Biocatalysis and Agricultural Biotechnology 18 (2019) 101001

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Medium optimization for polyhydroxyalkanoate production by *Pseudomonas pseudoalcaligenes* strain Te using D-optimal design

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ARTICLE INFO

Keywords:
Polyhydroxyalkanoate
Pseudomonas pseudoalcaligenes
Medium optimization

ABSTRACT

The hazardous effects of synthetic polymers encouraged investigators to find about biodegradable polymers among which microbial derived polyhydroxyalkanoate (PHA) have got attention during the two last decades. In the present study, isolation and identification of a PHA producing bacterial strain and optimization of cultural conditions for PHA biosynthesis were aimed. Several soil and spring samples were collected and screened for PHA producing bacterial strain according to the reported protocols. The most efficient isolate was identified using biochemical, morphological, and 16S rDNA gene sequencing methods. The related PHA production was then optimized using D-optimal statistical design. The obtained results of screening step using Nile blue containing culture plate together with biochemical and morphological studies as well as 16S rDNA gene analysis introduced *Pseudomonas pseudoalcaligenes* strain Te as the most efficient PHA producer. Evaluation of PHA productivity profile of *P. pseudoalcaligenes* strain Te revealed that maximum PHA biosynthesis was achieved at the stationary phase of growth. Assessment the influence of five parameters on PHA productivity of the selected strain revealed the significant effect of three factors including pH, K_2HPO_4, and temperature. The optimum level of these three factors (pH 8.6; K_2HPO_4, 4.7 g/L; and temperature of 25 °C) enhanced the PHA production to 5412.9 mM. Although, the capability of *P. pseudoalcaligenes* for PHA production was confirmed in the present study, it merits further investigations to find about the related pathways involved in PHA biosynthesis by this strain.

1. Introduction

The role of petrochemically derived polymers in our routine life is so considerable that even imagination of life without them is impossible. Everywhere we turn around, plastics can be observed even some industries will be crippled without plastics (Devi et al., 2015). However, the hazardous effects of such none degradable plastic materials (resistance to heat, pressure, chemical solvents, and UV light) for human and the environment encouraged investigators to search for new alternative groups of biodegradable and biocompatible polymers during the two last decades (Gholami et al., 2016; Albuquerque and Malafaia, 2018; Devi et al., 2015).

Polyhydroxyalkanoates (PHAs) are a great family of biodegradable (easily decompose to H_2O and CO_2) and biocompatible polyesters synthesized by a variety of microbial strains and plants and include 150 different types (Kourmentza et al., 2018). Generally, these polyesters accumulate intracellularly in the producing microorganisms under conditions of phosphate and nitrogen restriction and excess of carbon source as carbon and energy reserves (Gholami et al., 2016; Mahansaria et al., 2018; Nisha et al., 2012). PHAs were firstly discovered in *Bacillus subtilis* by Lemoigne (1925) where many reports have been published about the ability of different bacterial and fungal strains for PHAs biosynthesis (Kumar et al., 2018). For example, Vasheghani-Farahani et al. (2004) described about the optimization of *Ralstonia eutropha* cultural conditions which increased the production yield of poly (β-hydroxybutyrate) (PHB) to 92.36%. Taran (2011) investigated on the ability of *Haloarcula* sp. IRU1 (isolated from Urmia lake, Iran) for PHB production and optimized its production via Taguchi experimental

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https://doi.org/10.1016/j.bcab.2019.01.039
Received 14 September 2018; Received in revised form 8 December 2018; Accepted 22 January 2019
Available online 26 January 2019
1878-8181/

method. The *Comamonas testosteroni* was another Gram-negative bacterial strain which was reported to produce PHB on naphthalene containing medium (Thakor et al., 2003).

From the chemically structure point of view the PHAs are hydroxyalkanoic acid polyesters which classified as short (up to C_5 like PHBs), medium (C_6–C_{14} such as 3-hydroxyoctanoate), and long (more than C_{14}) chain length based on the size of the comprising monomers (Gholami et al., 2016; Raza et al., 2018; Samrot et al., 2011). So, the physicochemical properties and applications of PHAs depends on monomeric unit characteristics and their structure (Raza et al., 2018). As an example, short chain length PHAs are hard and brittle while medium chain length ones possess appropriate elasticity (Gholami et al., 2016). PHAs have been widely applied in drug delivery systems, tissue engineering, and medical implants as well manufacturing of bone plates, screws, nails, and in the treatment of osteomyelitis (Taran, 2011; Senthilkumar et al., 2017).

However, substrate cost and fermentation conditions increase the production costs of such bioplastics up to 4–9-fold of conventional polymers (Raza et al., 2018). Among different procedures applied for enhancing PHAs production, optimization of cultural conditions via experimental design approaches was recently used to achieve effective factors and their interactions involve in PHAs production using a limited number of experiments (Senthilkumar et al., 2017). Compared to such statistical methods, conventional experimental methods investigate one factor at a time while keeping other factors constant, resulting in too many time consuming tests unable to provide information about factors interaction (Mohan and Reddy, 2013). There are so many factors influencing the PHAs production like pH, temperature, carbon, nitrogen, and phosphorus resources (Mohan and Reddy, 2013; Pardakhty et al., 2012).

The main aim of the present study was to isolate and identify a PHA producing bacterial strain from soil samples followed by investigation on the parameters affects the PHA production using the statistical design methods.

2. Material and methods

2.1. Materials

Nile blue and Sudan black dyes as well as sodium hypochlorite and chloroform were provided by Merck chemicals (Darmstadt, Germany). Glucose, K_2HPO_4, and crotonic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Screening for PHA producing bacterial strain

During summer 2014 different water samples from local springs of Kerman province (30°15′ N, 56°58′ E, Kerman, Iran) were collected in order to isolate PHA producing bacterial strains. An amount of 5 mL of each sample was consequently added to sterile normal saline solution (15 mL) and 500 μL of each prepared diluted sample was streaked on M9 medium (Na_2HPO_4, 6 g/L; KH_2PO_4, 3 g/L; NaCl, 0.5 g/L; NH_4Cl, 1 g/L; $MgSO_4$, 0.5 g/L; glucose, 2 g/L; $CaCl_2$, 0.015 g/L) containing Nile blue (0.001% w/v). The plates were then incubated at 37 °C until colonies that showed bright halos under UV light were observed (Singh et al., 2011).

Axenic culture was then obtained by several subculturing of the selected bacterial colonies on M9 medium supplemented with Nile blue (0.001%). For confirmation the PHA biosynthesis by the isolates a heat fixed glass slide of the isolate biomass were stained for 10 min with Sudan black solution (0.3% w/v in 60% v/v ethanol). After 10 s decolorization by toluene, the slides were stained by Safranin O solution (0.5% w/v). Blue–black color of intracellular PHA were observed by optical microscope (Zeiss, Germany) equipped with a computer-controlled image analysis system in 10 × 100 magnifications (Mesquita et al., 2015). The selected isolate was consequently conserved at −80 °C in nutrient broth (NB) medium supplemented by glycerol (15%).

2.3. 16S rDNA gene analyses, morphological, and biochemical characteristics of the selected isolate

A freshly prepared culture of the selected isolate (100 μL) was subjected to centrifugation (11000 g, 5 min) and the produced biomass was subsequently washed repeatedly (three times) by sterile water followed by extraction of the genomic DNA assisted by GF-1 DNA isolation kit (Vivantis, South Korea) based on the instructions of manufacturer. The 16S rDNA gene of the selected isolate was then amplified using the attained genomic DNA as template and the primer pair of 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) as forward primer and 1525R (5′-AAGGAGGTGATCCAGCC-3′) as reverse primer (Ohadi et al., 2017; Shakibaie et al., 2015) assisted by a FlexCycler® thermal cycler (AnalytikJena, Jena, Germany). The related amplified ribosomal gene segment was then sequenced (Bioneer Corporation, South Korea) and compared with the GenBank deposited sequences using the BLAST software. For morphology study, the selected isolate at the exponential growth phase was treated with glutaraldehyde (2.5% in PBS 0.2 M) for 60 min at 4 °C. After 2 h fixation with osmium tetroxide (1%) at 4 °C, the cells were washed with PBS again and dehydrated using increasing concentration of ethanol (50, 75, 95, and 100%) and were dried at room temperature. Then, the sample was coated with gold in a sputter coater device (model SCD 005; Bal-Tec) for 5 min and the morphology of the selected bacterial isolate was analyzed using a field emission scanning electron microscope (HITACHI S-4160, Japan). The biochemical characterization was studied based on the method described by Bergey's Manual of Determinative Bacteriology (Vos et al., 2011).

2.4. General procedure for growth experiments and PHA production

For determination of PHA concentration, an indirect spectrophotometric method was used with some modification. This method was based on hydrolysis of PHA in acidic condition and subsequent measurement the absorbance of crotonic acid in 235 nm (Singh et al., 2011). Briefly, different concentrations of crotonic acid (0.25–2.5 mM) were prepared in concentrated H_2SO_4 and absorbance of the reaction mixture was measured at 235 nm using a Shimadzu UV–Vis Double Beam PC Scanning spectrophotometer (UV-1800, Shimadzu CO, USA). For drawing a suitable standard curve, these procedures were repeated three times on different days and the mean of absorbencies was used (Soflaei et al., 2012).

A set of growth experiments was carried out to study the ability of the selected isolate for PHA biosynthesis in M9 medium. 100 mL of this media was transferred to sterile 500 mL Erlenmeyer flasks and were inoculated with 1 mL of the fresh inoculums (OD_{600nm},0.1). For aerobic cultivation, the flasks were plugged with cotton and were incubated for 4 days at 37 °C in a shaker incubator (150 rpm). Samples were taken every 12 h, and absorbance was read at 600 nm. Then, the cells were harvested by centrifugation (7000 rpm, 8 min) and were treated with sodium hypochlorite 5% (50 °C, 1 h). After centrifugation of the dispersion (11000 rpm, 30 min), hot chloroform (65 °C) was added to the residuals and it was dried. Thereafter, 10 mL of concentrated H_2SO_4 was added and heated in a boiling water bath for 10 min and after cooling the reaction mixture the absorbance was read in 235 nm (Singh et al., 2011). These experiments were reiterated three times on various days and mean of the obtained results were reported.

2.5. Experimental design

A D-optimal designs, as response surface designs, was used to screen the most effective factors in PHA production by the selected strain and for developing a model for optimal condition. The parameters including

Table 1
Some biochemical characteristics of the isolated strain.

Characteristics	Result
Catalase production	+
Oxidase activity	–
Voges-Proskauer test	–
Methyl red test	–
Acid from:	
D-Glucose	+
D-Maltose	–
D-Lactose	–
D-Mannitol	–
Hydrolysis of	
Casein	–
Gelatin	+
Starch	–
Utilization of citrate	+
Nitrate reduced to nitrite	+
Formation of:	
Indole	–
Dihydroxyacetone	+
Growth in NaCl:	
2%	+
5%	+
7%	+
10%	–
Growth at:	
4 °C	–
30 °C	+
40 °C	+
50 °C	–

+: Positive, −: Negative.

glucose concentration (X_1), K_2HPO_4 concentration (X_2), NH_4Cl amount (X_3), pH of culture media (X_4), and temperature (X_5) were selected. The range and the levels of the variables are given in Table 1.

D-optimal design consisting of 56 factorial runs was designed using the software, Design Expert version 6.0.4 statistical software (Stat- Ease Inc., Minneapolis, MN) (Table 2). Coding of the variables was done according to the following equation:

$$x_i = (X_i - X_{i;0})/\Delta Xi \; i = 1,2,3, \ldots,k$$

where x_i is the coded value of an independent variable, X_i is the independent variable's real value, $X_{i;0}$ is the independent variable's real value at the center point, and ΔX_i is the step change value. The PHA concentration was taken as the dependent variable or response. The runs were randomly carried out trying to nullify the effect of nuisance variables. The chosen variables can be related to the response by model as shown in the following equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j$$

In this equation x_i (i = 1–5) is the variable of the experiment, Y is response function (here is PHA production), e is the error of the experiment, b_0 is the constant coefficient, b_i (i = 1–5) is the linear coefficient, b_{ij} ($i \neq j$) is the second-order coefficient, b_{ii} (i = 1–4) is the second-order interaction coefficient, and x are the independent variables, where in this study independent variables were coded as A, B, C, D, and E. At the end, the statistical model was validated for all variables within the design.

Table 2
Applied levels of independent variables in the DMD.

Variable	Component	Unit	Low level (−1)	High level (+1)
X_1	Glucose	g/L	1	10
X_2	K_2HPO_4	g/L	1	5
X_3	NH_4Cl	g/L	0.5	1.5
X_4	pH	–	5	9
X_5	Temperature	° C	30	37

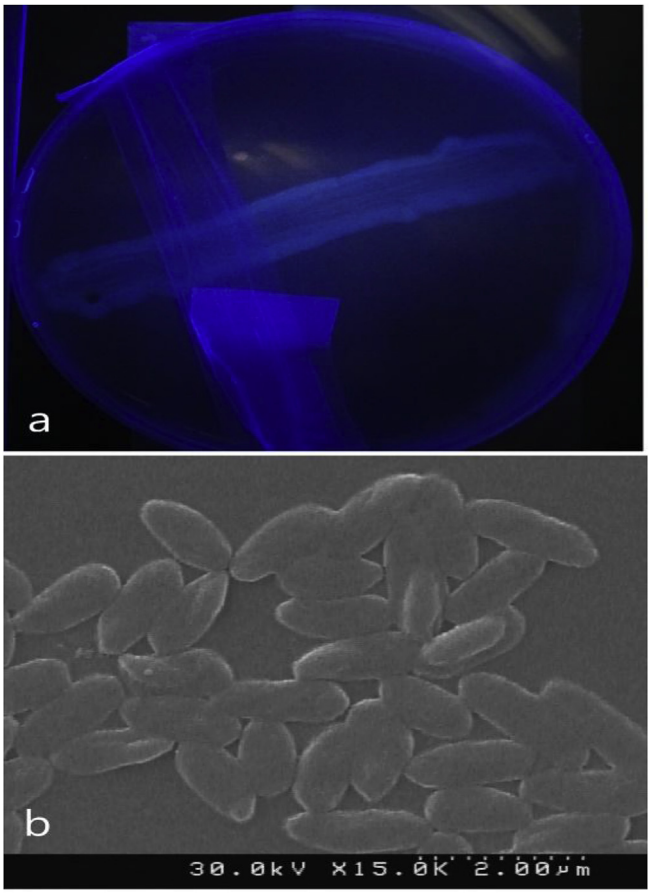

Fig. 1. (a) Fluorescent light of the selected isolate cultured on M9 medium supplemented by Nile blue under ultraviolet light. (b) Scanning electron micrograph of the *P. pseudoalcaligenes* strain Te.

2.6. Data analysis

The statistical analysis of the results was performed with the aid of Design Expert version 6.0.4 statistical software (Stat- Ease Inc., Minneapolis, MN). The qualities of the fitted models were examined by the coefficient of determination R^2. The PHA concentration was analyzed using the analysis of variance (ANOVA) combined with the F-test to evaluate if a given term had a significant effect ($p \leq 0.05$). The location of the optimum was determined by solving the set of equations derived by the differentiation of the final quadratic model.

3. Results and discussion

3.1. Isolation of PHA producing bacterial strain

The obtained results of the screening step revealed that one sample taken from Sirch hot spring in Kerman (30°15′N, 56°58′ E) contained a bacterium with the ability to produce PHA (Fig. 1a). The best time for observing the fluorescent light was 36–48 h after culturing and the light disappeared by the time where there was no light after 7–10 days. Sudan black staining confirmed the intracellular PHA accumulation in the isolate. In primary studies, the isolated strain showed up as a Gram-negative, and motile bacterium with yellowish colonies on the M9 agar medium and scanning electron micrograph of the isolate shown a rod-shaped bacterial strain (Fig. 1b). Table 1 represented the attained results of biochemical properties which together with morphological

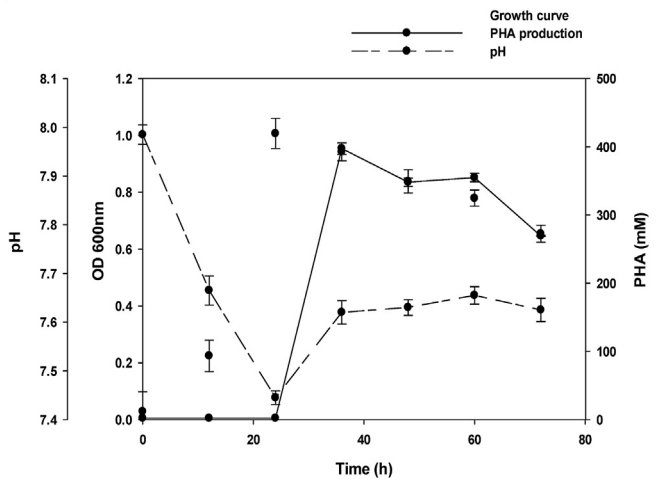

Fig. 2. Growth, pH, and PHA production profiles of in *P. pseudoalcaligenes* strain Te in culture medium.

results candidate the selected isolate as *Pseudomonas* strain. The alignment of the amplified 16S rDNA gene segment against the deposited genes of GenBank assisted by BLAST software showed a 99% identity score of the related gene with *Pseudomonas pseudoalcaligenes*. Afterwards, the related gene (1403 bp) of *P. pseudoalcaligenes* strain Te was deposited in GenBank under the accession number of KF055346.

The spectrophotometric measurement of the crotonic acid showed good linearity between the absorbance obtained at 235 nm and serial concentrations of crotonic acid. The time course study revealed that growth of *P. pseudoalcaligenes* strain Te was started at the beginning of cultivation and it reached to maximum after 24 h in the M9 broth (Fig. 2). In contrast, PHA production profile by the isolate showed that the biosynthesis process took place during the exponential phase of growth after 24 h and the maximum PHA production was occurred 36 h after the culturing in M9 medium (Fig. 2). Furthermore, the initial pH of the culture medium was decreased during the logarithmic phase and it shows a little increase during the stationary and death phase (Fig. 2).

3.2. Optimization of PHA production

The purpose of the first optimization step was to identify the component(s) of the medium that have a significant effect on PHA production by *P. pseudoalcaligenes* strain Te. Five medium ingredients were assessed at selected levels (Table 2). The D-optimal design and the results are summarized in Table 3 with the levels in coded units. The response range was from 65.5 mM to 5019.6 mM. So, a logarithm transformation was required.

To summarize the analysis of the response effects and show the significant model terms, the ANOVA table has been created (Table 4). For K_2HPO_4 (X_2) the measured p-value was less than 0.05 (Table 4), thus this parameter had a significant effect on PHA production by *P. pseudoalcaligenes*. Previously, Panda et al. (2006) reported that a minimal level of internal phosphate is essential for poly-ß-hydroxybutyrate synthesis in *Synechocystis* sp. PCC 6803. Protein synthesis was increased in high phosphorous concentrations, while phosphate deprivation leads to reduction of protein synthesis rate and diverts the pathway towards PHA accumulation (Mohan and Reddy, 2013). Analysis of variance results for pH (X_4) showed that this factor had significant influence on PHA production ($p < 0.05$). Filipe et al. (2001) reported that higher or lower extracellular pH induces electrical differences across the cell membrane, leading to the higher energy requirement for substrate uptake. Furthermore, initial pH around neutral was more favorable than high acidic or alkaline pH for enzymes involved in PHA biosynthesis (Panda et al., 2006). In the case of

Table 3
Experimental design and results of DMD.

Run	Coded levels					PHA concentration (mM)	
	x_1	x_2	x_3	x_4	x_5	Experimental	Predicted
1	−0.111	−1	+1	−1	−1	126.1	102.4
2	−0.111	−1	0	0	−1	590.2	726.6
3	−0.111	+1	+1	0	+1	743.8	734.3
4	+1	−1	0	+1	+1	247.4	368.3
5	+1	+1	0	+1	−0.167	5019.6	2916.8
6	−1	−1	−1	−1	−0.167	342.4	197.6
7	−0.111	−0.5	0	+1	−0.167	2123	880.4
8	−1	+1	+1	−1	+1	157.7	237.9
9	−1	−1	−1	0	−1	903.3	366.9
10	+1	+1	−1	+1	+1	3002.3	2570.8
11	+1	+1	0	0	+1	594.5	748.9
12	+1	−1	+1	0	+1	451.9	363.9
13	−1	−1	0	+1	−0.167	345.6	653.6
14	+1	+1	+1	−1	−1	216.9	424.9
15	−1	+1	−1	0	+1	583.8	443.5
16	+1	−0.5	−1	−1	−1	134.3	122.6
17	−1	−0.5	−1	+1	−0.167	346.6	824.2
18	+1	−0.5	+1	+1	+1	494.1	742
19	−1	−1	+1	0	−1	379.3	366.9
20	+1	−1	0	−1	−1	171.1	206.5
21	+1	−0.5	0	−1	−0.167	215.5	281.8
22	+1	−1	0	0	−0.167	640.6	445.3
23	−1	−0.5	0	0	−0.167	517.5	528
24	−1	+1	0	−1	−1	186	251.7
25	−1	−0.5	0	+1	+1	356.9	436.7
26	+1	+1	−1	0	−0.167	1513.2	1524.4
27	+1	−0.5	0	+1	−1	2644.2	3353.6
28	+1	−1	−1	+1	−0.167	696.6	695.2
29	−0.111	+1	−1	+1	−0.167	1885.5	1402.9
30	+1	−1	+1	−1	−0.167	365.1	232.7
31	−1	−1	0	−1	+1	91.7	90
32	−0.111	−1	0	−1	−0.167	245.2	235.3
33	−0.111	+1	−1	−1	−1	170.4	226.3
34	−1	+1	+1	−1	−0.167	253.4	283.7
35	−0.111	+1	0	0	−0.167	1725.9	898.6
36	−1	−0.5	+1	−1	−1	126.5	132.9
37	−0.111	+1	0	+1	+1	1474.2	743.4
38	−0.111	−1	+1	+1	−1	1772	1520.3
39	+1	−1	+1	+1	−1	1009.7	1664.8
40	−1	−1	+1	+1	+1	291.3	576.1
41	−0.111	−0.5	+1	−1	−0.167	194.9	266.2
42	−0.111	−1	−1	0	−0.167	517.5	367.3
43	+1	+1	+1	0	−1	2878.2	1637.4
44	−0.111	−1	−1	−1	+1	209.1	178.2
45	−1	+1	+1	+1	−1	1800.4	2029.3
46	−0.111	−0.5	−1	+1	−1	2062.7	1904.4
47	+1	−0.5	+1	0	−0.167	423.9	440
48	−0.111	−0.5	0	−1	+1	65.5	121.2
49	+1	−0.5	−1	0	+1	782.8	398
50	+1	+1	−1	+1	−1	3119.3	6309.1
51	−0.111	−0.5	+1	0	−1	707.6	494.2
52	−1	−1	−1	−1	−1	122.6	95.2
53	−1	−0.5	−1	−1	−1	96.7	132.9
54	−1	−0.5	+1	0	+1	443.1	431.4
55	−1	−1	0	−1	−1	164	175.3
56	−1	−0.5	−1	−1	−0.167	242	275.9

temperature (X_5), the measured *p*-value was less than 0.05 (Table 4), which means that this factor was also significantly affected the PHA production. However, it was found that glucose (X_1) and NH_4Cl (X_3) did not significantly influence ($p > 0.05$) on PHA biosynthesis within the tested levels. However, Mohan and Reddy (2013) reported that glucose concentration showed significant influence on PHA production with wastewater by a mixed culture. They showed that lower PHA accumulation (due to bacterial growth limitation) achieved in the absence of glucose while PHA was maximally synthesized in the presence of 6 g/L of glucose.

The sum of squares (SS) for each variable quantifies the variable's importance in the process, and as the value of the SS increased, the

Table 4
Analysis of variance for DMD refined model.

Source of variation	*SS*	*df*	*MS*	*F*	*P* > *F*
Model	9.83	8	1.23	33.07	< 0.0001
x_2	1.3	1	1.3	34.93	< 0.0001
x_4	0.49	1	0.49	13.12	0.0007
x_5	0.96	1	0.96	25.71	< 0.0001
x_1x_2	0.29	1	0.29	7.86	0.0073
x_4x_5	0.59	1	0.59	15.75	0.0002
$x_1x_2^2$	0.5	1	0.5	13.44	0.0006
$x_3^2x_5$	0.5	1	0.5	13.52	0.0006
$x_4x_5^2$	0.39	1	0.39	10.49	0.0022
Residual	1.75	47	0.037		
Cor Total	11.58	55			

significance of the corresponding variable in the process also increased. As shown in the ANOVA table (Table 4), x_2 was the most effective variable in this study, followed by x_5, x_4x_5, $x_1x_2^2$, $x_3^2x_5$, x_4, and $x_4x_5^2$.

The experimental results of the D-optimal designs were fitted with a second-order polynomial expression. The values of regression coefficients were calculated and the fitted equation (in the terms of coded values) for predicting PHA production was:

$$\text{Log}_{10}\ (\text{PHA concentration}) = 2.75 + 0.18\ x_2 + 0.21\ x_4 - 0.30\ x_5 + 0.11\ x_1x_2 - 0.16\ x_4x_5 + 0.14\ x_1\ x_2^2 + 0.27\ x_3^2\ x_4 + 0.22\ x_4\ x_5^2$$

The effects of x_2, x_4, x_1x_2, $x_1\ x_2^2$, $x_3^2\ x_4$, and $x_4\ x_5^2$ were positive (synergistic), while x_5 and x_4x_5 had negative (antagonism) effects on PHA production. The model F-value of 33 implies that the model is significant. Also, *P*-values less than 0.05 indicated model terms were significant at the probability level of 95%. The F-value of the model indicated that the model was significant with only 0.01% chance such that the "Model F-value" could have occurred due to the noise. The relatively high value of $R^2 = 0.8491$ implied that the model was significantly fitted. The adjusted R-squared and predicted R-squared values indicated variability in the observed and predicted response values, respectively. When the predicted R-squared and adjusted R-squared values are closer to one, a better fit is achieved (Anderson and Whitcomb, 2016).

The measured predicted $R^2 = 0.7813$ was in reasonable agreement with the adjusted $R^2 = 0.8235$. Adequate precision measured to be 23.8 which exhibited a desirable signal to noise ratio thus this model can be used to navigate the design space. Values mentioned above supported the hypothesis that the model equation is sufficient to describe the response of the experimental observations pertaining to PHA production.

The normality of the data was investigated by plotting a normal probability plot of the residuals (Fig. 3). As shown in Fig. 3 the data points on the plot fall fairly close to the straight line thus, the data are normally distributed by a nearly constant variance throughout the response range. The PHA concentrations, as predicted by the final quadratic model along with the corresponding observed values, represented in Table 3. Comparison of these values indicated that there was an excellent agreement between the model and experimental data.

The location of optimum levels by the selected model for PHA biosynthesis (5412.9 mM) was predicted to be 7.1 g/L of glucose, 4.7 g/L of K_2HPO_4, 1 g/L of NH_4Cl, pH 8.6, and temperature 25 °C.

To confirm the model adequacies for predicting PHA biosynthesis, four additional experiments using different medium composition were performed (Table 5). If the average of the results of the confirmation was within the limits of the confidence interval (CI), then the significant variables and appropriate levels for obtaining the desired results were properly chosen (Anderson and Whitcomb, 2016). The results of the confirmation experiments indicated that there are good agreements between the predicted and experimental results which indicated that the selected model was able to navigate within the design space.

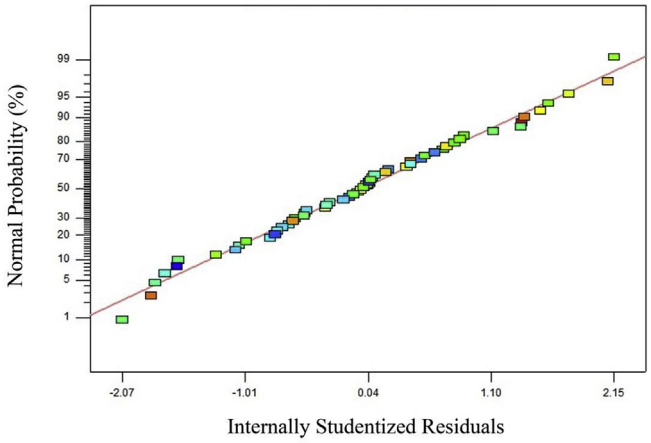

Fig. 3. The normal probability vs. residuals plot by final fitted model.

Table 5
Arrangement and results of confirmatory trials.

Trials	Variables					PHA concentration (mM)		Confidence interval (95%)	
	x_1	x_2	x_3	x_4	x_5	Observed value	Predicted value	Low	High
1	10	5	1	5	31	949.4	936.9	588.9	1490.5
2	1	2	0.5	7	31	464.3	470.1	402.9	548.5
3	5.5	3	1	7	31	526.4	558	491.2	633.9
4	7.1	4.7	1	8.6	25	5480.9	5522.4	3578	8523.5

The response surface graphs shown in Fig. 4a and b were based on the selected model, holding three factors constant, while varying the other two factors within their experimental range. Fig. 4a exhibited the response surface for the constant level of NH_4Cl (1 g/L), pH (9), and temperature (30 °C). The minimum response (653 mM of PHA) occurred when both glucose and K_2HPO_4 were at their lowest level. It seems that in high level of K_2HPO_4 (5 g/L), the response indicated a maximum nearly at the high level of glucose. Furthermore, when glucose was at low or high levels, the production of PHA by *P. pseudoalcaligenes* was not considerably changed at low level of K_2HPO_4 (1 g/L). Analysis of response at the different levels of the glucose revealed that glucose was not significantly affected the PHA biosynthesis but there is a remarkable interaction between glucose and K_2HPO_4 (Table 4). Fig. 4b exhibited the response surface for the constant level of glucose (10 g/L), K_2HPO_4 (5 g/L), and NH_4Cl (1 g/L). Minimum response (401 mM of PHA) was seen with low level of temperature (25 °C) and low level of pH (1) (Fig. 4b). When temperature was at low or high levels, the production of PHA was not significantly different at low level of pH ($p > 0.05$). Biosynthesis of PHA by *P. pseudoalcaligenes* in low level of temperature and high level of pH was considerable. Also, the results suggested the significant interaction between pH and temperature at the constant level of other factors.

4. Conclusion

The capability of an isolated bacterial strain (*P. pseudoalcaligenes* strain Te) for PHA production was inspected and the effect of five parameters on PHA biosynthesis was investigated among which three factors of pH, K_2HPO_4, and temperature were found to significantly affected the PHA production. At the optimum level of these three effective factors [pH 8.6, K_2HPO_4 (4.7 g/L), and temperature of 25 °C) an amount of 5412.9 mM PHA was produced by *P. pseudoalcaligenes* strain Te. However, more investigations must be performed to find about the related pathway involved in PHA biosynthesis.

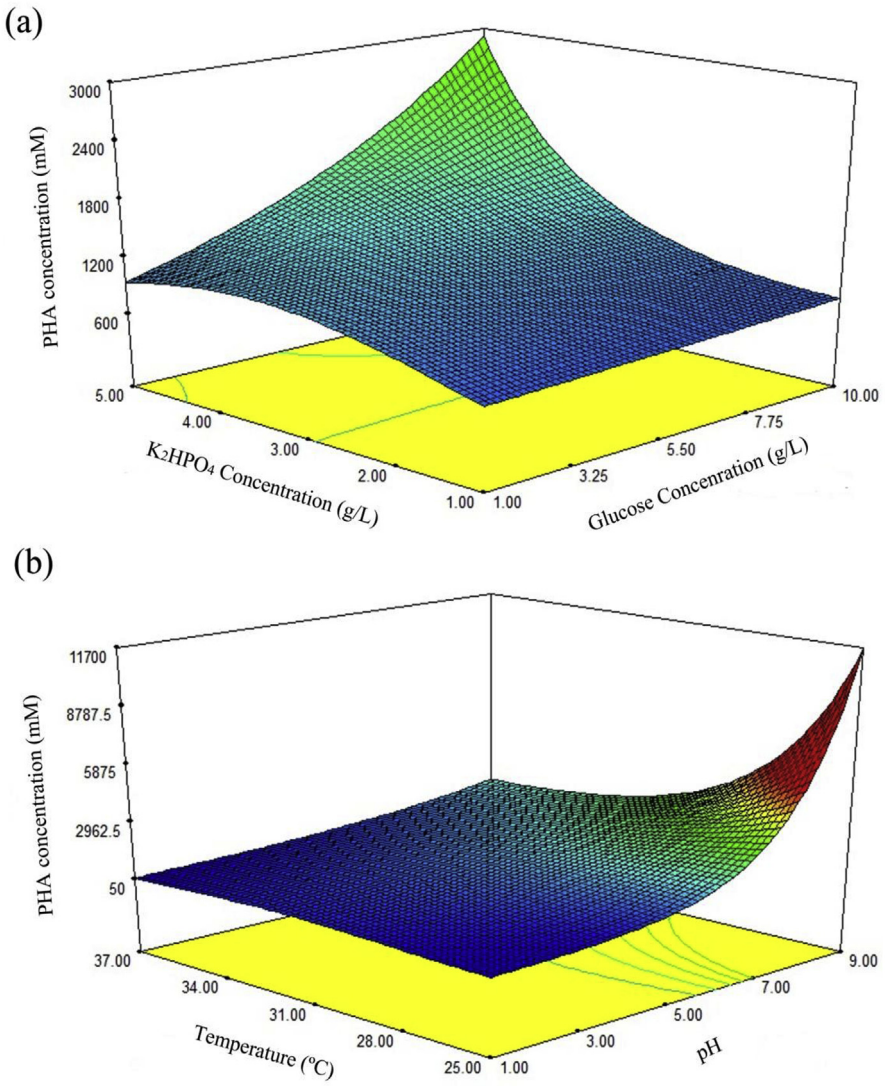

Fig. 4. Response surface of PHA concentration. (a) constant level of NH_4Cl (1 g/L), pH (9), and temperature (30 °C) and (b) constant level of glucose (10 g/L), K_2HPO_4 (5 g/L), and NH_4Cl (1 g/L).

Acknowledgements

This work was financially supported by a grant from deputy of research, Kerman University of Medical Sciences (Kerman, Iran).

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