African Journal of Microbiology Research Vol. 6(4), pp. 838-845, 30 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/ AJMR-11-1382
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Biosynthesis of polyhydroxybutyrate (PHB) biopolymer by *Bacillus megaterium* SW1-2: Application of Box-Behnken design for optimization of process parameters

Mahmoud M. Berekaa* and Ali M. Al Thawadi

Biology Department, College of Education, Dammam University, Dammam, Saudi Arabia.

Accepted 14 December, 2011

Bacillus megaterium **SW1-2 producing polyhydroxalkanoate (PHA), previously isolated from activated sewage sludge was selected for the study of PHB biopolymer production. Preliminary experiment showed that the strain capable of producing 36% cell dry weight (CDW) of the polymer during growth on basal E2 medium supplemented with glucose, followed by 28, 15 and 8% CDW during growth on Na-acetate, lactose or Na-succinate, respectively. Spectroscopic analysis by FT-IR revealed characteristic peaks at 1722.8 and 1276 cm^{-1} corresponding to the C=O and C-O stretching groups indicated that the polymer composed mainly of polyhydroxybutyrate (PHB). Statistically based experiments were applied to optimize the culture conditions for production of PHB. Four selected parameters namely; ammonium sulfate, glucose, KH_2PO4 and Na_2HPO_4, respectively, were further investigated using Box-Behnken design to define the optimal production condition. Based on statistical analysis, maximal PHB production (1.45 g/L) was reached using optimal medium composition, which is more than 1.8-folds the basal medium. Verification experiment was carried out to examine model validation and revealed more than 75% validity.**

Key words: Polyhydroxybutyrate, *Bacillus megaterium*, Box-Behnken design, optimization.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are polyesters naturally synthesized and intra-cellularly accumulated in numerous bacteria as intracellular energy storage materials during unbalanced growth (Du et al., 2001; Steinbüchel, 2001; Du and Yu, 2002; Reddy et al., 2009; Ojumu et al., 2004). They attracted considerable attention for many years because they are biodegradable and biocompatible thermoplastics (Madison and Huisman, 1999; Ojumu et al., 2004). Among PHAs, polyhydroxybutyrate (PHB) is the best known polyester, due to its similarity to synthetic petroleum–based plastics such as polypropylene (Mokhtari-Hosseini et al., 2009). PHB has many applications in medicine, veterinary practice, tissue engineering materials, food packaging and agriculture (van der Walle et al., 2001; Zinn et al., 2001; Luengo et al., 2003; Chen and Wu, 2005; Bucci et al., 2005).

Currently, PHB is produced in an industrial scale using Gram negative bacteria such as *Cupriavidus necator* (Vandamme and Coenye, 2004), *Alcaligenes latus* and recombinant *Escherichia coli* (Choi et al., 1998). However, PHB of those organisms contain the outer membrane lipopolysaccharide (LPS) endotoxins that may induce a strong immunogenic reaction and is therefore undesirable for the biomedical application of the PHAs (Chen and Wu, 2005). Possible removal of endotoxin during purification of poly(3-hydroxybutyrate) from Gram negative bacteria was reported by lee et al. (1999). On the other hand, Gram positive bacteria lack LPS, excreting proteins at high concentration and potentially use of cheaper raw materials, therefore considered as better source of PHAs to be used for biomedical applications (Valappil et al., 2007; Lopes et al., 2009; Singh et al., 2009).

*Corresponding author. E-mail: berekaa2005@yahoo.com. Tel: 00966-03-8274155. Fax: 00966-03-8269936.

A number of *Bacillus* species have been reported to accumulate 9 to 67% (Cell Dry Weight) CDW of PHA (Labuzek and Radecka, 2001; Wu et al., 2001; Belma et al., 2002; Borah et al., 2002; Hikmet et al., 2003; Tajima et al. 2003; Valappil et al., 2007; Adwitiya et al., 2009; Reddy et al., 2009). Furthermore, the genus *Bacillus*, in common with many other PHA-accumulating Gram-positive bacteria, accumulates co-polymers of 3-hydroxybutyrate when grown on different substrates (Chen et al., 1991; Caballero et al., 1995; Satoh et al., 2002; Tajima et al., 2003; Valappil et al., 2007 and 2008).

In spite of the advantages of PHAs compared with petroleum-derived plastics, their use is currently limited due to their high production costs (Ojumu et al., 2004; Nikel et al., 2005). In order to reduce the overall cost, it is important to produce PHA with high productivity and high yield. Recently, scientists have been exploring cultivation strategies involving inexpensive, renewable carbon substrates in order to reduce production cost and obtain high productivity (Ojumu, 2004). According to Lee et al. (1997), many carbon sources derived from wastes like whey, cane molasses and sugar beet molasses can be used in production. Recently, PHB proved to be produced from relatively cheaper substrates such as methanol (Kim et al., 2003; Mokhtari-Hosseini et al., 2009), carbon dioxide (Ishizaki et al., 2001), and agro-industrial by-product and rice bran and sea water (Nikel et al., 2005; Nath et al., 2008; Pandian et al., 2010).

Reducing the costs of PHB production by optimizing fermentation process is the basic research objective for industrial application. Medium optimization by application of statistical optimization method, compared to the common “one-factor-at-a-time” method, proved to be powerful and useful tool. Recently, application of statistical methods and response surface methodology (RSM) has gained a lot of impetus for medium optimization and understanding the interactions among various physicochemical parameters involved in biopolymer production (Khanna and Srivastava, 2005; Nikel et al., 2005; Berekaa et al., 2006; Sharma et al., 2007; Yu et al., 2008; Mu et al., 2009; Mokhtari-Hosseini et al., 2009; Pandian et al., 2010).

The main objective of this study was to isolate, characterize and identify PHB producing strain from sewage and soil sample in Eastern Province, Saudi Arabia. The biopolymer was identified by chemical characterization using FT-IR spectroscopic analyses. Furthermore, the effect of different carbon sources on PHB production was closely investigated. Special emphasis was given to the application of statistical experimental design (namely; Box-Behnken design) for optimization of PHB biopolymer production.

MATERIALS AND METHODS

Microorganism; enrichment and isolation

Screening was carried out by enrichment and isolation of bacteria from different soil and sewage samples from Eastern Province in Dammam, Saudi Arabia. For this purpose 1% of the sample was diluted and subsequently cultivated on nutrient agar (NA) medium. Separate colonies were purified by further subculture on NA medium. Purified strains were screened for PHB accumulation by staining with Sudan Black (0.3% (w/v) in 70% ethanol). Bluish-black colonies indicating PHB production and thus used for further studies (modified Belma et al., 2002). The potent isolate was maintained on nutrient agar slant composed of (g/L): peptone; 5, beef extract; 3, NaCl; 2 and agar; 20. Stock culture was subcultured at regular intervals of one month and stored under refrigeration. The bacterium was characterized and identified by 16S rRNA gene sequencing using universal primers as descirbed by (Soliman et al., 2005). The forward and reverse primers were of the following sequences, respectively: AGAGTTTGATCMTGGCTCAG and TACGGYACCTTGTTACGACTT. The 16S rRNA sequence was analysed using BLAST sequence analysis tool available in NCBI, to test for similarity with other 16S rDNA sequences. Subsequently, the sequence was deposited in the GenBank under the accession number HQ124332. Interestingly, the bacterium was recorded as polygutamate exopolymer producer (Berekaa and Al-Otaibi, 2011).

Growth and production conditions

The bacterium was grown in 50 ml aliquot of nutrient broth dispensed in 250 ml Erlenmeyer flask and incubated at 37°C for 24 h at 150 rpm. 1.5% inoculums of the overnight culture was used to inoculate basal salts E2 medium of the following composition (g/L): ammonium sulfate; 2.5, glucose; 20, K_2HPO_4; 1.5, Na_2HPO_4; 3.5, $MgSO_4.7H_2O$; 0.2, traces of yeast extract and 1 ml of trace element solution ($FeSO_4.4H_2O$, $CaCl_2.2H_2O$, $MnSO_4.4H_2O$, $ZnCl_2$ 1 mM each) at 37°C. To test the effect of different carbon sources on PHB production, glucose was replaced by 2% (w/v) Na-succinate, Na-acetate or lactose. 50 ml of the basal medium placed in 250 ml Erlenmeyer flasks were inoculated with 750 µl of the pre-culture. At the end of incubation period, PHB was determined and the cell dry weight was estimated.

Extraction of PHB from the isolate

PHB was extracted from the cell masses by using modified Hypochlorite method (Rawte and Mavinkurve, 2002). For this purpose, the isolate was grown in 250 ml Erlenmeyer flasks containing 50 ml of the basal E2 medium. At the end of incubation period, 1 ml of cell suspension was centrifuged at 6,000 rpm for 15 min. The cell pellet was washed once with 1 ml saline and was re-centrifuged. The cell pellet was then suspended in equal volume of sodium hypochlorite (5.5% active chlorine) and incubated at 45°C for 60 min. This extract was centrifuged at 8,000 rpm for 20 min and the pellet of PHB was washed with water and twice with ethanol:acetone mixture (2:1). The pellet was again centrifuged at 8,000 rpm to get purified PHB. Determination of PHB yield was performed routinely by dry weight estimation. The ultraviolet (UV) absorption spectrum of the polymer was analyzed following its conversion to crotonic acid by treatment with concentrated H_2SO_4, and the absorbence was scanned between 200 and 350 nm with UV-1800 spectrophotometer (Shimadzu Scientific, USA). For dry weight estimation, the pellet after extraction was dried to constant weight.

Analytical procedures

Cell dry weight

After centrifugation of the culture medium, the supernatant was discarded and the cell pellet was washed with distilled water. The washed pellet was resuspended in 1 ml distilled water, transferred

Table 1. Variables and their settings employed in Box-Behnken design for optimization of PHB production by *Bacillus megaterium* SW1-2.

Variable	Code	Level		
		-1	0	+1
Ammonium sulfate	X1	0.1	2.5	4
Glucose	X2	10	20	30
KH_2PO_4	X3	0.5	1.5	3
Na_2HPO_4	X4	1	3.5	5

to pre weighed boats and dried to constant weight at 60°C.

Characterization of extracted PHB by FT-IR

The presence and characterization of PHB in dry cell matter was confirmed by Fourier transform infrared spectroscopy (FT-IR) (Hong et al., 1999). Precipitated dry PHB polymer from *B. megaterium* SW1-2 was used to prepare KBr discs. Spectra were recorded between 400 and 4000 cm^{-1} using Nicolet 6700 FITR spectrometer from the Nicolet Instrument Corporation, USA.

Fractional factorial design

Box-Behnken design

For optimization of PHB production, statistical experimental design using response surface methodology, Box-Behnken design (BBD), (Box and Behnken, 1960) was applied (Figure 3). Normally, PHB is produced by bacteria in the presence of excess carbon, when at least one nutrient essential for growth, such as nitrogen, oxygen, or phosphorus becomes a limiting factor. Therefore, four critical variables namely; ammonium sulphate, glucose, KH_2PO_4 and Na_2HPO_4, were prescribed into 3 levels, coded -1, 0, +1 (Table 1). The design matrix of 27 trials experiment represented in Table 2. For predicting the optimal point, a second-order polynomial function was fitted to correlate relationship between independent variables and response represented by the amount of PHB produced. For the four factors the equation is:

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_{12}X_{12}+\beta_{13}X_{13}+\beta_{14}X_{14}+\beta_{23}X_{23}+\beta_{24}X_{24}+\beta_{34}X_{34}+\beta_{11}X_{11}+\beta_{22}X_{22}+\beta_{33}X_{33}+\beta_{44}X_{44} \quad (1)$$

Where Y is the predicted response, β_0 the model constant; X_1, X_2, X_3 and X_4 independent variables; β_1, β_2, β_3 and β_4 are linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are cross product coefficients and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients.

Microsoft Excel 97 was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2.

Statistical analysis of the data

The data of the PHB production were subjected to multiple linear regressions using MICROSOFT EXCEL 97 to estimate t-value, P-value and confidence level. The significance level (*P*-value) was determined using the Student's t-test. The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. Factors having highest t-value and confidence level over 95% were considered to be highly significant on PHB production. Optimal value of activity was estimated using the solver function of MICROSOFT EXCEL tools.

Table 2. Box-Behenken matrix representing the effect of significant variables affecting polyhydroxybutyrate (PHB) production by *B. megaterium* SW1-2.

Trail no.	X1	X2	X3	X4	PHB (g/L)
1	0	-1	-1	0	0.60
2	0	1	1	0	1.50
3	0	-1	0	1	0.70
4	0	1	-1	0	1.20
5	1	0	0	-1	0.50
6	0	0	1	-1	0.80
7	0	1	0	-1	1.50
8	0	1	0	1	1.80
9	-1	0	1	0	0.90
10	1	0	1	0	0.50
11	-1	0	0	-1	0.90
12	1	1	0	0	1.10
13	0	0	0	0	0.35
14	0	0	1	1	1.30
15	-1	-1	0	0	0.80
16	-1	0	-1	0	0.90
17	-1	0	0	1	1.00
18	0	-1	1	0	0.10
19	0	-1	1	1	0.80
20	1	0	0	1	0.80
21	0	0	-1	-1	1.10
22	0	0	-1	1	1.50
23	1	0	-1	0	0.30
24	0	-1	0	-1	0.80
25	1	-1	0	0	0.40
26	0	0	0	0	0.40
27	-1	1	0	0	2.00

RESULTS AND DISCUSSION

PHB accumulation by *Bacillus* sp. SW1-2

In a program for exploring the potential production of PHB biopolymers, group of 120 bacterial strains were enriched and isolated from soil and aeration tank in a sewage treatment unit in Dammam, Saudi Arabia. Purified strains were screened for PHB accumulation by staining with Sudan Black. Bluish-black colonies indicate PHB production. Among screened bacteria a potent biopolymer producing bacterium candidate, *Bacillus* sp. SW1-2 was recognized. To investigate phylogenetic affiliation of this strain, the complete 16S rRNA gene was amplified, sequenced and deposited in the GenBank with the accession number HQ124332. Comparison of the obtained sequence with other sequences available at

Table 3. Statistical analysis of Box-Behnken design showing coefficient values, t-Stat and P-values for each variable.

Variables	Coefficients	t-Stat	P-value
β_0	0.1069	-	-
β_1	0.0144	2.25	0.0456
β_2	-0.2199	-3.68	0.003
β_3	0.5103	9.13	1.806E-06
β_4	-0.0122	-0.22	0.827
β_{12}	0.1089	2.02	0.068
β_{13}	-0.0351	-0.34	0.737
β_{14}	0.1304	0.87	0.398
β_{23}	0.0176	0.18	0.856
β_{24}	0.2806	2.90	0.014
β_{34}	0.0334	0.36	0.727
β_{11}	-0.0125	-0.14	0.893
β_{22}	0.1041	1.15	0.272
β_{33}	0.4769	5.25	0.0002
β_{44}	0.2519	2.77	0.018

NCBI database revealed the greatest similarity to the corresponding sequence of *Bacillus megaterium*, thus the strain was given the name *B. megaterium* SW1-2, and was chosen for further analysis. Interestingly, a closely related PHB-producing isolate *B. megaterium* strain OU303A was isolated from municipal sewage sludge (Law et al., 2001; Reddy et al., 2009).

Effect of different carbon source on PHB accumulation

One of the most crucial variables affecting PHB biopolymer production is the carbon source. In this concern, the effect of different carbon sources on PHB production by *Bacillus* sp. strain SW1:2 were investigated. The strain exhibited nutritional versality in terms of varied growth and PHB production when tested on various carbon sources. Results in Figure 1 showed the growth and PHB accumulation measured after 48 h incubation. Obviously, the maximum PHB production was attained (36% CDW) when glucose was used as a sole carbon source. Amount of PHB was clearly decreased when glucose was replaced by Na-acetate, lactose or Na-succinate that was 28, 15 and 8% CDW, respectively. Similarly, *B. megaterium* OU303A, *Bacillus cereus* SPV and other bacilli produces PHA biopolymer composed mainly of PHB (97% CDW) during growth on E2 medium supplemented with glucose as a carbon source and in presence of traces of yeast extract (Valappil et al., 2007; Adwitiya et al., 2009; Reddy et al., 2009). However, lower PHB productivity was recognized when lactose or Na-acetate was used as carbon source (Adwitiya et al., 2009; Reddy et al., 2009, Gouda et al., 2001) supporting the results presented in this work.

Chemical analysis of the polymer

Preliminary analysis of polymeric material digested with concentrated H_2SO_4 and scanned with UV-Vis spectrophotometer revealed a sharp peak at 235 nm characteristic of crotonic acid indicating the presence of PHB biopolymer (data not shown).

FT-IR analysis

FT-IR analysis of PHA extracted from cell pellets revealed the C–H and carbonyl stretching bands characteristic to PHB (Figure 2). Absorption bands occurring at 2919.2 and 2851.7 cm^{-1} indicated the presence of aliphatic $-CH_3$ and $-CH_2$ groups. The absorption bands at 1722.8 and 1276 cm^{-1} in extracted PHB sample corresponding to the C=O and C–O stretching groups and were identical to PHB from some Bacilli (Hong et al., 1999, Pandian et al., 2010; Valappil et al., 2007).

Evaluation of factors affecting PHB production

Optimization of PHB production by application of Box-Behnken design

For optimization of PHB production a series of 27 experiments were carried out to obtain a quadratic model. As presented in Table 1, four crucial variables namely; ammonium sulphate, glucose, KH_2PO_4 and Na_2HPO_4, were prescribed into 3 levels, coded -1, 0, +1. The design of this experiment is given in Table 2 together with the experimental results. Regression analysis was performed to fit the response function (PHB production) with the experimental data. The analysis of variance *t*-stat and *p*-value for the four variables indicated that PHB production can be well described by a polynomial model with a relatively high coefficient of determination (R^2 = 0.93) (Table 3).

When presenting experimental results in the form of surface plot (Figures 3A to D) it can be seen that near to high levels of glucose, moderate to high levels of Na_2HPO_4, lower level of ammonium sulfate and KH_2PO_4 supported high PHB production. For predicting the optimal point, within experimental constrains, a second-order polynomial function was fitted to the experimental results of PHB production.

$$Y_{PHB\ (g/L)}=0.11+0.01X_1-0.21X_2+0.5X_3-0.01X_4+0.1X_{12}-0.04X_{13}+0.13X_{14}+0.02X_{23}+0.3X_{24}+0.04X_{34}-0.01X_{11}+0.1X_{22}+0.5X_{33}+0.3X_{44} \quad (2)$$

Where X_1, X_2, X_3 and X_4 represent codified values for ammonium sulfate, glucose, KH_2PO_4 and Na_2HPO_4, respectively.

At the model level, the correlation measures for the

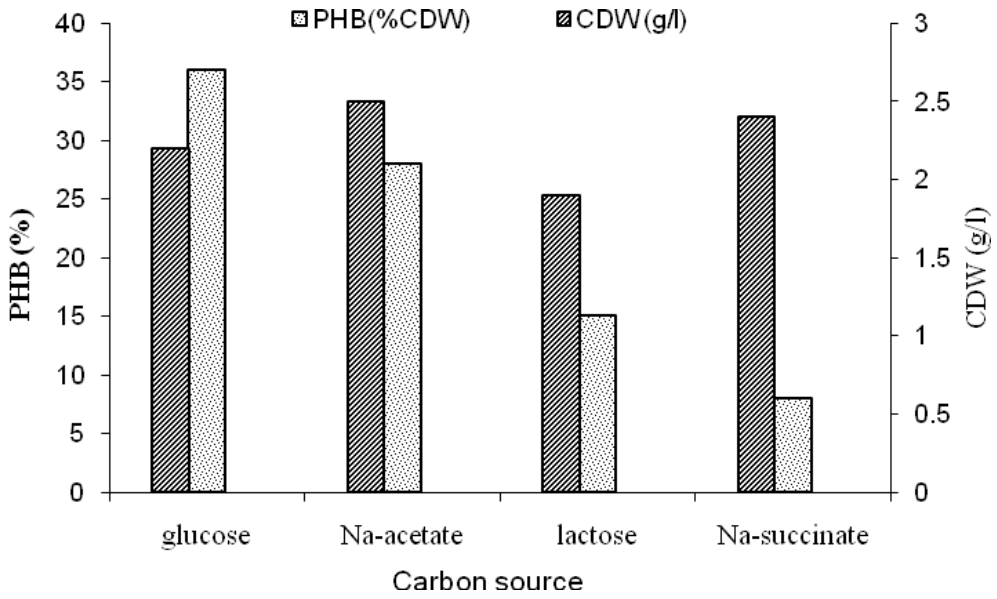

Figure 1. Effect of different carbon sources on growth (DCW) and polyhydroxybutyrate (PHB) production in culture of *B. megaterium* SW1-2.

estimation of the regression equation are the multiple correlation coefficients R and the determination coefficient R^2. The closer the value of R is to 1, the better is the correlation between the observed and the predicted values. In this experiment, the value of R was 0.96 for PHB production. This value indicates a high degree of correlation between the experimental and the predicted values. The value of determination coefficient R^2 = 0.93 being a measure of fit of the model, indicates that about 7% of the total variations are not explained by the activity model.

On analyzing Equation 2 with solver, based on non-linear optimization algorithm, with substitution and reduction, maximum PHB production was found to be 1.92 g/L when concentrations of tested variables were reached to the maximum. Interestingly, results presented in Figures 3A, C and D clearly indicated that PHB production was enhanced due to interaction between glucose with ammonium sulfate, K_2HPO_4 or Na_2HPO_4, respectively. Indeed, interaction between Na_2HPO_4 and KH_2PO_4 significantly affecting PHB production implies that its buffer role is very important for PHB accumulation, supported by the finding of Mokhtari-Hosseini et al. (2009). Furthermore, glucose concentration was the most significant factor in the overall design (*p* value=0.003). Interestingly, most of *Bacillus* strains such as *B. megaterium* OU303A, *B. cereus* SPV and *B. Thuringienesis* IAM 12077 showed maximum PHB production in presence of glucose and under limitation of ammonium sulfate concentration (Valappil et al., 2007; Adwitiya et al., 2009; Reddy et al., 2009) others such as *B. megaterium* NCIM 2475 and *B. megaterium* SRKP-3 do not (Otari and Ghosh, 2009; Pandian et al., 2010), supporting the results presented in this work.

Verification of the calculated optimum was done with a culture medium representing the optimal points and yielding 1.45 g/L. The results collectively showed that PHB production by *B. megaterium* SW1-2 was about 1.8-folds increased when cultivated in the optimal medium developed by BBD, as compared to basal E2 medium. Therefore, the statistical experimental design proved to be a powerful and useful tool for enhancing PHB production and confirm the necessity of the optimization process.

Conclusion

PHAs are synthesized and intracellularly accumulated as granules in many bacteria. Results analysis revealed that *B. megaterium* SW1-2 capable of producing PHB biopolymer and was proven by FT-IR spectroscopy as PHB. Furthermore, conditions for PHB production in *B. megaterium* SW1-2 is similar to the conditions of production in several bacilli that synthesize and accumulate PHA as carbon and energy storage materials under limitation in nitrogen source. RSM proved to be a powerful and useful tool for enhancing PHB production in this strain. Where, PHB production revealed approximately 1.8-folds increase in comparison with the production on basal E2 medium. Molecular characterization of the *pha*C gene synthase could be the next step that may allow for the application of this strain in biotechnological production of biodegradable PHB

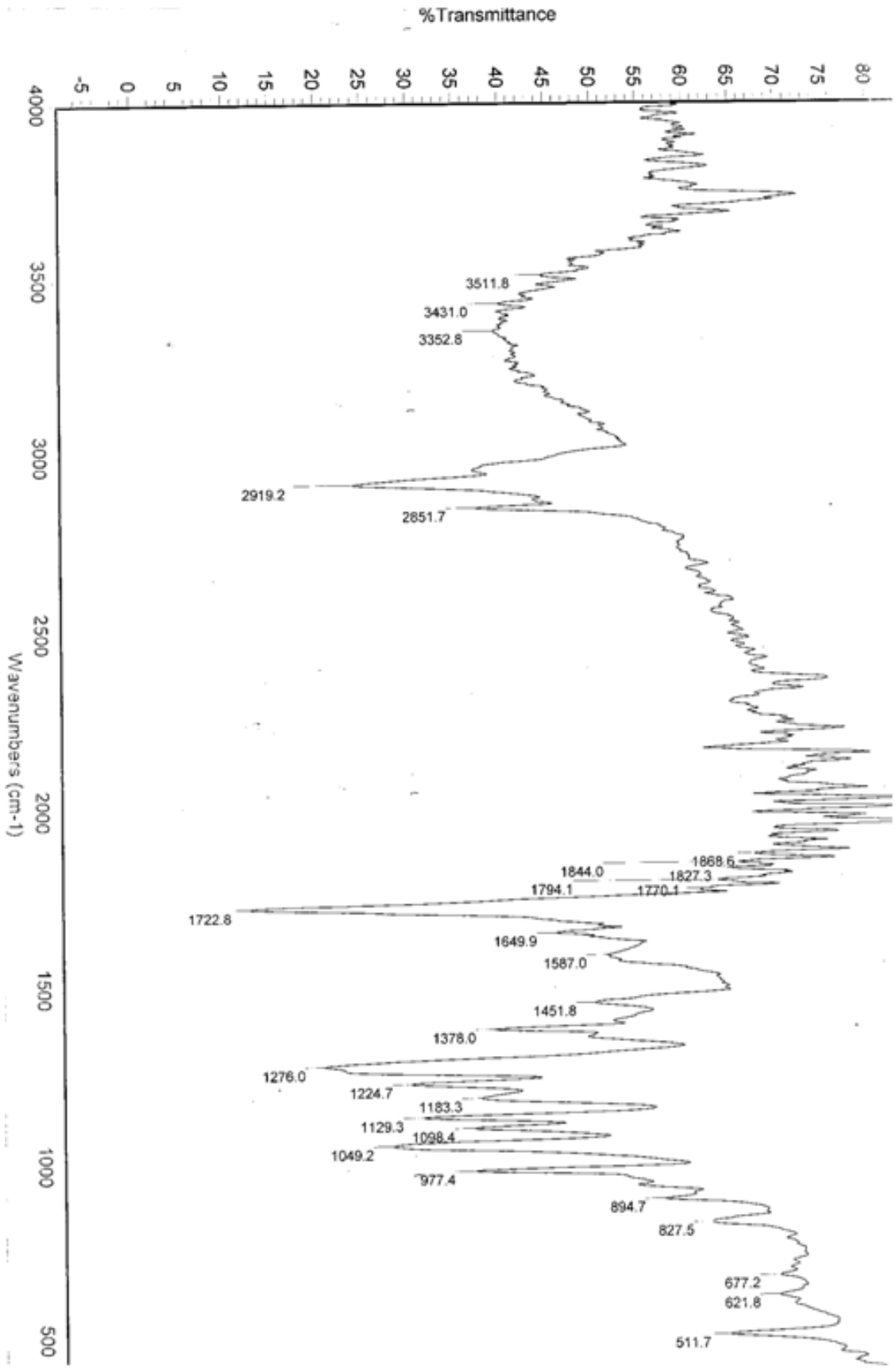

Figure 2. FT-IR spectroscopy of PHB biopolymer produced from *B. megaterium* SW1-2.

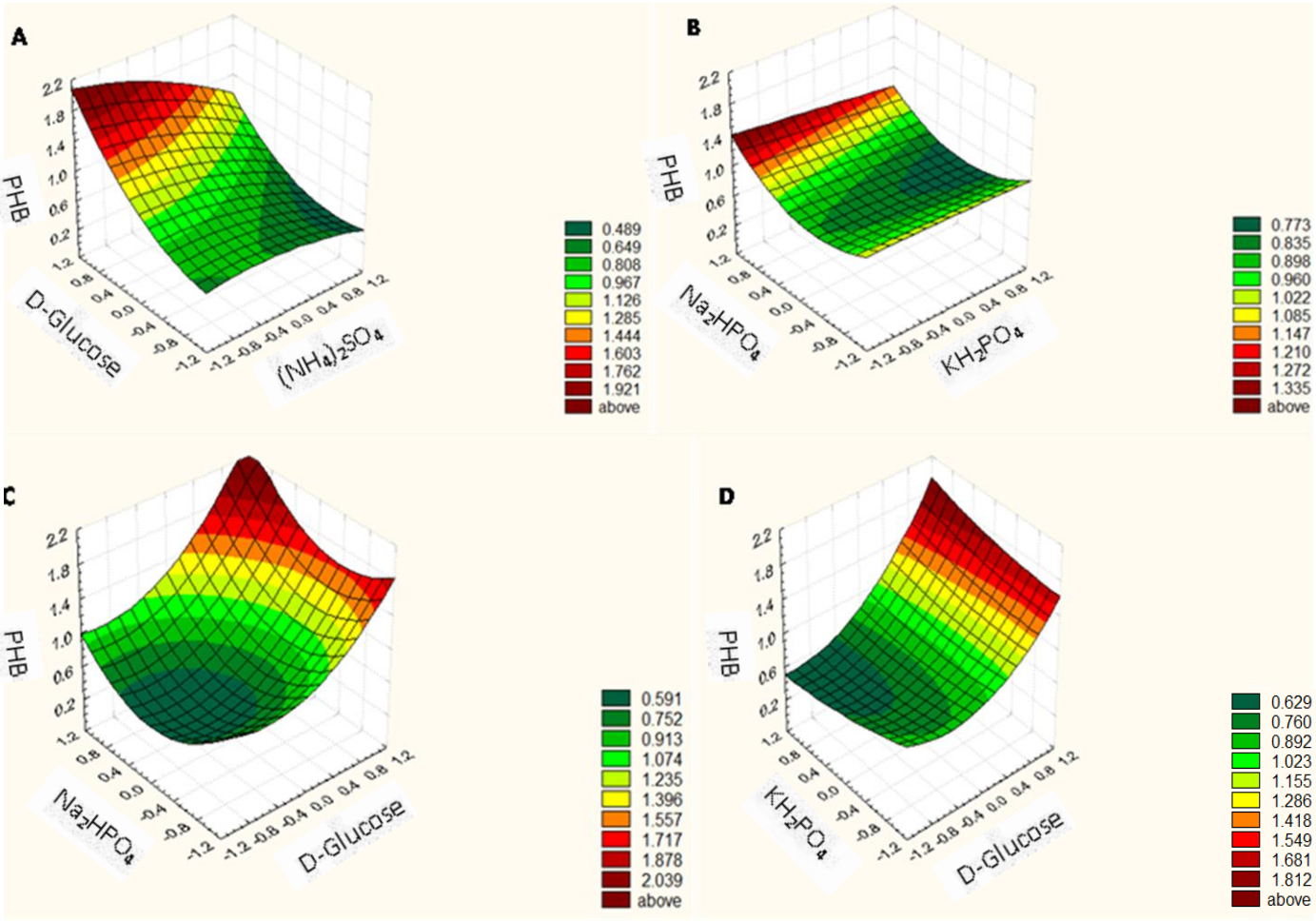

Figure 3A to D. Three dimensional response surface graphics showing the behaviour of PHB production by *B. megaterium* SW1-2 as affected by different culture conditions.

biopolymer.

ACKNOWLEDGEMENT

Authors are grateful for the financial support of this work by the Deanship of Scientific Research, Dammam University (Grant Application No-2011121).

REFERENCES

Adwitiya P, Prabhu A, Kumar A, Rajagopal B, Dadhe K, Ponnamma V, Shivakumar S (2009). Optimization of process parameters for maximum poly-Beta-hydroxybutyrate (PHB) production by *Bacillus thuringiensis* IAM 12077. Polish J. Microbiol., 58: 149-154.

Belma AY, Zehra N, Yavuz B (2002). Determination of PHB growth quantities of certain *Bacillus* sp. isolated from soil. Turk E. J. Biotechnol., Special issue: 24-30.

Berekaa MM, Abdel-Fattah YR, El-Sayed SM, El-Borai AM, El Aassar SA (2006). Optimization of culture conditions for production of polyamide biopolymer (Polyglutamate) by *Bacillus* sp. Strain-R. J. Biol. Sci., 6: 687-694.

Berekaa MM, Al-Otaibi SM (2011). Enhanced Production of Poly glutamic acid (PGA) by *Bacillus megaterium* SW1-2 using statistical experimental design. World J. Microbiol. Biotechnol., submitted

Borah B, Thakur PS, Nigam JN (2002). The influence of nutritional and environmental conditions on the accumulation of poly-bhydroxybutyrate in *Bacillus mycoides* RLJ B-017. J. Appl. Microbiol., 92:776–783.

Box GEP, Behnken DW (1960). Some new three level designs for the study of quantitative variables. *Technometrics* 2: 455-475.

Bucci DZ, Tavares LBB, Sell I (2005). PHB packaging for storage of food products. Polym. Test, 24: 564–571.

Caballero KP, Karel SF, Register RA (1995). Biosynthesis and characterization of hydroxybutyrate-hydroxycaproate copolymers. Int. J. Biol. Macromol., 17: 86–92.

Chen GQ, Konig KH, Lafferty RW (1991). Occurrence of poly-D (-)-3-hydroxyalkanoates in the genus *Bacillus*. FEMS Microbiol. Lett., 84:173–176

Chen GO, Wu Q (2005). The application of polyhydroxyalkanoates as tissue engineering materials. Biomater., 26: 6556–6578.

Choi JI, Lee SY, Han K (1998). Cloning of the *Alcaligenes latus* polyhydroxyalkanoate biosynthesis gene and use of these genes for enhanced production of poly(3-hydroxybutyrate)in *Escherichia coli*. Appl. Environ. Microbiol., 64: 4897-4903.

Du G, Yu J (2002). Metabolic analysis on fatty acid utilization by *Pseudomonas oleovorans*: mcl-poly-3-hydroxyalkanoates) synthesis versus oxidation. Process Biochem., 38: 325-332.

Du G, Si Y, Yu J (2001). Inhibitory effect of medium-chain-length fatty

acid on synthesis of polyhydroxyalkanoates from volatile fatty acid by *Ralstonia eutrophus*. Biotechnol. Lett., 23: 1613-1617.
Gouda MK, Swellam EA, Omar HS (2001). Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiol. Res., 156: 201–207.
Hikmet K, Belma A, Zehra KN, Nazime M, Yavuz B (2003). Production of PHB and differentiation of putative *Bacillus* mutant strain by SDS-PAGE of total cell protein. Afri. J. Biotechnol., 2: 147-149.
Hong K, Sun S, Tian W, Chen GQ, Huang W (1999). A rapid method for detecting bacterial polyhyroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. Appl. Microbiol. Biotechnol., 51:523-526.
Ishizaki A, Tanaka K, Taga N (2001). Microbial production of poly-3-hydroxybutyrate from CO_2. Appl. Microbiol. Biotechnol., 57: 6–12.
Kim P, Kim JH, Oh DK (2003). Improvement in cell yield of Methylobacterium sp. reducing the inhibition of medium components for poly-b-hydroxybutyrate production. World J. Microbiol. Biotechnol., 19: 357–361.
Labuzek S, Radecka I (2001). Biosynthesis of PHB terpolymer by *Bacillus cereus* UW85. J. Appl. Microbiol., 90: 353–357.
Law KH, Leung YC, Lawford H, Chau H, Lo WH, Yu PH (2001), Production of polyhydroxybutyrate by *Bacillus* species isolated from municipal activated sludge. Appl. Biochem. Biotechnol., 91: 515–524.
Lee SY, Middelberg APJ, Lee YK (1997). Poly (3-hydroxybutyrate) production from whey using recombinant Escherichia coli. Biotechnol. Lett., 19: 1033 – 1035.
Lee SY, Choi J-I, Han K, Song JY (1999). Removal of Endotoxin during Purification of Poly(3-Hydroxybutyrate) from Gram-Negative Bacteria. Appl. Environ. Microbiol., 65: 2762–2764.
Lopes MSG, Roch RCS, Zanotto SP, Gomez GC, de Silva LF (2009). Screening of bacteria to produce polyhydroxyalkanoates from xylose. World J. Microbiol. Biotechnol., 25:1751–1756
Luengo MJ, Garcıa B, Sandoval A, Naharro G, Olivera RE (2003). Bioplastics from microorganisms. Curr. Opi. Microbiol., 6:251–260.
Madison LL, Huisman GW (1999). Metabolic engineering of poly(3-ydroxyalcanoates): From DNA to plastic. Microbiol. Molecule Biol. Rev., 63: 21-53.
Mokhtari-Hosseini ZA, Vasheghani-Farahani E, Heidarzadeh-Vazifekhoran A, Shojaosadati SA, Karimzadeh R, Darani KK (2009). Statistical media optimization for growth and PHB production from methanol by a methylotrophic bacterium. Bioresour. Technol., 100: 2436–2443.
Mu W, Chen C, Li X, Zhang T, Jiang B (2009). Optimization of culture medium for the production of phenyllactic acid by *Lactobacillus* sp. SK007, Bioresour. Technol., 100: 1366–1370.
Nath A, Dixit M, Bandiya A, Chavda S, Desai AJ (2008). Enhanced PHB production and scale up studies using cheese whey in fed batch culture of *Methylobacterium* sp. ZP24. Bioresour. Technol., 99: 5749–5755.
Nikel PI, Pettinari MJ, Mendez BS, Galvagno MA (2005). Statistical optimization of a culture medium for biomass and poly(3-hydroxybutyrate) production by a recombinant *Escherichia coli* strain using agroindustrial by-products. Int. Microbiol., 8: 243–250.
Ojumu TV, Yu J, Solomon BO (2004). Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer. Afr. J. Biotechnol., 3: 18-24.
Pandian RS, Deepak V, Kalishwaralal K, Rameshkumar N, Jeyaraj M, Gurunathan S (2010). Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by *Bacillus megaterium* SRKP-3. Bioresour. Technol., 101: 705–711.
Rawte T, Mavinkurve S (2002) A rapid hypochlorite method for the extraction of polyhydroxy alkonates from bacterial cells. Indian J. Exptl. Biol., 40: 924–929.
Reddy VST, Thirumala M, Mahmood KS (2009). Production of PHB and P(3HB-co-3HV) biopolymers of *Bacillus megaterium* strain OU303A isolated from municipal sewage sludge. W. J. Microbiol. Biotechnol., 25: 391-397.
Satoh Y, Minamoto N, Tajima K, Munekata M (2002). Polyhydroxylakanoate Synthase from *Bacillus* sp. INT005 is composed of PhaC and PhaR. J. Biosci Bioengineer., 94: 343-350.
Sharma L, Singh AK, Panda B, Mallick N (2007). Process optimization for poly-hydroxybutyrate production in a nitrogen fixing cyanobacterium, *Nostoc muscorum* using response surface methodology. Bioresour. Technol., 98: 987–993.
Singh M, Patell SKS, Kalia VC (2009). *Bacillus subtilis* as potential producer for polyhydroxyalkanoates. Microbial Cell Factories, 8:38. doi:10.1186/1475-2859-8-38
Soliman NA, Berekaa MM, Abdel-Fattah YR (2005). Poly glutamic acid (PGA) production by *Bacillus* sp. SAB-26: application of Plackett–Burman experimental design to evaluate culture requirements. Appl. Microbiol. Biotechnol., 69:259-267.
Steinbüchel A (2001). Perspectives for Biotechnological Production and Utilization of Biopolymers: Metabolic Engineering of Polyhydroxyalkanoate Biosynthesis Pathways as a Successful Example. Biomolecular Biosci., 1: 1-24.
Tajima K, Igari T, Nishimura D, Nakamura M, Satoh Y, Munekata M (2003). Isolation and characterization of *Bacillus* sp. INT005 accumulating polyhydroxyalkanoate (PHA) from gas field soil. J. Biosci. Bioeng., 95: 77–81
van der Walle GA, de Koning GJ, Weusthuis RA, Eggink G (2001). Properties, modification, and application of biopolyesters. Adv. Biochem. Eng. Biotechnol., 71: 263–291.
Valappil PS, Misra KS, Boccaccini RA, Keshavarz T, Bucke C, Roy I (2007). Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterized *Bacillus cereus* SPV. Biotech., 132: 251–258.
Valappil SP, Rai R, Bucke C, Roy I (2008). Polyhydroxyalkanoate biosynthesis in *Bacillus cereus* SPV under varied limiting conditions and an insight into the biosynthetic genes involved. J. Appl. Microbiol., 104:1624–1635.
Vandamme P, Coenye T (2004). Taxonomy of the genus *Cupriavidus*: A tale of lost and found. Int. J. Syst. Evol. Microbiol., 54: 2285-2289.
Wu Q, Huang H, Hu G, Chen J, Ho PK, Chen QG (2001). Production of PHB by *Bacillus* sp. Jma5 cultivated in molasses media. Antonie Van Leeuwenhock. 80: 111-118.
Yu L, Lei T, Rena X, Pei X, Feng Y (2008). Response surface optimization of l-(+)-lactic acid production using corn steep liquor as an alternative nitrogen source by *Lactobacillus rhamnosus* CGMCC 1466. Biochem. Eng. J., 39: 496–502.
Zinn M, Witholt B, Egli T (2001). Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. Adv. Drug Deliver. Rev., 53: 5–21.
Otari SV, Ghosh SJ (2009). Production and Characterization of the Polymer Polyhydroxy Butyrate-copolyhydroxy Valerate by *Bacillus Megaterium* NCIM 2475. Cur. Res. J. Biol. Sci., 1(2): 23-26, 2009