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Optimizing Media of *Lactobacillus rhamnosus* for Lactic Acid Fermentation

Maizirwan Mel, Mohamad Ismail Abdul Karim,
Mohamad Ramlan Mohamed Salleh and Noraini Alamin Mohamad Amin
Bioprocess Engineering Research Group, Department of Biotechnology Engineering
Faculty of Engineering, International Islamic University Malaysia,
P.O. Box 10, Gombak 50728, Kuala Lumpur, Malaysia

Abstract: The aim of this study was to optimize the main media components that affect the production of lactic acid by *Lactobacillus rhamnosus* fermentation. In the experiment, the two variables (glucose and peptone) were optimized using the Central Composite Design (CCD) method by STATISTICA Software. The optimization studies were successfully carried out in shake flask experiments. The results indicated that the optimum concentration of glucose and peptone for optimum bacterial growth rate and lactic acid production in shake flask were 9.80 and 9.98 g L^{-1}, respectively. The optimum productivity of the lactic acid was 0.630 g g^{-1} h which correspond to optimum growth rate of the bacteria at 0.341 h^{-1}.

Key words: *Lactobacillus rhamnosus*, lactic acid, media optimization, fermentation, CCD method

INTRODUCTION

The efficiency and economics of the lactic acid production through microbial fermentation is still under problematic conditions if considered from many points of view and among them, media composition plays a vital role in the improvement of such a process (Stanbury *et al*., 2003). Fermentation of lactic acid by microbes such as bacteria using different media plays an important role in the final product of the process (Lee, 2005). Proper design of the media does affect the performance of microorganisms in optimizing the lactic acid production (Krishnan *et al*., 1998; Telez-Luiz *et al*., 2003).

Nowadays, research effort is focused on looking not only for new and effective nutritional sources but also for new progressive fermentation techniques which enable the achievement of both high substrate conversion and high production yields. The improvement of lactic acid production has been studied under the control of various factors and media components (Arasaratnam *et al*., 1996; Wenge and Methews, 1999; Hujanen *et al*., 2001; Molinier *et al*., 2004; Rao *et al*., 2004).

In our previous studies (Ismail *et al*., 2006), eleven components of media were screened using Placket Burman Design and the results indicated that, the main media components that affected the lactic acid production process by *L. rhamnosus* fermentation were glucose and peptone. The correlation between those two variables was analyzed using Response Surface Methodology (RSM). In this study, the media compositions of the fermentation process were optimized using the CCD method. The advantage of CCD method is that the effect of variable at a distance alpha from the design centre can be studied (Liew *et al*., 2005).

MATERIALS AND METHODS

Design of experiment (DOE): Experiment was conducted at Bioprocess Engineering Laboratory of IIUM Malaysia and was designed by Central Composite Design (CCD) using STATISTICA Software. CCD is a set of technique designed to find the best value of response.

Microorganism and media: The microorganism used in this study was *Lactobacillus rhamnosus* (*L. rhamnosus*, the homo-fermentative lactic acid bacteria (Takumi *et al*., 2001). The culture media used was the MRS medium containing glucose, peptone, yeast extracts, lactose, Tween 80, K_2HPO_4, sodium acetate, $(NH_4)_2SO_4$, $MnSO_4$, $MgSO_4$ and distilled water.

Inoculums preparation: The preparation of inoculums started with transferring the stock culture into a liquid MRS media. After the growth of culture, the microorganisms were transferred to a plate of solid MRS

Corresponding Author: Dr. Maizirwan Mel, Bioprocess Engineering Research Group, Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, P.O. Box 10, Gombak 50728, Kuala Lumpur, Malaysia Tel: 603-61964566 Fax: 03-61964442

medium. The plate was incubated at 37°C for 48 h in order to allow sufficient growth of colonies.

The grown colonies were either used to initiate a fermentation process or were stored back at 4°C as stock culture which can be prepared by culturing the colonies in slant agar followed by adding 30% of sterilized glycerol. The *L. rhamnosus* inoculums were prepared by inoculating a single colony of them into 10 mL broth media which was then incubated at 37°C for 24 h. One milliliter of inoculums was transferred into bijou bottle containing 9 mL media. Cultures were incubated for 10 h at 37°C before being transferred into shake flask.

Sampling: Sample in shake flasks were taken by using aseptic technique for every 2 h by flaming the cap swabbed with 70% ethanol. Twelve milliliter of sample was transferred into a bijou bottle, which was then being divided for measuring optical density (OD, $A_{660\ nm}$), product (lactate), substrate (glucose) and cell dry weight. The flasks then were transferred back to the thermostat rotary incubator shaker to continue the fermentation process.

Analytical method: Optical density analysis (OD), total cell number (TCN) and Cell Dried Weight (CDW) were analyzed as described by Maizirwan *et al*. (2006).

Glucose and lactate analysis: One milliliter sample was transferred into 1.5 mL Eppendorf tube and centrifuged at 3000 rpm for 10 min. The supernatant was transferred into a cuvette and analyzed using the YSI 2700 Biochemical Analyzer.

Experimental design for media optimization: The experiment for media optimization was designed using the Central Composite Design (CCD) method. From the media screening experiment, since peptone and glucose were found to be the most significant component that affects the production of lactic acid, they were selected as independent variables to be optimized in order to increase the productivity of lactic acid. Table 1 shows the actual parameters value used in media optimization experiment of media using CCD method.

Table 1: Experimental design for media optimization using central composite design

	Central composite design for optimization of *L. rhamnosus*			
Run	Variable (A)	Variable (B)	Glucose (A)	Peptone (B)
1	-1.00	-1.00	10	8.0
2	-1.00	1.00	10	12.0
3	1.00	-1.00	30	8.0
4	1.00	1.00	30	12.0
5	-1.41	0.00	4	10.0
6	1.41	0.00	34	10.0
7	0.00	-1.41	20	6.8
8	0.00	1.41	20	12.8
9	0.00	0.00	20	10.0
10	0.00	0.00	20	10.0

Fermentation in shake flask: In order to find the exact value of the optimum concentrations of peptone and glucose in producing the optimum concentration of lactic acid, experiments were carried out to find the growth kinetic of the bacteria and the productivity of lactic acid using the formulated media components by CCD method. For each run, 10 shake flasks were used. In each flask, 10 mL inoculums were transferred into 90 mL formulated media under aseptic condition. The shake flask was capped with cotton and swabbed with 70% ethanol and then incubated in a thermostat rotary incubator shaker for 30 h under setting temperature of 37°C and rotation speed of 150 rpm. The critical value or optimized value in percentage of peptone and glucose was studied by STATISTICA analysis.

RESULTS AND DISCUSSION

Media optimization: The results showed that the cell growth rates of *L. rhamnosus* are slightly different with the different media component (Fig. 1). At the beginning, the growth was quite similar but after certain time, the growth becomes slower not only due to the decrease of substrate concentration but also the resistance of these bacteria with acidic condition and other inhibitors.

From Fig. 2, it could be concluded that the production of lactate is different with different concentration of the peptone and glucose. The graph showed that Run 6 is the best run due to the high amount of lactate produced. The rate of productivity is increasing steadily. The substrate conversion (glucose) is decreasing as the fermentation getting longer (data not

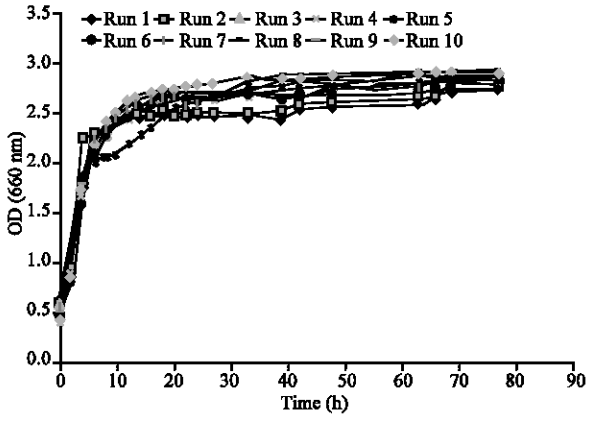

Fig. 1: The growth profile of *L. rhamnosus* in T-Flask culture

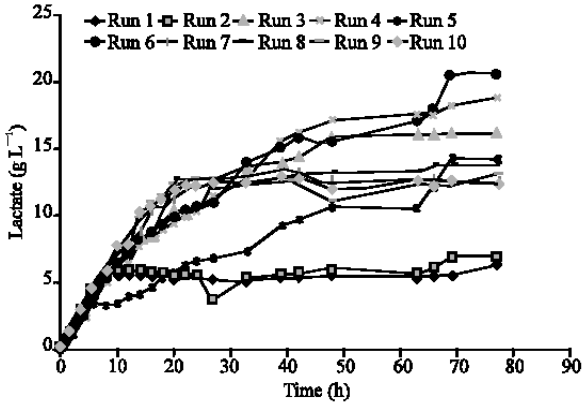

Fig. 2: The profile of lactic acid production for each run

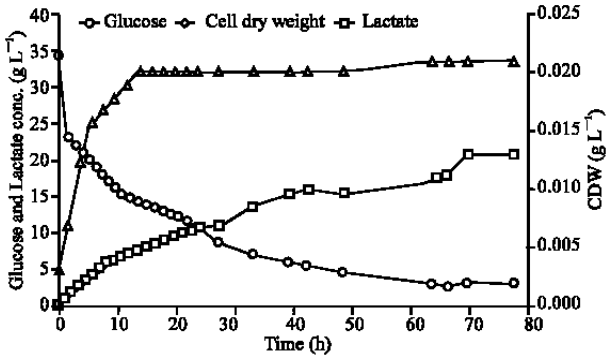

Fig. 3: The growth profile, glucose reducing and produced lactate for Run 6

shown). This mean that even though the cell growth is not much different but the lactate produced is significantly different. Many factors have been involved in this lactate production such as the combination of media quantity, pH and others environmental factors. Since *L. rhamnosus* is a gram positive, it needs longer time to secrete secondary metabolite compared to gram negative via metabolic pathway.

For run 6 (Fig. 3), the graph was extended to see the relation among the growth performance of the bacteria, glucose consumption and the lactate produced. The yield is increased as the substrate is decreasing. When all the substrates were consumed, the production was stopped. The *Lactobacillus* is non-growth associated, as can be viewed from the graph that even the cell growth had reached the stationary phase after 20 h of fermentation, the lactate still being produced with high rate. At this condition, the glucose has been converted to lactate via sugar metabolism.

The total of carbon source plays an important role in the lactate production. In many of the fermentation processes, sugars are controlled so that it will be

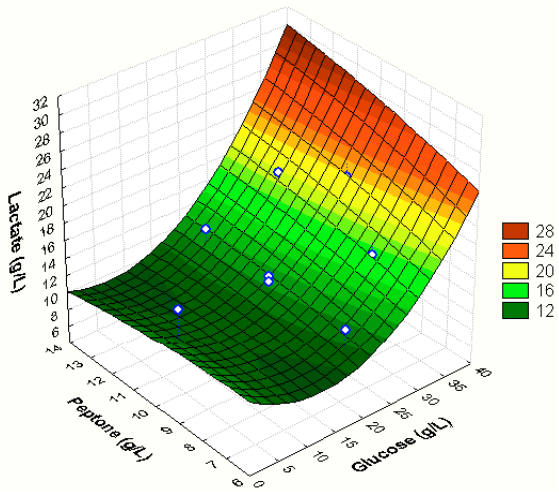

Fig. 4: Interaction pattern between glucose and peptone on Lactate production

Table 2: Critical value of glucose and peptone

Critical values; variable: lactate, solution: saddle point predicted value at solution: 10.08794

Factor	Observed minimum	Critical values	Observed maximum
Glucose	4.00	9.81	34.00
Peptone	6.80	9.99	12.80

Table 3: ANOVA table of quadratic regression model

ANOVA, Var Lactate, R^{-2} = 78391, Adj 51379 (Aini) 2 factors, 1 block, 10 runs, MS Residual = 9.985221 DV:Lactate

Factors	SS	DF	MS	F	p
(1) Glucose (L)	126.2143	1	12.64011	12.64011	0.023686
Glucose (Q)	20.0627	1	20.62700	2.00924	0.229313
(2) Peptone (L)	2.3520	1	2.35200	0.23555	0.652824
Peptone (Q)	0.2576	1	0.25760	0.02580	0.880174
1L by 2L	1.1025	1	1.10250	0.11041	0.756358
Error	39.9409	4	9.98520		
Total SS	184.8320	9			

completely metabolized to produce a targeted product but not the by-product as it will become the inhibitor for the cell itself. This homo-fermentative bacterium is hoped to produce lactate as a main product with less by-product such as ethanol and CO_2.

Critical value: From the Table 2, 9.8% of glucose and 9.98% of peptone was needed by *L. rhamnosus* in order to increase the production of acid lactic. This critical value was used for further optimization process in the bioreactor.

The ANOVA of quadratic regression model demonstrate the model was highly significant as shown in Table 3 with lower probability value (p-value). The pattern of interaction between the glucose and peptone (Fig. 4)

Table 4: Productivity and growth kinetic study of *L. rhamnosus* using optimized media

RUN	μ_{max} (h^{-1})	$Y_{P/S}$ (g g^{-1})	Productivity (g g^{-1} h)
1	0.277	0.565	0.228
2	0.335	0.579	0.216
3	0.272	0.651	0.086
4	0.348	0.736	0.065
5	0.341	2.097	0.630
6	0.321	0.710	0.058
7	0.341	0.732	0.110
8	0.370	0.767	0.118
9	0.391	0.721	0.128
10	0.394	0.698	0.111

was also indicated by this coefficient. Based on the p-value obtained from the ANOVA table, it shows that the glucose is the main factor to promote the lactic acid production, followed by the combination of glucose and peptone.

Growth kinetic study: From the Table 4, it shows that the cells grow fastest in Run 1 and 3 but with lower productivity. The highest productivity has been obtained at Run 5 with the value of 0.630 g g^{-1} h which corresponds to the fifth faster in the cell growth rate; it's about 0.341 h^{-1}. The value of growth rate is still within the range of value that had been obtained by Zannini *et al.* (2005); it ranged from 0.16 to 0.390 h^{-1}. The highest productivity has been achieved at lower concentration of glucose and shorter fermentation time. As reported by Serna and Rodríguez (2006), the productivity of lactic acid production using *Lactobacillus lactis* has been obtained at 0.29 g L^{-1}h using 20 g L^{-1} of glucose and 48 h of fermentation time. In this study, with low amount of substrate (<10 g L^{-1} of glucose) and short fermentation time (<40 h) has contributed to generate the high productivity of lactic acid production.

CONCLUSION

The Central Composite Design (CCD) is practical to be used in the optimization of fermentation media. The critical concentration of the most significant variables (glucose and peptone) which critically influence the cell growth and lactic acid production was 9.80 and 9.98 g, respectively. From ANOVA analysis, the most influence variable can be determined by p-value. The smaller p-value indicates the most significant variable toward the lactate production. The maximum lactic acid production was observed in Run 5 which produces 0.630 g L^{-1} of the acid which correspond to specific growth rate of 0.341 h^{-1}.

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