Journal of Scientific & Industrial Research
Vol. 78, September 2019, pp. 608-614

Medium Optimization using Response Surface Methodology for High Cell Mass Production of *Lactobacillus acidophilus*

A N Kepli[1], D J Dailin[1,2], R A Malek[1], EA Elsayed[3,4], O M Leng[5] and H A El-Enshasy[1,2,6]*

[1]Institute of Bioproduct Development, Universiti Teknologi Malaysia, 81310, Skudai, Johor, Malaysia

[2]Department of Bioprocess and Polymer Engineering, School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310, Skudai, Johor, Malaysia

[3] Bioproducts Research Chair, Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia

[4]Chemistry of Natural and Microbial Products Dept., National Research Centre, Dokki, 12622 Cairo, Egypt

[5]Harita Go Green SdnBhd, Johor Bahru, Johor, Malaysia

[6]City of Scientific Research and Technology Applications (CSAT), New Burg Al Arab Alexandria, Egypt

Received 18 April 2018; revised 17 October 2018; accepted 14 May 2019

Lactobacillus acidophilus belongs to probiotic microflora inhabiting human gut that provide beneficially enhances human health. Besides balancing the intestinal flora and inhibiting pathogenic microorganisms, the existence of *L. acidophilus* inside the intestine can restore gut flora following antibiotics treatments. However, usually microorganisms from lactic acid bacteria group are known as fastidious microorganism and naturally required complex nutrients to promote their cellular growth. Therefore, twelve reported cultivation media were screened for their capability to support cell growth of *L. acidophilus*. The most suitable medium was further optimized using response surface methodology (RSM) and Box-Behnken design to maximize cell growth of *L.acidophilus*. Using this statistical approach, about 2.5-fold increase in maximal cell dry weight was achieved (5.14 g L^{-1}) compared to the original medium (2.05 g L^{-1}).This increase was accompanied by a significant increase in cell growth rates as well. The new medium formulation composed of (g L^{-1}): glucose, 50; yeast extract, 20.91; ammonium citrate, 3.42; citric acid, 0.5; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.4; $MnSO_4.7H_2O$, 0.05; sodium acetate, 1; tween 80, 1.

Keywords: *Lactobacillus acidophilus*, high cell mass production, medium optimization, Box-Behnken design

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. The gut is one of the popular habitats becauseit contains the biggest population of microbes and a huge number of beneficial bacteria, known as probiotics. In order to maintain and restore probiotic population inside the human gut, probiotics should be taken in an appropriate amount. According to the Joint Food and Agriculture Organization and World Health Organization, FAO/WHO, probiotics are living microorganisms that, when administered in adequate amounts, can confer a health benefit to the host[1]. Commonly used probiotics belong to the group of lactic acid bacteria (LAB). In human intestine, *Leuconostocsp.* and *Lactobacillus sp.* Are usually the dominating lactic acid bacteria[2]. However, beside their probiotic applications, LAB was also used for the production of many bioactive metabolites due to their GRAS (Generally Recognized as Safe) status[3]. Thus, *L. acidophilus* is one of the important species that can support human health. However, this species generally requires complex nutrient compositions to enhance their cell massproducrion[4]. Response surface methodology is a useful mathematical technique because it contains variety of statistical method[5]. This method was applied to determine optimal conditions from multivariable system by studying the effect of several factors influencing the response and by varying them simultaneously in a limited number of experiments[6,7]. Furthermore, it also can be used to define the relationship between the response and independent variables. Box-Behnken statistical design has been previously used for optimization of different cultivation parameters, including medium

Author for Correspondence
E-mail: henshasy@ibd.utm.my

optimization as well as cultivation conditions[8-11]. Therefore, the current work focused on maximizing cell growth of *L. acidophilus* using statistical medium optimization by Box-Behnken Design. Initially, the suitability of 12 different cultivation medium, cited from literature, for maximal cell mas production of *L. acidophilus* was investigated. Box-Behnken design approach was then applied to optimize three main components of the most suitable medium. At the end, cell growth kinetics of *L. acidophilus* were compared under both optimized and un-optimized medium compositions.

Materials and Methods

Microorganism

L. acidophilus strain DSMZ 20079 was obtained from DSMZ-German Collection of Microorganisms and Cell Culture (Braunschweig, Germany). The cells were grown in de Man Rogosa Sharpe (MRS agar) and selected colonies were preserved in 50% glycerol solution and storedat -80°C.

Cell propagation

In shake flask experiments, 2ml of 50% of sterile glycerol plus cell suspension was transferred into 50 ml of MRS broth. The inoculated broth was incubated for 24 hours and 37°C at 200rpm in shaking incubator (Innova 4230, New Brunswick Scientific Co., NJ, USA) at 37°C for 48 h[12].Under aseptic conditions, 5 mL (10% vv^{-1}) of cultivation broth was used to inoculate the production medium under the same cultivation conditions.

Production media and cultivation conditions

The most suitable medium that promotes high cell mass of *L. acidophilus* DSM 20079 was choseby screening 12 different cultivation media from previous study. Table 1 represents the composition of different cultivation media obtained from literature. The pH of all media was adjusted to 6.5 before sterilization and carbon source was autoclaved separately before being added to the medium directly before inoculation. Under shake flask cultivation, all media were incubated at temperature 37°C and 200 rpm for 48 h.

Table 1 — Composition (g L^{-1})of different cultivation media used for *L. acidophilus* DSM 20079

Component	Medium No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Ammonium citrate	-	-	2.00	2.00	2.00	-	-	-	2.00	-	-	1.00
$Cacl_2$	0.9	-	-	-	-	-	-	-	-	-	-	-
Citric acid	-	-	-	-	-	-	-	-	-	-	-	0.50
Fructose	-	42.62	-	-	-	-	-	-	-	-	-	-
Glucose	-	-	333	-	-	20.0	20.0	-	13.4	20.0	-	30.0
Inulin	-	-	-	-	-	-	-	0.039	-	-	-	-
K_2HPO_4	-	-	-	-	-	-	5.00	-	2.00	5.50	-	-
KH_2PO_4	-	-	2.00	2.00	2.00	-	-	-	-	-	-	1.50
Lactose	17.8	-	-	333	-	20.0	-	9.500	-	-	140	-
Meat extract	-	-	-	-	-	-	-	-	7.20	-	-	-
$MgSO.7H_2O$	-	-	10.0	10.0	10.0	-	-	-	-	-	0.20	0.40
$MnSO.7H_2O$	-	-	0.20	0.20	0.20	-	-	-	-	-	-	0.05
$MnSO.H_2O$	-	-	0.05	0.05	0.05	-	-	-	-	-	0.04	-
$MnSO_4$	-	-	-	-	-	0.05	-	-	-	-	-	-
Peptone	-	-	10.0	10.0	10.0	-	-	-	-	4.00	150	-
Sucrose	-	-	-	-	333	-	-	-	-	-	-	-
Sodium acetate	-	-	5.00	5.00	5.00	5.00	10.0	-	5.00	-	-	1.00
Sodium chloride	-	-	-	-	-	-	-	-	-	10.0	-	-
Sodium pyruvate	-	-	-	-	-	-	-	-	3.40	-	-	-
Tri-sodium citrate	-	-	-	-	-	-	10.0	-	-	-	-	-
Tryptone	-	-	-	-	-	20.0	-	-	-	-	-	-
Tween 80	-	-	1.00	1.00	1.00	0.001	-	-	-	-	-	1.00
Urea	-	39.92	-	-	-	-	-	-	-	-	-	-
Yeast extract	18.6	-	5.00	5.00	5.00	-	20.0	9.600	-	10.0	5.0	6.00
Reference	13	14	15	15	15	16	17	18	19	20	21	12

Box Behnken statistical medium optimization

Three-level, there-factorial design was selected to evaluate the significant effect of glucose, yeast extract and ammonium citrate. This statistical technique was carried out to study both individual and mutual interaction effects between different components in selected medium on *L. acidophilus* DSM 20079 cell mass. The three levels (low, − 1; middle, 0; high + 1) for each component were as follows (gL^{-1}): glucose: 10, 30, 50; yeast extract: 5, 17.5, 30; ammonium citrate: 1, 3 and 5, respectively. Box-Behnken design was generated using MINITAB 16 software (Minitab, Ltd., Coventry, UK), to optimize medium composition. 45 experiments were formulated (Table 2), and data sets generated were statistically analyzed using ANOVA to determine their validity.

Box-Behnken design depends on the 2[nd]order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j \quad \ldots (1)$$

where Y is the predicted cellular growth (g L^{-1}), x_i and x_j are the parameters (glucose, yeast extract and ammonium citrate; g/L), β_0 is the intercept term, andβ_i, β_{ii}, and β_{ij} are the linear, squared, and interaction coefficients, respectively[7]. The predicted responses obtained from the Box-Behnken design were compared with the actual responses to estimate the accuracy of the model.

Optical density and pH determination

The resulting cell growth was determined by measuring the optical density (OD) of the medium at 600 nm immediately after sampling using spectrophotometer (SPECTRONIC 200E, Thermo Fisher Scientific, MA, USA)[22]. The OD of the culture was converted to dry cell mass through a liner correlation standard curve, where 1 OD_{600} is almost equivalent to 0.3 g L^{-1}.The pH of the culture was evaluated using pH meter (Hannah pH meter HI 8424, Hanna Instrument, RI, USA).

Statistical analysis

Each experiment was repeated three times, and the results were presented as mean±SD. SPSS 9.0 was used to analyze obtained data using ANOVA for comparison between different treatments, where $p \leq 0.05$ reflects statistical significance.

Results and Discussion

Cultivation using different production media

Twelve different media previously reported in literature (Table 1) were evaluated for obtaining high cell growth of *L. acidophilus*. Based on the results obtained (Figure 1), it can be seen that all media screened supported cell growth of *L. acidophilus*, however in different levels. Growth

Table 2 — Box Behnken design for three medium components and the cell biomass response

Run	Glucose (g L^{-1})	Yeast Extract (g L^{-1})	Ammonium Citrate (g L^{-1})	CDW (g L^{-1})
1	10.0	5.00	3.0	3.090
2	50.0	5.00	3.0	3.505
3	10.0	30.0	3.0	3.080
4	50.0	30.0	3.0	5.295
5	10.0	17.5	1.0	4.435
6	50.0	17.5	1.0	4.215
7	10.0	17.5	5.0	3.110
8	50.0	17.5	5.0	4.840
9	30.0	5.00	1.0	2.300
10	30.0	30.0	1.0	2.775
11	30.0	5.00	5.0	2.760
12	30.0	30.0	5.0	1.260
13	30.0	17.5	3.0	5.145
14	30.0	17.5	3.0	5.175
15	30.0	17.5	3.0	5.205
16	10.0	5.00	3.0	3.070
17	50.0	5.00	3.0	3.510
18	10.0	30.0	3.0	3.100
19	50.0	30.0	3.0	5.355
20	10.0	17.5	1.0	4.425
21	50.0	17.5	1.0	4.245
22	10.0	17.5	5.0	3.000
23	50.0	17.5	5.0	4.840
24	30.0	5.00	1.0	2.300
25	30.0	30.0	1.0	2.765
26	30.0	5.00	5.0	2.710
27	30.0	30.0	5.0	1.250
28	30.0	17.5	3.0	5.210
29	30.0	17.5	3.0	5.160
30	30.0	17.5	3.0	5.145
31	10.0	5.00	3.0	3.065
32	50.0	5.00	3.0	3.505
33	10.0	30.0	3.0	3.120
34	50.0	30.0	3.0	5.340
35	10.0	17.5	1.0	4.410
36	50.0	17.5	1.0	4.240
37	10.0	17.5	5.0	3.100
38	50.0	17.5	5.0	4.845
39	30.0	5.00	1.0	2.290
40	30.0	30.0	1.0	2.575
41	30.0	5.00	5.0	2.840
42	30.0	30.0	5.0	1.270
43	30.0	17.5	3.0	5.330
44	30.0	17.5	3.0	5.040
45	30.0	17.5	3.0	5.040

medium No. 12 was found to be the most suitable medium supporting higher biomass production after 48 h, which reached a maximal of 2.46 g L^{-1}. This medium was composed of (gL^{-1}): glucose, 30; yeast extract, 6; ammonium citrate, 1; citric acid, 0.5; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.4; $MnSO_4.7H_2O$, 0.05; sodium acetate, 1; tween 80, 1. Medium No. 9 was second to medium No. 12, where 2.34 g L^{-1} cell mass were produced (about 95% of cell growth obtained in medium No. 12. Accordingly, this medium was selected for further optimization using statistical medium optimization approach.

Box-Behnken design

Box-Behnken design (BBD) is an independent, no embedded factorial with rotatable quadratic design or fractional factorial points, where the variables combination is at the midpoints of the edges of the variable space and at the center[5]. The obtained response allows the development of model by generating smooth curves from response. The three investigated factors, i.e. glucose, yeast extract and ammonium citrate, were used in Box-Behnken design to determine their optimal concentration favoring high cell mass production. Table 2 shows the different combinations of the three components and the response for cell dry weight. Results showed that the maximal cell dry weight response ranging from 5.3 to 5.36 g $L^{-1}$was obtained in runs 4, 19 and 34. Multiple regression analysis of the results (Table 3) showed that the investigated model is highly significant based on the obtained *p*-values for the tested components. Accordingly, the experimental results were fitted and explained with the second order polynomial equation:

Cell mass, X (g L^{-1}) = 1.71218 – 0.03889A + 0.25041B + 0.99737C – $0.00129A^2$ – $0.01046B^2$ – $0.31738C^2$+ 0.00503 AB + 0.03145 AC – 0.01918BC

Results for ANOVA analysis of variance for Box-Behnken design (Table 4) demonstrated that the lack-of-fit of the regression model did not show any significant difference, while Fischer's F-test showed a high significance ($p < 0.05$) for the regression. Furthermore, to evaluate the efficiency of the model, R^2coefficient was determined. A value of R^2 = 0.9739, adjusted to R^2= 0.9671, for the production of cell mass showed that the model was able to explain 97.39% of the overall variations with the exception of only 2.61%. Accordingly, the model can be considered highly significant. Figure 2A-C represents the response surface plots inter-correlating investigated components with cell mass response. Figure 2A represents correlations between different concentrations of glucose (10.0-50.0g L^{-1}) and yeast extract (5.0-30.0g L^{-1}) at constant ammonium citrate concentration (3.0 g L^{-1}). It can be seen that highest cell mass (6.1g L^{-1}) will be obtained at 50.0 and 20.9 g $L^{-1}$of glucose and yeast extract, respectively. Figure 2B shows the interaction between variable concentrations of yeast extract (5.0-30.0 g L^{-1}) and ammonium citrate (1.0-5.0 g L^{-1}) at constant glucose concentration (30.0 g L^{-1}). Maximal cell mass of 5.5 g L^{-1}will be obtained at 20.0 and 3.4 g $L^{-1}$of yeast extract and ammonium citrate, respectively. Finally, Figure 2C shows the effect of variable concentrations of glucose (10.0-30.0 g L^{-1}) on ammonium citrate (1.0-5.0 g L^{-1}) at constant yeast extract concentration (17.5 g L^{-1}). Maximal cell mass of 6.1g L^{-1}will be obtained upon using 50.0 and 3.4 g L^{-1} of glucose and

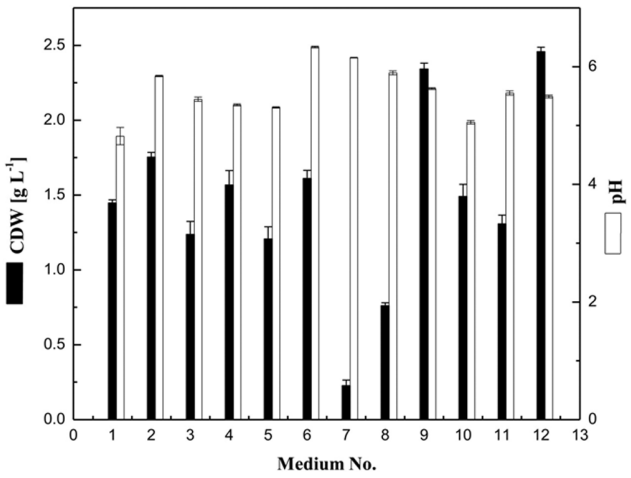

Fig.1 — Effect of different cultivation media on cell growth of *L. acidophilus.*

Table 3 — Estimated regression coefficients for cell mass production of *L. acidophilus* using Box-Behnken design

Terms	Coefficient	Standard Error	T value	*p* value
Constant	1.71218	0.490661	3.490	0.001
Glucose , A	-0.03889	0.017862	-2.177	0.036
Yeast extract, B	0.25041	0.027608	9.070	0.000
Ammonium citrate, C	0.99737	0.178624	5.584	0.000
A^2	-0.00129	0.000244	-5.287	0.000
B^2	-0.01046	0.000626	-16.707	0.000
C^2	-0.31738	0.024446	-12.983	0.000
AB	0.00503	0.000376	13.381	0.000
AC	0.03145	0.002349	13.390	0.000
BC	-0.01918	0.003758	-5.105	0.000

Table 4 — Analysis of variance (ANOVA) for cell mass production of *L. acidophilus* using Box-Behnken design

Sources	DF	Sequential SS	Adjusted SS	Adjusted MS	*F* value	*p* value
Regression	9	138.058	138.059	15.3399	144.83	0.00
Linear	3	52.1310	12.7410	4.24690	40.100	0.00
Square	3	45.2160	45.2160	15.0721	142.31	0.00
Interaction	3	40.7110	40.7110	13.5705	128.13	0.00
Residual Error	35	3.70700	3.70700	0.10590		
Lack-of-fit	3	3.60800	3.60800	1.20280	390.68	0.00
Pure Error	32	0.09900	0.09900	0.00310		
Total	44	141.766				

DF: Degree of freedom, SS: Sum of squares, MS: Mean sum of squares.

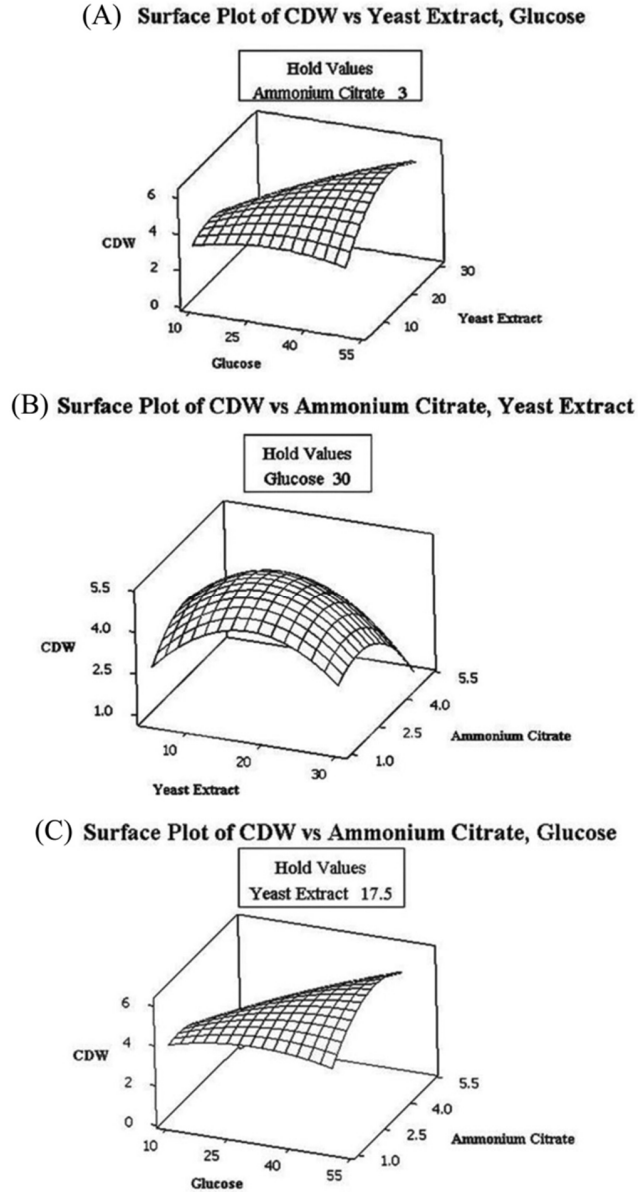

Fig. 2— Response surface 3-D plots of cell dry weight production of *L. acidophilus* showing the interaction between glucose, yeast extract and ammonium citrate.

ammonium citrate, respectively. Concluding, the optimum concentrations of glucose, yeast extract and ammonium citrate, to be applied, were found to be 50.0, 20.91, and 3.42 g L^{-1}, respectively. These optimal concentrations should afford the maximal production of 6.16 g $L^{-1}$of cell mass, according to the applied model. Glucose is widely known as carbon and energy and thus considered as the best nutrient to support biomass production. In LAB group, glucose is converted into lactic acid or acetic acid, depending on the cultivation conditions, strain used, and physicochemical conditions[23,24]. Beside glucose as a nutrient, yeast extract and ammonium citrate also produced significant impact ($p< 0.001$) towards *L. acidophilus* growth. Organic and inorganic nitrogen sources can promote cell growth of *L. acidophilus*. It has been reported that LAB usually requires complex nitrogen sources for better growth. Organic nitrogen sources provide growing cells with protein, free amino acids, peptides, fats, growth factors as well as vitamins, which support cell growth and different metabolic pathways[25,26]. In addition, yeast extract contains specific growth factors, which act as stimulators for cell growth and the production of secondary metabolites[27].

Cultivation of *L. acidophilus* under un-optimized and optimized medium composition

The final optimized medium composition was used to cultivate *A. acidophilus* cells in comparison to un-optimized medium composition. Cultivations were carried out for 48 hours and samples were taken for analysis every 3 hours. Results presented in Figure 3 shows that the newly optimized medium significantly improved cell mass production during cultivation. After inoculation, cells grew exponentially for the first 10 h in both cultivations. However, the cell growth rate under optimized conditions reached about 0.48 g $L^{-1}h^{-1}$, which is about 2.5-folds the cell growth

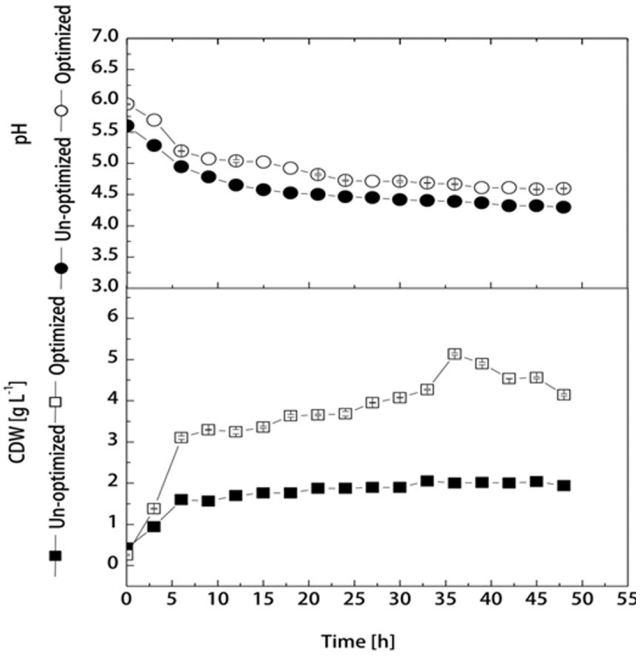

Fig. 3 — Growth kinetic and pH changes during cultivation of *L. acidophilus* using un-optimized and optimized medium

rate obtained under un-optimized medium cultivation (0.195 g $L^{-1}h^{-1}$). Such higher growth rate was reflected on the maximal cell mass obtained during this phase after 12 h, where cell mass concentration reached 1.7 and 3.25 g $L^{-1}$for un-optimized and optimized medium cultivations, respectively. After 12 h, cells growing under un-optimized medium continued to grow with lower growth rate (average 0.02 g $L^{-1}h^{-1}$), and reached a maximal cell mass of 2.05 g L^{-1} after 33 h. Afterwards, cell growth remained more or less constant till the end of cultivation. On the other hand, the mean average growth rate for cells growing under optimized medium was about 3.9-folds the corresponding average rate at un-optimized cultivation, where it reached 0.078 g $L^{-1}h^{-1}$. Accordingly, a maximal cell mass of 5.14 g $L^{-1}$was obtained at 36 h, after which, cell mass decreased and reached 4.14 g $L^{-1}$by the end of cultivation. This decrease can be attributed to the consumption of medium components and depletion of growth limiting components. The obtained optimized medium composition was evaluated for its efficiency in promoting maximal cell mass production by *L. acidophilus*. Results obtained (Figure 3) showed that the obtained results are in good agreement with the predicted model obtained from Box-Behnken design. Medium optimization significantly increased maximal cell mass obtained by about 150% than the maximal cell growth obtained using the initial un-optimized medium. Medium optimization through statistical approached has been previously applied for improving cultivation processes[7-11].

Conclusion

The present study aims at increasing cell mass production of *L. acidophilus* through statistical medium optimization approach. Firstly, twelve different growth media were surveyed for their suitability for cell mass production of *L. acidophilus* and the best suitable medium was chosen for further optimization. From Box-Behnken statistical design, the optimized concentrations for glucose, yeast extract and ammonium citrate were 50, 20.91 and 3.42 g L^{-1}, respectively. we found that the optimized medium composition consisted of (g L^{-1}): glucose, 50.0; increase cell mass from 2.05 to 5.14 g L^{-1}, from the initial medium. The second-order polynomial gave a satisfactory description of the experimental data. Interaction between major compositions like glucose, yeast extract, and ammonium citrate affecting cell growth gave significant effects (p<0.05) in the medium. Predicted and experimental results showed high agreement, which reflected the accuracy and applicability of RSM to optimized *L. acidophilus* production cultivations.

Acknowledgment

The authors would like to extend their sincere appreciation to Zamalah UTM, Harita Go Green SdnBhd, MOE, and UTM-RMC (Malaysia) through HICOE grant no. R.J130000.7846.4J262 for funding this reaseach project.

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