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# Optimization of Factors Affecting Glucuronic Acid Production in Yogurt Fermentation

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***Abstract:*** *Drinking yogurt fermentation with two bacteria strains Lactobacillus acidophilus and Gluconacetobacter nataicola was optimized to get maximal glucuronic acid concentration. A Blackett – Burman matrix was designed to screen the effect of seven factors to glucuronic acid concentration in yogurt. The design in Response surface methodology (RSM) with Central composite design (CCD) was applied to get maximum value of glucuronic acid concentration was 59.81mg/L in fermentation at 4.43 log CFU/mL of G. nataicola density, 5.1 log CFU/mL of L. acidophilus density, 9.96% sucrose, initial pH 5 and incubation time 32°C.*
***Keywords:*** *Glucuronic acid, Lactobacillus acidophilus, Gluconacetobacter nataicola, Plackett-Burman, RSM-CCD.*

## I. Introduction

Glucuronic acid ($C_6H_{10}O_7$) was a carbohydrate compound that condensed formula $HCO(CHOH)_4COOH$. It was formed by the oxidation of sixth carbon of glucose [1]. Glucuronic acid was found in *Glycyrrhiza* and some studies also mentioned that Kombucha contained glucuronic acid. It has been known as an anti-oxidation factor because it's combination with free radicals to form harmful component to enhance human immune system [2]. Additionally, glucuronic acid could be combined with fucose sulfate and manose sulfate in U-fucoidan which led to apotosis of cancer cells in gastrointestinal system.

Microbial synthesis of glucuronic acid has been concerned recently. In 2008, Khan et al enhanced the glucuronic acid production in tea fungus fermentation [3]. Yang et al (2010) proved that glucuronic acid concentration in Kombucha tea could be increased by combination of acetic acid bacteria and lactic acid bacteria. Optimization of glucuronic acid synthesis in Kombucha was mentioned in 2010 and 2011 by Yavari [5,6]. In 2014, Nguyen et al combined acetic acid bacteria and lactic acid bacteria in Kombucha for increasing glucuronic acid formation [7].

Study on glucuronic acid formation in the combination of lactic acid bacteria and acetic acid bacteria in yogurt is a new research that has supported for researches of bioactive components in probiotic yogurt fermentation. In this research, the screening with Plackett-Burman matrix design and the response surface methodology with central composite design were used to optimize affecting factors that influent to glucuronic acid production.

## II. Materials And Methods

### *2.1. Starters*

Microorganism used in this study was two identified strains: high probiotic activity *Lactobacillus acidophilus* and high capacity of glucuronic acid formation *Gluconacetobacter nataicola* that were 16S rDNA sequenced by Nam Khoa Biotek Company. The nucleotide sequencing was analyzed by free BLAST Search software.

### *2.2. Culture media*

In this research, sterilized fresh milk was used for culturing and fermenting. *L. acidophilus* was reserved in Man Rogosa Sharpe agar (MRS) and *G. nataicola* was reserved in Heschin-Schramm agar (HS) medium.

### *2.3. Experimental planning methods*

#### *2.3.1. Determination of factors affecting the glucuronic acid production*

Glucuronic acid production in yogurt fermentation was influenced by many factors. There were seven factors were selected including *G. nataicola* initial density, *L. acidophilus* initial density, sucrose concentration, fermentation temperature, initial pH, fermentation time, and shaking speed. The antecedent factors were properly selected for carrying out next experiments. Table I showed the ranges of each factor.

***Table I.*** *Experimental range of factors*

| **Factor** | Density of *G. nataicola* (log CFU/mL) | Density of *L. acidophilus* (log CFU/mL) | Sucrose (%) | Temperature (°C) | pH | Time (hours) | Shaking speed (rpm) |
|---|---|---|---|---|---|---|---|
| **Range** | 3 | 4 | 5 | 25 | 4 | 12 | 60 |
| | 4 | 5 | 7.5 | 30 | 4.5 | 24 | 90 |
| | 5 | 6 | 10 | 35 | 5 | 36 | 120 |
| | 6 | 7 | 12.5 | 40 | 5.5 | 48 | 150 |
| | | | 15 | 45 | 6 | | 180 |

***2.3.2. Plackett-Burman matrix design for screening factors affecting the glucuronic acid production***

Plackett-Burman matrix was designed base on the results of affecting factors that effected to glucuronic acid formation in yogurt fermentation in order to determine strong factors affecting glucuronic acid concentration and their influences [8].

In Plackett-Burman matrix, there were seven factors included *G. nataicola* initial density, *L. acidophilus* initial density, sucrose concentration, fermentation temperature, initial pH, fermentation time, and shaking speed. Base on experimental range of optimzed single factor in table I, these factors were studied with the highest (1) and the lowest level (-1), respectively. Important factors were examined base on 12 experimental design matrix (table II). Selected factors with p value lower than 0.05 were applied to response surface method with central composite design (RSM-CCD).

***2.3.3. Response surface method with central composite design (RSM-CCD)***

Factors with high statistical significance ($p < 0.05$) were selected by Plackett-Burman matrix has been carried out RSM-CCD for glucuronic acid concentration optimization. These selected factors were examined in five levels (-2, -1, 0, +1, +2) of CCD 28 experiments [9] (table III). Data was analyzed by Stagraphics Centurion XV.I. The most effective level of each factor for maximum glucuronic acid concentraion were determined base on the analyzation.

***2.4. Glucuronic acid quantified method***

Glucuronic acid concentration was determined by K-Uronic acid kits and measured absorption at 340nm by UV-Vis spectro 6000 spectrophotometer.

## III. Results And Discussion

***3.1. Optimization of single factors affecting glucuronic acid concentration***

Glucuronic acid was produced in the growth and development of *G. nataicola* in yogurt fermentation. This process was mainly affected by objective factors.

In this study, initial density of *G. nataicola* for fermentation media was firstly concerned because *G. nataicola* played the main role in the production of glucuronic acid. After 24 hours, highest glucuronic acid was 33.96 mg/L at 4 log CFU/mL of initial density. Increasing of density to 6 log CFU/mL, the concentration of acid dropped down to 29.84 mg/L. High population of *G. Nataicola* led to the decrease of glucuronic acid formation; because the bacteria competed for nutrition for growth and development. Therefore, suitable density of *G. nataicola* for fermentation was 4 log CFU/mL.

*L. acidophilus* played the main role in yogurt fermentation and also effectively stimulated glucuronic acid formation of *G. nataicola* [4]. *L. acidophilus* initial density for highest glucuronic acid was 5 log CFU/mL (34.33 mg/L); it was not significant with concentration of glucuronic acid at 6 log CFU/mL of *L. acidophilus* density (33.14 mg/L). The increasing of glucuronic acid followed *L. acidophilus* density had been mentioned by Yang (2010) in the rearch on the symbiosis of acetic acid bacteria and lactic acid bacteria in Kombucha [4]. The increase of glucuronic acid in the symbiosis of *L. acidophilus* and *G. nataicola* was importantly meaning to the biological activity enhancing in yogurt fermentation.

The effective level of each factor was found by measuring the maximal concentration of glucuronic acid by following step by step experimental factors. Glucuronic acid was 34.46 mg/L at 10% sucrose, highest in this experiment. This concentration was go up to 41.28 mg/L at 35°C and 44.14 mg/L at pH 5. Glucuronic acid concentration was double increase from 21.69 to 43.99mg/L in 12 and 24 hours. After 24 hours, the concentration remained around 44mg/L and was not significant in increasing. Jonas (1998) stated that efficient temperature of acetic acid bacteria was 30 - 35°C [14], and a study of Pederson (1995) also confirmed *Gluconacetobacter* can successfully develop at low pH. Therefore, this study once again confirmed

*Gluconacetobacter* fermentation condition [10]. Because after 24 hours the level of glucuronic acid had not significant, 24 hours was chosen as suitable time for high bio-activity yogurt fermentation. So, single affecting factors with 10% sucrose, pH 5, 35°C of incubation and 24 hours were selected for fementation.

Heath et al (2012) indicated that shaking speed affected cellulose formation of acetic acid bacteria [11]; also Khan et al (2008) stated there was a companion between cellulose formation and acid production of *Gluconacetobacter xylinus*, but there was not significant of acid concentration in different shaking speed for fermentation [3]. However, drinking yogurt would not be clumped with shaking speed at 120rpm was higher than other levels. Therefore, 120rpm was chosen for optimization.

Glucuronic acid formation was affected by experimental factors based on the level of glucuronic acid concentration after fermentation. As a result, these selected factors were used for screening by Blackett-Burman matrix and then optimization by RSM-CCD method (model) to find optimal conditions for glucuronic acid formation in yogurt fermentation.

### *3.2. Plackett-Burman design for sreening factors affecting glucuronic acid production*

Screening was a very important step in experimental planning when there were several factors affected to experimental samples. This process helped to determine real affecting factors and removed non or less affecting factors in order to simplified the study process.

The results of seven factors affecting the glucuronic acid was screenced by Plackett-Burman matrix based on glucuronic acid formation in fermentation were shown in table II. The results were analyzed variance (ANOVA) to determine affecting levels and p value (table III).

***Table II.*** *Plackett-Burman matrix in screening factors affecting glucuronic acid production*

| Expt | *G. nataicola* initial density (log CFU/mL) | *L. acidophilus* initial density (log CFU/mL) | Sucrose (%) | Temperature (°C) | pH | Time (hours) | Shaking speed (rpm) | Glucuronic acid concentration (mg/L) |
|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 15 | 45 | 6 | 12 | 180 | 7.79 |
| 2 | 6 | 3 | 15 | 25 | 4 | 12 | 180 | 37.42 |
| 3 | 2 | 7 | 15 | 25 | 6 | 12 | 60 | 20.53 |
| 4 | 6 | 7 | 5 | 45 | 6 | 12 | 180 | 17.6 |
| 5 | 6 | 3 | 15 | 45 | 4 | 36 | 60 | 30.62 |
| 6 | 6 | 7 | 15 | 25 | 6 | 36 | 60 | 36.85 |
| 7 | 2 | 3 | 5 | 25 | 4 | 12 | 60 | 24.87 |
| 8 | 2 | 7 | 5 | 25 | 4 | 36 | 180 | 29.11 |
| 9 | 6 | 3 | 5 | 25 | 6 | 36 | 180 | 17.42 |
| 10 | 2 | 7 | 15 | 45 | 4 | 36 | 180 | 30.15 |
| 11 | 2 | 3 | 5 | 45 | 6 | 36 | 60 | 6.28 |
| 12 | 6 | 7 | 5 | 45 | 4 | 12 | 60 | 36.19 |

***Table III.*** *The influence levels and p value of factors*

| Factor | Influence level | p value |
|---|---|---|
| *G. nataicola* initial density | 9.5617 | 0.0051 |
| *L. acidophilus* initial density | 7.6717 | 0.0112 |
| Sucrose | 5.315 | 0.0366 |
| Temperature | -6.262 | 0.0219 |
| pH | -13.65 | 0.0014 |
| Time | 1.005 | 0.5903 |
| Shaking speed | -2.642 | 0.1993 |

ANOVA results showed that seven factors affected to the glucuronic acid formation in yogurt fermentation. There were 5 factors had p value lower than 0.05 that really affected to the glucuronic acid production included *G. nataicola* initial density, *L. acidophilus* initial density, sucrose concentration, fermentation temperature, and initial pH. Therefore, these 5 factors were applied in RSM-CCD model to determine optimal points for maximal glucuronic acid.

There were 5 factors were selected to apply in RSM-CCD model included *G. nataicola* initial density, *L. acidophilus* initial density, sucrose concentration, fermentation temperature, and initial pH. In the optimization model, these were signed as $X_1$, $X_2$, $X_3$, $X_4$, $X_5$, respectively.

### *3.3. The RSM-CCD modeling for optimization of glucuronic acid production in yogurt fermentation*

RSM-CCD was used as design for modeling glucuronic acid formation in fermentation process. From this model, the optimal points for maximal glucuronic acid formation were pointed out. Glucuronic acid concentration when applied RSM-CCD model (table IV) was analyzed and evaluated affecting level and p value (table V) to determine regression equation.

***Table IV.*** *The RSM-CCD modeling in glucuronic acid optimization*

| Experiment | Factor | | | | | $Y_{axit\ glucuronic}$ (mg/L) Reality | $Y_{axit\ glucuronic}$ (mg/L) Model |
|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $X_5$ | | |
| 1 | 5 | 6 | 12.5 | 30 | 4.5 | 37.87 | 38.3756 |
| 2 | 4 | 5 | 10 | 25 | 5 | 35.02 | 36.9926 |
| 3 | 3 | 4 | 7.5 | 30 | 5.5 | 36.72 | 35.3364 |
| 4 | 4 | 5 | 10 | 35 | 6 | 10.57 | 15.3543 |
| 5 | 5 | 4 | 7.5 | 30 | 4.5 | 42.79 | 43.6714 |
| 6 | 5 | 4 | 12.5 | 30 | 5.5 | 40.85 | 38.5939 |
| 7 | 3 | 6 | 12.5 | 40 | 4.5 | 24.51 | 26.3456 |
| 8 | 5 | 4 | 7.5 | 40 | 5.5 | 19.07 | 18.5456 |
| 9 | 3 | 6 | 7.5 | 40 | 5.5 | 16.06 | 16.0322 |
| 10 | 3 | 4 | 7.5 | 40 | 4.5 | 21.32 | 23.5314 |
| 11 | 4 | 3 | 10 | 35 | 5 | 48.02 | 48.3259 |
| 12 | 4 | 7 | 10 | 35 | 5 | 48.08 | 47.3409 |
| 13 | 2 | 5 | 10 | 35 | 5 | 47.64 | 46.9526 |
| 14 | 3 | 6 | 7.5 | 30 | 4.5 | 30.15 | 31.5281 |
| 15 | 3 | 4 | 12.5 | 40 | 5.5 | 22.09 | 21.1639 |
| 16 | 3 | 4 | 12.5 | 30 | 4.5 | 30.43 | 30.9097 |
| 17 | 4 | 5 | 15 | 35 | 5 | 41.74 | 42.7976 |
| 18 | 5 | 6 | 7.5 | 30 | 5.5 | 42.25 | 40.8922 |
| 19 | 4 | 5 | 10 | 45 | 5 | 9.75 | 7.34427 |
| 20 | 5 | 4 | 12.5 | 40 | 4.5 | 27.19 | 28.5289 |
| 21 | 5 | 6 | 7.5 | 40 | 4.5 | 22.34 | 24.5772 |
| 22 | 6 | 5 | 10 | 35 | 5 | 54.28 | 54.5343 |
| 23 | 4 | 5 | 5 | 35 | 5 | 42.19 | 40.6993 |
| 24 | 4 | 5 | 10 | 35 | 4 | 25.15 | 19.9326 |
| 25 | 5 | 6 | 12.5 | 40 | 5.5 | 21.19 | 20.2897 |
| 26 | 3 | 6 | 12.5 | 30 | 5.5 | 40.06 | 38.3006 |
| 27 | 4 | 5 | 10 | 35 | 5 | 60.08 | 60.9816 |
| 28 | 4 | 5 | 10 | 35 | 5 | 59.45 | 60.9816 |

***Table V.*** *The influence levels and p value of factors*

| Factor | Influence level | p value | Factor | Affecting level | p value |
|---|---|---|---|---|---|
| $X_1$ | 3.79083 | 0.042 | $X_2$ $X_2$ | 6.19906 | 0.0065 |
| $X_2$ | -0.4925 | 0.7563 | $X_2$ $X_3$ | 1.52125 | 0.4425 |
| $X_3$ | 1.04917 | 0.5139 | $X_2$ $X_4$ | -0.63875 | 0.7426 |
| $X_4$ | -14.8242 | 0.0000 | $X_2$ $X_5$ | 0.96125 | 0.6229 |
| $X_5$ | -2.28917 | 0.1773 | $X_3$ $X_3$ | -9.24156 | 0.0007 |
| $X_1$ $X_1$ | -4.74406 | 0.0220 | $X_3$ $X_4$ | 2.36125 | 0.2469 |
| $X_1$ $X_2$ | -0.80875 | 0.6783 | $X_3$ $X_5$ | 0.83625 | 0.6681 |
| $X_1$ $X_3$ | -1.52375 | 0.4418 | $X_4$ $X_4$ | -19.0316 | 0.0000 |
| $X_1$ $X_4$ | -2.57375 | 0.2109 | $X_4$ $X_5$ | -4.44875 | 0.0489 |
| $X_1$ $X_5$ | -1.91875 | 0.3388 | $X_5$ $X_5$ | -21.2941 | 0.0000 |

ANOVA results indicated independent variables of regression equation in reality model included factors in table IV that had $p < 0.05$ (table V). Regression equation was $Y = -1777.9 + 44.5429X_1 + 30.0419X_3 - 2.37203X_1^2 + 3.09953X_2^2 - 0.73933X_3^2 - 0.38063X_4^2 - 0.88975X_4X_5 - 42.5881X_5^2$

Regression coefficient ($R^2$) was 0.981279 showed that 98.1279% experimental data fitted with expected data in model. According to Castillo (2007), $R^2 > 0.75$ had meaning that designed model fitted with reality [6]. So, there was a strong compatibility between studied factors and glucuronic acid formation, it indicated the accuracy of model and the exist of effective points.

The regression equation that showed all the factors are positively affected on the formation of glucuronic acid. The initial pH factor had the strongest influence level, and the results show that the lower the pH, the amount of glucuronic acid produced higher. High pH inhibited the biosynthesis of glucuronic acid in yogurt fermentation. This result is consistent with the conclusions of Hestrin (1954) about limitation in activity of acetic acid bacteria [12], and this result was again determined the study of Hwang et al (1999). In the study of cellulose acetic acid bacteria, Hwang et al found that this microbial strain grew effectively in low pH condition, the higher the pH the growth and development of them decreased.

Yoghurt fermentation temperature also had a huge impact on glucuronic acid production of *G. nataicola*. In the range of temperature from 25 to 45°C, when the yogurt fermentation temperature rose, the growth of bacteria was inhibited, and led to the decrease of glucuronic acid. This issue was also explained based on the conditions for microbial adaptation. Different microbial strains have different adaptation of temperature. Research the growth of *Gluconacetobacter* had shown that the proper temperatures for the growth of *Gluconacetobacter* were 12-35°C, while the optimal temperatures were 28-35°C [14]. *G. nataicola* was a bacteria of the genus *Gluconacetobacter*, so they fitted with this temperature range. Therefore, when temperatures rose too high (40-45°C), the growth and development of *G. nataicola* were inhibited, and they formed spores to protect themselves that led to the limitation of metabolic processes and glucuronic acid production was reduced.

During the fermentation process that enhance glucuronic acid production, *G. nataicola* was very important because it directly generated glucuronic acid. In this study, we found that when there were effects of other factors in the study, the concentration of acid produced depends greatly on the initial density of *G. nataicola*. As the density increased, the amount of glucuronic acid greatly generated. This was also been mentioned in the study of Khan (2008) [3], glucuronic acid content increased along with the increase of cellulose fibers of *Gluconacetobacter*. Earlier in 1992, Arie and colleagues found a correlation between the formation of cellulose and the density of acetic acid bacteria. Therefore, the results showed that initial density of *G. nataicola* had a positive impact on the formation of glucuronic acid in yogurt fermentation. However, if the density was too high, it led to nutritional competitiveness, also led to the limitation of glucuronic acid biosynthesis.

The regression equation also showed that the greater of *L. acidophilus* initial density, the higher of glucuronic acid production from *G. nataicola*. This result also coincided with experimental results of Yang et al in 2010 [4]. In research on the symbiosis of acetic acid bacteria and lactic acid bacteria in Kombucha, Yang found that the addition of lactic acid bacteria in the fermentation of acetic acid bacteria; greater glucuronic acid concentration was produced. Based on the growth and metabolic characteristics of *Gluconacetobacter* and lactic acid bacteria, the mechanism could be explained by the growth of lactic acid bacteria faster than the growth of *Gluconacetobacter*, so lactic acid bacteria fermented carbohydrates into simple carbon sources which *Gluconacetobacter* used as a nutrient source. Therefore, acetic acid bacteria did not need to use cellular enzymes to process glycolysis, they produced enzymes for pentose phosphate pathway; so, glucuronic acid greater generated. However, when applied a great number of density of *L. acidophilus*, led to the decrease of nutrient and nutrient competitiveness; also decreased the glucuronic acid biosynthesis of *G. nataicola*.

Sucrose concentration also affected the formation of glucuronic acid in yogurt fermentation because this is the nutrient source for microorganisms that involved in the fermentation were *L. acidophilus* and *G. nataicola*. Optimized results showed that when sucrose concentration rose up, the glucuronic acid concentration also increased, but until a certain limitation; the continued increase in nutrition could inhibit glucuronic acid generation. This was explained by the term about nutritional needs of microorganisms. Microorganisms require an adequate level of nutrition in the development process. So, when increasing the nutrient content that exceeds a certain limit, the enzyme activity is reduced due to substrate pressure on the biosynthesis of enzymes. Substrates increased while the maximum of enzymatical capacity remains constant, led to the decrease of glucuronic acid production.

Thus, the experimental results showed that all the examined factors in the optimization model had a strong influence on the formation of glucuronic acid in yogurt fermentation with varying degrees depending on each specific factor. Response surface plots (Figure 1a, 1b, 1c, 1d) showed the interaction of factorial pairs and the optimal value of each factor for maximum response function could be determined from the diagrams. The regression equation was found to reflect the degree of influence of each factor to glucuronic acid production. This confirmed the existence of the optimal point in the RSM-CCD modeling.

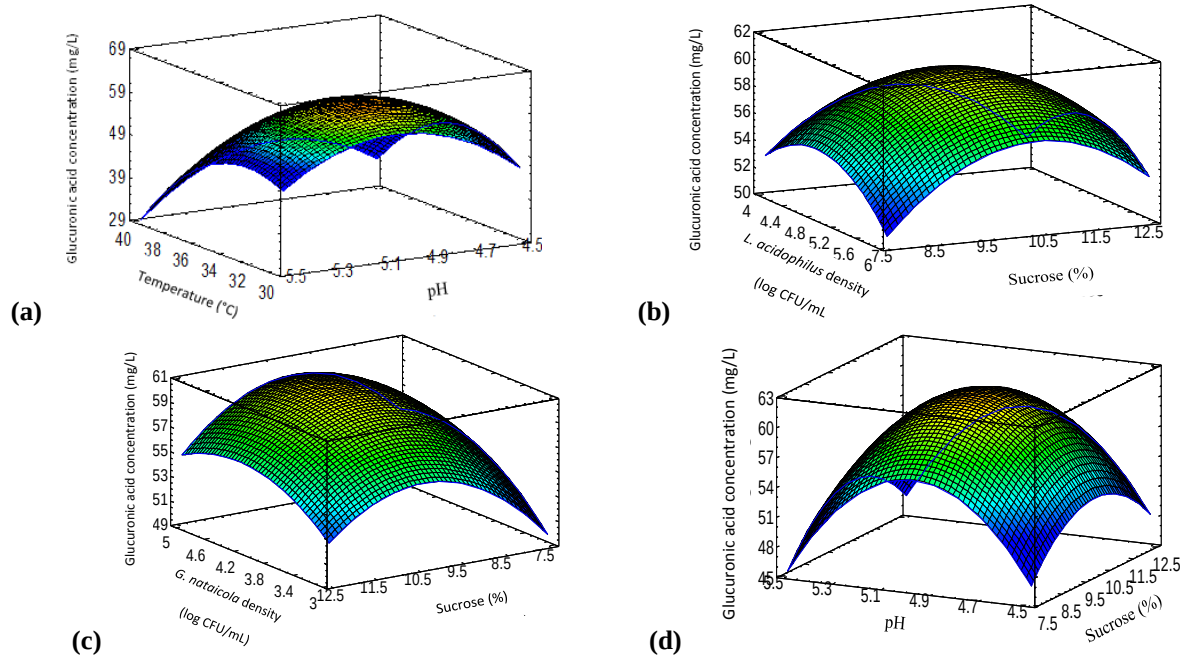


**Figure 1:** Response surface diagram of glucuronic acid concentration
(a) base on temperature and pH, (b) base on sucrose and *L. acidophilus* initial density, (c) base on sucrose and *G. nataicola* initial density, (d) base on sucrose and pH

From the response surface, optimal coordinates of factors were predicted for maximal glucuronic acid concentration was 62.2763 mg/L with an initial ensity of *G. nataicola* was 4.43 log CFU/mL. Initial density of *L. acidophilus* was 5.1 log CFU/mL, 9.96% sucrose, initial pH 5, and fermentation temperature was 32°C. Simulation the pattern at the optimal points, maximal 59.81mg/L glucuronic acid had obtained, reaching similar levels compared with the model was 96.04%. This result was higher than the result obtained by study about glucuronic acid of Yavari (2010) was about 32% [5], higher than glucuronic acid obtained when co-cultured lactic acid bacteria and acetic acid bacteria in Kombucha from 30-50% [7], and obtained 30% Vina results achieved after optimization of culture conditions was 178mg/L on Kombucha [1].

Therefore, the maximum concentration of glucuronic acid produced in the conditions had been optimized was 59.81mg/L with *G.nataicola* initial density was 4.43 log CFU/mL, the initial density of *L. acidophilus* was 5.1 log CFU/mL, 9.96% sucrose, pH 5, and fermentation temperature was 32°C. This results made yogurt product had one more biological activity of glucuronic acid beyond traditional probiotic activity in yogurt.

## IV. Conclusion

In optimization of condions for glucuronic acid production in yogurt fermentation, the Plackett-Burman matrix was used for screening factors affecting glucuronic acid formation and the response surface methodology with RSM-CCD was designed for modelling optimize value. As a result, maximal glucuronic acid concentration was 59.81mg/L. That was obtained with 4.43 log CFU/mL of *G. nataicola* initial density, 5.1 log CFU/mL of *L. acidophilus* density, 9.96% sucrose, pH 5, and fermentation temperature was 32°C. Traditional probiotic yogurt could incorporate a new biological activity of glucuronic acid by the presence of acetic acid bacteria (*G. nataicola*) combined with traditional probiotic lactic acid bacteria (*L. acidophilus*).

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