Enhanced degradation of polyhydroxyalkanoates (PHAs) by newly isolated *Burkholderia cepacia* DP1 with high depolymerase activity

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Abstract The contribution of microbial depolymerase has received much attention because of its potential in biopolymer degradation. In this study, the P(3HB) depolymerase enzyme of a newly isolated *Burkholderia cepacia* DP1 from soil in Penang, Malaysia, was optimized using response surface methodology (RSM). The factors affecting P(3HB) depolymerase enzyme production were studied using one-variable-at-a-time approach prior to optimization. Preliminary experiments revealed that the concentration of nitrogen source, concentration of carbon source, initial pH and incubation time were among the main factors influencing the enzyme productivity. An increase of 9.4 folds in enzyme production with an activity of 5.66 U/mL was obtained using optimal medium containing 0.028% N of di-ammonium hydrogen phosphate and 0.31% P(3HB-co-21%4HB) as carbon source at the initial pH of 6.8 for 38 h of incubation. Moreover, the RSM model showed great similarity between predicted and actual enzyme production indicating a successful model validation. This study warrants the ability of P(3HB) degradation by *B. cepacia* DP1 in producing higher enzyme activity as compared to other P(3HB) degraders being reported. Interestingly, the production of P(3HB) depolymerase was rarely reported within genus *Burkholderia*.

Therefore, this is considered to be a new discovery in the field of P(3HB) depolymerase production.

Keywords Biodegradable · Extracellular P(3HB) depolymerase enzyme · Polyhydroxyalkanoates (PHAs) · Poly(3-hydroxybutyrate) [P(3HB)] · P(3HB-co-4HB) · Response surface methodology (RSM)

Introduction

Polyhydroxyalkanoates (PHAs) are synthesized intracellularly by various microorganisms as energy and carbon storage during nutrients limitations with the excess of carbon sources (Kahar et al. 2004). Poly(3-hydroxybutyrate) P(3HB) is one of PHAs (Manna and Paul 2000; Knoll et al. 2009) that has attracted industrial applications since it is biodegradable in the natural environment through microbial enzyme activity (Jendrossek et al. 1996; Anderson and Dawes 1990).

Poly(3-hydroxybutyrate) development in numerous applications is accompanied with polymer degradation tests in order to handle polymer wastes with minimum environmental damage. Copolymer P(3HB-co-3HV) is extensively used in common packaging and household materials, where the management of these frequently discarded polymers is assisted by P(3HB) depolymerase enzyme via degradation of the P(3HB) ester bond (Choi et al. 2004). In addition, P(3HB) depolymerase enzyme hydrolyzes the P(3HB) chain on the amorphous surface of P(3HB-co-4HB) copolymer hence producing high 4HB content. Copolymers P(3HB-co-4HB) with high 4HB composition have advantageous on biodegradation and biocompatible characteristics with desirable mechanical

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properties for medical and pharmaceutical applications (Faezah and Amirul 2013).

The rate of P(3HB) degradation is influenced by polymer properties and the environment (Jendrossek and Handrick 2002). Temperature, moisture, pH, and nutrient availability are examples of environmental factors, whereas composition, crystallinity, additives, lamellar thickness, and surface area are examples of polymer properties (Sudesh and Abe 2000). In addition, microbial population and habitat types such as soil, lake, seawater and compost also influences degradation rate (Lee and Choi 1999). Moreover, PHAs with low molecular weight (M_w) and low melting point (T_m) are more favourable for biodegradation (Doi et al. 1990).

Several studies reported PHAs depolymerase secretion by microorganisms isolated from soil (Boyandin et al. 2012), sludge (Mergaert et al. 1993) and freshwater (Briese et al. 1994). Recently, the isolation of P(3HB)-degrading bacteria from the gastrointestinal tract of aquatic animals was carried out by Liu et al. (2010). Interestingly, the combination of the isolated P(3HB)-degrading bacterium and P(3HB) exhibits bio-control effects capable of inactivating acylhomoserine lactones, which regulates the virulence of pathogenic bacteria, thereby improving the gastrointestinal health of aquatic animals.

Although various studies were published on the disintegration of SCL-PHAs and production of P(3HB) depolymerase enzymes in *Acidovorax* sp. DP5 (Vigneswari et al. 2015), *Comamonas* sp. (Jendrossek et al. 1993), *Alcaligenes faecalis* T1 (Yamashita et al. 2001), *Pseudomonas mendocina* DSWY0601 (Wang et al. 2012) and *Arthrobacter* sp. strain W6 (Asano and Watanabe 2001), there were no similar investigations reported for *Burkholderia* sp. Boyandin et al. (2012) reported that P(3HB)-degrading bacteria from genus *Burkholderia* were among the common isolates from soil samples in Thailand. However, detailed study on the production of P(3HB) depolymerase enzyme by *Burkholderia* sp. has not been performed. Consequently, this study was conducted in order to optimize the medium culture conditions for P(3HB) depolymerase enzyme production by *Burkholderia cepacia* DP1. To the best of our knowledge, this is the first report describing the high-yield production of P(3HB) depolymerase obtained from genus *Burkholderia*.

Materials and methods

Isolation and screening of P(3HB)-degrading bacteria

Samples were collected from soil, sludge, lake and seawater. The isolation was done according to the method of Vigneswari et al. (2015) with slight modification. A volume of 1 mL of water or 1.0 g of soil and sludge sample

was enriched in the P(3HB) medium. The medium consisted of 2.56 g of K_2HPO_4 , 2.08 g of KH_2PO_4 , 1.0 g of NH_4Cl , 0.5 g of $MgSO_4 \cdot 7H_2O$ and 0.25% (w/v) P(3HB) (Sigma-Aldrich, Germany) with or without 1.5% (w/v) agar per litre. A volume of 2.5 mL of trace element solution was added into the medium after sterilization. After 24 h, serial dilution and spread plate were performed on the P(3HB) agar medium and incubated at 30 °C until bacterial growth and clear zone were observed. The pure colony was stabbed into a fresh P(3HB) agar plate, and the degradation index of each bacteria was calculated.

Enzyme activity assay

The activity of P(3HB) depolymerase enzyme was measured according to the method of Kobayashi et al. (1999) with slight modification. Standard enzyme assay mixture contained a stable suspension of 0.16% P(3HB) granules in 0.1 M phosphate buffer at pH 6.0. P(3HB) suspension was sonicated using sonic oscillator (QSonica, USA) (60 kHz, 250 W) for 15 min. One unit of P(3HB) depolymerase activity is defined as 0.001 OD decrease in absorbance at 650 nm per min under the assay conditions described. Enzyme activity was measured by monitoring the turbidity of P(3HB) suspension at 650 nm (Faezah and Amirul 2013). The amount of protein was measured using spectrophotometer at a wavelength of 750 nm via Lowry method with bovine serum albumin as the standard (Lowry et al. 1951).

Identification of isolated bacterium

Identification of DP1 was carried out using 16S rRNA sequence analysis. The DNA genome of P(3HB)-degrading bacterium was extracted according to the protocol described in the Wizard Genomic DNA Purification Kit (Promega, UK). The 16S rRNA amplification was carried out using two sets of primers: universal primers: 60F(5'-TNA NAC ATG CAA GTC GAK CG-3') and 1392R(5'-ACG GGC GGT GTG TRC AA-3'); and *recA* gene: *recAF* (5'-AGG ACG ATT CAT GGA AGA WAG C-3') and *recAR*(5'-GAC GCA CYG AYG MRT AGA ACT T-3'). The polymerase chain reaction (PCR) product was sent for DNA sequencing (MyTACG Bioscience), and the sequences obtained were then compared with reference strains in GenBank database. Phylogenetic analysis was performed to conclude the identity of the isolate.

Medium improvement for production of extracellular P(3HB) depolymerase enzyme

Extracellular P(3HB) depolymerase enzyme production was observed using selected parameters: inorganic nitrogen

[ammonium nitrate (NH_4NO_3), ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$, ammonium acetate ($\text{C}_2\text{H}_7\text{NO}_2$), di-ammonium hydrogen phosphate ($(\text{NH}_4)_2\text{HPO}_4$, ammonium chloride (NH_4Cl)], concentrations of nitrogen (0–0.052% N), initial pH (5.5–7.5), concentrations of P(3HB) (0–0.75%), carbon sources [P(3HB) Sigma, P(3HB), P(3HV-co-14%HV) and P(3HB-co-21%4HB) films] and concentrations of P(3HB-co-21%4HB) film (0–0.5%). The P(3HB), P(3HV-co-14%HV) and P(3HB-co-21%4HB) polymers were produced by *Cupriavidus* sp. USMAA2-4 and *Cupriavidus* sp. USMAA1020, respectively.

Optimization of P(3HB) depolymerase enzyme production using response surface methodology

The central composite design (CCD) was used to optimize extracellular P(3HB) depolymerase enzyme production through shake-flask fermentation (Shantini et al. 2012). Initial pH, concentration of nitrogen, concentration of substrate, and incubation time was selected as independent variables. Each variable was then tested for high (+) and low (–) levels according to the combination arranged by the Design Expert software 7.1.6 (Stat-Ease Inc). Experimental designs of 28 arrangements were formulated using the software as shown in Table 1. All experiments were performed in triplicates. The range for each variable was concentrations of di-ammonium hydrogen phosphate (0.013–0.039% N), concentrations of P(3HB-co-21%4HB) (0.125–0.5%), pH (6.0–7.5) and incubation time (28–48 h). In this experiment, the cell dry weight and enzyme production were categorized as responses. Model verification was conducted using the formula provided by the software.

Results and discussion

Isolation, screening and identification of P(3HB)-degrading bacteria

In this study, 21 samples were collected from various environments including soil, water and sludge in Penang, Malaysia. Based on the screening, five bacterial isolates showed good formation of halo zone on P(3HB) agar proving their ability to utilize P(3HB) as the carbon source. Halo zone formation was caused by the secretion of extracellular P(3HB) depolymerase enzyme that hydrolyzed the insoluble P(3HB) to water-soluble products. Clear zone method was employed as it was reported to be simple, fast and broadly used in isolation and screening of P(3HB)-degrading bacteria (Tokiwa and Calabia 2004). The degradation index of the five isolates indicated that each isolate had varying degrees of degradation. Among all the isolates, DP1 showed the highest degradation index

with a diameter of 5.0 mm, whereas DP4 showed the lowest degradation index (2.0 mm). However, higher degradation index does not represent higher P(3HB) depolymerase enzyme production (Jendrossek 2005). Therefore, the P(3HB) depolymerase enzyme produced by each isolate was assayed quantitatively for enzyme activity. P(3HB) depolymerase enzyme was assayed at 40 °C by decreasing the turbidity of P(3HB) suspensions. The diameter of P(3HB) granules decreased upon hydrolysis which resulted in a decrease of optical density of P(3HB) suspension. As shown in Fig. 1, isolate DP1 produced relatively high and consistent enzyme production (0.54–0.60 U/mL) within 24 h might be due to high production of P(3HB) depolymerase enzyme when the incubation time increased. It shows that the enzyme activity of DP1 was stable for the next following hours of incubation as compared to other isolates. The decrease in the enzyme activity might be due to the inhibition of depolymerase enzyme production (Manna and Paul 2000). Therefore, it can be concluded that the enzyme activity of other isolates which dropped drastically might be due to the presence of high protease enzyme on the culture medium. Therefore, the isolate DP1 was chosen for identification and optimization due to its remarkable capability of degrading P(3HB).

The 16S rRNA sequence of DP1 exhibited 99% similarity to *Burkholderia* sp. In genus of *Burkholderia*, there are several species of *Burkholderia* that have different genotypes but very similar phenotypes (genomovar) (Zhang and Xie 2007). They shared about 98–100% of similarity for 16S rDNA, 94–95% for *recA* sequence and moderate similarity for DNA–DNA hybridization (30–60%). Such properties grouped them as *B. cepacia* complex (Bcc). The *B. cepacia* complex comprises of *B. cepacia* (genomovar I), *B. multivorans* (genomovar II), *B. cepaciae* (genomovar III), *Burkholderia stabilis* (genomovar IV), *Burkholderia vietnamiensis* (genomovar V), *B. cepacia* (genomovar VI), *Burkholderia ambifaria* (genomovar VII), *Burkholderia anthina*, and *Burkholderia pyrrocinia* (Yoshie et al. 2002). A genetic scheme using *recA* gene proved to enhance the identification of *Burkholderia cepacia* complex species (Payne et al. 2005). Therefore, *recA* primers were used with universal primers to confirm the *Burkholderia* species (Spilker et al. 2009). From the results of *recA* primers, the sequence exhibited 99% similarity to *Burkholderia cepacia*. According to Bosshard et al. (2003), a bacterial isolate belongs to the same species when the nucleotide similarity is above $\geq 99\%$ and is assigned to the same genus when the nucleotide similarity is above $\geq 95\%$. Therefore, the isolate is designated as *Burkholderia cepacia* DP1. Figure 2 shows the phylogenetic analysis of *Burkholderia cepacia* DP1 with organisms of similar genus. The 16S rRNA sequence was deposited in Genebank with accession number *Burkholderia*KT867090.

Table 1 Experimental design as given by response surface methodology (RSM)

Std	Run	Type	Nitrogen (%N)	Substrate (%)	pH	Incubation time (hours)
1	27	Fact	0.013	0.13	6.00	28.00
2	22	Fact	0.039	0.13	6.00	28.00
3	19	Fact	0.013	0.50	6.00	28.00
4	21	Fact	0.039	0.50	6.00	28.00
5	1	Fact	0.013	0.13	7.50	28.00
6	12	Fact	0.039	0.13	7.50	28.00
7	25	Fact	0.013	0.50	7.50	28.00
8	14	Fact	0.039	0.50	7.50	28.00
9	5	Fact	0.013	0.13	6.00	48.00
10	17	Fact	0.039	0.13	6.00	48.00
11	11	Fact	0.013	0.50	6.00	48.00
12	24	Fact	0.039	0.50	6.00	48.00
13	26	Fact	0.013	0.13	7.50	48.00
14	6	Fact	0.039	0.13	7.50	48.00
15	13	Fact	0.013	0.50	7.50	48.00
16	8	Fact	0.039	0.50	7.50	48.00
17	20	Axial	0.000	0.31	6.80	38.00
18	15	Axial	0.052	0.31	6.80	38.00
19	23	Axial	0.026	0	6.80	38.00
20	18	Axial	0.026	0.69	6.80	38.00
21	9	Axial	0.026	0.31	5.25	38.00
22	10	Axial	0.026	0.31	8.25	38.00
23	4	Axial	0.026	0.31	6.80	18.00
24	7	Axial	0.026	0.31	6.80	58.00
25	2	Centre	0.026	0.31	6.80	38.00
26	28	Centre	0.026	0.31	6.80	38.00
27	16	Centre	0.026	0.31	6.80	38.00
28	3	Centre	0.026	0.31	6.80	38.00

Fig. 1 P(3HB) depolymerase enzyme activity from various isolates; values are the mean $\pm$ SD of three replicates

Enhancement of extracellular P(3HB) depolymerase enzyme production from *B. cepacia* DP1 using shake-flask fermentation

Enhancement of extracellular P(3HB) depolymerase enzyme production from *B. cepacia* DP1 was carried out

by manipulating the parameters in P(3HB) liquid medium. First, the effect of different inorganic nitrogen sources on enzyme production was investigated (Fig. 3). The result showed that di-ammonium hydrogen phosphate was the most favourable nitrogen source for enzyme production. Compared with the previous analysis utilizing 0.026% N of ammonium chloride, the enzyme production was increased by twofolds from 0.60 to 1.24 U/mL when di-ammonium hydrogen phosphate was supplemented as nitrogen source. Results suggested that the phosphate from di-ammonium hydrogen phosphate served as a putative nutrient supply for P(3HB) depolymerase production by *B. cepacia* DP1. Nevertheless, high P(3HB) depolymerase enzyme production was also achieved using other nitrogen sources on different bacterial species such as *Acidovorax* sp. DP5 (Urea) (Vigneswari et al. 2015). It is interesting to note that different types of nitrogen varying affect microbial P(3HB) depolymerase production.

Fig. 2 Phylogenetic tree based on 16S rRNA gene sequences, showing the positions of *Burkholderia cepacia* DP1 and the related genera using neighbouring joining tree method. Maximum sequence different = 0.02

Fig. 3 Effect of different inorganic nitrogen sources on the production of P(3HB) depolymerase enzyme by *Burkholderia cepacia* DP1; Values are the mean $\pm$ SD of three replicates

Subsequently, the effect comparison of different concentrations of di-ammonium hydrogen phosphate ranged from 0 to 0.052% N nitrogen was carried out. Based on the results (Fig. 4), the P(3HB) depolymerase production is dependent on the nitrogen source since enzyme production was zero when nitrogen supply was absent. The P(3HB) depolymerase production increased from 0.55 to 1.39 U/mL as the nitrogen source increased from 0.013 to 0.026% N, after which further increase in nitrogen source resulted in constant production. Lower production of P(3HB) depolymerase was obtained when supplied with 0.013% N di-ammonium hydrogen phosphate. This might be due to the depletion of nitrogen source for cell metabolism (Li et al. 2008). Therefore, 0.026% N of di-ammonium hydrogen phosphate was selected for subsequent analyses as it was the minimum concentration that

Fig. 4 Effect of different concentrations of di-ammonium hydrogen phosphate on the production of P(3HB) depolymerase enzyme by *Burkholderia cepacia* DP1, incubation time (36 h); Values are the mean $\pm$ SD of three replicates

yielded high enzyme production. Subsequently, the production of depolymerase enzyme by *B. cepacia* DP1 was further investigated by replacing the di-ammonium hydrogen phosphate with different types of organic nitrogen sources such as urea, peptone, lab lemco and yeast extract. One organic nitrogen source that exhibited depolymerase enzyme production was urea (data not shown). The lack of enzyme production using other organic nitrogen sources maybe be due to the presence of carbon or utilizable substrates in the nitrogen sources that repressed the production of depolymerase enzyme (Jendrosseck et al. 1993; Muller and Jendrosseck 1993). Then, the combination of two nitrogen sources, urea (organic) and di-ammonium hydrogen phosphate (inorganic), was evaluated for synergic effect on depolymerase enzyme production. The medium was supplied with different concentrations of urea from 0 to 0.042% with 0.026% N di-ammonium hydrogen phosphate. However, there was no significant difference on

enzyme production when the P(3HB) broth medium was added with different concentrations of urea (data not shown). Therefore, only di-ammonium hydrogen phosphate was added as the nitrogen source for the following analyses.

The effect of different concentrations of P(3HB) on depolymerase enzyme production with 36 h of incubation time is shown in Fig. 5. The enzyme production sharply intensified from P(3HB) concentrations 0.125 to 0.25%. Maximum enzyme production was observed with P(3HB) concentration of 0.25% (1.27 U/mL). However, enzyme production drastically dropped to 0.24 and 0.23 U/mL when the substrate concentration was increased to 0.50 and 0.75%, respectively. The result obtained in this study is similar to a paper reported previously for extracellular P(3HB) depolymerase enzyme from *Schlegelella thermodepolymerans* (Elbanna et al. 2004) and *Aspergillus fumigates* (Lodhi et al. 2011), where the optimum P(3HB) concentration for depolymerase enzyme production was at 0.20%. Results revealed that high percentage of carbon source repressed the production of P(3HB) depolymerase. Nandhman et al. (2012) reported the similar phenomenon whereby PHAs depolymerase production from *Aspergillus* sp. decreased when substrate concentration was increased. Correspondingly, various studies revealed similar trend of high concentration of P(3HB) concentration inhibited the production of P(3HB) depolymerase, such as in *Aspergillus fumigates* (Lodhi et al. 2011), *Streptomyces* sp. (Manna et al. 1999) and *Acidovorax* sp. (Vigneswari et al. 2015). The decrease in P(3HB) depolymerase enzyme production at high substrate concentration might be due to substrate-level inhibition such that the substrate concentration above optimum level as a rule inhibits the enzyme activity (Manna and Paul 2000).

As reported by Sonal et al. (2015), pH is one of the essential factors influencing P(3HB) depolymerase production in *Stenotrophomonas maltophilia*. Therefore, the P(3HB) depolymerase production was further improved by manipulating the initial pH of the medium. As shown in

Fig. 5 Effect of different concentrations of P(3HB) on the production of P(3HB) depolymerase enzyme by *Burkholderia cepacia* DP1, incubation time (36 h); Values are the mean $\pm$ SD of three replicates

Fig. 6, the highest yield was obtained at initial pH of 6.8 (1.50 U/mL). However, yield was low at initial pH of 5.0 (0.62 U/mL) and 7.5 (0.28 U/mL). Likewise, Magda et al. (2015) observed significant effect on the P(3HB) depolymerase production from *Streptomyces lydicus* MM10 by manipulating the initial pH. They described the production of P(3HB) depolymerase as high at pH 7.0, but low when initial pH was 10. In the same way, similar trend was observed by Lodhi et al. (2011) on the production of P(3HB) depolymerase enzyme from *Aspergillus fumigatus* using different initial pH. From the result, as the initial pH was increased to pH 7.0, the production of P(3HB) depolymerase also increased but it decreased at the initial pH of 9.0. According to Sandhya et al. (2005), maximum production of protease was recorded at pH 7.5. Thus, the enzyme yield decreased if the pH of medium was higher than pH 7.5. Therefore, the decrease in P(3HB) depolymerase production at pH 7.5 might be due to the presence of proteases that inhibited the enzyme production. Based on the results, *B. cepacia* DP1 produced high P(3HB) depolymerase enzyme at pH 6.8, thus the mentioned initial pH was selected for subsequent medium improvement experiments.

Effect of different carbon sources towards enzyme production is shown in Fig. 7. The P(3HB), P(3HB-co-14% 3HV) and P(3HB-co-21%4HB) films were produced using solvent-casting techniques and were cut into pieces with diameters between 1.0 and 2.0 mm. From the result, it was discovered that *B. cepacia* DP1 was able to utilize various carbon sources. Figure 7 shows that the depolymerase enzyme production increased steeply (4.67 U/mL) when P(3HB-co-21%4HB) was used as carbon source, followed by P(3HB-co-14%3HV) (1.57 U/mL) and P(3HB) [control] (1.39 U/mL) at 60 h of incubation. The production of P(3HB) depolymerase increased fourfold compared to the previous experiment. The lowest enzyme production (0.50 U/mL) was recorded when P(3HB) in the form of casted film was supplied as a carbon source. Different chemical

Fig. 6 Effect of different initial pH of medium on the production of P(3HB) depolymerase enzyme by *Burkholderia cepacia* DP1; values are the mean $\pm$ SD of three replicates

Fig. 7 Effect of different carbon sources on the production of P(3HB) depolymerase enzyme by *Burkholderia cepacia* DP1; values are the mean $\pm$ SD of three replicates

and physical properties of carbon sources influenced the enzyme production. Degree of crystallinity of the carbon source influenced the enzyme production whereby the enzymatic degradation of polymer decreases with increasing degrees of crystallinity (Tokiwa and Calabria 2004). Pure P(3HB) polymer has a high degree of crystallinity (Tsuge 2002), and therefore, the lowest enzyme production was recorded when pure P(3HB) films were used as carbon source. In contrast, copolymer P(3HB-co-21%4HB) films showed low degree of crystallinity and high flexibility compared to P(3HB) films. Therefore, it was easier for the P(3HB) depolymerase enzyme to hydrolyze the P(3HB-co-21%4HB) as the carbon source. In addition, other factors such as the different chemical structures of monomeric units, degradation conditions and sequential structure of polyester chain influenced the rate of enzymatic degradation (Yoshie et al. 2002). In this study, the lowest molecular weight was recorded with P(3HB-co-21%4HB) (94 ± 4 kDa) films compared to others. Polymers with a low molecular weight are more easily to be degraded. Therefore, low molecular weight of P(3HB-co-21%4HB) might contribute to high enzyme production. P(3HB-co-21%4HB) was selected for subsequent experiments as it was a suitable carbon source for the depolymerase enzyme production.

The effect of different concentration of P(3HB-co-21%4HB) was investigated in order to determine the best substrate concentration for P(3HB) depolymerase enzyme production. Figure 8 shows the effect of different concentrations of P(3HB-co-21%4HB) on enzyme production. From the figure, the P(3HB) depolymerase enzyme production increased from 3.16 to 5.99 U/mL as the concentration of P(3HB-co-21%4HB) increased from 0.125 to 0.50% (w/v) at 72 h of incubation. Maximum enzyme production was observed when supplied with 0.50% of P(3HB-co-21%4HB). The result revealed that as the concentration of P(3HB-co-21%4HB) increased, the

Fig. 8 Effect of different concentrations of P(3HB-co-21%4HB) on the production of P(3HB) depolymerase enzyme produced by *Burkholderia cepacia* DP1, incubation time (60 h); Values are the mean $\pm$ SD of three replicates

production of P(3HB) depolymerase enzyme also increased. Differing with previous experiment, the enzyme production elevated with increasing P(3HB-co-21%4HB). This might be due to the difference in chemical and physical characteristics of the P(3HB-co-21%4HB). Hence, P(3HB-co-21%4HB) concentration was chosen as one of the independent variables for medium optimization as it greatly influenced on the extracellular P(3HB) depolymerase enzyme production.

Optimization of extracellular P(3HB) depolymerase enzyme production using response surface methodology

In order to optimize the extracellular P(3HB) depolymerase production, central composite design (CCD) was applied. From preliminary studies, the concentration of nitrogen source, concentration of carbon source, initial pH and incubation times were the main factors influencing enzyme production, hence they were selected as the independent variables with enzyme activity as the dependent variable. Three tests were performed for each response namely tests significant for regression model, test significant for model term and the test for insignificance of lack-of-fit. The significance of the quadratic model was tested using analysis of variance (ANOVA).

The ANOVA result (Table 2) showed that the regression is statistically significant for enzyme production. The F -value of 34.39% implied that the model was significant with the values. In addition, values of “ $Prob > F$ ” less than 0.5 indicated that the model term was significant. From the result, A, D, AD, BC, A^2 , B^2 , C^2 and D^2 were significant model terms. The lack-of-fit F -value of 3.97 indicated the lack-of-fit are not significant relative to the pure error. In addition, the R^2 obtained for enzyme activity was 0.9454, which indicated that the experiments carried out were of high precision and reliability.

Figure 9 shows the 3D response surface towards enzyme production. From the figure, all the variables showed

Table 2 Analysis of variance and regression for enzyme activity

Source	Sum of squares	df	Mean square	F-value	Prob > F	Significant
Model	0.62	14	0.044	34.39	<0.0001	
A-nitrogen	0.014	1	0.014	10.92	0.0057	Significant
B-substrate	1.488E-003	1	1.488E-003	1.16	0.3020	
C-pH	1.000E-004	1	1.000E-004	0.078	0.7849	
D-incubation time	0.049	1	0.049	37.79	<0.0001	Significant
AB	1.958E-003	1	1.958E-003	1.52	0.2395	
AC	1.501E-004	1	1.501E-004	0.12	0.7383	
AD	9.073E-003	1	9.073E-003	7.04	0.0199	Significant
BC	7.098E-003	1	7.098E-003	5.51	0.0354	
BD	7.981E-004	1	7.981E-004	0.62	0.4454	
CD	1.106E-003	1	1.106E-003	0.86	0.3712	
A ²	0.18	1	0.18	140.62	<0.0001	Significant
B ²	0.30	1	0.30	232.15	<0.0001	Significant
C ²	0.29	1	0.29	228.52	<0.0001	Significant
D ²	0.13	1	0.13	99.98	<0.0001	Significant
Residual	0.017	13	1.288E-003			
Lack-of-fit	0.016	10	1.557E-003	3.97	0.1420	Not significant
Pure error	1.178E-003	3	3.927E-004			
Correction total	0.64	27				

elliptical contour for P(3HB) depolymerase enzyme production. It was suggested that optimum interaction occurred between the test parameters (Sonal et al. 2015). Higher concentration of substrate and nitrogen, pH and incubation time remarkably inhibited the production of P(3HB) depolymerase enzyme. Figure 9a represents the 3D response surface interaction between substrate and nitrogen concentration at pH 6.8 for 38 h of incubation. Two variables were investigated at a time while keeping the other one at a fixed value. Based on the results, the enzyme production increased along with P(3HB-co-21%4HB) concentration but decreased when the P(3HB-co-21%4HB) concentration was increased further. This result was also similar to those obtained from previous experiments whereby high concentration of substrate inhibited the production of P(3HB) depolymerase.

Figure 9b represents the 3D response surface interaction between concentration of nitrogen and time incubation using 0.31% substrate at pH 6.8. From the result, the enzyme production increased gradually from 0 to 38 h incubation and slowly decreases for the following hours of incubation time. From Fig. 9b, lowest enzyme production was obtained at 28 h as compared to 38 h of incubation. The enzymatic degradation process by depolymerase enzyme occurs in two steps, absorption and hydrolysis. During the process, the depolymerase enzyme absorb to the surface of the polymer and cleave the polymers chain into monomers (Doi et al. 1994). Most polymers are too large to pass through cellular membranes. Therefore, the polymers

must be broken down into smaller monomers before they can be absorbed and degraded within the microbial cells (Shah et al. 2008). It can be assumed that less than 28 h is needed for depolymerase enzyme to absorb to the substrate prior to hydrolyzing the substrate into simpler monomers. In addition, the production of depolymerase enzyme was decreased gradually after 38 h of incubation. The production of P(3HB) depolymerase enzyme is inhibited by lack of nutrient supply. Therefore, the decrease in enzyme production when prolonging the incubation times might be due to the lack of nutrients supply.

Figure 9c represents the 3D response surface interaction between concentration of nitrogen and pH using 0.31% substrate at 38 h of incubation. Figure 9c shows that the optimum enzyme production was obtained at pH 6.8. It clearly showed that the results correlated with the preliminary studies (Fig. 6).

Verification of the model

Verification of the RSM model was carried out with additional experiments under conditions predicted by the model. As shown in Table 3, great similarity was obtained between predicted (4.73 U/mL) and actual enzyme production (5.66 U/mL) indicating a successful model validation. The optimum P(3HB) depolymerase enzyme production was 5.66 U/mL. Based on the table, production of P(3HB) depolymerase from *B. cepacia* DP1 increased 9.4 folds after optimization. After optimization, the P(3HB-

Fig. 9 3D response surface Interactive effect of **a** different concentrations of nitrogen and substrate at pH 6.8, **b** different concentrations of nitrogen and pH at 0.31% of substrate and **c** different concentration of nitrogen and incubation time at 0.31% substrate on the production of P(3HB) depolymerase enzyme

co-21%4HB) was used at lower concentration (0.31%) when compared with non-optimized conditions where the highest enzyme production (5.99 U/mL) was achieved with 0.50% of P(3HB-co-21%4HB). In addition, the highest enzyme production was shortened to 38 h of incubation after optimization compared to 72 h using non-optimized conditions. This is the first report describing the high-yield

optimization of P(3HB) depolymerase enzyme production from the genus *Burkholderia*. From the result, the P(3HB) depolymerase enzyme production by *B. cepacia* DP1 was higher than that obtained by *Acidovorax* sp. DP5 (3.0 U/mL) (Vigneswari et al. 2015), *Stenotrophomona smaltophilia* (1.50 U/mL) (Sonal et al. 2015).

Table 3 Verification of the model using optimized condition given by the software for the maximized extracellular P(3HB) depolymerase enzyme

Variables	Responses		Enzyme activity (U/mL)		
	Before	After	Before	After	
				Predicted	Actual
Nitrogen (%N)	0.026	0.028	0.60	4.730	5.66
Substrate (%)	0.25	0.31			
pH	6.8	6.8			
Incubation time (hours)	72	38			

Conclusion

The production of P(3HB) depolymerase enzyme from *B. cepacia* DP1 was successfully optimized using statistical analysis. It has been concluded that the production of extracellular P(3HB) depolymerase enzyme by *B. cepacia* DP1 was influenced by several factors such as type of nitrogen supplied, concentration of nitrogen, concentration of substrate, incubation time and initial pH. Under optimized condition, the P(3HB) depolymerase enzyme production increased 9.4 folds using specific copolymer P (3HB-*co*-21%4HB) film as the carbon source. Interestingly, the copolymer P(3HB-*co*-21%4HB) film was fully degraded after 38 h of incubation, hence proving the efficiency of this strain to degrade PHAs. The degradation of P (3HB) was rarely reported within genus *Burkholderia*. Therefore, *B. cepacia* DP1 can be recognized as a potential bacterium that produces P(3HB) depolymerase enzyme with high enzyme activity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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