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OPTIMIZATION OF SKIM MILK BASED MEDIUM FOR BIOMASS PRODUCTION OF PROBIOTIC *LACTOBACILLUS ACIDOPHILUS* ATCC 4356 *USING* FACE CENTRAL COMPOSITE DESIGN-RESPONSE SURFACE METHODOLOGY APPROACH

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ABSTRACT

The use of commercial media to support probiotic bacterial growth is limited by its cost and off-flavour generated in food products. Thus, this study optimized skim milk and yeast extract concentration as a cultivation medium for optimal *Lactobacillus acidophilus* ATCC 4356 biomasses using FCCD-RSM. The statistical analysis showed that the optimal compositions of skim milk and yeast extract were at 9.46 g/L and 1.07 g/L, respectively by setting the goal of optimization for biomass production to be at maximum level and the factor concentration in selected range. Besides, pH profiling showed pH value declined from 4.262 to 4.061 during cultivation due to organic acid produced from carbohydrate metabolism at the predicted response in optimal conditions (0.979 OD600) generated by the RSM tool. However, the experimental and predicted results were not significant difference (P>0.05) with low error percentage. As conclusion, the optimized skim milk (10g/L) and yeast extract (1g/L) obtained from RSM model and experimental values can be used to support better biomass production for L. acidophilus and prepare the cells for further application.

Contribution/ Originality: This study provided easily prepared media using skim milk, which will enhance the growth of L. acidophilus ATCC 4356, in addition to the understanding the interaction for skim milk medium with bacterial growth in the process of cultivation L. acidophilus ATCC 4356 to maximum densities prior to further application in the food and pharmaceutical industries.

1. INTRODUCTION

It is well understood that the human digestive system has direct relationship and impact towards human health and their psychology as well. This is because human microbiota is made up with trillions of cells including different microorganisms. According to the Joint Food and Agriculture Organization and World Health Organization, FAO/WHO, Probiotics are described as selected viable microorganisms used as dietary supplements that have the potential to improve the health of humans or animals after ingestion. The gut is one of the most natural ecosystems for probiotics. Probiotics should be taken in a sufficient quantity to retain and regenerate the probiotic community within the human gut [1, 2]. Some lactobacilli are well known as beneficial bacteria for use as probiotics, and in the manufacture of some fermented milk products, they also have worldwide industrial use as starter cultures. Probiotic bacteria are belonging to Lactic acid bacteria are commercialized mainly as food supplements with dairy products

being the most frequently used [3, 4]. Probiotics are a good alternative to antibiotics that have been used in humans and animals ever since, and their efficacy has been largely concentrated in animals. Probiotics are viable single or mixed cultures of microorganisms that, when administered to animals or humans, beneficially affect the host by enhancing the qualities of the native bacteria [5, 6]. In the literature, *Lactobacillus* and *Bifidobacterium* are recognized probiotics bacteria used as food supplements. the consumption of probiotic has many health benefits such as, alleviation of lactose intolerance, antimicrobial activity, anti-carcinogenic properties, antidiarrheal properties, modulation of immune system, hypocholesterolemia and gastrointestinal diseases such as Crohn's disease and paucities [7-9].

Skim milk is a desirable medium to produce cell concentrates since the cells are adapted to the starting medium used in the dairy industry. For many microorganisms, milk containing casein, protein, fat, carbohydrates, minerals, and vitamins is a rather healthy means of development. A standard cow's milk composition consists of approximately 87.4% of water, 3.8% of fat, 4.7% of lactose, 3.3% of protein, 0.6% of minerals and 0.2% of citrate [10]. Skim milk is a potential medium to be used as a cultivation medium for probiotics. To date, the use of skim milk medium as the main medium remains scarce. This might be due to a lack of study on the optimization of the skim medium as a cultivation medium for probiotics [11]. In optimization of milk-based-medium for *L. acidophilus* growth, in order to promote lactose breakdown into more readily available sugar, it is important to consider the production of the enzyme by cells. In addition, to prevent nutrient wastage, the concomitant use of nitrogen sources is also necessary. To the best of our understanding, justification involving other results of growth activities of *L. acidophilus* bacteria in milk medium in order to make sure that the medium has been utilized proficiently is not available in the previous studies [12]. In recent years, probiotics have become one of the most used approaches in improving human health through the beneficial actions of various microorganisms such as Lactobacilli and Bifidobacterium, though *L. acidophilus* usually grows properly on commercial media, these media are unsuitable for large-scale production because of the chance of causing off-flavours in the food products and high cost of commercial media increase the manufacturing cost in large scale production [13].

Response surface methodology (RSM) has become increasingly favourable in optimizing compositions of microbiological media, parameters for food processes, and enzyme hydrolysis. A central composite design is one of the most commonly used designs for response surface optimization [14]. While rotatability is preferred characteristic in most central composite design. the design allows estimation of all the regression parameters required to fit a second-order model of given responses. Ratability character is the most preferred in any central composite design. This is because this characteristic provides constant variance of the estimated response corresponding to all new observation points that are at the same distance from the center point of the design (in terms of the coded variable) [15]. Therefore, the main objective of this study was to optimize skim milk-based medium using Response Surface Methodology for optimum biomass production of *L. acidophilus* ATCC 4356 and to verify the optimum skim milk-based medium for maximum biomass of *L. acidophilus* ATCC 4356.

2. METHODOLOGY

2.1. Microorganism and Inoculum Preparation

The bacteria cultures used in this study was *L. acidophilus* strain ATCC 4356 (American type culture collection, Manassas, USA). The strain was obtained from the Microbiology laboratory collection, Department of Biomolecular Science, University Technology MARA, Shah Alam. Stock culture of *L. acidophilus* ATCC 4356 was recultivated in de Man, Rogosa, and Sharp (MRS) broth. The medium used was incubated anaerobically in anaerobic jar (Merck, Darmstadt, German) at 37 ℃ for 24 hours. About 1mL overnight culture was further propagated by transferring into fresh prepared 9 mL MRS broth in universal bottle and further incubated using anaerobic jar at 37 ℃ for 24 hours. Then for enumeration of viable cells, samples were serially diluted in 0.1% (w/v) sterile peptone water (Merck, Darmstadt, Germany) and plated in duplicate onto MRS agar. Plates were incubated anaerobically at 37℃ for 24

hours. Anaerobic condition was achieved by placing the plates in anaerobic jars containing Anaerocult A (Merck, Darmstadt, Germany). All plates having 30 to 300 colonies were counted on a Quebec colony counter. Viability was expressed as log10 cfu mL^{-1}. Pure colony was then selected and recultivated for the preparation of bacterial stock solution and preservation in 50 % glycerol solution and stored at -80°C for further use.

2.2. Medium Preparation

The medium was prepared from two component namely skim milk and yeast extract with different concentrations using Face Central Composite Design- Response Surface Methodology (FCCD-RSM) in 250 mL SCHOTT DURAN bottle (Schott Duran, Mainz, Germany). All components were prepared in separate bottle and sterilized at 121∘C for 15 min then the different media kept to cooled at room temperature for further using.

2.3. Optimization using Face Central Composite Design- Response Surface Methodology (FCCD-RSM)

Optimization stage was employed using Face Central Composite Design-Response Surface Methodology (FCCD-RSM) to obtain the optimum concentration of the two significant factor affecting response. The final goal of the optimization was set, the independent factors concentration was chosen to be in the range and predicted optimal condition was generated by the software.

In this optimization, two significant factors were identified: Skim milk and Yeast extract with range from minimum 2 g/L (-1) to maximum 10 g/L (+1) and minimum 2 g/L (-1) to maximum 5 g/L (+1) respectively Table 1. Optimization step was performed with five replications at the center point (0) in duplicate samples to optimize the critical components and optical density growth. A FCCD-RSM containing 5 center runs, 4 factorials run, and 4 axial run was applied with *Lactobacillus acidophilus* ATCC 4356 biomass productions and Optical Density at 600 nm (OD_{600}) as response.

Table 1. Experimental range and levels of the independent variables used in the 2^3 full factorial design.

Independent variable	Unit	Range and Levels	
		-1	+1
Skim milk	g/L	2	10
Yeast extract	g/L	1	5

A quadratic model was generated as Equation 1:

$$Y_0 = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (1)$$

Here, x_1 and x_2 represent skim milk and yeast extract respectively and β_0, β_1, β_2, β_{11}, β_{22} and β_{12} are constant regression coefficients. Y_0 represents the response.

There were three coded factor levels: -1, 0, +1 in which −1 corresponds to the low level of each factor, +1 to the high level and 0 to the middle level. The coded value of each factor can be calculated using the Equation 2 as follows:

$$Coded\ value = \frac{\text{Actual level} - \left(\frac{High\ level + Low\ Level}{2}\right)}{\frac{High\ level - Low\ Level}{2}} \quad (2)$$

2.4. Verification Experiment

After optimization step, an experiment was done using suggested concentration of Skim milk and yeast extract in three replications to compare the experimental result and predicted result given by the RSM software in order to verify the adequacy of the predicted model.

2.5. Microbiological Analyses

The bacterial growth rate was determined by measuring the optical density (OD) of the medium at 600 nm immediately after sampling using spectrophotometer (SECOMAM, France). For the OD_{600} procedure, 0.5 ml of skim milk culture was mixed and incubated with 0.5 ml of 2 M borate-200 mM EDTA (pH 8.0) at 55°C for 10 min. The

cells were then harvested by centrifugation at 11000 rpm for 10 min and washed once with 1.0 ml of 2 M borate-200 mM EDTA (pH 8.0). The cell pellet was washed twice and resuspended with 1-ml samples of 100 mM bis-Tris buffer (pH 6.) The pH of the culture was evaluated using pH meter (Mettler-Toledo GmbH, Switzerland).

2.6. Statistical Analysis

The experimental results were analyzed using Minitab® 21.1 statistical package (MINITAB Inc, PA, USA) to perform data analysis, experimental design matrix and optimization procedure. The significant level was set at a value of P less than 0.05 to get high correlation and to fit the predicted model with the experimental data. P more than 0.05 indicate the replicated experimental data were not significant different which is close to the predicted data and low error percentage gave indication a good performance prognosis of the optimal formulation.

3. RESULTS

3.1. Optimization of Skim Milk and Yeast Extract Compositions in Basal Medium for Maximum L. acidophilus ATCC 4356 Biomass Production using Face Central Composite Design-Response Surface Methodology (FCCD-RSM)

The optimization experiments were carried out using a quadratic model consist of four axial runs and four factorial runs with five-time replication at the center point. The range of skim milk varies from 2 to 10 g/L and yeast extract from 1 to 5 g/L as shown in Table 2. It has been observed that the range biomass production was between 0.737 till 0.973 and pH was from 4.061 till 4.262 after 24 hours of cultivation under anaerobic at 37°C.

Table 2. Experimental design and results using face centered composite design response surface methodology (FCCD-RSM).

Run	Skim milk (g/L)	Yeast extract (g/L)	Biomass (OD600)	pH
1	2	1	0.745	4.061
2	2	3	0.737	4.101
3	2	5	0.756	4.120
4	6	1	0.931	4.221
5	6	3	0.911	4.243
6	6	3	0.897	4.233
7	6	3	0.913	4.262
8	6	3	0.914	4.254
9	6	3	0.916	4.212
10	6	5	0.920	4.183
11	10	1	0.973	4.112
12	10	3	0.968	4.175
13	10	5	0.937	4.137

The adequacy and fitness of the model choose were evaluated by ANOVA analysis (Analysis of Variance) as presented in Table 3. The regression equation coefficients were calculated Table and the data were fitted as polynomial Equation 3 as follows:

$$Y_0 = 0.912 + 0.107x_1 - 0.006x_2 - 0.065x_1^2 + 0.008x_2^2 - 0.012x_1x_2 \quad (3)$$

With Y_0 is the predicted response of bacteria growth optical density at 600 (OD_{600}) and x_1 is the coded value of the tested factor of skim milk and x_2 is the coded value of the tested factor of yeast extract. The F and P values determine the significant of each coefficient and indicate the interaction strength between two independent factors.

Table 3. ANOVA and regression analysis of optimization using FCCD-RSM.

Source	Sum of squares	Degree of freedom	Mean square	F value	P value
Model	0.0816	5	0.0163	183.70	< 0.0001
Lack of fit	0.0004	3	0.0001	2.26	0.2237
Pure error	0.0002	4	0.0001	-	-
Correlation total	0.0822	12	-	-	-
R^2	0.9924	-	-	-	-

The larger the magnitude of the F value and the smaller the P value, the more significant corresponding coefficient. It has been observed that the model was significant ($p<0.05$) as shown in Table 3. The R^2 obtained was 0.992 and lack of fit was 0.223 which showed that the data fitted the model well.

The response 3D surface model shown in Figure 1, was ploted to observed the interaction between skim milk and yeast extract in order to obtain high biomass production. It has been observed that adding of skim milk 2 g/L and yeast extract 3 g/L which is almost low concentration in basal medium had resulted lower cell biomass production (0.737 OD_{600}) at pH (4.101) after 24 hours of cultivation time. However, the increment of both important factors in the medium had resulted raise of cell biomass production. A combination of 10 g/L of skim milk and 1g/L of yeast extract the maximum bacterial growth of *L. acidophilus* ATCC 4356 (0.973 OD_{600}) at pH (4.112) was observed. However, the maximum biomass value obtained by increasing the concentration of skim milk and reducing the percentage of yeast extract in media. The responses criteria for the optimization process were maximum for *L. acidophilus* ATCC 4356 cell biomass production (OD_{600}).

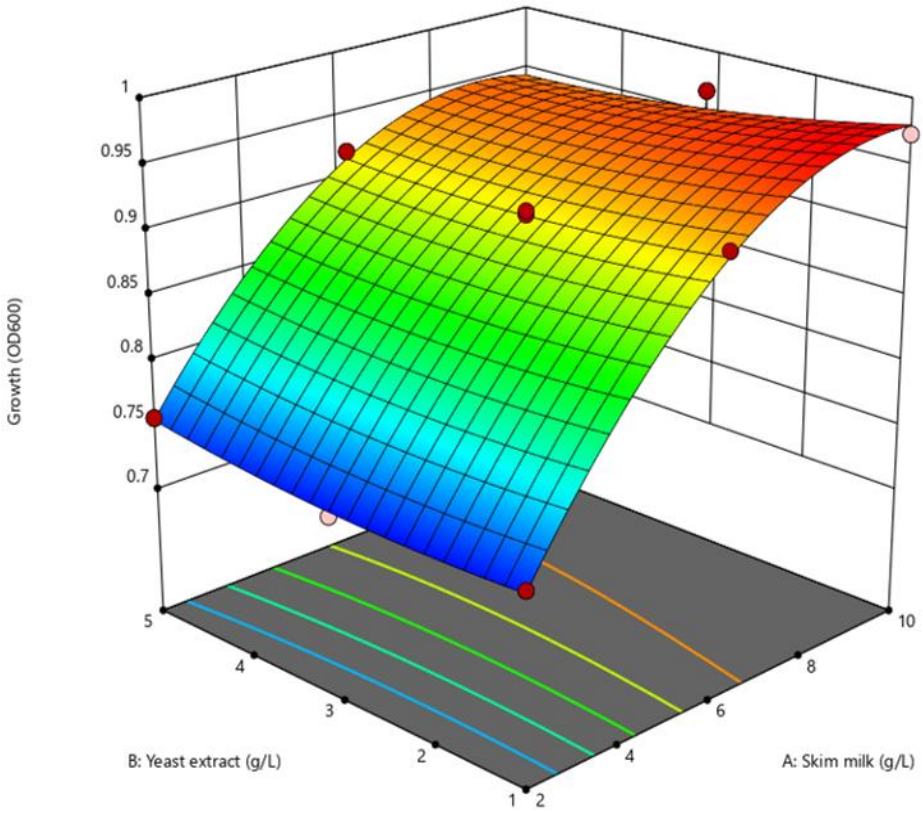

Figure 1. The 3D response surface plot shows the interaction of skim milk and yeast extract concentration on biomass production.

Table 4 presents the numerical value of skim milk and yeast's estimated coefficient, degree of freedom and F values which obtained from the 3D response surface plot (from Figure 1) at the optimum biomass yield condition.

Table 4. Regression analysis of optimization using FCCD-RSM with maximum biomass optical density at 600 nm (OD600) as the Response.

Factors	Coefficient estimate	Degree of freedom	F value	P value
Intercept	0.912	1	-	-
Skim milk	0.107	1	768.47	<0.0001
Yeast extract	-0.006	1	2.43	0.163
Skim milk^2	-0.065	1	131.22	<0.0001

3.2. pH Optimization of Basal Medium Containing Skim Milk and Yeast Extract for L. acidophilus ATCC 4356 using (FCCD-RSM)

The pH was observed during the experiment at the range of 4.061 to 4.262 as shown in Table 2. Anaerobic fermentation of lactic acid bacteria produces short-chain fatty acids (SCFA), that reduces the pH of the medium. The decline in the pH of the medium could indicate an active metabolism. Thus, the pH of the medium in the region of optimal growth was analyzed as a growth characteristic of *L. acidophilus* ATCC 4356 in skim milk. The appropriateness and suitability of the model for pH values were evaluated using ANOVA as stated Table 5.

Table 5. ANOVA and regression analysis of pH optimization using FCCD-RSM.

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value	Significant/ not significant
Model	0.0473	5	0.0095	15.81	0.0011	Significant
A-Skim milk	0.0028	1	0.0028	4.70	0.0667	-
B-Yeast extract	0.0003	1	0.0003	0.4454	0.5259	-
Skim milk and yeast extract	0.0004	1	0.0004	0.6681	0.4406	-
Skim milk^2	0.0271	1	0.0271	45.34	0.0003	-
Yeast extract^2	0.0032	1	0.0032	5.38	0.0535	-
Residual	0.0042	7	0.0006	-	-	-
Lack of Fit	0.0027	3	0.0009	2.44	0.2042	Not significant
Pure Error	0.0015	4	0.0004	-	-	-
Cor Total	0.0515	12	-	-	-	-

The regression equation coefficients were calculated, and the response surfaces of pH (Y_1) were generated based on the following Equation 4.

$$Y_1 = 4.24 + 0.0217x_1 + 0.0067x_2 - 0.01x_1^2 - 0.0991x_2^2 - 0.0341x_1x_2 \quad (4)$$

The Equation 4 is consisting of six parts. The first part is the constant value of intercept 4.24. increasing of X_1 (skim milk) and X_2 (yeast extract) in the second and third parts of the equation show the increment in the value of Y_1 response. On the other hand, the fourth, fifth and sixth parts of the equation will contribute the decrement in the value of Y_1 response.

As shown in Table 5, the F and P value of the combination of skim milk and yeast extract are 0.668 and 0.4406 respectively, which is not significant response to the pH. These results imply that the skim milk and yeast extract did not impact the pH value. Therefore, it could be concluded that the changes in the concentration of skim milk it does not make any huge change in pH.

The significance of each coefficient can be determined by the F and P value and the interaction strength between two independent factors can be indicated by it as well. The larger the magnitude of the F value and the smaller the P value, the more significant corresponding coefficient. It is stated in the obtained result that the model was around 0.0011which is significant ($p<0.05$) and lack of fit was 0.2042 which shows the data fitted the model well as shown in Table 5.

As shown in Figure 2, The 3D response surface plot for pH was attained to monitor the interaction and determine the optimum level of pH. It illustrates that by increasing skim milk to 6 g/L and yeast extract to 3 g/L, pH also increases to its highest value of 4.26. However, the experimental study achieved higher growth in the high concentration of skim milk despite the lower value pH. A low pH has been associated with inhibitory effects on microbial evolution due to inhibiting enzyme activities and cell functions. However, our results showed that growth was high in regions of low pH. *Lactobacilli* could produce >85% of the fermentation metabolites like lactic acid, with a small proportion as acetic acid [6]. The growth of *L. acidophilus* ATCC 4356 resulted in lactic acid, which was released into the medium. Higher production of SCFA indicates higher growth rates and a shorter period to attain maximal growth. The increasing of SCFA production causes a decrease in pH [16].

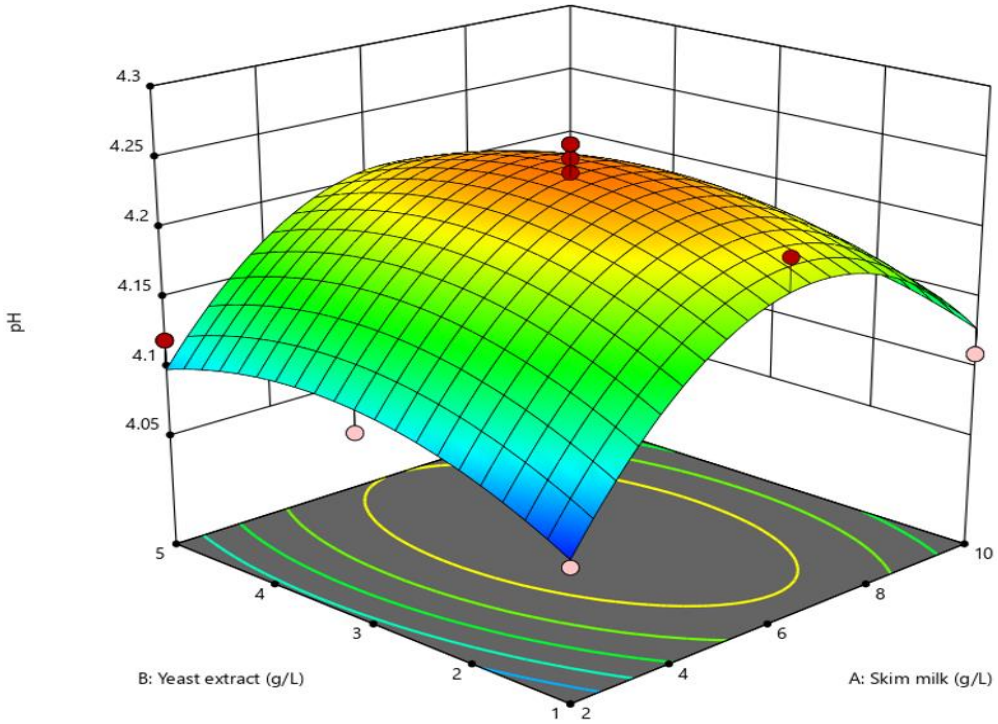

Figure 2. The 3D response surfaces for pH of *L. acidophilus* ATCC 4356 in skim milk and yeast extract medium.

The R^2 obtained was 0.918 as shown in Table 6 which indicates that the experimental result is near to the predicted data.

Table 6. Fit Statistics of pH optimization using FCCD-RSM.

Std. Dev.	0.0245	R^2	0.9187
Mean	4.18	Adjusted R^2	0.8606
C.V. %	0.5860	Predicted R^2	0.4253
-	-	Adeq Precision	10.3237

3.3. Verification of the Optimum Skim Milk and Yeast Extract in Basal Medium for Maximum Cultivation of L. acidophilus ATCC 4356

The optimum skim milk and yeast extract for the highest predicted *L. acidophilus* ATCC 4356 biomass production (0.979 OD_{600}) at predicted pH (4.153) was suggested as skim milk and yeast extract were at 9.463 g/L and 1.075 g/L, respectively. A further verification process was carried out to verify the adequacy of the predicted model. Based on the result obtained in Table 6, it has been observed that the use of optimum compositions skim milk and yeast extract in the basal medium had resulted in significant ($P<0.05$) *L. acidophilus* ATCC 4356 biomass production (0.971 OD_{600}) at pH 4.142 when it was compared to predicted data (0.979 OD_{600}) at pH 4.153 with 0.8% of error percentage between experiment and predicted data which indicates the accuracy of the agreement between a measured value and predicted value. The three replications of experimental data were done to check the accuracy of the experimental result and comparing with the predicted data. Based on Table 7, the result showed no significant difference between experimental and predicted data. Thus, the experimental data were close to the predicted one.

Table 7. Verification of experimental and predicted L. acidophilus ATCC 4356 biomass productions after using optimum skim milk and yeast extract composition in basal medium.

Source of result	Cell biomass production	pH
Experimental	0.971	4.142
Predicted	0.979	4.153
Error (%)	0.8%	-

As shown in Figure 1, the 3D response surface was plotted to evaluate the interaction of skim milk and yeast extract, subsequently locating the optimal level of each factor for maximal response. In detail, well-defined optimum factors were indicated by the convex response surface, which could be proved by the negative quadratic coefficient in Equation 3. From Table 2, the result shows that cell biomass production was considerably affected by varying skim milk and yeast extract concentrations used. *L. acidophilus* ATCC 4356 biomass production was decreased at the low range of skim milk and yeast extract compositions. Maximum biomass production was obtained at the high point of the response surface. The maximum biomass concentration was located at the high points, which was 0.973 (OD600) in skim milk and yeast extract medium with concentrations of 10 g/L and 1 g/L respectively. The value of determinations coefficient R2 for optimization was 0.992.

These indicated that 99.2% variability of *L. acidophilus* ATCC 4356 biomass productions (Y0) have a good relationship with different range of Skim milk and yeast extract concentrations used in basal medium. Based on ANOVA and regression analysis Table 3, the lack-of-fit value obtained was insignificant (0.223), which this indicate that the model was a good fit, and it is a good agreement between experimental and predicted values. From the optimization, the optimum concentration of skim milk and yeast extract were obtained at 10 g/L and 1g/L respectively. Based on previous study, the optimum concentration of lactose, yeast extract and calcium chloride medium for L. acidophilus has been reported 17.7g/L, 18.6 g/L and 0.9 g/L respectively [5]. Furthermore, another study has been conducted on skim milk based medium for *Bifidobacterium pseudocatenulatum* G4 which showed the optimum concentrations of the skim milk and yeast extract were 5.89% (w/v) and 2.31% (w/v), respectively [12]. *L. acidophilus* ATCC 4356 was reported as an acidic metabolite that produces lactic acid during fermentation. The reduction of the pH value was observed from pH 4.262 to pH 4.061 as a result of fermentation, which might be due to organic acids produced from the carbohydrate breakdown process by *L. acidophilus* ATCC 4356. In this study, the pH values were not much different in the skim milk-based medium incorporated with yeast extract. The decrease in pH (From 4.262 to 4.061) over time results from the breakdown of lactose to form lactic acid. Thus, the increasing acidity in the medium is attributed to the metabolism of the significant factors of the optimized medium, such as skim milk and yeast extract. The probiotic strain used in this study was observed to be remained to survive and continue to grow even though the medium pH throughout the cultivation process. The verification data obtained from Table 6 had revealed that the experiment results for *L. acidophilus* ATCC 4356 biomass production (0.971 OD_{600}) was an insignificant difference from the predicted data (0.979 OD_{600}), and the error between these two data was 0.8%. It has been reported that the minor error percentage between experiment and predicted data indicates the accuracy of the agreement between a measured value and an actual or predicted value [17].

4. DISCUSSION

4.1. Optimization Compositions in Basal Medium for Maximum L. acidophilus ATCC 4356 of Skim Milk and Yeast Extract Biomass Production using Face Central Composite Design-Response Surface Methodology (FCCD-RSM)

As shown in Figure 1, the 3D response surface was plotted to evaluate the interaction of skim milk and yeast extract, subsequently locating the optimal level of each factor for maximal response. In detail, well-defined optimum factors were indicated by the convex response surface, which could be proved by the negative quadratic coefficient in Equation 1. From Table, the result shows that cell biomass production was considerably affected by varying skim milk and yeast extract concentrations used. *L. acidophilus* ATCC 4356 biomass production was decreased at the low range of skim milk and yeast extract compositions. Maximum biomass production was obtained at the high point of the response surface. The maximum biomass concentration was located at the high points, which was 0.973 (OD_{600}) in skim milk and yeast extract medium with concentrations of 10 g/L and 1 g/L respectively.

The value of determinations coefficient R^2 for optimization was 0.992. These indicated that 99.2% variability of *L. acidophilus* ATCC 4356 biomass productions (Y_0) have a good relationship with different range of Skim milk and yeast extract concentrations used in basal medium. Based on ANOVA and regression analysis Table, the lack-of-fit

value obtained was insignificant (0.223), which this indicate that the model was a good fit, and it is a good agreement between experimental and predicted values. From the optimization, the optimum concentration of skim milk and yeast extract were obtained at 10 g/L and 1g/L respectively. Based on previous study, the optimum concentration of lactose, yeast extract and calcium chloride medium for *L. acidophilus* has been reported 17.7g/L, 18.6 g/L and 0.9 g/L respectively [5]. Furthermore, another study has been conducted on skim milk based medium for *Bifidobacterium pseudocatenulatum* G4 which showed the optimum concentrations of the skim milk and yeast extract were 5.89% (w/v) and 2.31% (w/v), respectively [12]. However, the used of skim milk with yeast extract combinations in basal medium for *Lactobacillus* strain cultivation has not been reported elsewhere. The optimization results were analyzed using statistical software and then the software gave the predicted data which was used to compare with the experimental result in the next step; validation step to obtain optimum concentration of the factors up to two decimal points. The modeling medium optimization for probiotic cultivation using Response Surface Methodology was recently shown by several previous studies end it has been proven effective and accurate [2, 18, 19].

4.2. Change in pH of Basal Medium Containing Skim Milk and Yeast Extract for L. acidophilus ATCC 4356 Using (FCCD-RSM)

The reduction of the pH value was observed from pH 4.262 to pH 4.061 as a result of fermentation, which might be due to organic acids produced from the carbohydrate breakdown process by L. acidophilus ATCC 4356. In this study, the pH values were not much different in the skim milk-based medium incorporated with yeast extract. The decrease in pH (From 4.262 to 4.061) over time results from the breakdown of lactose to form lactic acid. Thus, the increasing acidity in the medium is attributed to the metabolism of the significant factors of the optimized medium, such as skim milk and yeast extract. This scenario indicates that the L. acidophilus ATCC 4356 used in this study is able to tolerate in a low acidic environment, and this characteristic is one of the essential criteria for further application in foods industries. A previous study was reported that the threshold pH of skim milk containing 1% prebiotics P95 (an oligofructose), GR (inulin) and HP-Gel (inulin) and, the control skim milk with L. acidophilus (LA) (C-LA) and without LA (C-no LA) is 4.4 that shows that prebiotics had a more significant influence on the growth of L. acidophilus and a less pH lowering effect in a skim milk model system [20]. The combination of skim milk with prebiotics influences the overall favorable impact on the prebiotic. The activity and growth of pathogenic and probiotic bacteria are determined by the acidity of the gastrointestinal tract environment that affects human well-being.

4.3. Validation of the Experimental Design

The verification data obtained from Table had revealed that the experiment results for *L. acidophilus* ATCC 4356 biomass production (0.971 OD_{600}) was an insignificant difference from the predicted data (0.979 OD_{600}), and the error between these two data was 0.8%. It has been reported that the minor error percentage between experiment and predicted data indicates the accuracy of the agreement between a measured value and an actual or predicted value [17]. The good correlation between these results confirmed the validity of the predicted model, and the experimental data was proven to be adequate.

Several studies have been done to examine the potential of natural nutrition compounds' effects on probiotic bacterial growth. But many of them use MRS medium as probiotic cultivation medium supplemented with a studied natural source. MRS media is a selective medium for lactobacilli. Unlike this study, skim milk has been used as a medium for probiotic cultivation without being incorporated into commercial MRS medium. Earlier, Mustafa, et al. [12] revealed that skim milk medium in probiotic cultivation; *Bifidobacterium pseudocatenulatum* G4 resulting in high viable cell count; 1 x 10^7 (7.26 log10 CFU/mL) compared to MRS Media. In a recent study using skim milk, maltodextrin and Calcium Carbonate as a medium, the maximum biomass production was 1.945 x 10^{12} CFU/g, which shows significantly higher biomass production compared to the control group [21]. Even though the biomass rate from this study no significant difference ($P > 0.05$) compared to the study by Rohmatussolihat, et al. [21] but it was

proven that skim milk improved the selected probiotic growth as good as other reported natural food compounds for bacteria growth. From previous studies, these results show that the use of prebiotic substances either incorporated into commercial media or used alone is still able to improve the growth of many probiotic strains. Skim milk can act as a prebiotic source for probiotic bacteria, same as another natural prebiotic that has been reported.

5. CONCLUSION

Based on this research work, the highest biomass production was achieved when skim milk and yeast extract concentration at 10 g/L and 1 g/L, respectively. The cell production reached its maximum point when skim milk concentration was increased, and yeast extract remained at the lowest concentration. The result shows that a low concentration of both factors results in cell reduction. In the optimization step using Face Central Composite Design (FCCD-RSM), a maximum biomass cell production (0.971 OD_{600}) was achieved using the following optimized factors; 9.463 g/L of skim milk and 1.075 g/L yeast extract. In this study, the results showed that cell biomass production is dependent on both factors (skim milk and yeast extract), but mainly on skim milk.

The optimized media revealed that the number of cell biomass production is higher than the MRS medium, which is a selective medium for *Lactobacillus* species. Meanwhile, the pH results of this study showed that the reduction in pH value occurred after *L. acidophilus* ATCC 4356 cultivations due to lactic acid production from the fermentation of carbohydrates in skim milk medium with yeast extract supplementation. Data obtained from this study showed that the interaction between skim milk and yeast extract results as a good nutrition source for *L. acidophilus* ATCC 4356 in improving bacterial growth during the cultivation process. Thus, further applications of skim milk-based cultivation medium for probiotic growth can be made in order to replace commercial cultivation medium. Also, further exploration can be carried out on skim milk proximate analysis and compositions used in this study to understand more about the milk-based media.

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