pradana *et al.,* 2016). Similarly, steeping avocado and moringa leaves were processed into an herbal brew that can increase HDL levels in rats (Handayani *et al.,* 2017). However, this potential needs to be further investigated to also ensure its benefits in humans.

The fermentation of the kombucha beverage is one of the processes for developing products that are beneficial to health, due to its high antioxidant content which is useful in treating degenerative diseases (Srihari and Satyanarayana, 2012). Besides, phenolics are widely known to have antioxidant activity by scavenging free radicals and by inducing levels of antioxidant enzymes.

In this study, a healthy beverage made by fermenting katuk leaves with kombucha culture starter was optimized, and was proven to inhibit pancreatic lipase, as well as reduce lipid absorption in the case of hyperlipidemia or obesity (Kim *et al.,* 2020). Generally, natural products are considered to have lower side effects and toxicity compared to synthetic or factory drugs, hence, the utilization of polyphenols in natural sources is a potent alternative.

Kombucha is a traditional fermented tea product that involves the fermentation of sugar dissolved in tea with a symbiotic culture of bacteria and yeast (SCOBY). Its culture will modify the structure of polyphenols using enzymes produced by bacteria and yeast during fermentation (Jayabalan *et al.,* 2014). The formation of phenolic compounds process is presumably due to the biotransformation process by enzymes produced through the microorganisms in kombucha. During the fermentation process, yeast can produce several types of enzymes such as vinyl phenol reductase. Furthermore, ferulic acid reductase enzyme can form phenol due to decarboxylation of cinnamic acid and firulic acid produced by organic acid-producing bacteria. Cinnamic acid is also a phenolic compound that acts as an antioxidant as well as ferulic acid which is a derivative from the hydroxycinnamic acid group (Suhardini and Zubaidah, 2016). Fermentation is one way to improve bioactive compounds in various food products, for example, it can increase phenolics and the antioxidant activity of kombucha tea (Martínez Leal *et al.,* 2018).

The selection of katuk leaves is based on the use of green vegetables as functional food adjuvants in beverage products that are rich in nutrients and also have bioactive components such as total polyphenols. It also contains flavonoids, alkaloids and high antioxidants that produce secondary metabolic compounds and provide physiological effects in reducing the risk of cardiovascular disease. Traditionally, katuk leaves are consumed as fresh salads to increase and accelerate the production of mother's milk (Zhang *et al.,* 2020). Several studies also claimed that consuming these leaves can help in reducing weight as it contains adequate amounts of macroland micronutrients. The micronutrients include phenolics, carotenoids, antioxidant, vitamins, and minerals, while the antioxidant content showed extensive health benefits for humans (Khoo *et al.,* 2015).

Product development of fermented katuk leaves using the kombucha culture in functional beverage thrives as a prospective option. This is because kombucha is a well-known fermented beverage among other traditional products. The presence of symbiotic culture bacteria and yeast plays an important role in preventing microbial contamination (Vitas *et al.,* 2013). The predominant cultures present in kombucha brew are acetic acid bacteria which consume alcohol as the substrate to yield acetic acid. These bacteria, unlike yeast, require large amounts of oxygen for activity and growth (Lynch *et al.,* 2019). Therefore, the fermentation of kombucha steeped katuk leaves was examined in this study with different culture and sucrose concentrations, as well as fermentation time to determine the optimal antioxidant activity and lipase inhibitory activity.

This study aims to optimize the formulation of fermented beverage brewed katuk leaves (*Sauropus androgynus*) to increase the pancreatic lipase inhibitory activity which plays an important role in fat absorption using the response surface methodology (RSM). This is a very efficient procedure for optimizing multiple variables with a minimum number of experiments, using a series of techniques to yield an optimal, desired response (Myers *et al.,* 2016). RSM with Box-Behnken design was performed to optimize the process conditions of katuk leaves fermentation in kombucha culture and obtain the highest inhibition activity of pancreatic lipase.

MATERIALS AND METHODS

Materials

Katuk leaves were purchased from Modern market BSD city, SCOBY (kombucha culture) from

Indo Kombucha (www.indokombucha.com), while white sugar or sucrose was obtained from a local market. The kombucha culture SCOBY consisted of *Acetobacter xylinum*, *Glucono-bacter*, *Lactobacillus* sp, and *S. cerevisiae.*

Preparation of functional beverage from katuk leaves

The process of functional bevreage from brewed katuk leaves (*Sauropus androynous*) was prepared using Maryati and Susilowati (2020) method. Katuk leaves were sorted, washed, blanched, and then pasteurized with steamer cooker (Advance C30, China) for 10 min at 90-100°C with boiling water in a ratio of katuk leaves:water = 1:10 w/v, and filtered using 80 mesh. A total of 10-20% wt/vol sucrose was added to each filtrate and stirred. After brewing, the mixture was placed in a sterile jar and left air-cooled until it reached about 27-30°C. Kombucha starter culture of 10 to 20% wt/vol was introduced into the filtrate brewed at room temperature to prevent it from dying, then the jar was covered with sterile gauze. The fermentation process was carried out in a dark room at 28-30°C in an aerobic condition, for 1-5 days.

Determination of pancreatic lipase inhibitory activity

The potential of kombucha brewed from katuk leaves to inhibit pancreatic lipase was adapted from reports by Kim *et al*. (2016). Lipase from porcine pancreas at 25 mg/mL was dissolved in buffer solution (Sigma-Aldrich, USA) containing 1 mM EDTA (Sigma Aldrich, USA) and 10 mM morpholine-propane sulphonic acid (MOPS), as well as pH 6.8 (pH meters, pH 700, Eutech Instruments, USA) as an enzyme buffer solution. The assay of Tris buffer was prepared with 100 mM Tris-HCl and 5 mM $CaCl_2$ at pH 7.0, while 10 mM *p*-nitrophenylbutyrate (*p*-NPB) (Sigma Aldrich, USA) in dimethylformamide was used as the substrate. Next, 20 *µ*L orlistat as standard antiobesity drugs (Roche, Basel, Switzerland) or either vegetable extracts at the test concentration of 10 *µ*g/mL were mixed with 155 *µ*L Tris and 20 *µ*L enzyme buffer solution. After pre-incubation using Incubator Memmert INB 400 at 37°C for 15 min, the reaction was initiated by the addition of 5 *µ*L substrate to all tubes, while the enzymatic reaction was carried out for 30 min at 37°C. Orlistat antiobesity drugs (standard) act by reversibly inhibiting the gastric and pancreatic lipase enzymes responsible for the metabolism of fat. The mechanism of action is by breaking down triglycerides into absorbable free fatty acids and monoglycerides.

Lipase activity was determined based on the hydrolysis of p-NPB to p-nitrophenol, then the reaction results were measured using an ELISA reader (Varioskan Flash, Thermo Scientific) at a wavelength of 405 nm. The decrease in OD percentage of pancreatic lipase incubated with the test material represents the lipase inhibitory activity. The Lipase inhibition (%) was calculated as follows:

$$\text{Inhibition (\%)} = 100-[(A_B-A_b)/(A_A-A_a)]\times 100 \text{ ..... (1)}$$

A_A is the Absorbance of the activity solution without inhibitor; A_a is the negative control without inhibitor; A_B is the Absorbance of the activity solution with inhibitor; A_b is the negative control with inhibitor, and the results were calculated as an average (n = 3).

Determination of total polyphenol content

Total polyphenol content was measured according to Folin-Ciocalteu's method (Akhter *et al.,* 2013). About 0.1 mL of sample was dissolved in 0.7 mL of distilled water, 1 mL of saturated Na_2CO_3 (Merck KGAa, Germany) solution and 0.5 mL of Folin-Ciocalteu reagent (Merck KGAa, Germany). Next, the mixture was added with 1.4 mL of distilled water and mixed by the vortex shaker (Thermolyne Maxi Mix Plus™, USA) for a few seconds. It was stored in a dark room temperature for 30 min, and the absorbance was measured at 760 nm against a blank, using a UV-Vis spectrophotometer (Agilent Technologies, Cary 60 UV-Vis, USA). Gallic acid (Sigma Aldrich, USA) was used as a standard microgram of gallic acid equivalents (GAE)/mL.

Antioxidant assay by DPPH radical scavenging activity

Free radical scavenging activity of kombucha from brewed katuk leaves was measured by performing DPPH (2.2-Diphenyl-1-picrylhydrazyl) scavenging activity assay as previously reported by (Mazidi *et al.,* 2012). A total of 500 *µ*L aliquot of each fermented product was added to 500 *µ*L of 0.1 mM DPPH solution (Sigma Aldrich, USA) and 0.4 mg/mL methanol. Afterward, the solution was added with 1,500µL methanol (Merck KGaA, Germany), shaken vigorously (Thermolyne Maxi Mix Plus™, USA), and stored in a dark room at room temperature for 30 min. The absorbance of the DPPH activity was measured using a spectrophotometer (Agilent Technologies, Cary 60 UV-Vis, USA) at 515 nm. The calculation was carried out using the equation:

$$\text{Scavenging Capacity (\%)} = \frac{\text{Abs control-Abs sample}}{\text{Abs control}}\times 100 \text{ ..... (2)}$$

Measurement of total acid

The total acid of fermented beverages brewed from katuk leaves was measured using the method previously reported by Mergypta *et al.* (2014). The

sample was allowed to stand for 30 min and stirred, then it was filtered and pipetted up to 10 mL into a glass beaker. The solution was added with 2-3 drops of phenolphthalein and titrated with 0.1 N NaOH until it turned red. Next, the total amount of titrated acid was calculated using the formula:

$$TA=\frac{a\times b\times c\times d}{e}\times 100\% \quad \ldots\ldots (3)$$

TA= Total Acetic Acid (%); a= Amount of NaOH required in the titration; b= Normality of NaOH (0.1 N); c= Weight of lactic acid equivalent (60); d = Diluent factor (10); e= Weight of sample (mg).

Design of experiment

The Box-Behnken experimental set-up in RSM (Suberu *et al.*, 2019) was employed to determine the effects of three independent variables on response function and to identify the optimal conditions by maximizing the percentage inhibition of lipase, anti-oxidants, polyphenols, as well as to minimize the percentage of total acidity. The RSM design provided 17 experimental treatments. Table 1 presents the ranges and levels of the experimental parameters.

The DX 7® RSM *Box-Behnken Design* programmed to be randomized and the detailed experimental design are presented in Table 2. After randomization of the factor combination was carried out, 17 treatments were analyzed as shown in Table 2. The response functions were inhibitory activity of lipase, DPPH, total polyphenol content, and total acid. In the response analysis stage, each variable was analyzed by ANOVA which was used based on the suggestion by the program, namely the model that had the highest level of accuracy and produced significant values. ANOVA models found in this design were Linear, 2-factor interaction, and Quadratic. The model that yielded significance and non-significance on lack of fit was determined for the analysis of corresponding variables.

Sensory test

Sensory testing used the hedonic method on fermented beverage products from brewed katuk leaves compared to kombucha tea. The parameters assessed were color, aroma and taste, while the rating scale ranged from 1–5, namely 1= dislike, 2= dislike slightly, 3= neither like nor dislike, 4= like slightly, 5= like. The test was carried out on 25 untrained panelists.

RESULTS AND DISCCUSION

Optimization of fermentation conditions with RSM

The interdependence between fermentation process conditions of brewed Katuk leaves and the observed responses of lipase inhibitory activity, DPPH interception activity, total polyphenol content, and total acidity is exhibited in Table 3.

Table 1. The range of independent variable values

Factor	Name	Units	Type	Low Actual	High Actual	Low Coded	High Coded	Mean	Standard Deviation
A	Sucrose	%	Numeric	10	20	-1	1	15	3.43
B	Culture	%	Numeric	10	20	-1	1	15	3.43
C	Fermentation Time	days	Numeric	1	5	-1	1	3	1.37

Table 2. Design of the fermentation and brewing of katuk leaves variations

Std	Run	Block	Factor 1 A:Sucrose	Factor 2 B:Culture	Factor 3 C:Fermentation Time
4	1	Block 1	10	20	3
3	2	Block 1	20	20	3
11	3	Block 1	15	15	3
6	4	Block 1	20	15	1
14	5	Block 1	15	20	5
17	6	Block 1	15	15.	3
7	7	Block 1	15	10	5
5	8	Block 1	15	15	3
2	9	Block 1	15	15	3
13	10	Block 1	20	15	5
12	11	Block 1	10	15	1
9	12	Block 1	20	10	3
8	13	Block 1	15	20	1
10	14	Block 1	10	10	3
16	15	Block 1	15	15	3
1	16	Block 1	15	10	1
15	17	Block 1	10	15	5

The analysis results of ANOVA presented in Table 4 indicate that the model selected to analyze the response of lipase inhibitory activity was the two-factor interaction, Quadratic was used for DPPH scavenging activity and total acid, while the total polyphenol content followed the Linear model. A significant value of $p<0.05$ was found for all responses, while the ANOVA results also showed that each component including sucrose, culture concentration, and fermentation time had a significant effect on the response.

Lack of Fit (F-Value) responses to lipase inhibitory activity at 0.11, DPPH scavenging 0.48, total polyphenol content of 0.97, and total acid of 0.09 were not significant. Insignificant F-Value indicates a good model as it shows the compatibility of the yield response data with the model (Domingo *et al.,* 2019).

The coefficient of determination (R^2) was 0.82, meaning that 82.37% of the sample variation in lipase inhibition was related to the independent variable. This value also indicates that 17.63% of the variation cannot be explained by the model. It was concluded that the regression model is suitable for predicting the optimum value of lipase inhibition due to the small difference between the experimental and predictive values (Chrisnasari *et al.,* 2011).

Effects of the fermentation process of katuk leaves as a functional drink on pancreatic lipase inhibitory activity

The lipase inhibitory activity of fermented drinks from brewed katuk leaves with various treatments ranged from 28.1891–95.0372% as shown in Figure 1. The optimum condition was determined regarding the maximum value of pancreatic lipase inhibitory activity.

Table 3. Box-Behnken design for responses

Factor			Response			
A	B	C	Y1	Y2	Y3	Y4
Sucrose	Culture	Fermentation Time	Lipase inhibitor	DPPH	Poliphenol	Total Acid
10	20	3	54.732	92.888	0.980	1.645
20	20	3	62.269	91.533	1.183	0.608
15	15	3	59.864	90.669	0.794	0.701
20	15	1	58.531	90.755	1.179	0.567
15	20	5	52.961	92.028	0.890	1.588
15	15	3	71.079	91.300	1.130	0.641
15	10	5	95.037	93.013	1.033	1.364
15	15	3	59.288	88.785	1.201	0.688
15	15	3	65.737	91.178	1.067	0.761
20	15	5	76.265	91.640	0.964	1.375
10	15	1	41.688	90.334	1.175	0.644
20	10	3	92.602	93.203	1.052	1.436
15	20	1	68.052	89.864	1.160	0.816
10	10	3	69.960	96.334	1.090	1.000
15	15	3	58.939	91.173	1.201	0.821
15	10	1	28.189	89.932	1.267	1.019
10	15	5	53.761	93.533	0.727	1.548

Table 4. Analysis of the model for the responses of lipase inhibition, DPPH antioxidants, polyphenols, and total acid

Name Response	Units	Model	Math Equation	*Significant* ($p<0.05$)	*Lack of fit* ($p>0.05$)	R^2
Lipase inhibition	% inhibition	2 factor interaction	Y1=80.36946+3.57929A+7.21 680 B+33.7022C 0.15105AB + 0.14152AC-2.04849 BC	0.0026	0.1124	0.8237
DPPH	% inhibition	Quadratic	Y2= +118.09596 -2.17663A - 1.85937 B+2.79644 C +0.017767 AB -0.057848 AC - 0.022940 BC + 0.064490 A^2 +0.050248 B^2 -0.1669C^2	0.0362	0.4758	0.8432
Polyphenol	mg GAE/mL	Linear	Y3 = +1.21729 +0.010120 A - 5.735 E-003 B -0.072961 C	0.0158	0.9720	0.5370
Total acid	%	Quadratic	Y4 = +5.33817 - 0.025750 A - 0.019000 B - 6.04167E-003 C	0.0003	0.0928	0.9652

Figure 1 is a 3D response surface model of total polyphenols, in which the concentration of sucrose, and culture, as well as fermentation time influenced lipase inhibition. Additionally, color differences in the graph indicate the change in the lipase inhibitory response rate. The blue contour shows the lowest response rate namely 28.19% at 15% sucrose, 10% culture concentration, and 1 day fermentation, while the red indicates the highest inhibition response of 95.04% at 10% sucrose, 15% culture, and 5 days fermentation. The predicted optimum conditions for kombucha from brewed katuk leaves was 86.85%.

The graph suggests that lipase inhibition based on ANOVA was significantly influenced by sucrose concentration, fermentation time, and the interaction between culture and fermentation time, but not significantly influenced by culture concentration, interaction between sucrose and culture, as well as the interaction between sucrose and fermentation time. Meanwhile, the culture concentration and fermentation time affected the pancreatic lipase inhibitory response. Optimum fermentation time will produce organic acids, such as glucuronic acid as secondary metabolites that induce inhibition, as well as polyphenol activity which increases during fermentation. Polyphenols are effective in inhibiting pancreatic lipase in vitro (Yang *et al.,* 2014), while phenolics are useful for reducing the accumulation of dietary fat absorption. Furthermore, glucoronic acid (GlcUA) plays a role in increasing the bioavailability of polyphenols. Phenol conjugates with GlcUA, thereby increasing its transport and bioavailability. Other bioactive compounds such as flavonoids are obtained during the fermentation process due to the degradation of some active compounds.

DPPH radical scavenging activity (%)

Scavenging activity based on the analysis results ranged from 88.78-96.33% as shown in Figure 2. The optimum condition was determined regarding the maximum value of DPPH scavenging activity.

Figure 2 shows a 3D response surface model in which the concentration of sucrose, culture, and fermentation time affected DPPH scavenging activity. The color difference in the graph indicates the change in the response rate. Blue shows the lowest DPPH inhibitory response rate of 88.78% at 15% of sucrose, 15% of culture, and 3 days fermentation time, while red indicates the highest rate namely 96.33% at 10% of sucrose, 10% of culture, and 3 days fermentation. The predicted optimum conditions for inhibiting DPPH in kombucha from brewed katuk leaves was 96.33%.

From the results, it was concluded that scavenging activity based on ANOVA was significantly influenced by fermentation time, but not sucrose and culture concentration, nor the interactions between these factors. Antioxidant activity in kombucha of katuk leaves primarily comes from the metabolism of microorganisms during fermentation. According to Massoud *et al.* (2022), fermentation time affects the free radical scavenging properties of kombucha. Goh *et al.* (2012) also reported that more metabolic products were produced during fermentation. A substantial amount of sugar present in the tea broth might explain this result as high metabolic products might lead to inhibition. Another possible explanation is unequal transport of critical cell materials or nutrients and rates of the nutrients' utilization, as well as the breakage of phenolic compounds by kombucha culture leading to loss of stability in the activity of antioxidants due to the enzymes produced.

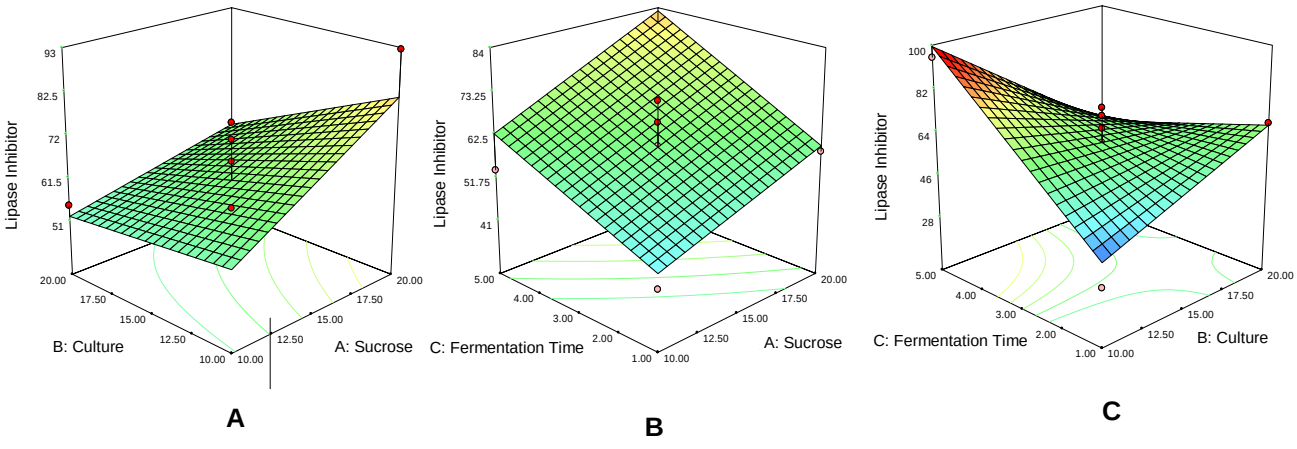


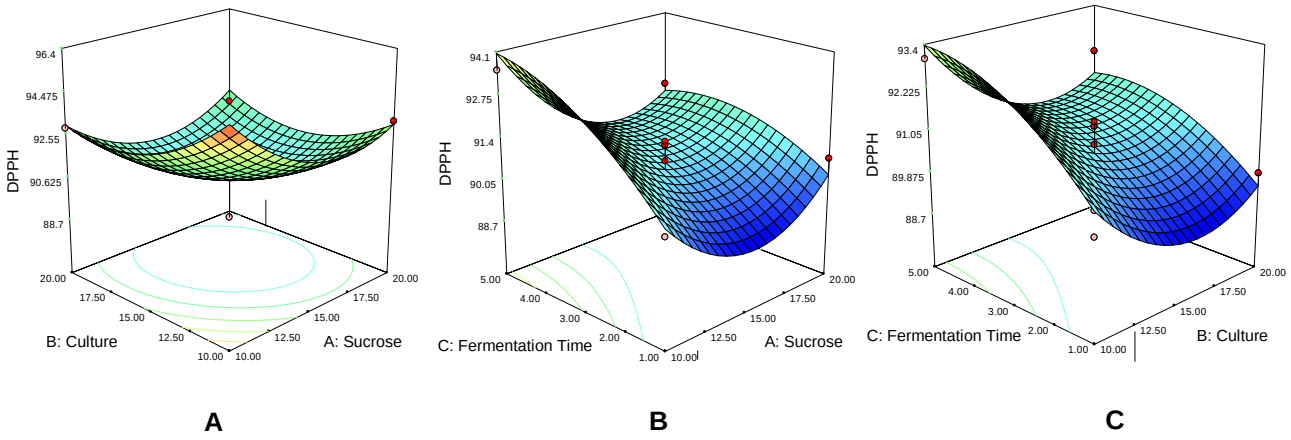
Figure 1. Profile of surface response 3-D plot for (A) the effect of sucrose and culture, (B) the effect of sucrose and fermentation time and (C) the effect of culture concentration and fermentation time, on lipase inhibitory activity

Total polyphenol content

The total polyphenol of the fermented beverage with variations in treatments ranged from 0.73-1.27 mg GAE/mL as shown in Figure 3. The optimum condition was also determined regarding the value of the target range.

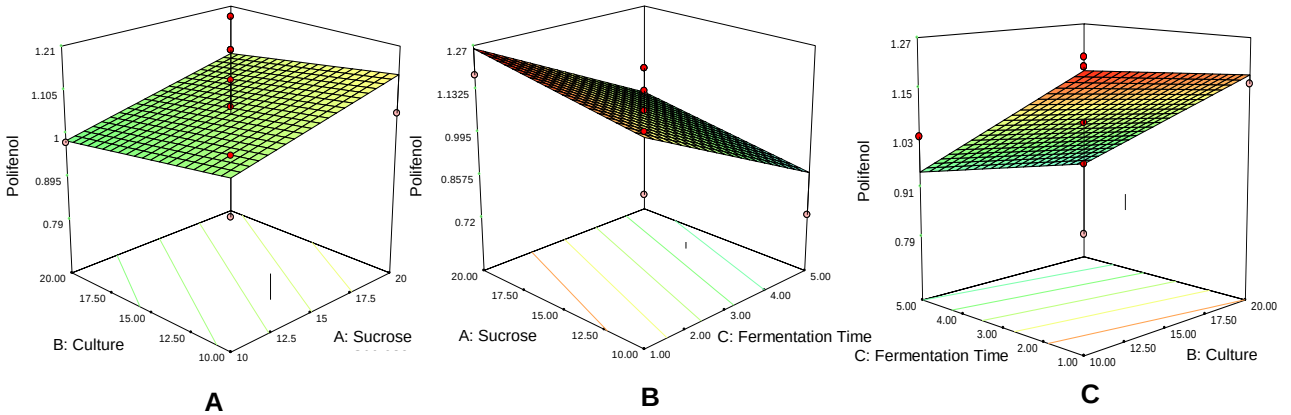
Figure 3 is a 3D response surface, in which sucrose and culture concentrations, as well as fermentation time influenced the total polyphenol content. The color differences in the graph show the change in response rate. Blue represents the lowest polyphenol response rate of 0.73 mg of GAE/mL at 10% of sucrose, 15% of culture, and 5 days of fermentation time, while red shows the highest response of 1.27 mg GAE/mL at 15% of sucrose, 10% of culture, and 1 day of fermentation. The predicted optimum conditions for total polyphenol content in kombucha from brewed katuk leaves was 0.90 mg GAE/mL. However, based on the ANOVA results, it showed a mathematical equation model with the highest desirability level at a sucrose concentration of 10.43%, culture 10.00%, and a fermentation time of 5 days with a total polyphenol of 0.9007 mg GAE/mL.

The surface response graph implied that total polyphenols content was significantly influenced by the fermentation time, but not sucrose and culture concentrations. This occurred because the fermentation process causes polyphenols and other components in katuk leaves including polysaccharides, starch, and protein to degrade into simple molecules and several bioactive compounds, such as organic acids, acetaldehyde, carbon dioxide, and alcohol, which are also responsible for the formation of the specific aroma of kombucha.



A B C

Figure 2. Profile of surface response 3-D plot for (A) the effect of sucrose and culture, (B) the effect of sucrose and fermentation time and (C) the effect of culture concentration and fermentation time, on DPPH scavenging activity



A B C

Figure 3. Profile of surface response 3-D plot for (A) the effect of sucrose and culture, (B) the effect of sucrose and fermentation time and (C) the effect of culture concentration and fermentation time, on total polyphenol content

Total acid

Figure 4 shows a 3D response surface model, in which sucrose, culture concentration, and fermentation time influenced total acid content. The color differences in the graph show the change in the total response rate. Blue indicates the lowest total acid response rate of 0.57% at 20% of sucrose, 15% of culture, and 1 day of fermentation time, while red shows the highest response rate of 1.64% at 10% of sucrose, 20% of culture, and 3 days of fermentation. The predicted optimum condition of the total acid content was 1.64%, based on the RSM.

The graph shows the significant influence of sucrose, fermentation time, and interaction between sucrose and culture concentration on the total acid content. However, the culture concentration, along with the interaction between sucrose and fermentation time, as well as culture and fermentation time had no significant effect. This is possibly due to the close range of the sugar and culture concentrations, hence, changes in total acid during fermentation were influenced by the sugar substrate in alcohol and acetic acid products. Changes in the acetic acid were caused by the higher rate of product formation, which further inhibits the decomposition reaction of the substrate. It is apparent that as fermentation occurs, complex sugar are broken down into simple molecules. Furthermore, sugar is converted into alcohol which in turn, will be transformed by the kombucha culture into organic acid through oxidation. Therefore, the total acid level tends to increase with higher rates of fermentation.

Optimum point and verification for optimum models

Optimum conditions for the fermentation of katuk leaves with kombucha culture were examined by analyzing the experimental data using Design-Expert 7 software. Based on Table 5, the range of response values for lipase inhibitory activity was 28.19 to 95.04%, DPPH scavenging activity at 88.78 to 96.33%, total polyphenol content of 0.727 to 1.27 mg GAE/mL, and total acid of 0.567 to 1.645%.

Afterward, the software automatically provided the optimum solution based on the existing criteria as shown in Table 6. Verification was further performed by comparing the results to the actual experiment. A total of three factors were used namely sucrose and kombucha culture concentrations, as well as fermentation time, with four responses observed. The desired criteria were to maximize the percentage of lipase inhibition, antioxidants, and polyphenols, as well as to minimize the percentage of total acid.

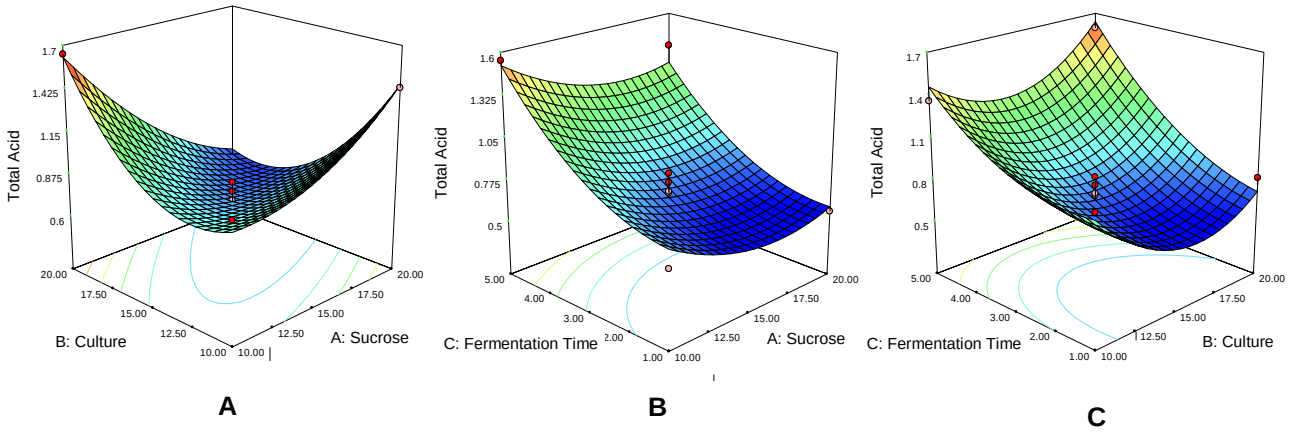


Figure 4. Profile of surface response 3-D plot for (A) the effect of sucrose and culture, (B) the effect of sucrose and fermentation time and (C) the effect of culture concentration and fermentation time, on total acid

Table 5. Response value at optimum condition

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Sucrose	is in range	10	20	1	1	3
Culture	is in range	10	20	1	1	3
Fermentation Time	is in range	1	5	1	1	3
Lipase inhibitor	maximize	28.1891	95.0372	1	1	5
DPPH	maximize	88.7848	96.3344	1	1	5
polyphenol	is in range	0.726942	1.26702	1	1	3
Total acid	is in range	0.5666	1.64452	1	1	3

The verification stage carried out along with the predictions of each response is exhibited in Table 7. The results of the actual response showed a value of 76.13% for lipase inhibition, 96.27% antioxidants DPPH, 0.97 mg GAE/mL polyphenols and 1.11% total acids. Based on the comparison of the results between verification and prediction data, the values obtained from the four responses satisfied 95% of the confident and prediction intervals respectively. Therefore, the proposed equation is considered reliable to determine the optimum process in achieving the desired criteria.

Sensory acceptability (Hedonic test)

The hedonic or preference test was carried out on 25 untrained panelists with parameters in the form of taste, aroma, and color. The scale used ranged from 1–5, namely 1= dislike, 2= dislike slightly, 3= neither like nor dislike, 4= like slightly, and 5= like. The results showed that the taste, aroma, and color of the drink as a whole had an average score of 4.12, 4.24, and 4.08, respectively, implying they are in the preferred range, compared to kombucha tea with values of 3.44, 2.88, and 3.32 which are in the neutral and less favorable range. According to Gunawan *et al.* (2021), taste and aroma are two organoleptic parameters that are interconnected and also influence each other.

Table 8. Sensory score of fermentation and brewing of katuk leaves compare with tea kombucha

Parameters	Hedonic Test	
	Brewed Katuk Kombucha	Tea Kombucha
Taste	4.12	3.44
Aroma	4.24	2.88
Color	4.08	3.32

CONCLUSIONS

The potential of fermented vegetables to produce a functional beverage product with high antioxidants and capable of inhibiting pancreatic lipase enzymes as antiobesity has been widely developed. Several studies have reported the prospective of katuk leaves as antioxidant and anti-obesity. RSM revealed that the optimal process to ferment brewed katuk leaves into a beneficial functional beverage was 10.43% sucrose, 10% kombucha culture concentration, and fermentation for 5 days. These conditions produced the best responses, namely 76.13% inhibition of lipase, 96.27% antioxidant DPPH, 0.97mg GAE/mL polyphenol, and 1.11% total acid.

Table 6. Optimum point solution from the selected criteria

Sucrose	Culture	Fermentation Time	Lipase inhibitor	DPPH	Polyphenol	Total Acid	Desirability	Criteria
10.43	**10.00**	**5.00**	**86.851**	**96.334**	**0.901**	**1.366**	**0.937**	**Selected**
10.37	10.07	5.00	86.320	96.334	0.899	1.363	0.933	
10.42	10.00	4.94	85.957	96.335	0.905	1.349	0.930	
10.32	10.13	5.00	85.939	96.334	0.899	1.362	0.929	
10.06	10.00	5.00	85.806	96.700	0.897	1.369	0.928	
10.00	10.00	4.97	85.154	96.745	0.899	1.359	0.923	
10.39	10.00	4.86	84.658	96.335	0.912	1.324	0.919	
12.34	10.00	5.00	92.140	94.774	0.920	1.378	0.871	
13.13	10.00	5.00	94.345	94.261	0.928	1.395	0.847	
20.00	10.00	4.18	100.23	93.321	1.057	1.644	0.775	
19.99	10.00	4.16	99.918	93.319	1.059	1.639	0.775	
10.00	13.70	5.00	68.873	94.516	0.875	1.420	0.680	
20.00	20.00	2.43	64.341	92.262	1.127	0.567	0.499	
20.00	19.84	2.47	64.325	92.203	1.126	0.566	0.495	
10.00	20.00	2.62	54.230	92.705	1.013	1.540	0.450	
10.00	20.00	2.59	54.392	92.681	1.015	1.534	0.450	
10.00	18.01	3.46	52.047	93.036	0.963	1.403	0.448	

Table 7. Prediction and verification Results of the responses in optimum conditions

Response	Prediction	SE Mean	95% CI low	95% CI high	SE Pred	95% PI low	95% PI high	Verification Result
Lipase inhibitor	86.8548	8.95	66.92	106.79	12.42	59.18	114.53	86.13
DPPH	96.3344	1.17	93.57	99.10	1.58	92.60	100.06	96,27
Polyphenols	0.900734	0.074	0.74	1.06	0.14	0.61	1.19	0,97
Total Acid	1.36584	0.12	1.08	1.65	0.16	0.98	1.75	1,11

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