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Optimization of Antioxidant Activity of Water-Soluble Fraction of Wader Pari Fish (*Rasbora Argyrotaenia*) Fermentation Using Response Surface Methodology

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A B S T R A C T

Rusip processed by fermentation can potentially contain bioactive peptides as antioxidants and antimicrobials. This study aims to optimize the bioactive content in producing rusip from wader pari fish with the *Response Surface Methodology* (RSM) method. There were 20 recommended formulas based on factors such as sugar concentration, salt concentration, and fermentation duration. The results showed that salt concentration, fermentation duration, and sugar concentration significantly affected antioxidant activity in rusip. The optimal formulation produced involves the addition of liquid palm sugar with a concentration of 11.22%, salt concentration of 15%, and fermentation duration of 8.9 days. Verification of activity response showed results with antioxidant activity of 56.78%.

1. INTRODUCTION

1.1. Research Background

Wader pari fish (*Rasbora lateristriata*) is one of the high sources of protein, fluorine, and Omega-3 and has a uniqueness and good taste so it has high economic value. Wader pari fish can be processed into various types of food, one of which is *rusip*. *Rusip* is a fermented product made from fish [1]. In general, *rusip* are made using anchovies, but some studies report making *rusip* from other basic ingredients such as shrimp [2], catfish [3], and petek fish [4]. *Rusip* has a characteristic light brown to dark gray color, sweet taste, sour and salty are peculiar [5]. The protein content is high, reaching 34.86% or 14.71 to 18.39 mg/mL [9].

Processing *rusip* begins with washing, adding salt and sugar, and incubating at room temperature with anaerobic conditions for 7 to 14 days [6]. The turnover of salt and sugar used ranges from 10-25% and 5-15% respectively [6]. In *rusip*, salt plays a role in absorbing water, which is helpful as a substrate for microbial growth, while palm sugar acts as a source of carbohydrates for microbial growth. Therefore, both significantly affect the type and growth of microbes during the fermentation process [7].

During fermentation, the microbes that grow and develop predominantly are lactic acid bacteria (BAL) [8]. BAL like *Streptococcus sp*, *Lactococcus sp*, and Leuconostoc sp grow naturally during incubation [9]. BAL is known to produce proteolytic enzymes that can break down proteins into peptides or amino acids. Rusip is reported to contain bioactive peptides with biological activities such as antioxidants [10], ACE (Angiotensin Converting Enzyme) inhibitors or antihypertensives [11], and anticholesterol.

Response Surface Methodology (RSM) is a set of mathematical models and statistical techniques that aims to model and analyze responses influenced by several factors [12]. RSM also aims to optimize response parameters influenced by controlled parameters [13]. Based on this, this study was conducted using the RSM approach to optimize the production of bioactive peptides with antioxidant activity from rice made from wader ray fish.

1.2. Literature Review

Rusip is a fermented product originating from Bangka Belitung, Indonesia. Rusip has a light brown to dark grey sensory characteristics a distinctive sweet, sour, and salty taste [1]. Rusip has a water content of 62.19%, salt 17%, fat 1.82%, total protein of 15.45%, total microbes 8.23-13.45 lof cfu/g, total mold 1.70-6.49 cfu/g and total lactic acid bacteria 7.62-10 log cfu/g [16]. Common rusip are made using anchovies, but some studies report making rusip from other basic ingredients such as shrimp [5], trash fish [2], catfish [3], and petek fish [4]. Another basic material that can be used in making rusip is wader pari fish. Wader pari fish is a freshwater fish with a high protein content ranging from 15-20% per 100 grams, omega-3 and vitamin B12, and important minerals such as selenium, phosphorus, magnesium, and zinc [18].

Waderfish is made with added sugar and salt, which is further fermented. Sugar acts as a source of carbohydrates, the main nutrients for microbial growth, while salt acts as a control agent that prevents the growth of pathogenic microbes [19]. Salt can create controlled conditions so that only salt-resistant (halophilic) microorganisms can live. BAL is an oba mice that grows and develops dominantly during Fermentation of rusip. BAL can produce proteolytic enzymes that break down proteins into peptides and amino acids [20]. Peptides are known to have biological activity called bioactive peptides. Bioactive peptides have physiological effects on the body including antioxidants, antimicrobials, antihypertensives, immunomodulators, and anticancer), as well as antidiabes [21]. The bioactivity of such peptides can be multifunctional and highly dependent on the molecular weight, composition, and sequence of the constituent amino acids [22].

Response Surface Methodology (RSM) is a set of mathematical models and statistical techniques that aim to create models and analyze responses influenced by several factors [12]. In general, RSM aims to optimize response parameters influenced by controlled parameters [23]. RSM aims to see and measure the relationship between variable interactions in a system with a quadratic effect that can provide an accurate surface picture [14]. In RSM analysis, fixed and changing variables are determined. Fixed variables are variables whose values do not change in each formula, while variable changes are factors that are assumed to influence the resulting response [15].

1.3. Research Objective

This study aims to optimize the fermentation process of Wader Pari Rusip by examining the effects of sugar, salt concentration, and fermentation duration on antioxidant activity using the Response Surface Methodology (RSM).

2. MATERIALS AND METHODS

2.1. Materials and Tools

The main ingredients for making *rusip* are wader pari fish obtained from Mojokerto, palm sugar, and salt. Materials for analysis are Lowry Reagent, Bovine Serum Albumin (BSA), aquades, PP indicator, NaOH 0.1 N, formaldehyde, CH_3COOH, perchloric acid 70%, K_2CO_3, HCl 37%, boric acid, filter paper, pH paper. PCA (*Plate Count Agar)* media, sterile physiological salt, MRSA (Methicillin-Resistant Staphylococcus aureus) media, SMA media (*skim milk agar)*. The tools used in this study include balance, pH meters, jars, spoons, *autoclaves*, incubators, desiccators, Petri dishes, Conway dishes, measuring cups, and other analyzers.

2.2. Design of Experiment and Analysis

This research uses a Response Surface Method (RSM) research design with a Central Composite Design using 3 factors: salt concentration, palm sugar concentration, and fermentation time. The 3-factor response surface method uses repetition at the midpoint (X=0) 6 times. The data obtained will be processed using the Microsoft Excel 2021 computer and analyzed using IBM SPSS 25. The data will be analyzed using the One-Way ANOVA and Duncan's advanced tests with a significant difference value ($P<0.05$).

2.3. Analytical methods

Data processing and analysis were carried out using Design Expert v13.0.0 software. Data were entered in a centralized composite design with three factors. Then, the response used includes antioxidant activity.

3. RESULT AND DISCUSSION

3.1. Optimization of Sugar Concentration, Salt Concentration and Fermentation Time

This study aims to optimize the making of wader pari fish rusip to produce bioactive peptides with antioxidant activity. Efforts to achieve this goal are carried out by controlling several parameters: sugar concentration, salt concentration, and fermentation time during production. The added sugar and salt concentration has great potential to influence the physicochemical properties of wader pari fish. The length of fermentation time affects the formation of bioactive compounds in wader pari fish *rusip*, such as bioactive peptides. Bioactive peptides are known to have high potential antioxidant activity. In this study, the main response was antioxidant activity, based on the potential of wader pari fish rusip to produce bioactive peptides in the form of antioxidants.

Table 1. Literature study

Types of Fish	Types of Sugar and Salt		Concentration		Fermentation Duration
	Sugar	Salt	Sugar	Salt	
Anchovies	Palm sugar	Not mentioned	10%	25%	14 Hours
Ruach fish	Liquid palm sugar	Not mentioned	5%, 10%, 15%	20%, 25%, 30%	7 Hours
Bilis Fish	Palm sugar	Wholesale salt	5%	7%	7 Hours
Petek Fish	Palm sugar	Not mentioned	0%	20%, 25%, 30%	14 Hours
Shrimp	Not mentio ned	Not mentioned	0%	25%	12 Hours
Wader Fish	Not mentio ned	Not mentioned	0%	30g	7 hari

Reference: [6], [9], [11], [4], [7], dan [18]

Table 1. shows literature on the fermentation of fish products, sugar concentration, salt concentration, type of sugar, type of salt, and the length of fermentation time. This is done to determine the

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middle point based on the results of previous studies that previous researchers have published. In this context, salt concentrations are set in the range of 15% to 25%, sugar additions are set at 5% to 15%, and fermentation time is in the range of 7 days to 21 days.

This study adjusted the number of treatment combinations provided by DX13 software to a predetermined midpoint. Before determining the number of combinations of factors, a midpoint must first be set to identify each factor's lower and upper bounds.

Table 2. Results of antioxidant activity by software Design Expert 13.0.0

Sample Code	Independent Variable: Concentration of Aren Sugar (%)	Independent Variable: Salt concentration (%)	Independent Variable: Long Fermentation (day)	Antioxidant Activity (%)
A	5.00	15.00	7.00	58.45
B	15.00	15.00	7.00	58.81
C	5.00	25.00	7.00	38.88
D	15.00	25.00	7.00	29.98
O	5.00	15.00	21.00	37.37
P	15.00	15.59	21.00	40.04
Q	5.00	25.00	21.00	27.76
R	15.00	25.00	21.00	48.75
E	2.00	20.00	14.00	42.53
F	18.00	20.00	14.00	57.65
G	10.00	12.00	14.00	34.52
H	10.00	28.00	14.00	39.50
S	10.00	20.00	2.00	25.62
T	10.00	20.00	26.00	59.88
I	10.00	20.00	14.00	50.53
J	10.00	20.00	14.00	50.80
K	10.00	20.00	14.00	55.52
L	10.00	20.00	14.00	54.36
M	10.00	20.00	14.00	54.00
N	10.00	20.00	14.00	50.27

The results of the data analysis (Table 1) are re-entered into the Design Expert 13.0.0 software to obtain optimal conditions, and then validation. The results obtained showed that the antioxidant activity in the T sample with the addition of sugar with a concentration of 10%, salt concentration of 20%, and a fermentation time of 26 days in determining the formula of the wader pari fish rusip product resulted in higher antioxidant activity of 59.88% when compared to sample S with the addition of sugar with a concentration of 10%, salt with a concentration of 20% and a fermentation time of 2 days. This is thought to be due to several main factors, the first factor is an increase in sugar concentration which provides more carbohydrate sources that can be used by microorganisms during fermentation, which can increase the production of antioxidant compounds such as lactic acid and phenolic compounds.

Increased antioxidant activity in fermentation has been associated with increased concentrations of organic acids. The second factor, the length of fermentation time, which is too short may not be enough to produce lactic acid bacteria that can produce proteolytic enzymes that can break down complex components in anchovies, such as proteins, into simpler components, such as peptides or amino acids [7]. Peptides are known to have various functional properties or bioactivity. Peptides with a wide range of biological activities are called bioactive peptides. Bioactive peptides have physiological effects on the body, including antioxidants and antimicrobials [8].

The addition of palm sugar that has been melted first will support the growth of lactic acid bacteria because palm sugar that undergoes a heating process breaks down into simple sugars so that it can be used as an energy source for lactic acid bacteria so that the higher the addition of palm sugar concentration, the total BAL will increase. Increased total BAL will affect pH. This is due to the activity of BAL in the fermentation of simple sugars into lactic acid. This is supported by [9] that lactic acid bacteria can convert carbohydrates contained in rusip raw materials, such as sugar into lactic acid. This process helps lower the pH, preventing pathogenic microorganisms' growth and unwanted spoilage.

The addition of sugar and salt in manufacturing rusip affects the production of bioactive peptides. Lactic acid bacteria, one of the important microorganisms in fermentation, utilize sugars as a carbon source for their growth and activity. Therefore, as the sugar concentration increases, lactic acid bacteria have more raw materials to produce bioactive peptides with antioxidant properties. In addition, salt concentration also plays a role in regulating the fermentation environment, which is suitable for the growth of lactic acid bacteria. Excessive salt concentration can inhibit the growth and activity of bacteria, disrupting peptide formation. Therefore, maintaining the right balance between sugar and salt concentrations becomes a significant factor in ensuring optimal production of antioxidant peptides in the rusip fermentation process [20]. The length of fermentation time in making rusip is important in producing bioactive peptides with antioxidant properties [6].

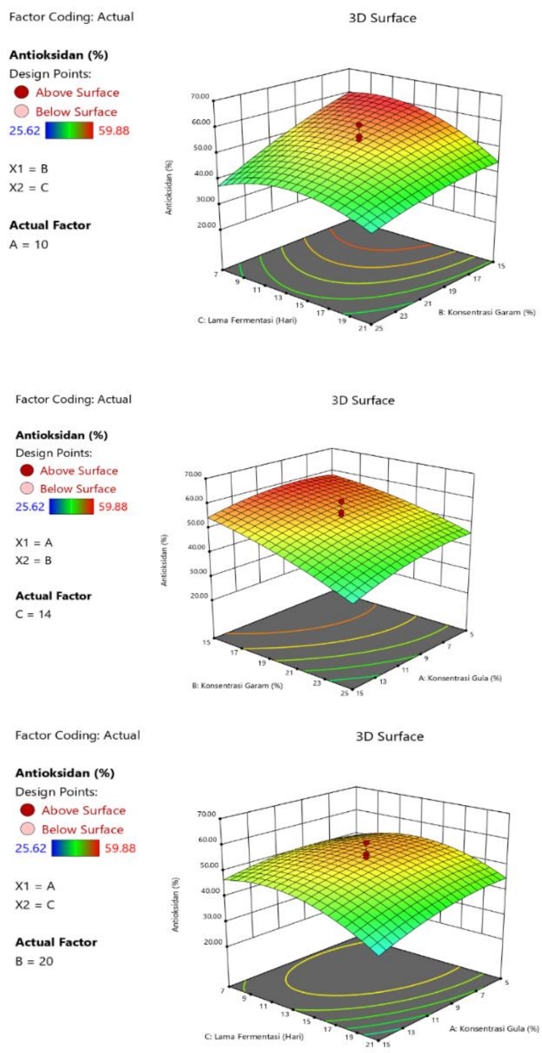

Figure 1. 3D graph- the surface of the relationship of the three factors to antioxidant activity response (a); the relationship of sugar and salt (b); relationship between fermentation duration and sugar cons (c); the relationship between fermentation duration and salt concentration

Based on Figure 1. shows that the x-axis and y-axis indicate the optimized variable. The lines within the plot contour are visual representations used to show the level of response in research. The outermost line shows the lowest response value while the deeper line shows the highest response value. The optimal response value is usually indicated by the presence of a flag or indicated by the presence of points (*nodes)* on the contours. The contour shows that the closer the area that has yellow to orange, the optimization value is said to be better, and if the response value is closer to the red area, the point or sign has a higher response value.

The surface response to sugar addition factors by concentration, salt, and length of fermentation time to the response of antioxidant activity variable concentrations of sugar, salt, and length of fermentation time. Based on the graph, the response of antioxidant activity for all three factors is significant.

3.2. Optimization Results Based on Software

Table 3. Optimum solution based on *Design Expert 13.0*

Parameter	Prediction Standards
Sugar Concentration (%)	11,22
Salt Concentration (%)	15
Duration of Fermentation (days)	8,9
Antioxidant (%)	56,78
Desirability	0,99
Information	*Selected*

The predictive results of the overall response to the rusip product produced follow quality standards or predetermined limits so that the optimal solution verified results from computing the data with limits based on these standards. The optimum point value is obtained from the results of possible deviations. The predictive results of the optimum and minimum solutions are presented in (Table 4).

Table 4. Prediction of optimum and minimum solutions from software

Parameter	Predictions	SE Predictions	Prediction Lower Limit	Upper Limit of Predictions
Antioxidant (%)	60.79	60.79	56.82	64.76

The lower and upper limits of the overall optimum value of the response calculated by the *software* are used as limitations of the verification results. If the experimental results are between these ranges, it can be said that the optimization results are appropriate and verified [6].

3.3. Verify the Optimum Condition of the Model Prediction Results With the Actual Model

The verification process involves retesting antioxidant activity following the formula suggested by the software and then comparing the data between the results of the software predictions and the verification results carried out in a row.

The difference between the verified antioxidant activity response value and the value produced by the *software* was 3.32%, with a precision level of 94.20%. The resulting difference does not exceed 5%, this indicates that the verification results on the antioxidant activity of wader pari fish rusip are acceptable and indicates that the model provided by the *software* is quite correct.

Table 5. Comparison data of actual verification results with predictions

	Sugar Concentration (%)	Salt Concentration (%)	Duration of fermentation (days)	Antioxidant activity
Predictions	11.23	15	8.9	60,79
Verification	11.23	15	8.9	56,78
The lower limit of predictions	-	-	-	56,82
The upper limit of predictions	-	-	-	64,76
Precision Level (%)				94. 20

4. CONCLUSION

The optimal formulation recommended by *software design expert 13.0.0* for wader pari fish rusip products was obtained by adding a sugar concentration of 11.22%, a salt concentration of 15%, and a fermentation duration of 8.9 days. The verification of the response of antioxidant activity was significant by 56.78%.

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