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Optimization of pulsed electric field processing time and hydrolyzed bovine collagen concentration in pasteurized milk

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KEYWORDS

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Optimization
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Viscosity

ABSTRACT

Milk is a highly perishable food due to its nutritional composition for microbial growth. Improper milk handling practices cause nutritional reduction and microbial contamination in milk. Collagen drinks are currently a growing commercial product. Therefore, this study aimed to determine the effect of hydrolyzed bovine collagen concentration and pulsed electric field (PEF) time on the physical, microbiological, and organoleptic qualities of milk enriched with hydrolyzed bovine collagen, as well as to determine the best treatment. Central composite design (CCD) for Response Surface Methodology (RSM) was used in this experimental design to explore optimal response based on the relationship between collagen concentration and PEF processing time. This CCD experiment was proposed to optimize TPC and viscosity and obtained a total of 13 experimental designs. The model results suggested by RSM-CCD are quadratic models. The result showed the optimization of the supplemented milk using a concentration of 2.837% hydrolyzed bovine collagen and PEF processing time of 116.369 seconds were the optimal designs with the desirability value of 0.809. Validation results using three repetitions produced an average TPC of 3.38 log CFU/mL and viscosity results of 4.56 mPas. Under these conditions, the error rate value of both responses is less than 5%, indicating that the model optimization can be accepted.

Introduction

According to the World Food and Agriculture Organization (FAO), 83% of commercial milk is dominated by cow's milk (FAO, 2021). Milk is perishable food, meaning this liquid is easily spoiled and may generate food poisoning associated with foodborne diseases. Thus, food processing is required to improve food safety and prolong the shelf-life time. Degradation of nutritional values in milk is caused by its suitability of milk composition for microbial growth medium. Food contamination can reduce in the quality of nutritional composition, physical properties, and organoleptic, influencing food safety consumers. Visual characteristics of damaged milk include sour smell, slimy, unstable emulsion (marked by two separate parts), and color changes.

Pasteurization is a method known in the milk processing industry to kill microorganisms using high temperatures (thermal). This thermal pasteurization causes damage to chemical components (such as proteins, vitamins, and minerals). Thus, non-thermal pasteurization technology is needed as it is safe and can maintain nutrients and other quality attributes (Putranto et al., 2014). Pulsed Electric Field (PEF) is a non-thermal technology that utilizes high-voltage electric pulses to create an electroporation process on cell membranes. Cell membranes damaged by this non-thermal process then become downstream processes that have been widely studied, such as the biorefinery process of bioactive compounds (e.g. phenols, anthocyanins, carotenoids) from food and non-food waste, drying processes, freezing processes, osmotic dehydration and inactivation of pathogenic microorganisms (Putranto et al., 2014; 2018; 2020; Izza et al., 2016; Dewi et al., 2019). Processing fresh milk using PEF with a voltage of

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15.9 – 26.2 kV/cm, previously preheated at 55°C for 24 seconds, can reduce the amount of contamination from 3.43 log CFU/mL to 2.82 – 2.12 log CFU/mL (Sharma et al., 2014a).

Milk processed by PEF has a weakness in organoleptic, which is unacceptable. The final milk product has a strong "fishy" aroma, possibly due to the absence of heat. Thus, there is no change in the chemical components of milk or the common language known as "ripe". Therefore, it is necessary to have some pretreatment to remove unwanted odors. A potential solution is pre-heating at the right time and within the temperature limit, aiming to achieve a ripe aroma without causing damage to the nutritional components. Research on various temperature treatments such as thermization (60°C for 10 seconds), pasteurization (65°C for 30 minutes), and sterilization (110°C for 10 minutes) had a significant effect on the sensory properties produced (Haq et al., 2014). PEF can also be combined with the initial heat before the electric shock is carried out; this process is called the initial temperature pre-heating. Research on the combination of PEF and variations in pre-heating temperature of 4 – 55°C showed effectiveness in reducing *P. aeruginosa*, *E. coli*, *S. aureus*, and *L. innocua* (Sharma et al., 2014b).

On the other hand, commercial dairy products are often added with several other components in the context of commercialization, diversification, and product development in a milk processing industry as an effort to exist. Some fortified components such as omega-3, prebiotics, probiotics, antioxidants, vitamins, and minerals that are not found in milk or replace and add nutrients to milk are marketing strategies for business expansion in the food industry. Therefore, the trend of collagen hydrolyzate-enriched drinks is currently being developed to have a tangible effect on the level of elasticity and moisture of the skin in humans if consumed by 2.5 - 5 g per day (Proksch et al., 2014).

The addition of collagen hydrolyzate can improve the functional properties of food products. Collagen hydrolyzate is a food ingredient that is high in peptide content. Peptides consumed regularly can increase the inhibitory activity of angiotensin-I converting enzyme (ACE) in the human body (Priyanto et al., 2021a). A previous study on non-thermal pasteurization of collagen-supplemented milk using PEF and pre-heating obtained a TPC value of 3.299 ± 0.003 log CFU/mL, viscosity of 4.48 ± 0.08 cP, pH of 6.6, and several parameters were better when pasteurized with a concentration of 2% bovine-collagen and a PEF time of 2 minutes (Priyanto et al., 2021b). In addition, a previous study reported that various hydrolyzed collagens as food ingredients led to enhance functional properties, such as water binding, surface active, and film-forming (Gómez-Guillén et al., 2011; Mohammad et al., 2014). The pH number of milk changes continuously over time. This phenomenon is due to the bacteria in milk transforming the lactose as sugar into lactic acid (Marouf and Elmhal, 2017). This pH number describes the quality of raw milk due to the population of microbes induces the acid formation. However, it is still unclear the optimal conditions for application at an industrial scale. Therefore, this research aimed to optimize the PEF time factor and collagen concentration on the TPC, pH value, and viscosity responses. Those parameters are required to identify the conditions of non-thermal pasteurized milk because TPC describes the microbial population, pH value as acid-forming in food, and viscosity as texture profile. It is hoped that the non-thermal technology pasteurization process can produce bovine collagen supplemented milk within a short time and with a low TPC value, as well as feasible for commercialization.

Research Methods

Material and Equipment

Fresh cow's milk was obtained from SW Dairy Farm, Sidoarjo, East Java, Indonesia. The raw materials in the milk processing were hydrolyzed bovine collagen (Halavet, Turkey) and granulated sugar (Gulaku, Indonesia). In addition, some analytical materials were plate count agar (Himedia M091, India), aquadest, methylene blue, phenolphthalein, 70% alcohol, 1% H2O2, cotton, plastic wrap, aluminum foil, and Whatman filter paper. The equipment for pasteurizing fresh cow's milk was a PEF machine with a double jacket system pasteurization vessel. The equipment used in the analysis were analytical balance (Metler Toledo, US), oven (Universal Oven UF55, Memmert GmbH + CmbH + Co. KG, Germany), micropipettes (Socorex Acura 825, Switzerland), autoclave (Hirayama HVE-85, Japan), vortex mixer (Gemmy VM-300, Taiwan), biosafety cabinet (Thermo Scientific, US), viscometer NDJ-8S rotor #1, plastic petri dish (OneMed, Indonesia), lactodensimeter, tasting booth, sensory evaluation questionnaire sheet, and some glassware (such as test tubes, funnels, glass beakers, and erlenmeyer).

Table 1. The central composite design

Name	Unit	Low	High	-alpha	+alpha
Collagen concentration	%	1	3	0.59	3.41
PEF Time	seconds	60	180	35.14	204.85

Non-thermal Pasteurization Process using PEF

The pasteurization process was conducted based on Priyanto et al. (2021a) study. First, the fresh cow's milk was poured into a pasteurization vessel. Sugar (3% (w/v)) and collagen (1 - 3% (w/v)) were added to fresh cow's milk before pasteurization. Then, all ingredients were mixed using a stirrer in a pasteurization vessel at 50 rpm. Prior to pasteurization using PEF, a pre-heating process was carried out at a temperature of 55°C with a holding time of 30 minutes. High-voltage electric pulses were given when the pre-heating time had been completed. The electric field strength was set up at 18 kV/cm with a frequency of 8 kHz and a pulse width of 69 µs. The PEF time was set at 60-180 seconds, following the optimization design (Table 1). The configuration of the electrodes and stirrer in the PEF treatment chamber adopts the scheme, as reported by Putranto et al. (2020).

Modelling and Optimization using Response Surface Method

The RSM optimization process was conducted using Design Expert 13 (DX13) and a Central Composite Design (CCD) because it uses two factors i.e., the bovine-collagen concentration and PEF time (see Table 1). The variation of concentration was based on previous research, as reported Priyanto et al. (2021a), with the best combination of bovine-collagen concentration of 2% and PEF time of 120 seconds. The best combination was taken as the middle value.

A common problem with the response surface method was the unknown relationship between the response variable and the independent variable. Therefore, the first step in the response surface method was to find the form of the relationship between the response and several independent variables through an appropriate approach. The form of a linear relationship was a form of relationship that was tried first because it was the simplest form of relationship (low-order polynomial). If the relationship between the response and the independent variable was a linear function, the function approach was called a first-order model, as shown in the following equation:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots\cdots + \beta_k x + \varepsilon_i \quad (1)$$

If the relationship was a quadratic, then a higher degree polynomial was used for the function approach, namely a second-order model such as the following equation:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i X_i^2 + \cdots + \sum_{i<j} \sum \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

Almost all problems in the response surface method use one or both of the above models. After obtaining the most suitable relationship, the next step was to optimize the relationship. If the most suitable surface was searched through a sufficient approximation, then the results of this analysis would be close to the actual function.

Model Validation

The validation process of the model formed was used to test the accuracy of the model. Validation was conducted by comparing the results of optimal variables (collagen concentration and PEF time) based on predicted values from RSM and actual or experimental study results. The level of difference or error rate from the experimental results with the predicted value of the program was a maximum of 5%. If the error rate was under 5%, then the optimum variable value suggested by the program (collagen concentration and PEF time) was valid according to the desired responses.

Results and Discussion

Modelling and Optimization

The observation data can be seen in Table 2. The lowest TPC results are shown in run 11 with the addition of 2% bovine-collagen and 120 seconds of PEF time, which produces a TPC value of 3.02 log CFU/mL. The highest viscosity shown in run 10 and 13 with the viscosity value of 4.48 mPas. In the RSM method with the CCD model, four statistical models that will be suggested to obtain the optimum response. These statistical models offered include the quadratic model, the linear model, the 2FI model, and the so-called two-factor interaction, and the cubic model. The selection of the model is intended to obtain a model based on the research design and the response results obtained to obtain optimal results.

Table 2. TPC, viscosity and pH result using RSM-CCD

Std	Actual Variables		Coded Variables		Responses	
	Collagen Concentration (%)	PEF Time (second)	X_1	X_2	TPC (log CFU/mL)	Viscosity (mPas)
1	1	60	-1.000	-1.000	3.77	3.52
2	1	180	1.000	-1.000	4.58	3.93
3	3	60	-1.000	1.000	3.26	4.04
4	3	180	1.000	1.000	3.69	4.04
5	2	35.147	-1.414	0.000	4.47	3.67
6	2	204.853	1.414	0.000	4.35	3.76
7	0.585	120	0.000	-1.414	4.59	3.15
8	3.414	120	0.000	1.414	3.78	4.70
9	2	120	0.000	0.000	3.45	4.46
10	2	120	0.000	0.000	3.74	4.48
11	2	120	0.000	0.000	3.02	4.17
12	2	120	0.000	0.000	3.65	4.08
13	2	120	0.000	0.000	3.79	4.48

Table 3. Fit summary of model selection from TPC response

Source	Sequential p-value	Lack of fit p-value	Adjusted R^2	Predicted R^2	
TPC response:					
Linear	0.1483	0.1678	0.1808	-0.1879	
2FI	0.6974	0.1350	0.1058	-0.3103	
Quadratic	**0.1186**	**0.2097**	**0.3747**	**-0.8685**	Suggested
Cubic	0.5133	0.0999	0.3296	-8.7175	Aliased
Viscosity response:					
Linear	0.0517	0.0704	0.3365	0.0443	
2FI	0.5933	0.0569	0.2870	-0.2858	
Quadratic	**0.0348**	**0.1673**	**0.6488**	**-0.0963**	Suggested
Cubic	0.0643	0.6717	0.8359	0.6818	Aliased

Based on all inputted data from Table 2, the selection of statistical models was carried out. This step was done based on the three categories such as the sum of the squares of the model sequence (Sequential Model Sum of Squares), model inaccuracy testing (Lack of Fit Tests), and statistical model summary (Model Summary Statistics). The three categories are summarized in the fit summary table for TPC and viscosity responses, as shown in Table 3.

Based on the TPC and viscosity responses (Table 3), the DX13 software suggests a quadratic model with a p-value of 11.86% and 3.48%, respectively. The parameter of the statistical model selection by testing the sum of the square model is the p-value of the selected model, which is the model with the smallest p-value (p-value<5%). If the p-value is less than 5%, this means the inaccuracy value is also less than 5% (Putranto, 2014). Although the p-value in the TPC responses was more than 5%, this value was also the lowest p-value compared with the other three models. Thus, the quadratic model was suggested.

The subsequent model evaluation was based on the model inaccuracy test (Lack of Fit Tests). The model would be considered suitable if the deviation test of the model is not accurate or not statistically significant at a certain level, in this case 5% or 0.05 is used. If the model has a p-value of more than 0.05, then the model has no significant effect and was considered suitable for the response. Based on Table 3, the quadratic model has the highest p-value for TPC response and viscosity of 20.97% and 16.73%, respectively. In addition, the following model evaluation method was based on the standard deviation value and R^2 value. The parameters used to select the best model were the highest adjusted R^2 and predicted R^2 (Hendrawan et al., 2020). Based on Table 3, it can be seen that the software suggests a quadratic model since the adjusted R^2 and predicted R^2 for both TPC and viscosity responses have the highest value compared with other models. Although in viscosity response, a cubic model has higher adjusted R^2 and predicted R^2 values than the quadratic model, the p-value of the cubic model is larger than the quadratic model.

Table 4. ANOVA analysis based on quadratic model of TPC and viscosity responses

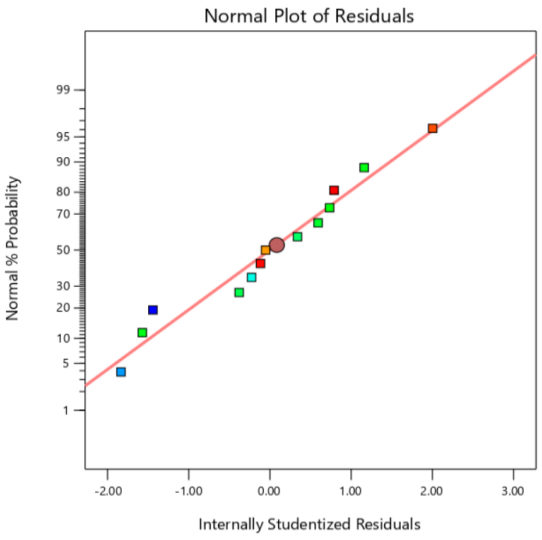
Source	Sum of Squares	df	Mean Square	F-value	p-value	
TPC response:						
Model	1.91	5	0.3817	2.44	0.1383	not significant
A- Collagen concentration	0.8100	1	0.8100	5.17	0.0571	
B- PEF time	0.1432	1	0.1432	0.9149	0.3707	
AB	0.0361	1	0.0361	0.2306	0.6457	
A^2	0.3050	1	0.3050	1.95	0.2054	
B^2	0.7207	1	0.7207	4.60	0.0690	
Residual	1.10	7	0.1565			
Lack of Fit	0.7030	3	0.2343	2.39	0.2097	not significant
Pure Error	0.3926	4	0.0982			
Cor Total	3.00	12				
Viscosity response:						
Model	1.83	5	0.3670	5.43	0.0233	significant
A- Collagen concentration	0.9955	1	0.9955	14.74	0.0064	
B- PEF time	0.0361	1	0.0361	0.5343	0.4885	
AB	0.0420	1	0.0420	0.6223	0.4561	
A^2	0.2482	1	0.2482	3.67	0.0968	
B^2	0.6008	1	0.6008	8.90	0.0204	
Residual	0.4727	7	0.0675			
Lack of Fit	0.3228	3	0.1076	2.87	0.1673	not significant
Pure Error	0.1499	4	0.0375			
Cor Total	2.31	12				

Therefore, based on overall analysis, a quadratic model was suggested as the most suitable model, while the cubic model was aliased from the DX13 software. The aliased mark indicates that the model was not recommended by the program. Therefore, it was not used to determine the response since there are some parameters are not in accordance with the design that uses two variables (Montgomery, 2001).

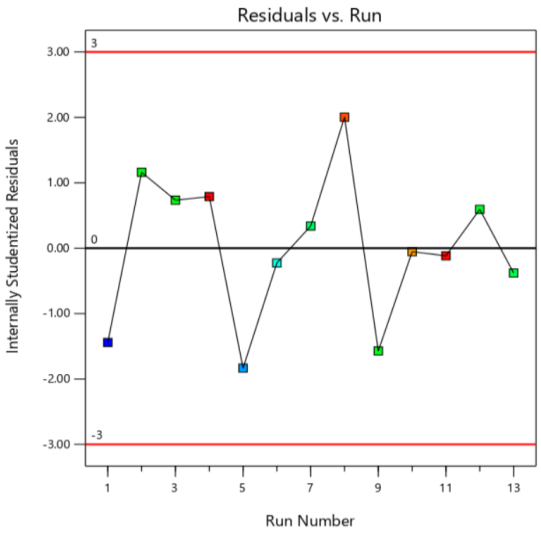
Based on the DX13 software, the quadratic model was appropriate and recommended. Furthermore, the quadratic model was analyzed using the analysis of variance (ANOVA). The ANOVA analysis aims to determine the effect of bovine-collagen concentration and PEF processing time on the TPC response and collagen-supplemented milk's viscosity. The suggested quadratic model is also analyzed to determine whether the model is significant or not to the study (Putranto, 2014). Analysis of the model using ANOVA on the TPC and viscosity responses can be seen in Table 4.

Based on Table 4 above, it can be seen that the quadratic model suggested by the software has an F-value for the TPC and viscosity responses of 2.44 and 5.43, respectively. For the TPC response, the model was not significant (F-value <5%). There was a 13.83% chance that this condition may possibly due to noise. For viscosity response, the F-value of 5.43 implies that the model was significant (F-value>5%) and there was a 2.33% chance that this could also occur due to noise. The p-values are less than 5%, thus the model was considered significant to the study. The trend was in contrast to the TPC response. However, the quadratic model was chosen because it was the most recommended and close to the desired responses.

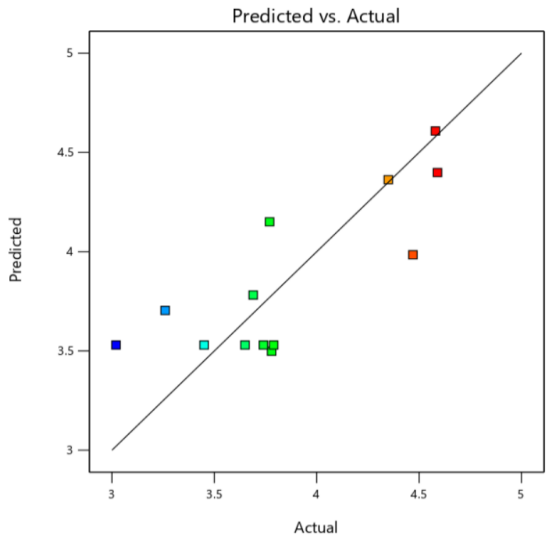
In addition, the factor of collagen concentration has a strong influence or has a significant effect on the viscosity response. It was indicated by the highest F-value of collagen concentration (14.74) and the p-value less than 0.05 or 0.0064. The smaller p-value was preferred, which suggested the suitability of corresponding variables. Particularly, the factors at a 95% confidence level ($p < 0.05$) indicates a strong influence on the response. Whereas, the factor of PEF time has no influence or insignificant effect on both TPC and viscosity responses. Table 4 shows that the lack of fit for TPC and viscosity responses was insignificant, suggesting that the model has a good agreement for the model fitting.



(a)

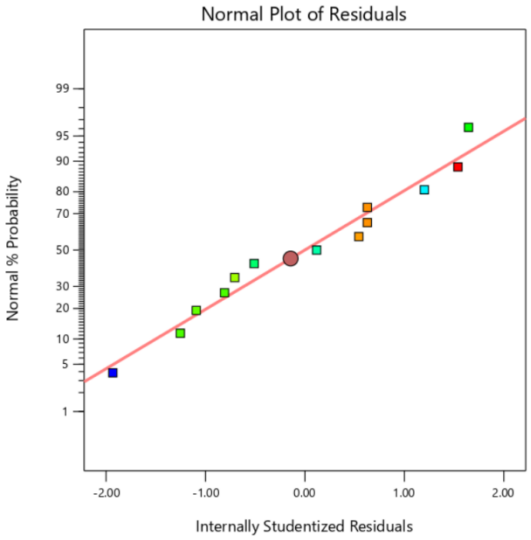


(b)

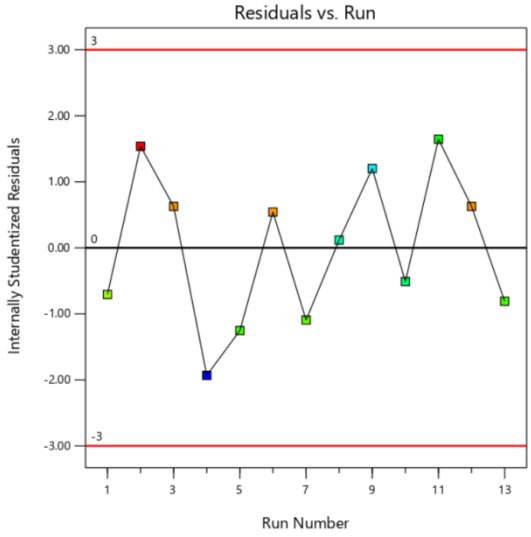


(c)

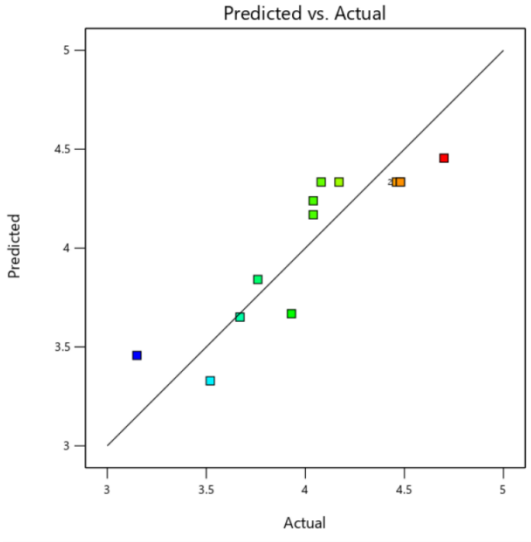
Figure 1. Normal probability plot of residual (a), residual plot (b) and predicted versus actual plot (c) of TPC response



(a)



(b)



(c)

Figure 2. Normal probability plot of residual (a), residual plot (b) and predicted versus actual plot (c) of viscosity response

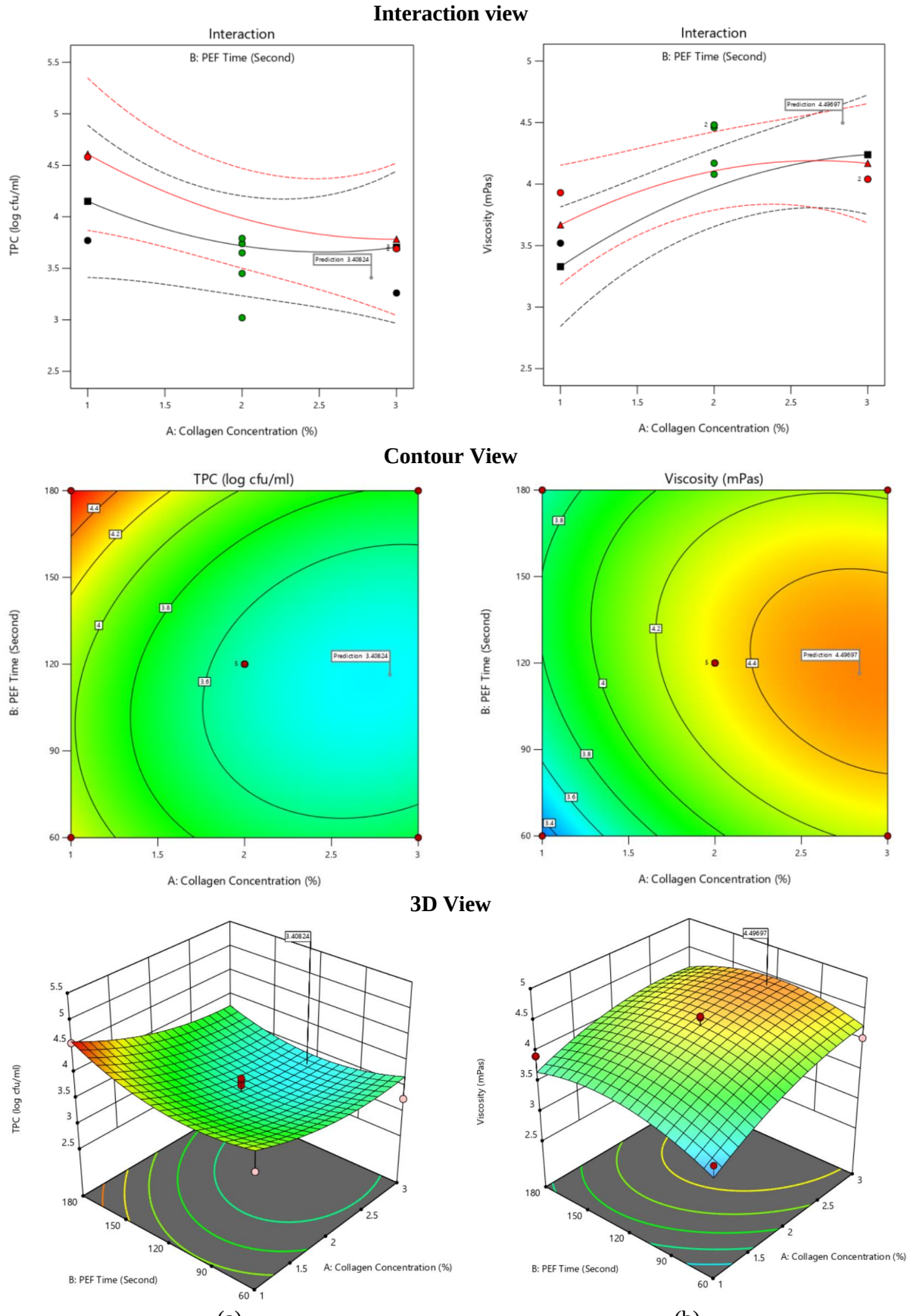


Figure 3. The optimal graphic based on several views (interaction, contour and 3D views) of TPC response (a) and viscosity response (b)

Table 5. Optimization constraint

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: Collagen Concentration	is in range	1	3	1	1	3
B: PEF Time	is in range	60	180	1	1	3
Total Plate Count	minimize	3.02	4.59	1	1	3
Viscosity	maximize	3.15	4.7	1	1	3

Table 6. Optimization solutions

Number	Collagen Concentration (%)	PEF Time (second)	TPC (log CFU/mL)	Viscosity (mPas)	Desirability
1	2.837	116.369	3.408	4.497	0.809

Moreover, for both the TPC and viscosity responses, there are three graphics include normal probability plot of residual, residual plot, and predicted versus actual plot (demonstrating the fitting data based on the selected model). Figure 1 and 2 show the data representative of TPC and viscosity responses, respectively. Figure 1(a) and 2(a) illustrate the normal probability plot of residuals where the clusters were closely arranged through the diagonal line. It was indicated that the error was normally distributed (Putranto, 2014). The internally studentized residuals value toward the input data and residual plot in Figure 1(b) dan 2(b) also supported the good fitting of the quadratic model. Based on the quadratic model, the accuracy level can be known by comparing the study's actual value with the model's predicted value. Based on Figures 1(c) and 2(c), the actual point is approaching the normal line (straight line), where according to Myers et al. (2016), if the actual data points are getting closer to the normal line, the data distribution is normal, the prediction results from the DX13 software will be in accordance with actual results.

The optimization using DX13 software with the collagen concentration and PEF time factor resulted in the desired optimization based on the limits for each variable following the desired criteria. The constraint's criteria for generating the optimal value can be seen in Table 5.

Based on the results obtained, the optimum point of TPC and viscosity of bovine-collagen supplemented milk was obtained when using collagen concentration of 2.837% and PEF time of 116.369 seconds. The optimum point of TPC and viscosity of bovine-collagen supplemented milk were 3.408 log CFU/mL and 4.497 mPas, respectively (Table 6). The desirability value generated is 0.809, which means that this study has a level of accuracy of 80.9%. The desirability value is used to determine the outcome of the solution. The desirability value of 1 indicates a perfect response, whereas the desirability value of 0 indicates that the response must be discarded (Putranto, 2014).

The modeling results obtained from RSM are polynomial equations in the form of coded variables. Model accuracy can be calculated from the comparison of the actual and predicted values. Based on ANOVA analysis, the collagen concentration and PEF time significantly affected on the response of TPC and viscosity of collagen supplemented milk which was marked by the outer lines on the graph, which shows the higher response value. The optimal response value was indicated by the peak point on the contour of the graph.

Figure 3 shows the interaction between factors, contour plot, and 3D views of the responses, indicating the influence of quadratic variables on the response of TPC and viscosity. The contour plot of the graph indicates that the concentrations of collagen and PEF time had a significant effect on the TPC and viscosity of collagen-supplemented milk.

The outermost line is blue, indicating the lowest response value, and inward indicates a higher response value. The response will decrease parallel to an increase in the collagen concentration and PEF time addition. Based on Figure 3, it can be illustrated that the minimum value of TPC response is predicted at the value of 3.40824 log CFU/mL, while the viscosity response predicted at 4.49697 mPas. The results were higher than Priyanto et al. (2021a) who study on a pre-heating process with PEF before non-thermal pasteurization. Their study showed that the bovine-collagen supplemented milk has TPC and viscosity of 3.498 ± 0.010 log CFU/mL and 4.49 ± 0.17 mPas. Furthermore, it suggested that a decrease in TPC of milk using PEF could be increased until it reaches less than 2 log cycles, as reported by several studies (Sharma et al., 2014a; Sharma et al., 2014b; Yulianingsih et al., 2021).

Table 7. Validation of response under optimal conditions

Responses	Model equations	Predicted value	Actual value (experimental)	Error rate value
TPC (log CFU/mL)	$Y = 3.53 – 0.12A + 0.13B – 0.09AB – 0.01A^2 + 0.40B^2$	3.408	3.38	0.82%
Viscosity (mPas)	$Y = 4.33 + 0.08A + 0.07B – 0.10AB + 0.02A^2 – 0.36B^2$	4.497	4.56	1.40%

Various options could be done to reduce the total microbes include increasing preheating temperature and PEF treatment time on pasteurized milk, yet this needs further investigated. Putranto et al. (2022) reported that by pre-heating for 10 minutes at a temperature of 70°C and continuing with the PEF process for 3.9 minutes, the optimum TPC on milk pasteurization decreased to 2,126 log CFU/mL. However, in this study, the lowest (optimum) TPC value is still acceptable since the maximum TPC value in pasteurized milk was 3.48 log CFU/mL (Miskiyah, 2011).

In addition, Priyanto et al. (2021a) reported that the results of sensory analysis of milk with the addition of 2% collagen had a good value and were well received by consumers from the parameters of taste, aroma, flavour, and color. In addition, the non-thermal pasteurization process with a short time of less than 2 minutes can also reduce energy costs. This is because energy requirements during the pretreatment process using PEF is an important parameter (Putranto et al., 2020). In this study, the optimal PEF time value (116 seconds), hence the process of making collagen-supplemented milk is strongly recommended using non-thermal pasteurization using PEF to produce products with good quality and low processing costs. This is in line with Donsi et al. (2010) study, that PEF was one of the promising non-thermal technologies to achieve several keys to success in the food industry, such as improving food quality, introducing new foods to the market, and optimizing the processing procedures while reducing energy costs.

Model Validation

The validation experiment was carried out by adding a bovine-collagen concentration of 2.837% and PEF time of 116.369 seconds with a desirability value of 0.809. The validation experiment was repeated five times, and the results showed a good agreement for all repetitions. Based on Table 7, the TPC and viscosity results from the experimental (actual value) were 3.38 log CFU/mL and 4.56 mPas, respectively. When the result was compared with the software calculation based on the model predictions, the deviation or error rate of TPC and viscosity responses were 0.82% and 1.40%, respectively. Since the error rate value was less than 5%, the model that RSM has obtained can be accepted and considered valid. The confidence level of the response value from the experimental results (actual value) with the predicted value was more than 95%, indicating that the independent variable value was quite appropriate to produce optimal responses (Putranto, 2014).

Conclusion

The results of this study indicated that milk enriched with bovine collagen hydrolyzate is optimal in the collagen-concentration of 2.837% and PEF time of 116.369 seconds with the desirability value of 0.809. The validation model showed the error rate value both of responses was less than 5% and model was accepted. The findings confirmed that the non-thermal technology pasteurization process could be potential and feasible to be applied at an industrial scale. Thus, the hydrolyzed bovine collagen supplemented milk can be produced within a short time with a low TPC value.

Declarations

Conflict of interests The authors declare no competing interests.

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