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Bioconversion of waste cooking oil glycerol from cabbage extract to lactic acid by *Rhizopus microsporus*

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ABSTRACT

Glycerol from spent oil was processed by transesterification for biodiesel production. Although glycerol contains many types of impurities, it can be used as a C-source for lactic acid production by fungi, such as *Rhizopus microsporus*. In this study, we found that wild type *R. microsporus* (LTH23) produced more lactic acid than the mutant strains on cabbage glycerol media (CG media). More lactic acid was produced on CG media than on cabbage extract media (C media) by about two-fold in batch fermentation conditions. In addition, we found that lactic acid production in a fed-batch process was also slightly higher than in a batch process. To study the combined effects of pH, urea, and glycerol waste concentration on lactic acid production, a response surface methodology was used. The optimum pH, urea, and glycerol waste concentrations were pH 6.5, 3.75 g/L, and 17 g/L, respectively. The maximum lactic acid production predicted by this equation model was 4.03 g/L.

Introduction

Some routes of chemical synthesis, such as those for citric acid and lactic acid synthesis are complex and expensive. Furthermore, more chemicals are being produced using biotechnology due to the need for a reduction in natural gas and oil dependence and a reduction in the environmental impact from the chemical industry. Bio-renewable lactic acid can be produced using fermentation. The global production of lactic acid is expected to reach 1960 kilo tons by 2020[1]; of this amount, approximately 90% of worldwide lactic acid production is derived from fermentation processes. Due to the human body being adapted to assimilate only the L-(+)-form of lactic acid, it is safe and used by the food and drug industries. Lactic acid is widely used across many industries, including food, beverage, personal care, textile, and polymer industries. Currently, biodegradable polymer polylactic acid

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(PLA)is an environmentally friendly replacement for plastics obtained from petrochemical materials.

Various microorganisms can produce lactic acid, such as lactic acid bacteria species, including *Bacillus* sp.[2,3] *Amylomyces rouxii*,[4] and *Rhizopus* species. A sole isomer of L-lactic is generated by *Rhizopus* fungi. In addition, this fungus is capable of using a wide range of substrates for lactic acid production, including glucose,[5] molasses, starch,[6] lignocelluloses,[7,8] office paper,[9] and crude glycerol.[10] Currently, biodiesel is an important alternative fuel that is used in many counties in the world to replace petro oil. Glycerol is a by-product from biodiesel manufacture. Higher biodiesel production means that an abundant glycerol supply could be an attractive feedstock for value-added chemical production. Many microorganisms can convert glycerol to ethanol, butanol, citric acid, hydrogen, 1,3-propanediol, polyhydroxyalkanoate,[10,11] succinic acid,[12] and lactic acid.[13–15] *Rhizopus* species have been recognized as attractive candidate microbes for lactic acid production through fermentation, due to its growth on nitrogen-limited media, its production of optically pure L-(+)-lactic acid, and the ease of separating fungal mycelium from fermentation products (leading to less expensive downstream processing).[16] However, there are only a few reports on lactic acid production using glycerol from waste cooking oil. In this study, we screened for a new strain with the ability to convert waste cooking oil glycerol into lactic acid, focusing on investigating lactic acid production by *Rhizopus microsporus* from waste cooking oil glycerol derived from biodiesel production.

Materials and methods

T-glycerol from waste cooking oil glycerol

Waste cooking oil glycerol was obtained from the Faculty of Engineering, Khon Kaen University. It is a by-product of biodiesel production using cooking oil processed with caustic soda (NaOH) as a catalyst. Impurities including free fatty acids, were removed by pretreatment with 6N HCl until the pH was 7 (modified from the method of Tianfeng et al., 2013).[17] The fatty acid phase was easily removed from the mixture by heating to 70 °C and cooling to room temperature in a separating funnel. Three distinct phase layers of this solution were then separated. Organic salts (bottom phase) and free fatty acids (top phase) were removed. The middle phase, T-glycerol, was kept and analyzed for its glycerol content using the Bondioli–Bella method (2005).[18]

Microorganisms and inoculum

R. microsporus LTH23 was isolated from soil and identified by sequencing of the Internal transcript spacer (ITS) region compared with a previous study by Yuwa-amornpitak and Chookietwatana (2014).[6] The mutant strains of *R. microsporus* 2T5, *R. microsporus* 5T3, *R. microsporus* 10T5, and *R. microsporus* H120M4 were derived from the wild type *R. microsporus* LTH23 by UV and chemical treatments. The genomic sequence of *R. microsporus* LTH23 was submitted to NCBI gene bank under the accession number KU358721. The fungi was cultivated on modified YM media, referred to as G-YM (glycerol 9/l, yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, and glucose 1 g/L), and incubated at 35 °C for 3–5 d. Strains were stored temporarily at 4 °C but were transferred to new media every 2–4 weeks. Fungal spores were collected with sterilized water containing 0.05% tween 80 and filtered through glass wool. Cabbage-glycerol media was inoculated with spore suspension at a spore concentration of 1×10^6 spores/mL.

Table 1 – Values of parameters used in Box–Behnken's response surface design.

Parameters	Symbol code	Levels	
		Low (−1)	High (+1)
pH	X_1	4	9
Urea, g/l	X_2	0.5	7
Glycerol	X_3	4	30

Cabbage-glycerol media (CG media)

Cabbage extract (1 L) was prepared from 300 g of chopped green cabbage that was boiled in distilled water for 10 min. Inorganic salts with normal dosages[7] were used as follows: 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L urea, and 0.05 g/L $ZnSO_4 \cdot 7H_2O$. The amount of T-glycerol (equivalent 55% pure glycerol) (20 g/L) was added to the CG media.

Statistical analysis

Response surface methodology with a Box–Behnen design was used for determining the role of pH, urea, and media glycerol content in lactic acid production from *R. microsporus* LTH23 (Table 1). A total of 17 experiments were performed using CG media, each with three replicates. Lactic acid was estimated using the response surface methodology (RSM) by Design Expert® Software version 7.15 (Stat-Ease, Inc.).

Batch fermentation

Batch fermentation processes were carried out in a fermenter (Biostat B, Sartorious, Germany) containing 1 L of CG media, and after adding waste glycerol, the pH was adjusted to 6.5 before being autoclaved. A seed inoculum of *R. microsporus* LTH23 was prepared as above, and a spore suspension of 1×10^6 spores/mL was inoculated to make the total volume 1.1 L. Fermentation was conducted at 35 °C, with an agitation speed of 200 rpm, and 0.05 vvm of air was supplied continuously. Glycerol, lactic acid, and sugar concentrations were determined using cell-free fermentation broth.

Fed batch fermentation

The fed batch fermentation process was performed in a fermenter (Biostat B, Sartorious, Germany) with an initial CG media volume of 600 mL before being autoclaved. A spore suspension of 1×10^6 spores/mL (100 mL) was inoculated and fermentation was run at 35 °C, with agitation speed of 200 rpm, and 0.05 vvm of continuously supplied air (microaerobic phase). After 24 h, 400 mL of fresh CG media (the same composition as in the fermenter) was added by continuous

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feeding for 4 h. Reducing sugar, glycerol, and lactic acid levels were determined from the supernatant collected at fixed time intervals.

Analytical methods

The sugar residues from clear samples at the indicated times were estimated using the dinitrosalicylic acid method (dissolve 1 g 3,5-dinitrosalicylic acid in 20 mL of 2 N NaOH and 30 g $KNaC_4H_4O_6{\cdot}4H_2O$ in 100 mL) using glucose as a standard.[19]

Glycerol was measured as described by Bondioli-Bella (2005).[18] Briefly, a 0.25 mL sample was mixed with 0.75 mL of working solvent (50% ethanol) and 0.6 mL of 10 mM sodium periodate for 30 s. Then, 0.6 mL of a 0.2 M acetylacetone solution was added and incubated at 70 °C for 1 min. This was then immediately transferred to cooled water. The optical density of the resulting yellow color was measured with a spectrophotometer at 410 nm.

Lactic acid was analyzed by a modified Barker–Summerson method (1941).[20] Briefly, a 0.5 mL sample was mixed with 0.25 mL of 4% $CuSO_4{\cdot}5H_2O$ and 3 mL of conc. H_2SO_4. The mixture was boiled for 5 min in a water bath and cooled down. The color of the mixture was developed by adding 50 μL of 0.75% p-hydroxydiphenyl and measured at an optical density of 560 nm using magnesium lactate as a standard.

Results

Selection of lactic acid producing fungi using glycerol

Rhizopus species are able to utilize various types of carbon sources, such as sugars and starches, including cellulose. Currently, glycerol is an abundant C-source derived from biodiesel industries that few microbes can use for lactic acid production. Cassava starch can easily be converted to lactic acid by *R. microsporus* LTH23,[6] but only limited lactic acid was produced from on glycerol media. We then developed mutant strains by UV and chemical treatments. The selected mutant strains were then compared with the wild type strain (*R. microsporus* LTH23). Glycerol media supplemented with cabbage extract was used for lactic acid production, as shown in Fig. 1. Initially, the reducing sugar was consumed rapidly followed by glycerol and all strains produced lactic acid at low concentrations. A low amount of reducing sugar remained at 72 h, whereas glycerol concentrations remained high. However, the mutant

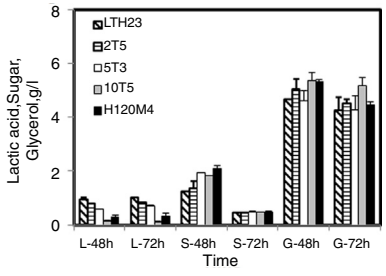

Fig. 1 – Lactic acid production by *R. microsporus* LTH23 and mutant strains using CG. Media L = lactic acid, S = sugar, G = glycerol, 48 h = time at 48 h, 72 h = time at 72 h.

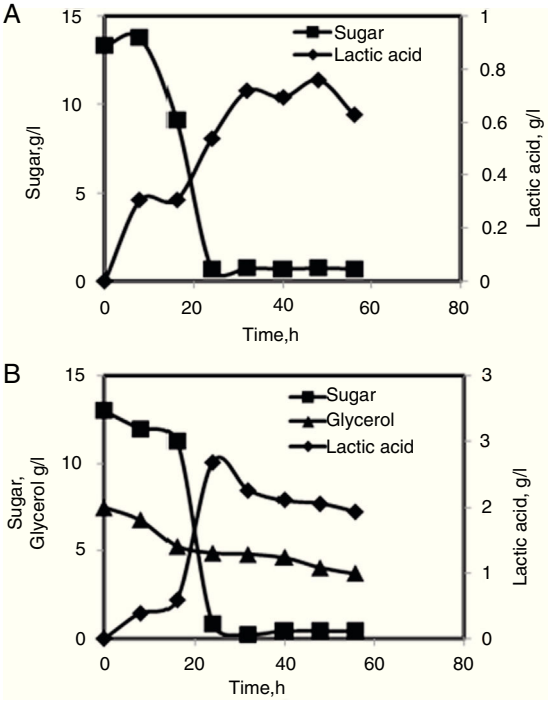

Fig. 2 – Comparative study of batch fermentation process for lactic acid production by *R. microsporus* LTH23 using cabbage extract media (C medium: A) and CG media (cabbage extract with glycerol: B).

strains *R. mirosporus* 2T5, *R. mirosporus* 5T3, *R. mirosporus* 10T5, and *R. mirosporus* H120M4 produced lower lactic acid concentrations than wild type at both 48 h and 72 h. Therefore, the wild type strain *R. mirosporus* LTH23 was selected for further study.

Batch fermentation for lactic acid production

An analogous study was conducted for lactic acid production by *R. microsporus* LTH23 using cabbage extract with and without glycerol added. Cabbage is a vegetable that contains sugar and various minerals,[21] meaning that cabbage extract is suitable for microbial growth. The results of this study are shown in Fig. 2. We observed that the reducing sugar in the medium was rapidly used within 24 h, and low levels of lactic acid (0.76 g/L) were produced at 48 h (Fig. 2A). Concurrently, a higher lactic acid level (2.69 g/L) was achieved using the same substrate with the addition of glycerol (Fig. 2B). However, reducing sugars were quickly consumed by the fungi, and glycerol levels slowly decreased to almost 50% of the initial levels at 56 h. The average degree of reduction per carbon in glucose ($K_{glucose}$) and glycerol ($K_{glycerol}$) were 4 and 4.7, respectively; therefore, all sugar present was easily reduced and assimilated in a short time. This finding is supported by results from Wang et al. (2015).[15]

Fed-batch fermentation for lactic acid production

The fed-batch process was performed by adding 40% fresh media containing glycerol at the same concentration into the

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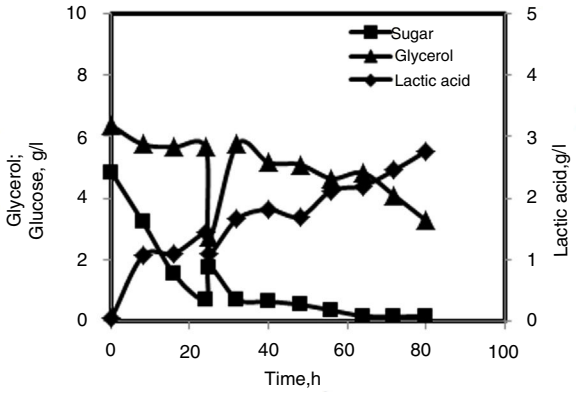

Fig. 3 – Fed-batch process for lactic acid production by *R. microsporus* LTH23 using CG media with 40% fresh media added at 24 h.

fermenter at 24 h. These results are shown in Fig. 3. The reducing sugar from cabbage extract was rapidly decreased after glycerol addition at 24 h. The glycerol was slowly but continuously consumed, and 50% of the initial glycerol amount remained at the end of the fermentation process. Lactic acid increased and the highest production observed was 2.76 g/L after an 80-h fermentation run. This indicated that a fed-batch fermentation technique enhanced the accumulation of lactic acid using this fungus. However, the production lactic acid was still low and needed production needed optimization.

Response surface model

A total of 17 runs for the Box–Behken design were performed and a polynomial equation for the lactic acid concentration was generated as a model (Eq. (1)), using Design Expert Software 7.15. The model fit the experimental data and the predicted values were plotted (data not shown). Predictions for all experiments from Eq. (1) were performed and are presented in Table 2.

$$\text{Lactic acid (g/l)} = -8.19258 + 1.62599A + 2.16336B + 0.25950C - 0.0004307AB - 0.00883AC - 0.000349BC - 0.11701A^2 - 0.28654B^2 - 0.00307737C^2 \quad (1)$$

where "A" is pH, "B" is urea, and "C" is glycerol.

Analysis of variance (ANOVA) was used to optimize the results and is shown in Table 3. The coefficient of determination (R^2) for the model was 0.8552 (85.52% of variability in

Table 2 – Lactic acid production using Box–Behnken response surface design.

Runs	Parameters			Measured lactic acid, g/l	Predicted lactic acid, g/l
	pH (A)	Urea g/l (B)	Glycerol g/l (C)		
1	9	0.50	17	0.275	0.139
2	9	7	17	0.325	0.168
3	6.5	3.75	17	3.11	4.029
4	4	0.5	17	0.211	0.367
5	4	7	17	0.275	0.410
6	4	3.75	30	5.432	4.432
7	6.5	0.5	4	0.064	−0.800
8	6.5	7	30	0.872	1.736
9	9	3.75	4	0.698	1.697
10	6.5	7	4	0.109	−0.734
11	6.5	3.75	17	4.979	4.029
12	4	3.75	4	0.65	1.358
13	6.5	0.5	30	0.886	1.729
14	6.5	3.75	17	2.98	4.029
15	9	3.75	30	4.332	3.623
16	6.5	3.75	17	4.152	4.029
17	6.5	3.75	17	4.926	4.029

Table 3 – ANOVA for lactic acid production by *R. microsporus* LTH23 using glycerol.

Variables	Sum of squares	df	Mean square	*F* value	*p*-value $p > F$
A-pH	0.11	1	0.11	0.08	0.786
B-Urea	0.0026	1	0.0026	0.0019	0.9664
C-glycerol	12.5	1	12.5	9.05	0.0197
AB	0.000049	1	0.000049	0.000035	0.995
AC	0.33	1	0.33	0.24	0.6402
BC	0.00087	1	0.00087	0.000629	0.9807
A^2	2.25	1	38.57	1.63	0.2424
B^2	38.57	1	2.25	27.92	0.0011
C^2	1.14	1	1.14	0.82	0.3941
Model	57.11	9	6.35	4.59	0.0284

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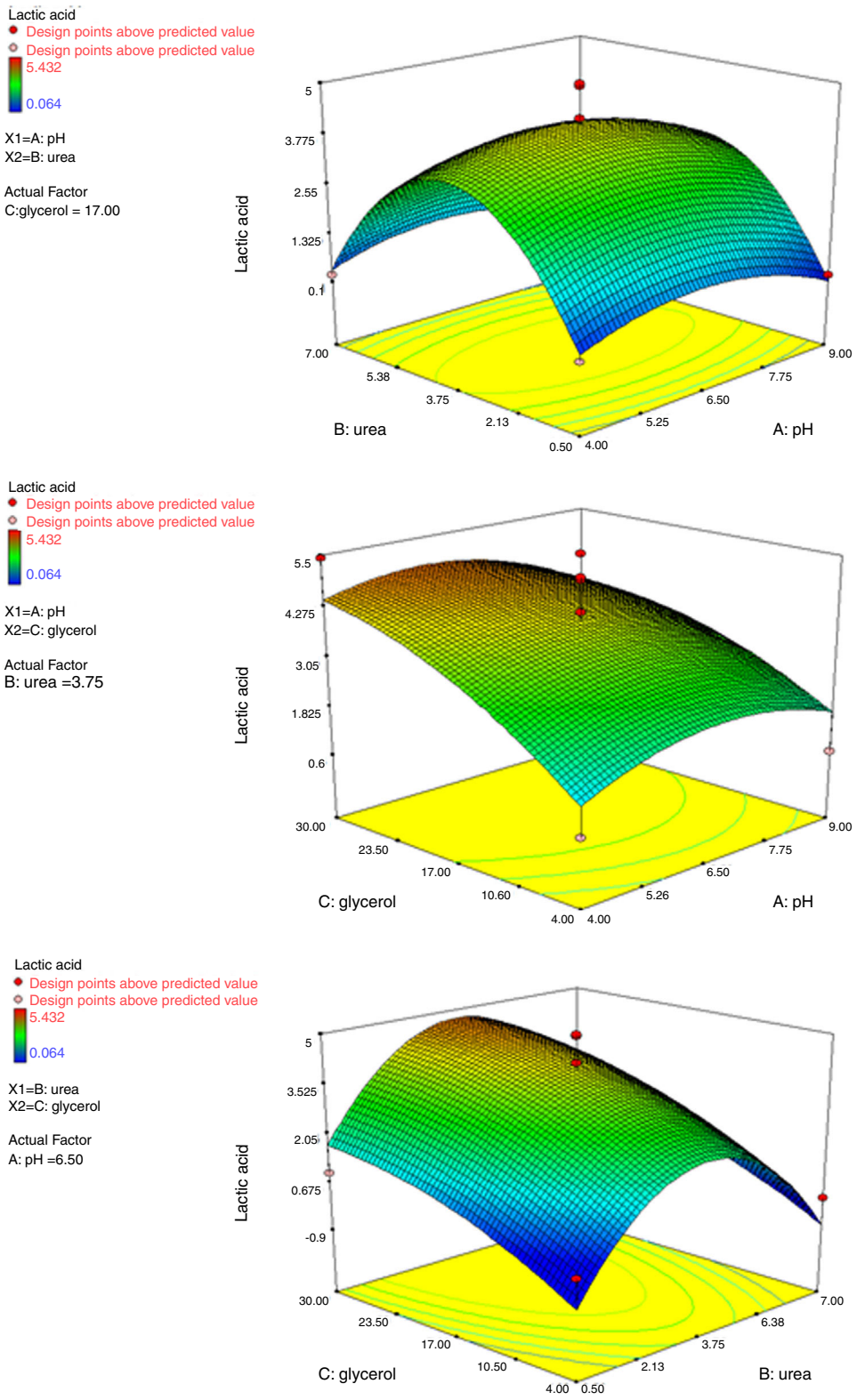

Fig. 4 – Response surface and contour curve plot showing effect of pH, urea, and glycerol concentration on lactic acid production by *R. microsporus* LTH23.

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the data predicted by the model). The experimental data were then analyzed by fitting to a quadratic model. The fit was not significant while the p-value of the model was significant with a *p*-value of 0.024. In addition, it was also shown that glycerol had a greater effect on lactic acid production than pH and urea (lower *p* value).

The three-dimensional response surface plots are shown in Fig. 4. These indicate that urea was an influencing factor on lactic acid production. The optimum urea concentration was 3.75 g/L, although we found that at a low urea concentration of 0.5 g/L and at a higher one of 7 g/L, lactic acid production was decreased. Furthermore, the optimum pH and glycerol concentrations were 6.5 and 17 g/L, respectively. Most fungi prefer acidic conditions for growth. Coban and Demirci (2015)[22] studied lactic acid production by *Rhizopus oryzae* from glucose by the RSM method, with an optimum pH of 6.22. Furthermore, their findings suggested that microparticles, such as talcum, enhanced lactic acid production. Unfortunately, we found that talcum was not suitable for lactic acid production by *R. microsporus* LTH23 (data not shown).

Discussion

Generally, the impurities found in crude glycerol derived from biodiesel production are inorganic salts, soap, free fatty acids, and alcohol. Venkataramanan et al. (2012)[23] reported that free fatty acids such as linoleic, stearic and oleic acid influence the synthesis of metabolites and the diffusion of the substrates across the plasma membrane.[24] However, greater production of lactic acid was obtained from crude glycerol rather than pure glycerol using some microbial strains,[13,25] because some free fatty acids are used as nutrients for microbial growth. The results from this current study demonstrated that more lactic acid was produced by the wild type of *R. microsporus* LTH23 using cabbage glycerol media than in crude glycerol media. This result is in line with those from Vodnar et al. (2013),[13] who reported that a higher specific fungal biomass was gained using crude glycerol, compared with pure glycerol. Furthermore, higher lactic acid production was observed using biodiesel crude glycerol supplemented with lucerne green juice rather than just the crude glycerol media, due to the presence of various minerals and sugars (16.8 g/L) in lucerne juice. Moreover, the process of lactic acid production may be further improved by using a fed-batch process rather than a batch process for fermentation.

This is the first report on lactic acid production by a fungus using waste cooking oil glycerol. These findings indicate that treated waste cooking oil glycerol can be used as a C-source for lactic acid production by *R. microsporus* LTH23. However, it was found that the fungi favored glucose over glycerol, similar to the results of Wang *et al.* (2015).[15] They found that there was no lactic acid accumulation using glycerol as a sole C-source by *Rhizopus oryzae* ATCC 9363. Due to glycerol having a higher degree of reduction per carbon than glucose, under aerobic conditions, there is no lactic acid accumulation using glycerol. Additionally, they also found that the intracellular pyruvate concentration could enhance lactic acid formation when 40 mM sodium pyruvate was added to glycerol media every 24 h. Higher lactic acid levels of 1.33 g/L at 37 °C and 0.67 g/L at 30 °C were produced under microaerobic conditions. However, we demonstrate here that the addition of cabbage extract to glycerol media promoted more lactic acid accumulation under microaerobic conditions.

Response surface methodology is a powerful tool to determine the optimum parameters for production of molecules by fermentation. In the present study, a quadratic model was fitted to a second-order polynomial equation. The optimum values were determined to be pH 6.5, urea 3.75 g/L, and glycerol 17 g/L using this model. The predicted maximal production of lactic acid from the model was 4.03 g/L. Lactic acid production experimentally (corresponding with the optimum condition) had an average of 3.99 g/L, in good correlation with the model's predictions.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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