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Optimization of polyhydroxyalkanoate (PHA) production by *Burkholderia cepacia* BPT1213 utilizing waste glycerol as the sole carbon source

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ABSTRACT

Aims: This study was carried out to optimize the fermentation conditions using statistical approach for polyhydroxyalkanoate (PHA) production by a local isolate, *Burkholderia cepacia* BPT1213, in the shake flask system.
Methodology and results: Throughout this study, *B. cepacia* BPT1213 was grown in minimal salt medium (MSM) supplemented with 2% of waste glycerol (86.70% purity). The strain can produce up to 1.33 g/L cell dry weight (CDW) with 22.21% of PHA content, thus giving a total PHA concentration 0.30 g/L before optimization. A factorial design experiment that was carried out showed all parameters KH_2PO_4, $Na_2HPO_4 \cdot 2H_2O$, carbon-to-nitrogen ratio (C/N), initial pH of medium, and temperature significantly affected the growth (cell dry weight, CDW) and PHA content. Response surface methodology (RSM) using central composite design (CCD) was then applied to optimize these parameters. The optimum conditions suggested were at 2.5 g/L KH_2PO_4, 4.5 g/L $Na_2HPO_4 \cdot 2H_2O$, 30 (g/g) C/N ratio, initial medium pH of 8.5 and 37 °C cultivation temperature, with a predicted CDW of 3.43 g/L and PHA content of 45.71% contributing to 1.57 g/L total PHA concentration. The verification experiment resulted in 3.60 g/L of CDW with 48.08% of PHA content contributing to 1.73 g/L total PHA concentration.
Conclusion, significance and impact of study: The statistical approach using factorial design and RSM have succeeded in increasing the production of PHA by *B. cepacia* BPT1213 using waste glycerol as the sole carbon source which is a promising renewable and cheaper feedstock.

Keywords: Bulkholderia cepacia, polyhydroxyalkanoate (PHA), response surface methodology (RSM), optimization, glycerol

INTRODUCTION

Conventional petrochemical plastic possesses versatile properties that fit a lot of applications, somehow making the use of plastic difficult to be controlled due to the growing population (Kathirvale *et al.*, 2003). Its excellent properties which are durable and highly resistant to the environment give it a very long lifespan, causing it to be accumulated as solid wastes after being discarded (Loo and Sudesh 2007). Polyhydroxyalkanoate (PHA) produced by microorganism has emerged as the best candidate to replace the conventional chemically synthesized polymers. PHA synthesis occurs when excess carbon source is available with at least one of the nutrients such as nitrogen, phosphorus, sulphur or oxygen present in limited concentration, restricting microbial growth (Anderson and Dawes, 1990; Poirier *et al*., 1995; Lee, 1996). Biosynthesis of PHA under stress conditions by limiting factors such as nitrogen or phosphate during microbial fermentation, was the most suggested by researchers (Papaneophytou and Kyriakidis, 2012). Apart of being fully biodegradable, PHA is of biological origin produced from renewable sources, thus allowing for a lower environmental impact. The PHA polymers have similar characteristics as that of the commercially used conventional plastic (Lageveen *et al*., 1988). PHAs also have numerous applications in medicine, pharmacy (implants, covering of pharmaceuticals), and packaging (Lemos *et al.*, 1998).

Carbon source is reportedly one of the major contribution to the high cost in PHA production. Waste glycerol has been suggested as a promising cheaper and renewable carbon source for PHA production. The production of biodiesel generally produces 10% glycerol waste as a major by-product (Chatzifragkou and Papanikolaou, 2012). Rapid expansion of this biodiesel industry consequently leads to a glut of waste glycerol, which giving rise to environmental concern. The application of waste glycerol as the feedstock for various value-added products can evade the problems in the waste glycerol management and at the same time reduces the feedstock cost (Yazdani and Gonzalez 2007;

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Santibáñez *et al*., 2011). *Burkholderia cepacia* is a potential industrial PHA producer as it can utilize various carbon substrates such as glycerol, levulinic acid, glucose, xylose and lactose as well as palm oil derivatives and fatty acids, producing between 22-70 % PHA content. A previous study using *Burkholderia* sp. USM JCM15050 able to produce up to 60-70% of PHA using glycerol and various palm oil derived products (Chee *et al*., 2010). Zhu *et al*., (2010) reported that *B. cepacia* mostly synthesized 3HB and the continuous feeding of levulinic acid as co-substrate to glycerol leads to the production of P(3HB-co-3HV) co-polymer by *B. cepacia* ATCC 17759 (Zhu *et al*., 2010; Zhu *et al*., 2013).

Since PHA production is influenced by multivariable factors, RSM can provide a systematic and efficient research strategy by limiting the number of experimentation (Grothe *et al*., 1999; Papaneophytou and Kyriakidis, 2012). This study was aimed to optimize the fermentation conditions for production of PHA by a local isolate, *B. cepacia* BPT1213 in MSM supplemented with 2% of waste glycerol (86.70% purity), a by-product of the production biodiesel from palm oil, as the sole carbon source. The factors that affect *B. cepacia* BPT1213 growth and PHA production were screened using factorial design. The optimization of PHA production was carried out using RSM. Central Composite Design (CCD) was used to develop a mathematical model by identifying the combination of significant factors for the design of the optimization experiment.

MATERIALS AND METHODS

Bacteria strain and inoculum preparation

The strain was maintained on nutrient agar containing (g/L in distilled water): Trypton, (10); Yeast extract (5); NaCl (7), and agar (15) as well as in glycerol (25% v/v) stocks stored at –20 °C (Sudesh *et al*., 2004). The inoculum was prepared by streaking the PHA producing bacteria from the stock culture onto the nutrient agar and incubating it at 30 °C for 24 h. The bacteria cells were activated in 30 mL nutrient broth and grown at 180 rpm, 30 °C for 12-24 h. Then, one mL of activated culture was transferred into mineral salt medium (MSM) supplemented with 2% (v/v) waste glycerol and grown at the same condition for inoculum preparation. Cultures obtaining an optical density (OD) = 1.5 was then inoculated to the sterile fresh MSM for PHA biosynthesis.

Fermentation medium

The MSM containing (g/L): $Na_2HPO_4{\cdot}2H_2O$ (4.5); KH_2PO_4 (1.5), $(NH_3)_2SO_4$ (0.5), 0.1 M $MgSO_4{\cdot}7H_2O$ (10.0 mL/L) (autoclaved separately); and trace element (1.0 mL) containing (g/L): $FeSO_4{\cdot}7H_2O$, (2.78); $MnCl_2{\cdot}4H_2O$, (1.98); CoSO4·7H2O, (2.81); $CaCl_2{\cdot}2H_2O$, (1.67); $CuCl_2{\cdot}2H_2O$, (0.17), and $ZnSO_4{\cdot}7H_2O$, (0.29) (filtered using a 0.2 µm sterile filter) (Sudesh *et al*., 2004). The MSM was supplemented with 2% (v/v) of glycerol (87.67%) as the sole carbon source. The final pH was adjusted to 7 using either 2M NaOH and 2M H_2SO_4. All separate components were autoclaved at 121 °C for 15 min.

PHA fermentation

The microbial cultivation was carried out in shake flask culture containing 50 mL of MSM and shaken at 180 rpm. The other cultivation condition was done according to experimental protocol designated by the statistical software. The cell was harvested after 72 h of fermentation.

Screening for the factors affecting the growth and PHA production by *B. cepacia* BPT1213

As a preliminary study of the factors that affect the growth and PHA production, a two-level factorial design from Design Expert 7.00 (Stat-Ease Inc., Minneapolis, USA) software package was used. The selected variables were cultivation temperature (27-37 °C), initial pH of the medium (5-9), carbon-to-nitrogen (C/N) ratio (15-35 g/g), concentration of $Na_2HPO_4{\cdot}2H_2O$ (3.0-6.0 g/L), and KH_2PO_4 (0.5-2.5 g/L). The experimental design was constructed by the 2^{5-1} fractional factorial design where all the parameters were investigated at high (+1) and low (-1) levels. The CDW and PHA percentage were determined as the response and their significance were analyzed by the software to a confidence level above 95 % ($P < 0.05$). All the significant factors were used in the subsequent optimization step.

Optimizing the growth and PHA production using Central Composite Design (CCD)

A rotatable CCD (Alpha= 1.5) from the same software package was used to identify the interactive effects between the significant variables. The culture was carried out according to the respective factor combination in central composite rotary design. Analysis of variance (ANOVA) of the responses was used to find out the interactive effects of the selected variables. The optimal point of each factor was predicted, and validated by actual experiments.

Verification of optimum point suggested by RSM

Burkholderia cepacia BPT1213 was cultivated at the optimal point suggested by the software. The CDW and PHA content were also determined every 6h for 72h of fermentation. The percentage of deviation between suggested optimal point and actual experimental result was compared, and kinetic parameters (biomass production rate, PHA production rate, biomass yield coefficient ($Y_{x/s}$), product yield coefficient ($Y_{p/s}$) were also determined.

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Analytical method

After fermentation, the cells were harvested by centrifugation at a speed of 7000 g for 10 min, and were subsequently freeze dried. The freeze-dried cells (25 mg) were methanolyzed in a mixture of 2 mL acidic methanol [15% (v/v) H_2SO_4] and 2 mL chloroform in screw-cap test tube at 100 °C for 140 min using digital dry bath. The reaction product was separated and dehydrated using Na_2SO_4 (Sudesh *et al*., 2004; Anis *et al*., 2012). The sample was analyzed using gas chromatography (Shimadzu GC-2014, Japan) with Flame Ionization Detector (FID) by using a capillary column SBP-1 (30 × 0.25 mm × 0.25 µm film thickness) according to the Braunegg method (Braunegg *et al*., 1978)**.** Caprilic methyl ester (1:500 caprylic acid in chloroform) was used as the internal standard (Lageveen *et al*., 1988; Zhu *et al*., 2010; Anis *et al*., 2012).

RESULTS AND DISCUSSION

Two-level-factorial design

Table 1: The experimental design and response for CDW and PHA content by *B. cepacia* BPT1213 in two-fractional factorial study.

Run	Factor					Response	
	A (g/L)	B (g/L)	C (g/g)	D	E (°C)	CDW (g/L)	PHA content (%)
1	0.5	3.0	15	5	37	0.16	27.45
2	2.5	3.0	15	5	27	0.23	11.44
3	0.5	6.0	15	5	27	0.17	21.74
4	2.5	6.0	15	5	37	0.19	27.03
5	0.5	3.0	35	5	27	0.53	25.83
6	2.5	3.0	35	5	37	0.56	38.66
7	0.5	6.0	35	9	37	0.26	27.07
8	2.5	6.0	35	5	27	0.41	21.19
9	0.5	3.0	15	9	27	0.75	25.65
10	2.5	3.0	15	9	37	3.47	23.24
11	0.5	6.0	15	9	37	3.27	34.29
12	2.5	6.0	15	9	27	2.02	12.49
13	0.5	3.0	35	9	37	0.8	39.29
14	2.5	3.0	35	9	27	1.42	31.66
15	0.5	6.0	35	5	27	0.65	32.21
16	2.5	6.0	35	9	37	2.99	46.08
17	1.5	4.5	25	7	32	1.07	24.87
18	1.5	4.5	25	7	32	1.1	27.09
19	1.5	4.5	25	7	32	1.23	26.77
20	1.5	4.5	25	7	32	1.19	25.88

A: KH_2PO_4, B: $Na_2HPO_4{\cdot}2H_2O$, C: C/N, D: Initial pH, E: Temperature

Twenty experiments were conducted following a combination of factors designed by 2^{5-1} fractional design consisting of 16 factorial points and 4 center points. From the result (Table 1), *B. cepacia* BPT1213 showed cell dry weights (CDW) in the range of 0.16-3.47 g/L and PHA content 11.44 - 46.08% from the total weight of dried cell. As supported by a previous study (Suhaila 2014), the growth of *B. cepacia* BPT1213 was ineffective at low initial pH of the culture medium as compared to high pH medium and temperature. Run no. 10 yielded the highest biomass of 3.47 g/L but lower PHA content (23.24%) while run no. 16 gave the highest PHA content, (46.08%) but showed slightly lower CDW (2.99 g/L). The growth of *B. cepacia* BPT1213 and PHA production were enhanced by high pH (9) and high temperature (37 °C).

Analysis of variance (ANOVA) for factorial design

Table 2: Summary of analysis of variance (ANOVA) by factorial design for CDW and PHA content in screening of factors affecting *B. cepacia* BPT1213 PHA production.

Model term	p-value	
	CDW	PHA
Model	0.0004	0.0018
A	0.0006	0.0121
B	0.0065	0.7970>
C	0.0031	0.0003
D	< 0.0001	0.0015
E	0.0004	0.0003
AB	0.0054	0.3234>
AC	0.0133	0.0012
AD	0.0008	0.0378
AE	0.0903>	0.0030
BC	0.9024>	0.0250
BD	0.0023	0.0622>
BE	0.0186	0.0491
CD	0.0006	0.0056
DE	0.0045	0.9118>
ABD	0.0003	0.8394>
Curvature	0.5259	0.0570
R^2	0.9992	0.9977
Std. Dev.	0.075	1.000

A: KH_2PO_4, B: $Na_2HPO_4{\cdot}2H_2O$, C: C/N, D: Initial pH, E: Temperature. > Denotes insignificant term (p-value>0.05)

Table 2 shows a summary of analysis of variance (ANOVA) for the screening of factors affected PHA production by *B. cepacia* BPT1213 using waste glycerol. Each single parameter was presented in coded term A, B, C, D and E. All the independent factors significantly affected both the CDW and PHA content, except $Na_2HPO_4{\cdot}2H_2O$ with *p*-value 0.7970 (*p*-value >0.05). As suggested by Johar *et al*., (2012) the insignificant factor can be eliminated if the *p*-value>0.05. However, $Na_2HPO_4{\cdot}2H_2O$ concentration (B) was a significant term to CDW and its interaction with the other individual variables also significantly affected CDW and PHA content. Thus, $Na_2HPO_4{\cdot}2H_2O$ concentration also was selected for the optimization process.

Optimization of the growth and PHA production using central composite design (CCD)

Following the preliminary experiments, all the significant factors were optimized using CCD order to obtain the optimum condition for PHA production by *B. cepacia* BPT1213. The same range was used for all the factors except for temperature (changed to 31-37 °C). The CCD (Table 3) consisted of 32 sets of experimental

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combinations including 16 of factorial points (run no.1-16), 10 axial points (alpha value, α=1.4) (run no. 17-26) and 6 repeated central points (run no. 27-32). The highest CDW (5.10 g/L) was obtained by run no. 13 but PHA content (33.65%) was comparatively low. Thus, the conditions used 2.5 g/L KH_2PO_4, 6 g/L $Na_2HPO_4 \cdot 2H_2O$, C/N ratio of 15 g/g, initial PH of 9, and 37 °C seemed favourable for microbial growth. On the other hand, run no. 16 showed the highest PHA content, (54.15%) but a low CDW of 3.03 g/L. This result confirms that limitation of nitrogen source (C/N = 35) promoted the PHA accumulation. The PHA content might also be enhanced by the limitation of one of the phosphate sources, KH_2PO_4, and $Na_2HPO_4 \cdot 2H_2O$.

Results revealed that low concentrations of phosphate elements and nitrogen did not promote the growth of *B. cepacia* BPT1213 (0.78 g/L) and PHA accumulation (39.52%) as shown in run no.15. In this instance, the presence of high nitrogen concentration (C/N=15) at low phosphate concentration did not improve PHA (0.83 g/L) and CDW (23.33%) as seen in run no.5. This proves that *B. cepacia* BPT1213 favours the limiting of nitrogen compared to phosphate limitation for increased PHA production. Khatipov *et al*., (1998) agreed that C/N ratio plays an important role in both growth and PHA production (Khatipov *et al*. 1998). In addition, the concentrations of nitrogen and phosphate must be sufficient to support the microbial growth and at the same time provide a stress condition (limiting factor) to trigger the PHA synthesis. Otherwise, it would just lead to the built up of biomass instead of PHA production (Sangkharak and Prasertsan, 2008; Papaneophytou and Kyriakidis, 2012). The growth of *B. cepacia* BPT1213 was found to be suppressed in the low pH medium where low CDW (0.20-0.47 g/L) was obtained, coupled with poor PHA content (23.33-42.05%). On the other hand, *B. cepacia* BPT1213 showed excellent growth and PHA content when grown in medium culture at pH 9. The growth of *B. cepacia* BPT1213 was also enhanced at higher temperature (37 °C). Johar *et al*., (2012) also suggested that the initial pH of medium and cultivation temperature affected the PHA production by *Camamonas* sp. EB172 based on their work using RSM (Johar *et al*., 2012).

Table 3: The experimental design and response for CDW and PHA content by *B. cepacia* BPT1213 in CCD for optimization experiment in shake flask culture.

Run no.	Point type	Factor					Response			
		A (g/L)	B (g/L)	C	D	E (°C)	CDW (g/L)		PHA content (%)	
							Actual	Predicted	Actual	Predicted
1	Fact	2.5	3.0	15	5.0	31	0.24	0.22	27.85	28.15
2	Fact	0.5	6.0	15	5.0	31	0.45	0.40	33.09	33.51
3	Fact	0.5	3.0	35	5.0	31	0.46	0.33	25.96	26.03
4	Fact	2.5	6.0	35	5.0	31	0.20	0.34	26.97	26.81
5	Fact	0.5	3.0	15	9.0	31	0.83	0.79	23.33	23.68
6	Fact	2.5	6.0	15	9.0	31	3.11	3.21	21.69	21.78
7	Fact	2.5	3.0	35	9.0	31	2.03	2.03	39.63	39.53
8	Fact	0.5	6.0	35	9.0	31	0.76	0.74	46.44	46.32
9	Fact	0.5	3.0	15	5.0	37	0.34	0.40	27.52	27.84
10	Fact	2.5	6.0	15	5.0	37	0.47	0.42	29.94	29.97
11	Fact	2.5	3.0	35	5.0	37	0.45	0.34	42.05	41.79
12	Fact	0.5	6.0	35	5.0	37	0.47	0.52	34.94	34.89
13	Fact	2.5	6.0	15	9.0	37	5.10	4.94	33.65	33.80
14	Fact	0.5	6.0	15	9.0	37	3.33	3.48	36.85	36.92
15	Fact	0.5	3.0	35	9.0	37	0.78	0.72	39.52	39.31
16	Fact	2.5	6.0	35	9.0	37	3.03	3.15	54.15	53.78
17	Axial	0.1	4.5	25	7.0	34	1.11	1.18	33.80	36.25
18	Axial	2.9	4.5	25	7.0	34	2.41	2.43	34.54	37.81
19	Axial	1.5	2.4	25	7.0	34	1.55	1.57	28.07	27.79
20	Axial	1.5	6.6	25	7.0	34	2.44	2.04	31.64	31.65
21	Axial	1.5	4.5	11	7.0	34	1.43	1.43	22.79	22.25
22	Axial	1.5	4.5	39	7.0	34	0.46	0.46	33.11	34.67
23	Axial	1.5	4.5	25	4.2	34	0.24	0.41	25.95	25.53
24	Axial	1.5	4.5	25	9.8	34	3.17	3.20	33.76	33.91
25	Axial	1.5	4.5	25	7.0	30	1.29	1.30	25.27	23.71
26	Axial	1.5	4.5	25	7.0	38	2.30	2.31	33.94	33.21
27	Center	1.5	4.5	25	7.0	34	1.85	1.81	28.64	28.67
28	Center	1.5	4.5	25	7.0	34	1.78	1.81	27.85	28.67
29	Center	1.5	4.5	25	7.0	34	1.87	1.81	29.09	28.67
30	Center	1.5	4.5	25	7.0	34	1.79	1.81	25.96	28.67
31	Center	1.5	4.5	25	7.0	34	1.60	1.81	27.26	28.67
32	Center	1.5	4.5	25	7.0	34	1.88	1.81	28.11	28.67

A: KH_2PO_4, B: $Na_2HPO_4 \cdot 2H_2O$, C: C/N, D: Initial pH, E: Temperature

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ANOVA result of optimization using CCD

Table 4: ANOVA result from CCD for response CDW of *B. cepacia* BPT1213, optimization in shake flask culture.

Model term	p-value	
	CDW	PHA
A	< 0.0001	0.0463
B	< 0.0001	<0.0001
C	< 0.0001	< 0.0001
D	< 0.0001	< 0.0001
E	< 0.0001	< 0.0001
A^2	-	< 0.0001
B^2	-	0.0052
C^2	< 0.0001	-
D^2	-	0.0052
E^2	-	0.0121
AB	< 0.0001	< 0.0001
AC	-	< 0.0001
AD	< 0.0001	-
AE	< 0.0001	< 0.0001
BC	-	-
BD	0.0037	0.0001
BE	-	-
CD	< 0.0001	< 0.0001
CE	< 0.0001	0.0256
DE	< 0.0001	0.0056
Lack of fit	0.2766 not significant	0.7881 not significant
R-Squared	0.9922	0.9915
Adj R-Squared	0.9873	0.9823
Pred R-Squared	0.9693	0.9533

A: KH_2PO_4, B: $Na_2HPO_4{\cdot}2H_2O$, C: C/N, D: Initial pH, E: Temperature

The ANOVA result for CDW (Table 4) showed all the five individual factors were significantly affecting the growth of *B. cepacia* BPT1213, as indicated by the *p*-value < 0.05, together with cross product terms AB, AD, BD, CD, CE,and DE, were the significant model term. Only linear term C^2 was significant giving a quadratic model, as suggested by the RSM software (Equation 1). The R^2 value (0.9922) supports the goodness of fit of the model, indicating that the responses fit the model with 99.22% certainty supported by adjusted R^2 (0.98730). The model terms for PHA content showed that the p-value for all individual factors were also *p*<0.05 indicating that they were highly influential in PHA production by *B. cepacia* BPT1213. The terms AB, AC, AE, BD, CD, CE and DE were found to be significant for cross product while A^2, B^2, D^2 and E^2 were significant for the linear terms. A quadratic model was also suggested for PHA content. Based on the R^2 value (0.9915), the variation on PHA content fit the model equation with a certainty of 99.15% with adjusted R^2 (0.9823) and predicted R^2 (0.9533).

The mathematical models for CDW (Equation 1) and PHA content (Equation 2) fit the second-order polynomial equation as given below:

Y [CDW(g/L)] = 1.80 +0.44A +0.16B -0.34 C +0.99D +0.36E -0.20AB +0.49AD+0.12BD -0.36CD -0.20CE +0.31DE- $0.43C^2$

(Equation 1)

Y [PHA (%)] = +27.86 +0.47 A + 1.48 B + 4.53 C + 2.91D +3.30 E -2.84AB +1.41 AC +2.10 A E +1.34 BD +3.29 C D +0.60 C E +0.78 DE ++$3.21A^2$ +$1.01B^2$+$1.01D^2$ + $0.88E^2$ (Equation 2)

Response surface plot and contour plot of the interaction between quadratic significant terms interaction with other terms are represented in Figure 1 for response CDW (quadratic term, C^2), CD (a) and CE (b), for PHA content (quadratic term A^2, B^2, D^2, and E^2) for interaction AB, AC, AE, BD, CD, CE and DE (c-i). A maximum CDW was obtained at pH 9, temperature 37 °C and when the C/N ratio was reduced, as high nitrogen concentration supports the growth of *B. cepacia* BPT1213. However further increase of nitrogen concentration (smaller C/N) did not enhance further increase of CDW otherwise reduced the CDW. All the interaction terms (AB, AC, AE, BD, CD, CE and DE) for PHA content showed minimum surface plot. Further increase or decrease of any values of that term will increase the PHA content due to unfavourable growth condition or depletion of nutrient.

Verification of predicted optimum point by CCD in actual experiment

Growth of *B. cepacia* BPT1213 and its attendant PHA production at the optimized conditions were compared to the values obtained prior to optimization (Table 5). For non-optimum condition (Hamizah, 2012) (1.5 g/L KH_2PO_4, 4.5 g/L $Na_2HPO_4{\cdot}2H_2O$, C/N ratio = 25, initial medium pH 7 and 30 °C) *B. cepacia* BPT1213 only obtained 1.33±0.04 g/L CDW and 22.21±1.56% of PHA content, resulting in 0.33 g/L total PHA concentration. Using the mathematical model attained from CCD, the optimal condition for both CDW and PHA content at 2.5 g/L KH_2PO_4, 4.5 g/L $Na_2HPO_4{\cdot}2H_2O$, C/N ratio = 30, initial pH of medium 8.5 and cultivation temperature of 37 °C were predicted to be 3.43 g/L and 45.71% respectively. From the actual fermentation, the CDW and PHA content were found to be 3.60±0.08 g/L and 48.08±0.23%, comparable to the predicted values with deviation of 4.96% and 5.18% respectively. The total PHA concentration at the optimal condition was 1.73±0.03g/L which was 5.77 folds higher than non-optimum total PHA concentration (0.33 g/L). Kinetic parameters (Table 5) for non-optimum condition are, biomass production rate; 0.02 g/Lh, PHA productivity; 4.16 $\times10^{-3}$ g/Lh, biomass yield ($Y_{x/s}$); 0.05 and PHA yield ($Y_{p/s}$); 0.05. All the kinetic values calculated in optimal condition were higher than for the condition before optimization. The kinetic values obtained were biomass production rate; 0.05 g/Lh, PHA productivity; 24.00 $\times10^{-3}$ g/Lh, $Y_{x/s}$; 0.14 (3 times higher), and $Y_{p/s}$; 0.07 (7 times higher).

Figure 2 shows the CDW, PHA content and total PHA concentration of the strain under optimal condition for 72 h. The sampling was taken after 24 h to obtain adequate amount of cells for quantification analysis. All the results showed the highest CDW, PHA content and total PHA concentration were obtained at 72 h fermentation.

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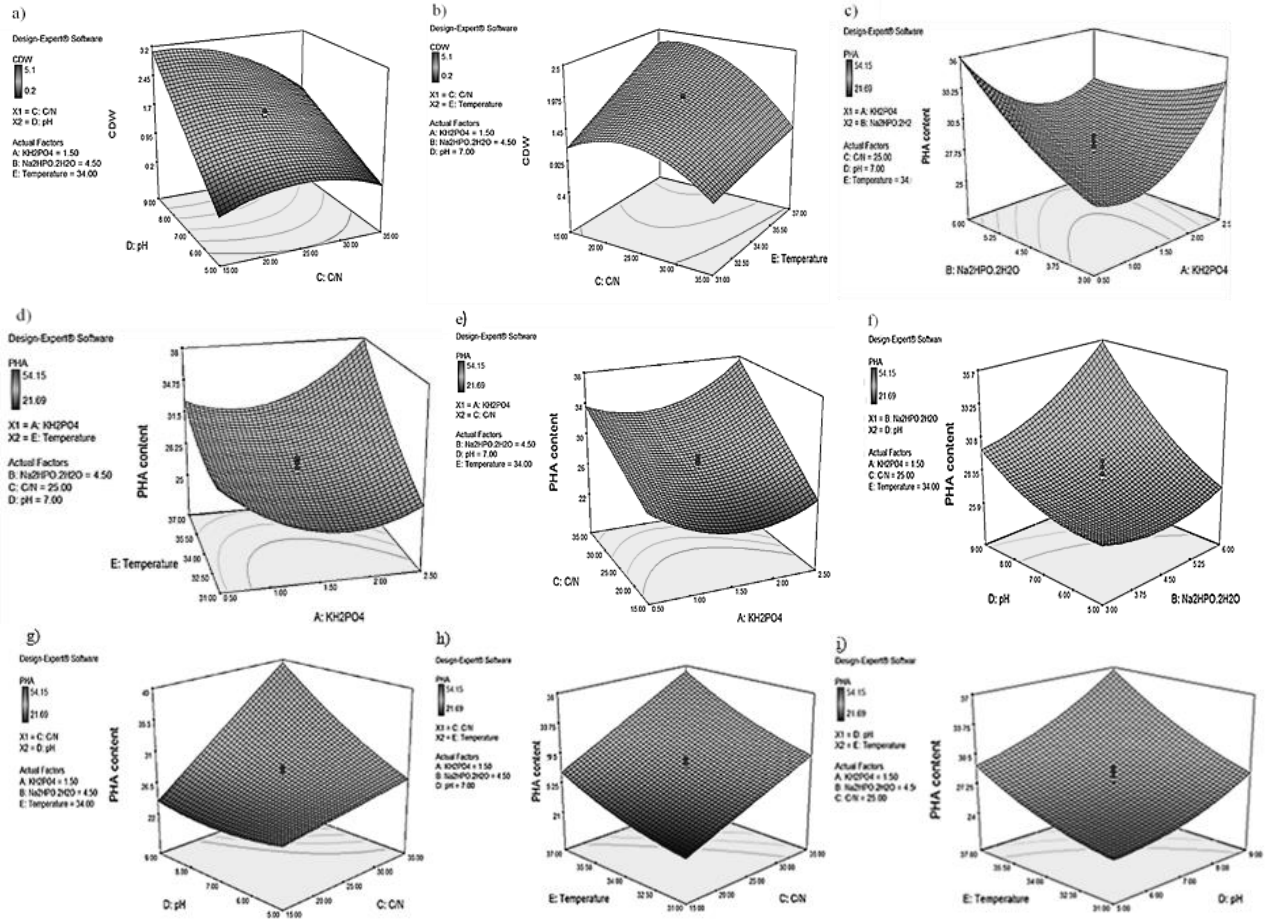

Figure 1: 3-Dimensional contour plot for CDW (a and b) and PHA content (c, d, e, f, g, h, and i) from the significant interaction between significant individual factor, A: KH_2PO_4, B: $Na_2HPO_4 \cdot 2H_2O$, C: C/N, D: Initial pH, E: Temperature.

Table 5: Summary of PHA production of *B. cepacia* BPT 1213 cultivated at condition before and after optimization in shake flask culture after 72 h.

Condition	CDW (g/L)	PHA content (%)	Total PHA concentration g/L	Biomass production rate g/Lh	PHA productivity g/Lh	Biomass yield $Y_{x/s}$	Product yield $Y_{p/s}$
Before optimization	1.33±0.04	22.21±1.56	0.30±0.03	0.018	4.16×10^{-3}	0.05	0.01
Optimal point	3.60±0.08	48.08±0.23	1.73±0.03	0.05	24.00×10^{-3}	0.14	0.07

*The kinetic value was calculated according to the average responses of CDW, PHA content and total PHA concentration

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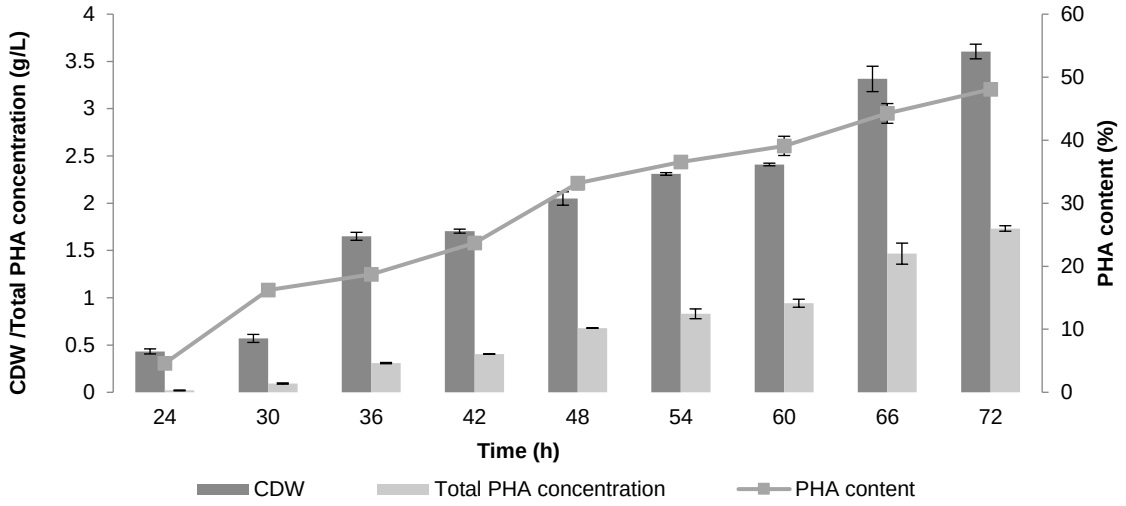

Figure 2: Plot of CDW, PHA content and total PHA concentration versus time by *B. cepacia* BPT1213 grown under optimal condition.

CONCLUSION

Through this study, *B. cepacia* BPT1213 produced up to 1.33 g/L cell dry weight (CDW) with 22.21% of PHA content (0.30 g/L total PHA concentration) before optimization. The factorial design experiment showed that all parameters KH_2PO_4, $Na_2HPO_4{\cdot}2H_2O$, carbon-to-nitrogen ratio (C/N), initial medium pH, and temperature significantly affected the CDW and PHA production. Using the optimum points suggested by CCD (2.5 g/L KH_2PO_4, 4.5 g/L $Na_2HPO_4{\cdot}2H_2O$, 30 (g/g) C/N ratio, initial medium pH of 8.5 and 37 °C cultivation temperature), 3.60 g/L of CDW with 48.08% of PHA content (1.73 g/L total PHA concentration) were obtained. The optimization improved PHA production 5.7 folds compared to the non-optimized condition.

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REFERENCES

Anderson, A. J. and Dawes, E. A. (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological Reviews* **54, 450-472.**

Anis, S. N. S., Nurhezreen, M. I., Sudesh, K. and Amirul, A. A. (2012). Enhanced recovery and purifucation of P(3HB-co-3HHx) from recombinant *Cupriavidus necator* using alkaline digestion method. *Applied Biochemistry and Biotechnology* **167, 524-535.**

Braunegg, G., Sonnleitner, B. and Lafferty, R. M. (1978). A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. *European Journal of Applied Microbiology and Biotechnology* **6, 29-37.**

Chatzifragkou, A. and Papanikolaou, S. (2012). Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes. *Applied Microbiology and Biotechnology* **95, 13-27.**

Chee, J. Y., Tan, Y., Samian, M. R. and Sudesh, K. (2010). Isolation and characterization of a *Burkholderia* sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty acids and glycerols. *Journal of Polymers and the Environment* **18, 584-592.**

Grothe, E., Moo-Young, M. and Chisti, Y. (1999). Fermentation optimization for the production of poly(beta-hydroxybutyric acid) microbial thermoplastic. *Enzyme and Microbial Technology* **25, 132-141.**

Hamizah, N. (2012). Study on isolation and screening of PHA producing bacteria from waste glycerol. Final Year Project Thesis, Universiti Sains Malaysia.

Johar, N. A. M., Hassan, M. A., Zakaria, M. R., Yee, P. L., Shirai, Y. and Ariffin, H. (2012). Evaluation of factors affecting polyhydroxyalkanoates production by *Comamonas* sp. EB172 using central composite design. *Malaysian Journal of Microbiology* **8, 184–190.**

Kathirvale, S., Muhd Yunus, M., Sopian, K., and Samsuddin, A. (2003). Energy potential from municipal solid waste in Malaysia. *Renewable Energy* **29, 559-567.**

Khatipov, E., Miyake, M., Miyake, J. and Asada, Y. (1998). Accumulation of poly-β-hydroxybutyrate by *Rhodobacter sphaeroides* on various carbon and nitrogen substrates. *FEMS Microbiology Letters* **162, 39-45.**

Lageveen, R. G., Huisman, G. W., Preusting, H., Ketelaar, P., Eggink, G. and Witholt, B. (1988). Formation of polyesters by *Pseudomonas oleovorans*:

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkenoates. *Applied and Environmental microbiology* **54, 2924–32.**

Lee, S. Y. (1996). Bacterial polyhydroxyalkanoates. *Biotechnology and Bioengineering* **49, 1-14.**

Lemos, P. C., Viana, C., Salgueiro, E. N., Ramos, A. M., Crespo, J. P. S. G. and Reis, M. A. M. (1998). Effect of carbon source on the formation of polyhydroxyalkanoates (PHA) by a phosphate-accumulating mixed culture. *Enzyme and Microbial Technology* **22, 662-671.**

Loo, C. and Sudesh, K. (2007). Polyhydroxyalkanoates : Bio-based microbial plastics and their properties. *Malaysian Polymer Journal* **2, 31-57.**

Papaneophytou, C. P. and Kyriakidis, D. A. (2012). Optimization of polyhydroxyalkanoates production from *Thermus thermophilus* HB8 Using response surface methodology. *Journal of Polymers and the Environment* **20, 760-773.**

Poirier, Y., Nawrath, C. and Somerville, C. (1995). Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. *Nature Biotechnology* **13, 142-150.**

Sangkharak, K. and Prasertsan, P. (2008). Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. *Electronic Journal of Biotechnology* **11, 1-12.**

Santibáñez, C., Varnero, M. T. and Bustamante, M. (2011). Residual Glycerol from Biodiesel Manufacturing, Waste or Potential Source of Bioenergy: A Review. *Chilean Journal of Agricultural Research* **71, 469-475.**

Sudesh, K., Few, L. L., Azizan, M. N. M., Majid, M. I. A., Samian, M. R. and Najimudin, N. (2004). Biosynthesis and characterization of polyhydroxyalkanoate blends accumulated by *Pseudomonas* sp. USM 4–55. *Jurnal Biosains* **15, 15-28.**

Suhaila, S. (2014). Study on optimization of PHA production by *Burkholderia cepacia* (s11b) in shake flask and 2 L bioreactors. Final Year Project Thesis, Universiti Sains Malaysia.

Yazdani, S. S. and Gonzalez, R. (2007). Anaerobic fermentation of glycerol: A path to economic viability for the biofuels industry. *Current Opinion in Biotechnology* **18, 213-219.**

Zhu, C. C., Chiu, S., Nakas, J. P. and Nomura, C. T. (2013). Bioplastics from waste glycerol derived from biodiesel industry. *Journal of Applied Polymer Science* **130, 1-13.**

Zhu, C., Nomura, C. T., Perrotta, J. A., Stipanovic, A. J. and Nakas, J. P. (2010). Production and characterization of poly-3-hydroxybutyrate from biodiesel-glycerol by *Burkholderia cepacia* ATCC 17759. *Biotechnology Progress* **26, 424-430.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538