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ORIGINAL PAPER

Biosynthesis of 1,3-propanediol from recombinant *E. coli* by optimization process using pure and crude glycerol as a sole carbon source under two-phase fermentation system

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Abstract The environmental and nutritional condition for 1,3-propanediol (1,3-PD) production by the novel recombinant *E. coli* BP41Y3 expressing fusion protein were first optimized using conventional approach. The optimum environmental conditions were: initial pH at 8.0, incubation at 37 °C without shaking and agitation. Among ten nutrient variables, fumarate, $(NH_4)_2HPO_4$ and peptone were selected to study on their interaction effect using the response surface methodology. The optimum medium contained modified Riesenberg medium (containing pure glycerol as a sole carbon source) supplemented with 63.65 mM fumarate, 3.80 g/L $(NH_4)_2HPO_4$ and 1.12 g/L peptone, giving the maximum 1,3-PD production of 2.43 g/L. This was 3.5-fold higher than the original medium (0.7 g/L). Two-phase cultivation system was conducted and the effect of pH control (at 6.5, 7.0 and 8.0) was investigated under anaerobic condition by comparing with the no pH control condition. The cultivation system without pH control (initial pH of 8.0) gave the maximum values of 1.65 g/L 1,3-PD, the 1,3-PD production rate of 0.13 g/L h and the yield of 0.31 mol 1,3-PD/mol crude glycerol. Hence, using crude glycerol as a sole carbon source resulted in 32 % lower 1,3-PD production from this recombinant strain that may be due to the presence of various impurities in the crude glycerol of biodiesel plant. In addition, succinic acid was found to be a major product during fermentation by giving the maximum concentration of 11.92 g/L after 24 h anaerobic cultivation.

Keywords Glycerol · 1,3-Propanediol · Recombinant *E. coli* · Optimization · Response surface methodology

Introduction

Glycerol can be bio-converted to 1,3-propanediol (1,3-PD), a value-added chemical used as a monomer for the synthesis of poly(propylene)terephtalate (PPT) (Zeng et al. 1993) which is widely used in the polymer industry. The microbial production of 1.3-PD involves two parallel pathways: oxidative and reductive pathway. Through the oxidative pathway, glycerol is dehydrogenated to dihydroxyacetone which is phosphorylated to dihydroxyacetone phosphate before entering glycolysis. In the reductive pathway, glycerol is dehydrated to 3-hydroxypropionaldehyde (3-HPA) which is further reduced to 1,3-PD with the consumption of reducing power NADH. 1,3-PD is not metabolized further, but excreted into the medium (Sun et al. 2003). To improve 1,3-PD production, we proposed to use a fusion protein of the related protein as a new approach for a more efficient coupling of the enzymes needed for 1,3-PD production (Rujananon et al. 2011). When protein structure of glycerol dehydratase (DHAB) is fused to 1,3-propanediol oxidoreductase isoenzyme (YQHD), the occurrence of 3-HPA that is converted from

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glycerol by DHAB should be immediately reduced to 1,3-PD by YQHD. From our previous work, we have constructed the recombinant *E. coli* BP41Y3 by assembling genes *dha*B1–*dha*B2 from *Clostridium butyricum* VPI1718 and *yqh*D from *E. coli*. The fusion gene of *yqh*D encoding NADP-dependent 1,3-propanediol oxidoreductase isoenzyme (PDORI) and *dha*B1 encoding the coenzyme B_{12}-independent glycerol dehydratase (GDHt) was assembled in the upstream of *dha*B2 encoding the coenzyme B_{12}-independent glycerol dehydratase (GDHt) activator (GDHtAc), yielding the recombinant strain *E. coli* BL21 (DE3)//pET22b+::*yqhD-dha*B1_*dha*B2 (strain BP41Y3). The strain BP41Y3 gave 10-fold higher 1,3-PD concentration than *E. coli* BL21 (DE3)//pET22b+::*yqhD-dha*B1_*dha*B2 (strain BP31Y2) expressing the recombinant enzymes simultaneously but in a non-fusion mode. This work aims to find out the optimum environmental and nutritional conditions for 1,3-PD production by combining the conventional and statistical methodology. In addition, the cultivation system under different pH control was also investigated in order to achieve the highest efficiency for 1,3-PD production.

Materials and methods

Bacterial strains and growth conditions

The novel recombinant strain *E. coli* BL21(DE3):: pET22b+//*yqh*D-*dha*B1_*dha*B2 (BP41Y3) expressing YqhD and DhaB1 in fusion mode from our previous work (Rujananon et al. 2011) was used in this study. Modified Riesenberg medium (mRb) (Riesenberg et al. 1991) for aerobic stage was consisted of (per liter): 7 g glycerol, 13.3 g KH_2PO_4, 4.0 g $(NH_4)_2HPO_4$, 1.7 g citric acid, 8.4 mg EDTA, 1.2 g $MgSO_4{\cdot}7H_2O$ and trace element. The trace element contained (per liter): 2.5 mg $CoCl_2{\cdot}6H_2O$, 15 mg $MnCl_2{\cdot}4H_2O$, 1.5 mg $CuCl_2{\cdot}2H_2O$, 3.0 mg H_3BO_3, 2.5 mg $Na_2MoO_4{\cdot}2H_2O$, 13 mg $Zn(CH_3COO)_2{\cdot}2H_2O$ and 100 mg Fe(III)citrate. The medium was supplemented with 100 μg/mL ampicillin to avoid the contamination during cultivation. This recombinant strain contained the plasmid harboring ampicillin resistance gene. So, addition of ampicillin had no effect on the growth and product formation of the recombinant strain. The fermentation culture was performed in a shake flask by incubation on a rotary shaker (150 rpm) for 16 h at 37 °C. Whole cells were harvested by centrifugation at 6,000×*g* for 15 min and resuspended with the medium for an anaerobic production stage. The mRb medium for the second stage was supplemented with 45 mg/L thiamine, 50 mM K^+–Na^+-tartrate, 50 mM di-Na^+-fumarate and 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultivation was performed in serum bottles and incubated at 37 °C for 24 h. 1,3-PD and other metabolite products such as succinic acid were then determined in triplicate.

Optimization of environmental condition for 1,3-PD production by conventional methodology

The effect of initial pH (5, 6, 7, 8 and 9) incubation temperature (25, 30, 37 and 45 °C), shaking speed (0, 40, 70 and 150 rpm) and agitation speed (0, 150 rpm) were stepwise investigated in mRb medium to study their effect on 1,3-PD production. The influences of all nutrients were simultaneously investigated under the optimum environmental condition. Nutrient variables were glycerol (4, 7, 10 and 15 g/L), fumarate (0, 10, 50 and 100 mM), glucose (0, 2, 4 and 6 g/L), methionine (0, 5, 10 and 15 mM), inorganic nitrogen [$(NH_4)_2HPO_4$, NH_4Cl, $(NH_4)_2SO_4$ at 4 g/L] and organic nitrogen (yeast extract, peptone and urea at 4 g/L). The selected inorganic and organic nitrogen sources were varied at 2, 4 and 6 g/L. Samples from each parameter were taken after 24 h cultivation to determine 1,3-PD concentration. The nutrients affecting 1,3-PD production were selected to study their interaction effect at a fixed glycerol concentration by using the response surface methodology (RSM).

Interaction of selected medium for 1,3-PD production by using the RSM

Compared with conventional method, RSM is a time and labor saving method which displays the interaction between the components of medium and also find out the optimum levels (Ghadge and Raheman 2006). The central composite design (CCD) was used in this study. It allows estimating the second degree polynomial of the relationships between the factors and the dependent variable and gives information about interaction between variables in their relation to the dependent variable (Zheng et al. 2008). The levels and codes variable for CCD is shown in Table 1. A 2^3-factorial CCD with six axial points ($\alpha = 0.5$) and six replications at the centre points ($n_0 = 6$) leading to a total number of 20 experiments was employed, to determine the selected nutrients for the maximum production of 1,3-PD.

Table 1 Levels and codes variable for central composite design

Variables	Coded and actual levels				
	−1	−0.5	0	0.5	1
$(NH_4)_2HPO_4$ (g/L), X_1	1	2	3	4	5
Peptone (g/L), X_2	1	2	3	4	5
Fumarate (mM), X_3	10	32.5	55	77.5	100

The variables were coded according to the following equation, Eq. (1).

$$x_i = \frac{(X_1 - X_0)}{\Delta X}, \quad i = 1, 2, \ldots, k \tag{1}$$

where X_i is the coded value of the variable, X_1 the real value of the variable, X_0 the real value of X_1 at the center point, and ΔX the step change value. A quadratic model, Eq. (2), including all interaction term, was used to evaluate the predicted response.

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \beta_{ij} x_i x_j \tag{2}$$

where Y_i is the predicted response, k the number of factors, x_i and x_j the input variables, β_0 the offset term, β_i the linear effects, β_{ii} the squared effects, and β_{ij} the interaction term.

The experiments were designed by using the Design Expert software, version 8.0.7.1 (Stat Ease Inc. Minneapolis, USA, trial version) and performed in duplicate. The polynomial equations for the three responses were validated by the statistical test called analysis of variance (ANOVA), for determination of the significance of each term in equations and also to estimate how well a model fit in each case. The design expert software was performed for regression and graphical analysis of data obtained. The optimum level of selected nutrients was obtained by solving the regression equation and also analysis the 3D response surface. F-test was employed to evaluate the statistical significance of the quadratic model; its quality was expressed by the determination coefficient of correlation (R^2).

Production of 1,3-PD from crude glycerol with different pH control system under two-phase fermentation system

The novel recombinant strain BP41Y3 was cultivated in a 3-L stirred tank bioreactor under two-phase fermentation process. In the first phase, the cells were cultivated in the mRb medium for an aerobic growth phase using pure glycerol as a substrate. Resazurin of 0.0005 % (v/v) was added as an indicator of aerobic-anaerobic condition. The cultivation was performed under agitation speed of 400 rpm, aeration rate of 1.5 vvm at 37 °C for 8 h. After that, the aeration was stopped and the culture was flushed with nitrogen gas for 30 min until the pink color of resazurin became colorless which indicated an anaerobic condition. IPTG was then added into the culture medium to a final concentration of 0.5 mM to induce protein expression. The culture medium was replaced by the optimum mRb medium (from RSM study) for an anaerobic 1,3-PD production phase using crude glycerol as substrate. The influence of pH control (at 6.5, 7.0 and 8.0) on 1,3-PD production was investigated by comparing with the no pH control (initial pH of 8.0) condition. Samples were taken every 6 h for 24 h to determine for 1,3-PD, 3-HPA and succinic acid.

Analytical methods

The biomass concentration was determined by measuring optical density at 600 nm and dry cell weight (grams dry weight per liter). Glycerol, 1,3-PD and succinic acid were determined by HPLC (Agilent 1200). The culture medium was centrifuged (at 12,000×g for 15 min), and the supernatant was filtered through a nitrocellulose membrane with 0.2-μm pores. The HPLC apparatus included: a quaternary pump; a manual injector; a refractive index detector; an online vacuum degasser; a thermostatted column compartment; an Aminex HPX-87H ion exclusion column (300 mm × 7.8 mm) (Bio-Rad, USA); and ChemStation Software. Operation conditions were: 20 μl sample volume; 5 mM H_2SO_4 as a mobile phase; flow rate of 0.7 ml/min; and a column temperature of 65 °C. 3-HPA was analyzed based on tryptophan colorimetric method according to the procedure as described by Circle et al. (1945).

Results

Optimization of environmental condition for 1,3-propanediol production by conventional methodology

The effects of the initial pH (pH 5–8), incubation temperature (25–45 °C), shaking speed (0–150 rpm) and agitation speed (0, 150 rpm) on 1,3-PD production from the novel recombinant strain BP41Y3 are shown in Fig. 1. The optimum initial pH was at alkaline pH of 8 giving the highest 1,3-PD of 1.36 g/L. Temperature outside the optimum range had a great influence on 1,3-PD production. The highest 1,3-PD production from the recombinant *E. coli* was achieved at 37 °C (1.39 g/L) but not significant difference from the production at 30 °C (1.30 g/L). No growth was observed at 45 °C. We hypothesized that remained oxygen in culture medium might be consumed quickly and homogeneous cultivation might occur by shaking or agitation. However, the results revealed that shaking speed (0–150 rpm) gave no significant difference in 1,3-PD (1.43 g/L) when compared with no shaking (1.40 g/L). Similar result was observed with agitation speed.

The optimum environmental conditions (initial pH of 8.0, incubation temperature of 37 °C without shaking and agitation) were used for subsequent experiment. The

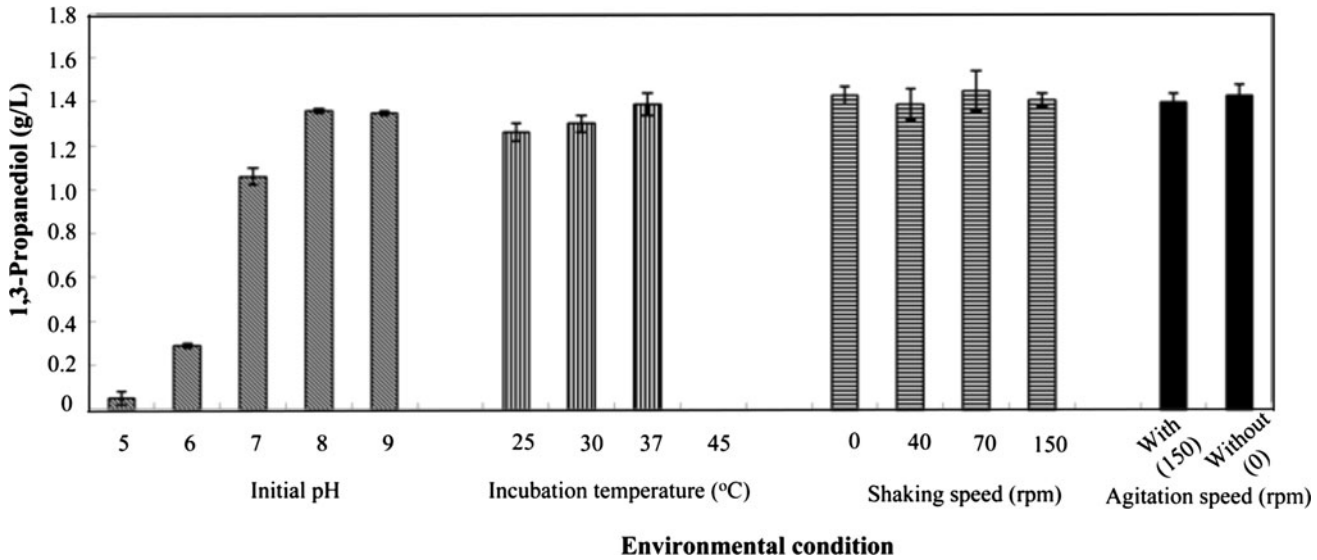

Fig. 1 A summary of the effects of the environmental condition for 1,3-propanediol production from recombinant *E.coli* strain BP41Y3 in mRb medium after 24 h cultivation under anaerobic fermentation step. The *error bars* are standard deviations from the average values of duplicate determinations

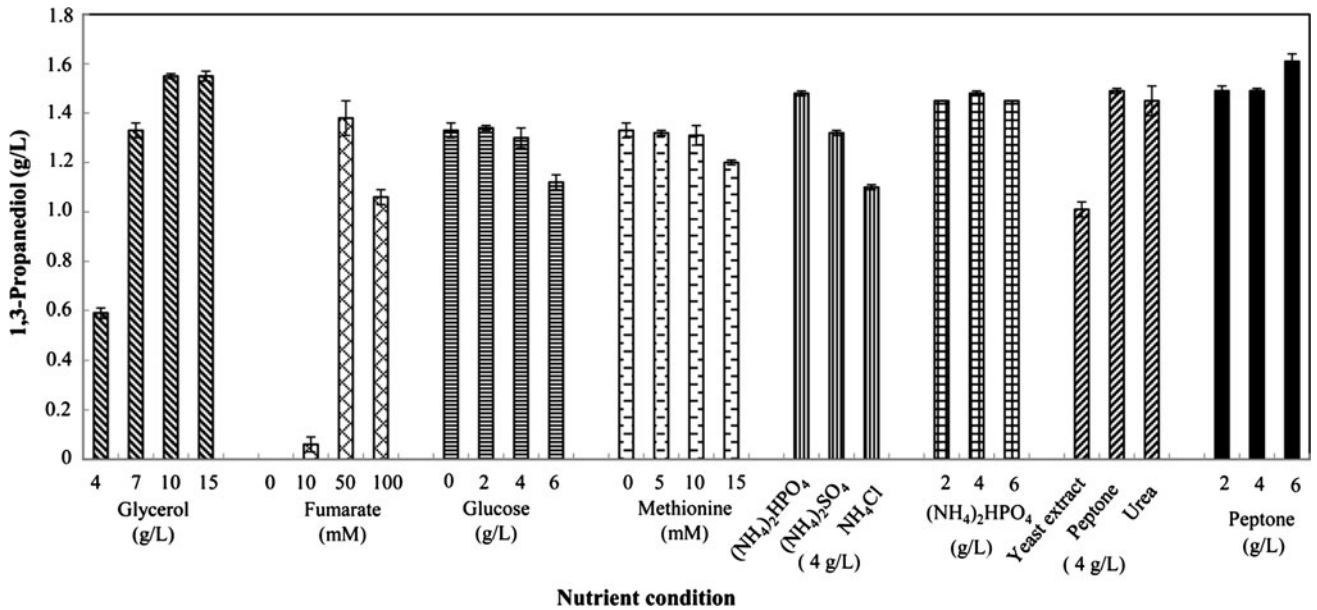

Fig. 2 A summary of the effects of various nutrients concentrations on 1,3-propanediol production from the recombinant *E.coli* strain BP41Y3 in mRb medium after 24 h cultivation under the optimum environmental condition from anaerobic production step. The *error bars* are standard deviations from the average values of duplicate determinations

effects of various nutrients (glycerol, fumarate, glucose, methionine and nitrogen) on 1,3-PD production are shown in Fig. 2. The recombinant *E. coli* could grow and produce 1,3-PD at every glycerol concentration tested (4–15 g/L). The optimum glycerol concentration was 10 g/L, giving the maximum 1,3-PD of 1.55 g/L. At different fumarate concentrations (0–100 mM) tested, the highest 1,3-PD (1.38 g/L) was obtained at 50 mM fumarate and no production of 1,3-PD without fumarate supplementation. In addition, fumarate could enhance glycerol consumption rate and 1,3-PD productivity as well as decreased 3-HPA accumulation. Therefore, fumarate is one of the key factors influencing 1,3-PD production. The role of glucose as co-substrate of glycerol for growth and product formation by the recombinant strain was tested. Addition of 0–4 g/L glucose had no effect on 1,3-PD production (1.30–1.33 g/L) while 6 g/L glucose caused the negative effect. S-adenosyl methionine (SAM) has been reported to enhance GDHt.

However, SAM is a high-cost molecule, looking for a cheaper alternative molecule such as methionine as a precursor of SAM was considered. Results indicated that addition of methionine (0–15 mM) had no significant effect on enhancing 1,3-PD production. Among inorganic nitrogen sources, $(NH_4)_2HPO_4$ at 4 g/L gave the maximum 1,3-PD (1.48 g/L). In case of organic nitrogen source, peptone and urea gave similar maximum 1,3-PD (1.49 and 1.45 g/L, respectively). Therefore, $(NH_4)_2HPO_4$ and peptone were selected to study their interaction effect with fumarate by using RSM.

Interaction of selected medium for 1,3-PD production by using statistical methodology

The effect of three variables [$(NH_4)_2HPO_4$ (A), peptone (B), fumarate (C)] on 1,3-PD production from the recombinant *E. coli* was studied. The experimental design matrix and result of CCD are presented in Table 2. In the Design Expert software, the response data were analyzed by default. The effects for all model terms were calculated and the statistical values, such as F value, lack of fit and coefficient of determination (R^2 value), were compared and a quadratic model was selected. The ANOVA results for all responses (Table 3) indicated that the lack-of-fit showed significance and the model was highly significant with very low probability values ($P < 0.0001$). The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental variables and their interactions. The data showed high R^2 value (0.9790). The low value of coefficient of variation (CV) (5.98) indicated good precision and reliability of the experiment. The 3D response surface is generally the graphical representation of the regression equation. Figure 3 represented the 3D response surface for the nutrient optimization of 1,3-PD production, calculated by setting the partial derivatives of Eq. (3) to zero with respect to the corresponding variables. The 3D response surface showed the effect of $(NH_4)_2HPO_4$ and peptone interaction (Fig. 3a), fumarate and peptone interaction (Fig. 3b), as well as $(NH_4)_2HPO_4$ and fumarate interaction (Fig. 3c) on 1,3-PD production.

It could be deduced from Fig. 3 that $(NH_4)_2HPO_4$ and fumarate had significant interactive influence on 1,3-PD production, but peptone had no interactive effect. However, if peptone was not added in the culture medium, it gave lower 1,3-PD (1.49 g/L) than that obtained from the addition of peptone (2.43 g/L) (Table 4) Therefore, peptone was one of the components influencing on 1,3-PD production.

According to the results of the statistical design, verification experiment was performed at the predicted condition and another two conditions to confirm the quadratic model given in Eq. (3). The 1,3-PD concentration from the experiment was in close agreement with the predicted value as shown in Table 4. It gave the percentage of deviation from predicted value at 10.99–15.24 %. This result confirmed that the model was adequate.

$$Y = 0.70 + 0.39(X_1) - 0.16(X_2) + 0.05(X_3) - 0.09(X_1)^2 + 0.03(X_2)^2 - 0.0004(X_3)^2 + 0.002(X_1X_2) + 0.002(X_1X_3) - 0.0008(X_2X_3) \quad (3)$$

Table 2 Experimental design matrix and results of CCD

Run	Actual levels[a]			1,3-Propanediol (g/L)	
	A	B	C	Predicted	Experimental
1	1.00	1.00	10.00	1.36	1.42 ± 0.087
2	5.00	1.00	10.00	0.74	0.65 ± 0.026
3	1.00	5.00	10.00	1.34	1.30 ± 0.075
4	5.00	5.00	10.00	0.75	0.76 ± 0.013
5	1.00	1.00	100.00	2.14	2.13 ± 0.007
6	5.00	1.00	100.00	2.09	2.14 ± 0.002
7	1.00	5.00	100.00	1.84	1.93 ± 0.043
8	5.00	5.00	100.00	1.83	1.77 ± 0.183
9	2.00	3.00	55.00	2.60	2.45 ± 0.132
10	4.00	3.00	55.00	2.44	2.66 ± 0.038
11	3.00	2.00	55.00	2.67	2.69 ± 0.006
12	3.00	4.00	55.00	2.60	2.66 ± 0.095
13	3.00	3.00	32.50	2.17	2.35 ± 0.224
14	3.00	3.00	77.50	2.63	2.53 ± 0.125
15	3.00	3.00	55.00	2.61	2.59 ± 0.157
16	3.00	3.00	55.00	2.61	2.66 ± 0.061
17	3.00	3.00	55.00	2.61	2.55 ± 0.054
18	3.00	3.00	55.00	2.61	2.50 ± 0.023
19	3.00	3.00	55.00	2.61	2.55 ± 0.020
20	3.00	3.00	55.00	2.61	2.60 ± 0.160

[a] $(NH_4)_2HPO_4$ (A), peptone (B), fumarate (C)

Table 3 Summary of the analysis of variance result for the response models for 1,3-propanediol production

Source	Sum of squares	Degree of freedom	Mean square	F value	*P* value
Model	7.66	9	0.85	51.86	<0.0001
Residual	0.16	10			
Lack of fit (LOF)	0.15	5	0.030	10.39	0.0113
Pure error	0.014	5	2.883×10^{-3}		
Total	7.83	19			

Coefficient of determination $R^2 = 0.9790$; Adj $R^2 = 0.9601$; CV = 5.98; Adeq precision = 21.310

where Y is the response value, as the 1,3-propanediol, X_1 = ammonium phosphate (g/l), X_2 = peptone (g/l), X_3 = fumarate (mM).

Production of 1,3-PD from crude glycerol with different pH control system under two-phase fermentation system

The cultivation system without pH control showed the accumulation of 3-HPA (1.25 mM) after 6 h anaerobic cultivation and decreased sharply from 6 to 12 h (0.30 mM) (Fig. 4). 3-HPA was not produced again led to no significant increase in 1,3-PD production (1.56 g/L) after 12 h and slightly increased (1.67 g/L) at 24 h cultivation. Under this system, the initial pH of 8.0 decreased to 6.38 after 6 h cultivation (data not shown). The formation of 3-HPA after 6 h in the anaerobic phase resulted in a decrease of cell growth at the same cultivation time. Meanwhile, the system with pH control at 7.0 showed

Table 4 Confirmation experimental design

Trial	Parameters			1,3-Propanediol (g/L)		% deviation
	X_1	X_2	X_3	Predicted	Experimental	
1	2.10	1.30	57.99	2.77	2.37 ± 0.07	14.44
2	2.83	4.89	62.11	2.69	2.28 ± 0.02	15.24
3	3.80	1.12	63.65	2.73	2.43 ± 0.07	10.99

X_1 = $(NH_4)_2HPO_4$ (g/L), X_2 = Peptone (g/L), X_3 = Fumarate (mM)

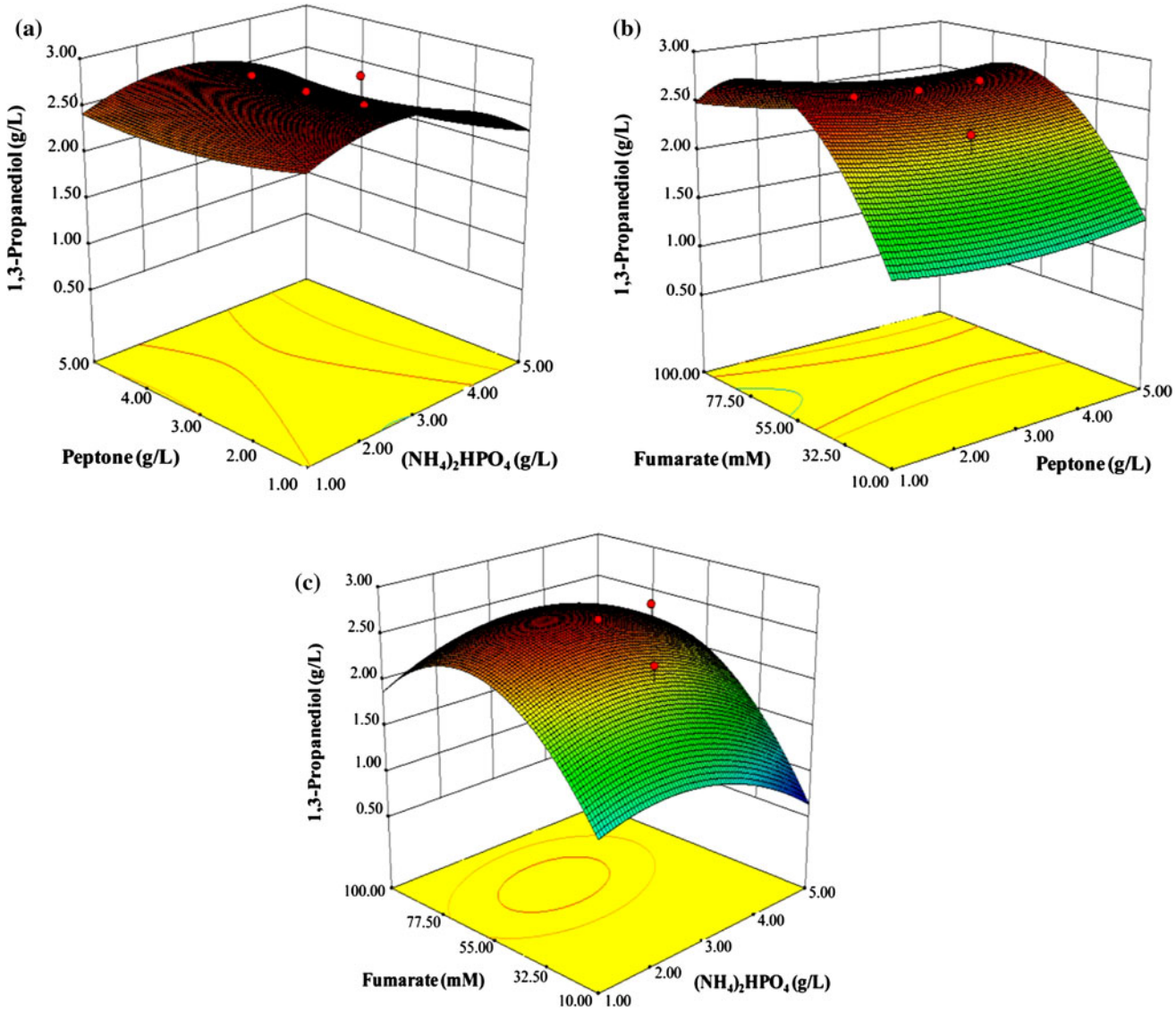

Fig. 3 3D response surfaces show the influence of **a** peptone and $(NH_4)_2HPO_4$, **b** fumarate and peptone, **c** fumarate and $(NH_4)_2HPO_4$ on the 1,3-PD production from the recombinant *E.coli* strain BP41Y3 cultivated in mRb medium with the initial pH of 8.0 and incubated at 37 °C for 24 h under anaerobic condition

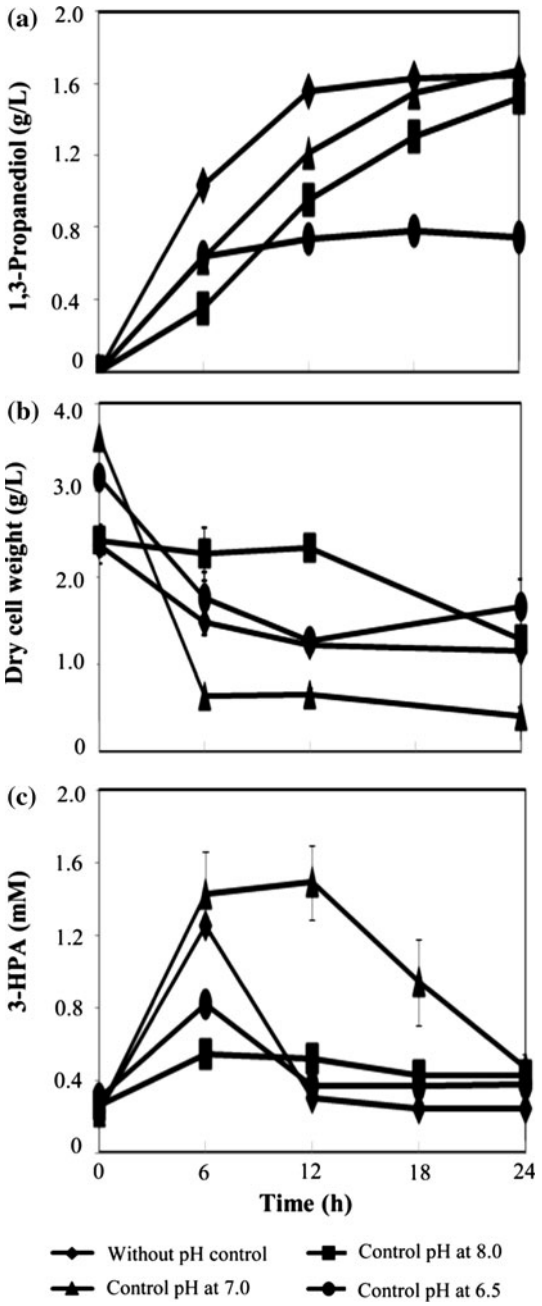

Fig. 4 Time course of 1,3-PD production (**a**), cell growth (**b**), and 3-HPA formation (**c**) during anaerobic cultivation of the recombinant *E.coli* strain BP41Y3 in the optimum mRb medium containing crude glycerol as a sole carbon source at 37 °C under different pH control systems

1.43 mM 3-HPA after 6 h and slightly increased to the peak level at 1.49 mM after 12 h before gradually decreasing to 0.47 mM at 24 h. The formation of 3-HPA led to the dramatically decrease of cell growth. However, the remained cells (0.5 g/L dry cell weight) could reduce the 3-HPA by gradually converting it to the 1,3-PD (1.67 g/L at 24 h cultivation). It was noticed that the level of 3-HPA at 6 h showed no significant difference with that after 12 h but 1,3-PD concentration increased twofold. Noticeably, the value of 3-HPA in the cultivation system with pH control at 8.0 was less than those of the other systems. This system showed the low peak level of 3-HPA but the production of 1,3-PD was gradually increased. This meant that there was no 3-HPA accumulation in the system with the balanced ratio of related enzyme activities that involve in the conversion of glycerol to 3-HPA and subsequently to 1,3-PD. When there was no 3-HPA accumulation, the cell growth did not decrease. In the case of cultivation system with pH control at 6.5, the peak level of 3-HPA was 0.82 mM leading to the 1,3-PD production of 0.64 g/L after 6 h. Both 3-HPA and 1,3-PD did not produce thereafter. The cell growth also declined at the same cultivation time. From this experiment, it was noticed that the cell growth from all cultivation systems decreased when 3-HPA formation was over 0.54 mM. Controlling of pH at 8.0 seemed to be the best condition for 1,3-PD production because there was no 3-HPA accumulation and no decrease of cell growth. However, it gave the lowest production rate when compared with the other conditions (data not shown). Therefore, cultivation system without pH control was the best condition for 1,3-PD production in this study. It exhibited the highest production rate of 0.13 g/L h, the maximum yield of 0.31 mol 1,3-PD/mol crude glycerol and maximum 1,3-PD of 1.65 g/L after 12 h. Succinic acid was found to be a major product during fermentation by giving the maximum concentration of 11.92 g/L after 24 h anaerobic cultivation.

Discussion

Optimization of environmental condition for 1,3-propanediol production by conventional methodology

The pH is one of the key factors affecting growth and 1,3-PD production because the catalytic activity of the enzyme and the metabolic activity of the microorganism depend on the extracellular pH (Pirt 1975). The optimum pH at 8.0 for the recombinant *E.coli* was the same as that of *K. pneumoniae* (pH 8.0) (Zhang et al. 2007). This was due to the fact that a higher pH value was contributed to 1,3-PD formation while the low pH inhibited the cell growth and product formation (Zhang et al. 2007). According to the report of BRENDA database, the optimum pH value for the GDHt activity of *Klebsiella* sp. was in the range of 7.5–9.5 while that of recombinant *E.coli* was pH 8.5 (Qi et al. 2006). The optimum pH for PDORI activity from *Clostridium* sp. and *Klebsiella* sp. were in the range of 9–9.72 while pH 7–8 was the optimum pH for *Enterobacter agglomerans* (Barbirato et al. 1995). However, the optimum

pH value of GDHt from *Clostridium* and 1,3-PDORI from *E. coli* have not been reported so far.

The recombinant *E. coli* had the optimum incubation temperature for production of 1,3-PD at 37 °C and no growth was observed at 45 °C. This was similar to complete inhibition of the 1,3-PD forming activity of *K. pneumoniae* at 45 °C (Zhang et al. 2007). Other recombinant *E.coli* showed high efficiency for 1,3-PD production when the cultivation temperature was shifted from 30 to 42 °C that might be because its expression vector (pBV220) could function well at high temperature (Tang et al. 2009). In this study, the expression vector (pET22b+) can normally function at 37 °C or lower than that (18–25 °C) (Novagen, Germany). No growth at 45 °C of this strain was contrary to the report that the optimum temperature of GDHt from recombinant *E.coli* was at 45 °C (Qi et al. 2006).

The recombinant *E. coli* produced 1,3-PD under strict anaerobic condition (Raynaud et al. 2003). The recombinant strain (in this study) harbored *dha*B1 gene coding for vitamin B_{12}-independent glycerol dehydratase from *C. butyricum* that was extremely sensitive to oxygen. Therefore, the anaerobic condition was created by flushing with N_2 gas. The results clearly indicated that shaking (0–150 rpm on a rotary shaker) and agitation (0 and 150 rpm on a magnetic stirrer) had no effect on 1,3-PD production from the recombinant *E. coli.*

A substrate metabolism is required to maintain the redox balance of the cell, to provide the ATP necessary for maintenance processes (Meinander et al. 1996). Pure and crude glycerol were used as a substrate acting as a carbon and energy source for 1,3-PD synthesis from the recombinant strain. The optimum glycerol concentration was found to be 10 g/L, giving the 1,3-PD of 1.55 g/L. It should be noted that this recombinant strain could tolerate 15 g/L glycerol while the other recombinant *E.coli* could not tolerate over 5 g/L (Tong et al. 1991; Skraly et al. 1998; Zhu et al. 2002).

Unlike glycerol, fumarate is used as the external electron acceptor for cultivation of anaerobic microorganism. It was used to speed up the metabolic flux of 1,3-PD by increasing the activities of the key enzymes and decreasing of NAD+/NADH ratio (Lin et al. 2005). Fumarate is a precursor of TCA cycle relating to the available of reducing power that needed for PDORI activity. Not only the availability of reducing powers from TCA cycle, ATP generation also affects on 1,3-PD production as it influenced on the cells to maintain homeostasis (the property of a system that regulates its internal environment and tends to maintain a stable, constant condition of properties like temperature or pH). The optimum fumarate concentration for production of 1,3-PD from the recombinant *E. coli* was 50 mM. This was 10 times higher than the amount of 5 mM fumarate, resulting in the increase of glycerol dehydratase (160 %) and PDOR (25 %) activities (Lin et al. 2005). In addition, fumarate could enhance the glycerol consumption rate and the 1,3-PD productivity as well as decrease 3-HPA accumulation. It also gave more than 30-fold increase in the specific activity of anaerobic G3P dehydrogenase and a significant decrease in concentrations of intracellular G3P resulting in better cell growth and an improved production of 1,3-PD (Zhu et al. 2002). Therefore, fumarate is one of the key factors influencing 1,3-PD production.

Glucose was reported to be one of the medium components that could be used as co-substrate to produce 1,3-PD from glycerol (Tong et al. 1991; Skraly et al. 1998; Wang et al. 2007). Glucose catabolism is used by the cells to produce energy and reducing power (NADH), whereas glycerol is used to produce 1,3-PD (Abbad-Andaloussi et al. 1998). Co-substrate fermentation in this study revealed the negative effect of glucose at 6 g/L on 1,3-PD production which might be because glucose had an inhibitory effect on the induction of enzymes for glycerol metabolism (Malaoui and Marczak, 2001). This was supported by Abbad-Andaloussi et al. (1998) that GDHt and PDOR activity were not detected when glucose was used as a sole carbon source. Production of 1,3-PD from glycerol and glucose as co-substrate by recombinant *E.coli* harbouring 1,3-PD operon from *Klebsiella pneumoniae* M5al gave higher yield of 1,3-PD (0.63 mol/mol) than without adding glucose (0.46 mol/mol). However, the 1,3-PD production was inhibited at too high glucose concentration (Biebl and Martin 1995).

All the native 1,3-PD producing strain, such as *K. pneumoniae* and *Clostridium pasteurianum*, requires vitamin B_{12} as a coenzyme for GDHt enzyme during conversion of glycerol to 1,3-PD.

One of the most development in 1,3-PD production is the recognition of a new superfamily of enzymes that utilizes S-adenosyl methionine instead of adenosylcobalamin (vitamin B_{12}) to catalyze radical reactions at enzymatic sites (Frey and Magnusson 2003, Cheek and Broderick 2001). SAM is synthesized from adenosine triphosphate (ATP) and methionine by methionine adenosyltransferase. In the presence of 4 mM SAM, the specific activity of GDHt in the culture medium increased 2.5-fold (Raynaud et al. 2003). In this study, the addition of methionine (10–15 mM) gave no significant increase of 1,3-PD production which might be due to the inadequate of ATP in the system to interact with methionine to synthesize SAM. This is the first report to study the effect of methionine on 1,3-PD production although it still had no evidence to confirm its reaction.

Among all nitrogen sources tested, $(NH_4)_2HPO_4$ and peptone gave the maximum production of 1,3-PD. Besides

being a nitrogen source, $(NH_4)_2HPO_4$ also kept the medium pH constant and increased the quantities of key enzymes for 1,3-PD production (Oh et al. 2008). Phosphate could improve the efficiency for 1,3-PD production and stimulate the complete metabolism of bacteria (Garg and Jain, 1995). $(NH_4)_2HPO_4$ was also found to be the best inorganic nitrogen source for 1,3-PD production from *K. pneumoniae* SU6 (Sattayasamitsathit et al. 2011) whereas Zhang et al. (2007) reported $(NH_4)_2SO_4$ was the best source. During fermentation, the elements of organic nitrogen (peptides and free amino acids) are taken up from the medium by the cell and directly assembled into proteins or transformed into other cellular nitrogenous compounds. Peptone contains many biological compounds (such as peptides, diketopiperazines and amino acids) which play an important role in cell metabolism by supplying essential amino acids and metabolic energy. In contrast to inorganic nitrogen source, the cell consumes more energy and time in assembling amino acids for protein synthesis (Mongi et al. 2005). Therefore, $(NH_4)_2HPO_4$ and peptone were selected to study their interaction effect with fumarate by using RSM.

Interaction of the selected medium for 1,3-PD production by using statistical methodology

Statistical values such as F value, lack of fit and R^2 value were used for comparing the models and consequently a quadratic model was selected. The model terms in the equations are calculated after elimination of some insignificant variables and their interactions which have the lowest F value (Aghaie et al. 2009). Lack of fit is not desirable, so a low F value and probability >0.1 are desired (non-significant). The R^2 value closer to 1.0 (0.9790) indicated the better the model predicts the response (Liu and Chiou, 2005). The low value of coefficient of variation (5.98) indicates good precision and reliability of the experiment (Zinatizadeh et al. 2006). Validation of the models by cultivation the recombinant *E. coli* gave the highest 1,3-PD production under the optimum medium composition of 3.80 g/L $(NH_4)_2HPO_4$, 1.12 g/L peptone and 63.65 mM fumarate in the mRb medium. It was 3.5 fold higher 1,3-PD concentration than that from the original medium (0.7 g/L).

Production of 1,3-PD from crude glycerol with different pH control system under two-phase fermentation system

3-HPA is a toxic intermediate metabolite that converted from glycerol, then reduced to 1,3-PD by PDORI with the association of cofactor NADPH. Therefore, avoiding 3-HPA accumulation could be achieved by either the enhancement of PDORI activity or the increment of NADPH as a cofactor. In this study, 3-HPA and pH values were determined. Without pH control, the pH dropped to pH 6.38 (from the initial pH of 8.0). This pH value might influence on the GDHt activity because the optimum pH for this enzyme is mainly alkaline pH (pH 7.5–9.5 as stated earlier). Control of pH at 7.0 gave the significant twofold increase of 1,3-PD while no significant increase of 3-HPA during 6 and 12 h cultivation. This might be due to the balance of GDHt and PDORI leading to the balance conversion of glycerol to 3-HPA and 3-HPA to 1,3-PD. Cultivation system with control of pH at 8.0 gave lower 3-HPA to 1,3-PD than the other systems. 3-HPA accumulation was reported to be more likely to occur at low pH value than at high pH value (Barbirato et al. 1996). Low production of 1,3-PD at the pH control of 6.5 was due to the fact that the pH value in culture broth was not suitable for the activities of GDHt and PDORI. The low yield of 1,3-PD was anticipated to be due to the accumulation of 3-HPA during cultivation which has an inhibitory effect on glycerol consumption (Rasch 2002) and also GDHt activity (Barbirato et al. 1996). In addition, 3-HPA also related to the ratio of GDHt and PDOR activities (Zhuge et al. 2010). At higher level of 3-HPA, PDOR activity was more sensitive than GDHt (Saxena et al. 2009). PDOR and its isoenzyme are NADH- and NADPH-dependent enzymes, respectively (Laffend and Nagarajan 1997). The inadequate amount of these reducing equivalents available from the oxidative pathway of glycerol as a cofactor might affect on the catalyzation of the enzyme for the conversion of 3-HPA to 1,3-PD completely which led to the accumulation of 3-HPA. Therefore, the accumulation of 3-HPA may be because of the following reasons; (1) the unbalanced ratio of GDHt and PDOR activities and (2) the amount of reducing equivalent available from the oxidative pathway of glycerol as a cofactor for PDOR or PDORI activity.

In conclusion, the combination of conventional and statistical methodology were used to reach the maximum production of 1,3-PD from the novel recombinant *E. coli* BP41Y3 expressing fusion protein. Under the optimized condition, the concentration of 1,3-PD increased 3.5 folds (2.43 g/L) comparing to that using the original medium (0.7 g/L) (Rujananon et al. 2011). Cultivation system under two-phase fermentation process (aerobic-anaerobic) without pH control was found to be the best condition for 1,3-PD production from this study.

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