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Optimization For Improving Biomass And Antioxidant Activity From Lactobacillus Acidophilus

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ABSTRACT: *Lactobacillus acidophilus* is an important probiotic in many countries. The demand for biomass of this bacterium is huge. In recent years, the antioxidant activity of *Lactobacillus* is concerned and exploited. However, the low yield severely hindered advance study and application of itsantioxidant activity. In this study, we conducted fermentation experiment using *Lactobacillus acidophilus* in de Man-Rogosa-Sharpe. The glucose content in this environment is replaced by 6% sucrose solution. Optimal fermentation conditions were determined by experimental planning method and Box-Wilson method including pH 5.1, temperature at 36.6^{o}C andreaction time in 40hour. At this fermentation conditions, the biomass obtained was 11.589±0.09 log (CFU/ml) (6.4% increase). *Lactobacillus acidophilus* BK4 showed reducing power was 0.551±0.04 (37.8% increase) and 1,1-diphenyl 2-picrylhydrazyl (DPPH) scavenging activities, with inhibition rates of 53.63% (12.02% increase). The biomass and the yield of antioxidant activity from *Lactobacillus acidophilus* BK4 is significantly improved which satisfies the requirement for advance study and application.
KEYWORDS: Antioxidant activity, *Lactobacillus acidophilus*,Stress oxidative

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I. INTRODUCTION

Probiotics are living microorganisms which confer health benefit for the hostwhen administered in adequate amounts [1]. The majority of probiotics are lactic acid bacteria such as *L. acidophilus*, *L. casei*,... Because of their benefits for digestive system, immune support, cholesterol control, probiotic biomass has been researched and applied in many fields.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are activated forms of oxygen and nitrogen, respectively. ROS and RNS have been implicated in the aetiology of numerous pathological conditions such as diabetes mellitus, cardiovascular, gastrointestinal and neurodegenerative diseases. Oxidative stress happens when the oxidant/antioxidant ratio tilts in favour of oxidant factors, it is involved in the aging process and also causes inflammation [2]. Free radicals attack cellular components lead to the oxidation of lipids, proteins and DNA, thus causes structural and functional changes to these molecules [3, 4]. Antioxidant research has become a major scientific pursuit because of the evidence linking oxidative stress with many diseases and because of potential food preservative applications. *L. acidophilus*have also been shown to possess antioxidant activities. In in-vitro studies, *L. acidophilus*improved nutrition and efficacy of the food product [5-8]. In in-vivo studies, *L. acidophilus*enhancing intestinal immune function, increased antioxidant enzymes, reduced arthritis, and improved type 2 diabetes [5, 9-12].

The quality and productivity of the biomass production and the antioxidant activity of the bacteria depend on the conditions of the culture medium as well as on the cost of production. Therefore, studies have been conducted to improve the bacterial strain and culture environment, especially the use of cheap raw materials for the development of fermentation technology and the antioxidant activity of *L. acidophilus* BK4 [13].

The aim of the present study is to optimum fermentation conditions for maximum biomass and antioxidant activity by experimental planning method and Box-Wilson method [14]. *L. acidophilus*BK4 was incubated in de Man-Rogosa-Sharpe (MRS) with sucrose instead of glucose. This result will assess the antioxidant activity of *L. acidophilus*BK4 and provides potential biotechnology applications.One of the possible applications in food is yogurt and fruit juice which improved antioxidant activity.

II. MATERIALS AND METHODS

2.1 Materials

The *L. acidophilus*BK4 used in this study is derived from standard microorganisms in the Department of Biotechnology, Faculty of Chemical Engineering, Bach Khoa University.

The fermented environment MT01 isde Man-Rogosa-Sharpe (MRS) Broth.The fermented environment MT02, MT03 and MT04are similar to MT01, but substituted glucose with6%, 8% and 10%sucrose.All fermented environmentsused for propagation, breeding and surveying *L. acidophilus* BK4.

2.2 Methods

2.2.1 Determinationof sucrose concentration

The experiment was conducted to investigate the effect of sucrose concentration on bacterial biomass and antioxidant activity. The amount 5ml of *L. acidophilus* BK4 was incubated in 45ml MT01,MT02, MT03 and MT04at 37^{o}C for 24 hours. The optical density of the solution was measured at 610nm. The antioxidant activity of *L. acidophilus* BK4 was determinated after 24 hours.

2.2.2 Determination of antioxidant activity

2.2.2.1 Reducing power

Total reducing power was determined as described by [15] with some modifications.The amount 5ml of *L. acidophilus* BK4 was incubated in 45ml MT01,MT02, MT03 and MT04at 37^{o}C for 24 hours. The solution was then centrifuged 4000rpm for 20 minutes. The amount 2,5ml of the solution in 1ml of ethanol were mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]; the mixture was then incubated at 50ºC for 20 minutes. 2.5 ml of trichloroacetic acid (10%) was then added to the mixture, which was then centrifuged at 650rpm for 10 minutes. 2.5 ml of supernatant was mixed with 2.5ml of distilled water and 0.5ml $FeCl_3$ (0.1%), and the absorbance was measured at 700nm.MT01 within *L. acidophilus* BK4has been used as the control sample. Ascorbic acid sigma (µg/ml) has been used as a standard.

2.2.2.2 2,2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging activity of samples and standard reference compound, ascorbic acid was analyzed by the DPPH assay as described by [16] with minor modification.5ml of *L. acidophilus* BK4 incubated in 45ml MT01,MT02, MT03 and MT04at 37^{o}C for 24 hours. The solution was then centrifuged 13000rpm for 5 minutes. 40µl of the solution mixed with 460µl ethanol 95% and 1.5ml DPPH 0.8mM which was then incubated at 37^{o}C for 30 minutes in darken. Ascorbic acid sigma (µg/ml) has been used as a standard. The optical density of the solution was measured at 517nm using CT-2200 Spectroiphotometer. The DPPH radical scavenging activity was calculated from the absorption value by the following equation:

$$\text{Inhibition}(\%) = \left(\frac{OD_{Control} - OD_{Sample}}{OD_{Control}}\right) x100 \quad (1)$$

where $OD_{Control}$ is the absorbance of the control sample (MT01 within *L. acidophilus*) and OD_{Sample} is the absorbance of test sample.

2.2.3 Optimization ofbiomass and the yield of antioxidant activity

The experimental planning methodology and the Box-Wilson methodology was employed to determin the effect of 3 reaction parameters (pH, temperature, reaction time) on biomass and the yield of antioxidant activity from *L. acidophilus*BK4. Procedures for the construction of the 2^3 orthogonal design matrix, for the mathematical statistical treatments, and for the determination of optimal conditions followed instructions described by Pham Hong Hai (2007) [14]. Regression validation was performed using the Student t-test and the Fisher F-test.

2.3 Statistical analysis

All the data have been expressed as mean ± Standard deviation (SD) for n=3. Statistical significance of the data was determined by Student's t-test. The probability of occurrence was selected at p-value ≤0.05.

III. RESULTS AND DISCUSSION

3.1 Determination of sucrose concentration

After 60 hour, the growth curve of *L. acidophilus* BK4 in MT02, MT03 and MT04 shows that sucrose affects the growth and development of bacteria. In MT02 and MT03, the growth of *L. acidophilus* BK4 increased gradually as the fermentation started and reached a maximum after 20 hours. The equilibrium phase began 2 hours earlier than in MT01. However, in MT03, the equilibrium phase is short. The degradation phase begins after 30 hours. In MT04, the equilibrium phase is just 2 hours (from 18th hour to 20th hour). After that, thebiomass decreases rapidly due to the depletion of nutrients. In absence ofMT02, equilibriumphaseof *L. acidophilus* BK4was highly stable, at least for 60 hours, similar in absence ofMT01 (Figure1).

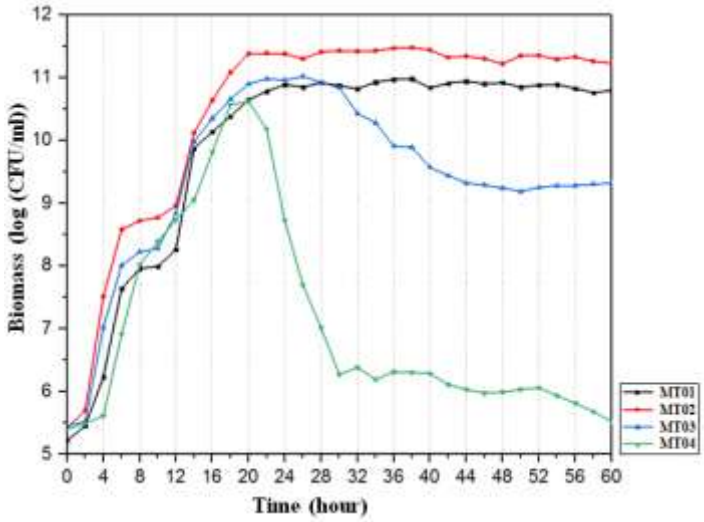

Figure1:The growth curve of*L. acidophilus* BK4 in MT01, MT02, MT03 and MT04

Microorganisms need carbon sources to make skeletal biosynthesis of various substances in the body.According to Ismail et al. (2010), glucose is the best source of carbon for the growth of *L. acidophilus* NRRL-4495 [17]. For*L. acidophilus* BK4, the growth rate in glucose is similar in sucroseat a concentration of 6%. After 24 hours,the maximum biomass was 10.89 log(CFU/ml) in MT01 and 11.43 log (CFU/ml) in MT02. For *L. acidophilus* BK4, sucrose is a suitable source of carbon for growth, which can be used as a substitute for glucose in production. When using sucrose instead of glucose at different concentrations, the antioxidant activity of the bacteria also changes. After 24 hours, the antioxidant activities of *L. acidophilus* BK4 in 6%, 8% and10% of sucrose solutions are shown in Table 1.

Table1:The antioxidant activity of*L. acidophilus* BK4 after 24 hours

Samples	Reducing power (OD_{700})	DPPH radical scavenging activity(OD_{517})	Inhibition (%)
MT02 (6%sucrose)	0.49 ± 0.02^{a}	1.22 ± 0.03^{a}	50.81
MT03(8%sucrose)	0.56 ± 0.03^{b}	1.17 ± 0.03^{b}	52.82
MT04(10%sucrose)	0.39 ± 0.03^{c}	1.36 ± 0.05^{c}	45.16
MT01 (glucose)	0.40 ± 0.02^{a}	1.20 ± 0.03^{a}	51.61
Ascorbic acid	0.92 ± 0.03	0.18 ± 0.02	92.74
The control sample	0.07 ± 0.02	2.48 ± 0.02	0

All the points represent Mean ± SD of triplicate samples.^{abc} The probability of occurrence was selected at p-value ≤ 0.05.

After 24 hours fermentation period,the measurement of reducing power and radical scavenging activity showedthat *L. acidophilus* BK4 has the ability to produce antioxidants in all determinated environments. The DPPH removal capacity was inhibition over 50%. The highest development of reducing power and DPPH scavenging activity was observed with the *L. acidophilus* BK4in MT03.The reducing power was 0.56 ± 0.03 and the DPPH scavenging activity was 1.17 ± 0.03 (52.82%inhibition). The least activity was shown by *L.acidophilus* in MT04. The reducing power was 0.39 ± 0.03 and the DPPH scavenging activity was 1.36 ± 0.05 (45.16%inhibition)(Table 1). The antioxidant activity of *L. acidophilus* BK4 in MT03 was higher than MT02 at p-value ≤ 0.05. In a similar assay, ascorbic acid as standard reference compound scavenges up to 92.74%.However, the biomass was highest in MT02 and the equilibrium phase was stable for 60 hours.This is the first priority of the study.The MT02solution with the highest biomass were selected for further studies.

3.2 Optimization ofbiomass(Y_1)

3.2.1 Construction of the design matrix

Based on our prior experiments, 3 reaction parameters, including pH (Z_1), temperature (Z_2), reaction time (Z_3) were chosen as independent variables for the design of experiments (Table 2) because of their observed effects on the outcome of the process. The target function (Y_1) is the optical density (OD_{610}) corresponding to the bacterial density (log (CFU/ml)).

Table 2: Real values of the independent variables at their corresponding levels in the design

Variables	Coded levels			Variable rangeΔ_i
	-1	0	+1	
Z_1 (pH)	4.5	5.5	6.5	1.0
Z_2 (^oC)	31	37	43	6
Z_3 (hour)	20	30	40	10

The experimental planning was described by a equation as followings:

$Y = b_0+b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3+b_{23}X_2X_3+b_{123}X_1X_2X_3$(2)

Three replicates of experiments at center point were performed.Because the design matrix is orthogonal, the coefficients bj and their variances S_{bi} can be directly estimated according to Equation (3) and (4).

$b_0 = \frac{\sum_{i=1}^{N} Y_i}{N}; b_{ik} = \frac{\sum_{i=1}^{N}(X_iX_k)Y_i}{N}; \quad b_i = \frac{\sum_{i=1}^{N} X_{ij}Y_i}{N}; \; b_{123} = \frac{\sum_{i=1}^{N}(X_1X_2X_3)_iY_i}{N}$(3)

$S_{bi} = \frac{S_{th}}{\sqrt{N}}$ (4)

Table3:The design matrix ofbacterial density (Y_1)

N^o	Variables			Coded levels			The biomass
	Z_1 (pH)	Z_2 (^oC)	Z_3 (hour)	X_1	X_2	X_3	Y_1(log(CFU/ml))
1	4.5	31	20	-1	-1	-1	11.079
2	6.5	31	20	+1	-1	-1	10.835
3	4.5	43	20	-1	+1	-1	11.397
4	6.5	43	20	+1	+1	-1	11.405
5	4.5	31	40	-1	-1	+1	11.489
6	6.5	31	40	+1	-1	+1	11.452
7	4.5	43	40	-1	+1	+1	11.513
8	6.5	43	40	+1	+1	+1	11.435
9	5.5	37	30	0	0	0	11.563
10	5.5	37	30	0	0	0	11.481
11	5.5	37	30	0	0	0	11.529

The design matrix of bacterial density (Y_1)shown in Table 3. The estimated variances of the regression coefficients: b_0= 11.326; b_1= -0.044; b_2= 0.112; b_3= 0.147; b_{12}= 0.026; b_{13}= 0.015; b_{23}= -0.110; b_{123}= -0.037.

We have S_{bi}= 0.015. The t-values for the regression coefficients:t_0= 777.542; t_1= 3.012; t_2=7.681;t_3= 10.066;t_{12}= 1.811; t_{13}= 1.038;t_{23}= 7.560; t_{123}= 2.514; $t_{0.05}$= 4.303. The values of t_0, t_2,t_3,t_{23}were higher than the tabulated value $t_p(f) = t_{0,05}(2)$ = 4.303, indicating that the regression coefficients in Equation (5) were significant.The experimental planning was described by the equation as followings:

$Y_1=11.326+0.112X_2+0.147X_3-0.110X_2X_3$(5)

We have S^2_{du}= 0.0116. F-value of 19.25, which is lower than the tabulated value $F_{(1-p)(f1,f2)}= F_{(0.05)(4,2)}$ = 6.82, indicating that the mathematical model was well fitted to the experimental data.

From Equation (5), the growth rate of bacterial cells is proportional to the temperature and reaction time. Temperature affects bacteria when it affects the biochemical reactions and activity of the enzyme in the cell. The pH of the medium affects the metabolism of the cell membrane. Temperature and pH too low or too high will inhibit the growth of bacteria. As the temperature increases, the fermentation time must be reduced to ensure sufficient nutrient supply for the growth of bacteria.As the yield of antioxidant activity from *L. acidophilus*BK4 is also the goal, Equation (5) was used for the optimization of the process.

3.2.2 Determination of the optimal conditions

Determination of the optimal conditions by the Box-Wilson method, equation of movement steps (Table 4) as followings:

$\delta_i = \delta_{cs}\frac{b_i\Delta_i}{b_{cs}\Delta_{cs}}$ (6)

Table 4:Movement steps $\delta_i$ofbiomass target (Y_1)

Parameters	Variables		
	Z_1 (pH)	Z_2 (^oC)	Z_3 (hour)
Center point	5.5	37	30
Variable rangeΔ_i	1	6	10
Regression coefficientsb_i	-0.044	0.112	0.147
Movement steps δ_i	-0.15	2.29	5

Table 5:The Box-Wilson method in biomass target (Y_1)

N°	Variables			The biomass	Antioxidant activity
	Z_1(pH)	Z_2(°C)	Z_3(hour)	Y_1(log (CFU/ml)	Y_2(OD_{700})
12	5.5	37	30	11.469	
13	5.3	39	35	11.572	0.409
14	5.1	41	40	11.416	
15	4.9	43	45	11.373	

In Table 5, the optimal fermentation conditions were determined includingpH = 5.3; T = 39°C andτ = 35h. The 13th experiment using these parameters gave 11.572 log (CFU/ml) biomass and 0.409 reducing power. The reducing powerwas not the highest. Subsequent experiments were conducted to the optimization of the process for the antioxidant activity.

3.3 Optimization ofantioxidant activity(Y_2)

3.3.1 Construction of the design matrix

Based on our prior experiments, 3 reaction parameters, including pH (Z_1), temperature (Z_2), reaction time (Z_3) were chosen as independent variables for the design of experiments (Table 2) because of their observed effects on the outcome of the process. The target function (Y_2) is the optical density (OD_{700}) corresponding to the the yield of antioxidant activity from *L. acidophilus*BK4.

Table6:The design matrix ofantioxidant activity (Y_2)

N°	Variables			Coded levels			Antioxidant activity
	Z_1 (pH)	Z_2 (°C)	Z_3 (hour)	X_1	X_2	X_3	Y_2 (OD_{700})
1	4.5	31	20	-1	-1	-1	0.315
2	6.5	31	20	+1	-1	-1	0.281
3	4.5	43	20	-1	+1	-1	0.298
4	6.5	43	20	+1	+1	-1	0.245
5	4.5	31	40	-1	-1	+1	0.441
6	6.5	31	40	+1	-1	+1	0.379
7	4.5	43	40	-1	+1	+1	0.447
8	6.5	43	40	+1	+1	+1	0.396
9	5.5	37	30	0	0	0	0.459
10	5.5	37	30	0	0	0	0.474
11	5.5	37	30	0	0	0	0.465

The design matrix of antioxidant activity (Y_2)shown in Table 6. From estimated according to Equation (3) and (4), the variances of the regression coefficients: b_0= 0.350; b_1= -0.025; b_2= -0.004; b_3= 0.066;b_{12}= -0.001; b_{13}= -0.003; b_{23}= 0.01; b_{123}= 0.004.We have S_{bi}= 0.0027. The t-values for the regression coefficients: t_0= 131.216; t_1= 9.366; t_2=1.405;t_3= 24.539;t_{12}= 0.375;t_{13}= 1.218;t_{23}= 3.559; t_{123}= 1.405; $t_{0.05}$= 4.303.The values of t_0, t_1,t_3 were higher than the tabulated value $t_p(f) = t_{0,05}(2) = 4.303$, indicating that the regression coefficients in Equation (7) were significant.The experimental planning was described by a equation as followings:

$Y_2 = 0.35 - 0.025X_1 + 0.066X_3$(7)

We have S^2_{du}= $1.042.10^{-3}$. F-value of 19.16, which is lower than the tabulated value $F_{(1-p)(f1,f2)}$= $F_{(0.05)(5,2)}$ = 18.28, indicating that the mathematical model was well fitted to the experimental data.From Equation (7), the regression equation demonstrating the antioxidant activity by *L. acidophilus* BK4 was dependent on pH and reaction time. pH of environmental affect the growth and membrane transport of cells. As the time increases, antioxidants have time to disperse into the environment.

3.3.2 Determination of the optimal conditions

Determination of the optimal conditions by the Box-Wilson method, from estimated according to Equation (6), theequation of movement steps shown in Table 7.

Table 7: Movement steps $\delta_i$ofantioxidant activity target (Y_2)

Parameters	Variables		
	Z_1 (pH)	Z_2 (°C)	Z_3 (hour)
Center point	5.5	37	30
Variable rangeΔ_i	1	6	10
Regression coefficients b_i	-0.025	-0.004	0.066
Movement steps δ_i	-0.19	-0.17	5

Table 8:The Box-Wilson method in antioxidant activity target (Y_2)

N°	Variables			Antioxidant activity	The biomass
	Z_1 (pH)	Z_2(°C)	Z_3(hour)	$Y_2(OD_{700})$	Y_1(log (CFU/ml)
12	5.5	37	30	0.452	
13	5.3	36.8	35	0.497	
14	5.1	36.6	40	0.553	11.581
15	4.9	36.4	45	0.483	
16	4.5	36.2	50	0.478	

In Table 8, the optimal fermentation conditions were determined includingpH = 5.1; T = 36.6°C and τ = 40h. The 14th experiment using these parameters gave 0.553reducing powerand 11.581 log (CFU/ml) biomass. The biomasswas the highest.An experiment performed using these parameters gave 11.589± 0.09log (CFU/ml) biomass,0.551± 0.04reducing power and 1.15 DPPH scavenging activities (53.63%inhibition),which was close to the optimal value.The biomass6.4% increase, the reducing power 37.8% increase and the DPPH scavenging activities 12.02% increase as compared with control experiment (MT01).After determination of optimal fermentation conditions by experimental planning method and Box-Wilson method, the biomass and the yield of antioxidant activity from *L. acidophilus* BK4 is significantly improved.

IV. CONCLUSION

The antioxidant activity of *L. acidophilus* BK4 is investigated by two different free radicals scavenging capacities combined with reducing power analysis.The application of 6% sucrose solutionin combination with the application of the experimental planning method and the Box-Wilson method substantially improved biomass andantioxidant activity yield. The biomass 6.4% increase, the reducing power 37.8% increase and the DPPH scavenging activities 12.02% increase as compared with control experiment.

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