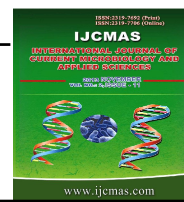
Int.J.Curr.Microbiol.App.Sci (2013) 2(11): 227-241



International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 Volume 2 Number 11 (2013) pp. 227-241
http://www.ijcmas.com

Original Research Article

Optimization of Polyhydroxybutyrate (PHB) production by *Azotobacter vinelandii* using experimental design

Abd El-Raheem R. El-Shanshoury[1, 2,*], El-Refaie Kenawy[3], Amro A. Amara[4,5], Afrah F. Salama[6] and Shaimaa S. Kishk[6]

[1]Bacteriology Research Unit, Botany Department, Faculty of Science, Tanta University, Tanta 31527, Egypt.
[2]Biotechnology Department, Faculty of Science, Taif University, P.O. Box 888, Taif 21974, Kingdom of Saudi Arabia
[3]Polymer Research Unit, Chemistry Department, Faculty of Science, Tanta University, Tanta 31527, Egypt
[4]Protein Research Department, Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technology Applications, Alexandria, Egypt;
[5]Microbiology Division, Pharmaceutics Department, Faculty of Pharmacy at King Saud University (KSU) Riyadh, kingdom of Saudi Arabia
[6]Biochemistry Unit, Chemistry Department, Faculty of Science, Tanta University, Tanta 31527, Egypt
**Corresponding author*

Keywords

Azotobacter vinelandii; Polyhydroxybutyrate; Plackett-Burman and Box-Behnken; Optimization.

A B S T R A C T

In this study, thirty local isolates of *Azotobacter* spp. were isolated from Egyptian soil and screened for PHB production. Plackett-Burman and Box-Behnken optimization methods have been used to optimize PHB production from the best locally isolated producer. The most producer isolate was identified to the molecular level using 16S rRNA as *Azotobacter vinelandii*. Nine variables represent the medium constituents and the environmental conditions were randomized. The accumulation of PHB granules in cells of *A. vinelandii* significantly depended on the tio of C- and N- sources in the culture medium, minerals, temperature and aeration. According to the main effect analysis, all the above variables have a positive effect on PHB production. The most effective and promising variables included shaking rate with confidence 90.04%, followed by $FeSO_4$, sucrose and NaNO3 with confidence levels 81.16, 78.88 and 77.27%, respectively. The maximum yield of PHB was 0.88 gm/gm dry cells. ANOVA analysis of the regression model showed significant interaction between the various variables that reflect the powerful of Plackett-Burman and Box-Behnken optimization methods. The present data indicated that strategies included in this study could be used for PHB production by this bacterium with gh HB content and adequate properties of the biopolymer

Introduction

Azotobacter vinelandii is an obligate aerobic bacterium that able to fix atmospheric nitrogen and grow under microaerophilic conditions. This

microorganism is able to synthesize three molecules of important biotechnological and biochemical applications; the extracellular polysaccharide alginate, sidephores compounds and polyhydroxybutyrate (PHB). The Latter is biodegradable thermoplastic polyester analogous or better than those of chemically synthesized and petroleum-based polymers such as polyethylene and polypropylene (Byrom, 1987; Doi, 1990). PHB is a biopolymer that has been implicated in supporting nitrogen fixation (Segura *et al*., 2003), biodegradable thermoplastic material for management including plastics, films, and fiber strategies, and biocompatibility in the medical devices (Steinbüchel, 1995; Gouda *et al*., 2001; Lenz and Marchessault, 2005). This polymer is accumulated due to depletion of nitrogen, phosphorous or oxygen to form carbon and energy reserve material (Anderson and Dawes, 1990; Aldor and Keasling, 2003; Burns *et al*., 2007; Galindo *et al*., 2007; Khanna and Srivastava, 2005; Suzuki *et al*., 2008).

Production of PHB has been reported using microorganisms such as yeast (Lee *et al*., 1997), *Alcaligenes latus* (Grothe and Chistri, 2000), *A. eutrophus* (Song *et al*., 2001), *Pseudomonas* species (Ashby *et al*., 2002), wide range of bacterial and archaeal species (Aldor and Keasling, 2003), *Rhodobacter sphaeroides* (Chen *et al*., 2006), *Ralstonia eutropha* (Verlinden *et al*., 2007) *Bacillus subtilis* and *Bacillus megaterium* (Nur Yüksekdag *et al*., 2004; Chaijamrus and Udpuay, 2008), recombinant *Escherichia coli* (Yu *et al*., 2002; Khanna and Srivastava, 2005; Li *et al*., 2007) and *Azotobacter* species (Page and Knosp, 1989; Segura *et al*., 2003; Kishk, 2009; Diaz-Barrera and Soto, 2010).

The microbial large scale production of PHB is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to petrochemical plastics (Doi and Steinbüchel, 2001; Sims, 2003; Apostolis *et al*., 2006). Various nitrogen-rich media containing casein hydrolysate, yeast extract, tryptone, casamino acids, corn steep liquor and collagen hydrolysate (Lee and Chang, 1995; Bormann *et al*., 1998; Ghaly, 2003; Khanna and Srivastava, 2005; Chaijamrus and Udpuay, 2008) have been used for PHB production. Carbon-substrate rich media such as molasses, whey, hemicelluloses, palm oil, starch, glucose, fructose, sucrose, maltose, gluconate or glycerol accumulates PHB in bacterial cells (Page, 1989; Page and Knosp, 1989; Quagliano *et al*., 1994; Alias and Tan, 2005; Chaijamrus and Udpuay, 2008; Kishk, 2009) and have been used as substrates for PHB production. However, unrefined carbon sources such as corn syrup, cane molasses, beet molasses, or malt extract, also supported PHB formation, obtaining yields of PHB comparable to, even better than the refined sugars.

Production of PHB by fermentation has been normally operated as a two stage fermentation fed-batch processes, an initial growth phase followed by a PHB formation stage (Chen and Page, 1997). Among factors influencing production costs is the recovery of the product. In this context, Page and Cornish (1993) reported that beside the substrate cost, the PHA extraction method from inside the cells, and the treatment of the fermentation wastes are cost effective factors. The substrate cost affects the overall cost but the cheapest substrate is not always the ideal choice concerning the downstream

processes. When the PHB productivity increased from 1.98 to 3.2 g/h, the PHB production cost decreased from $5.37/kg to $4.91/kg (Lee and Choi, 1998). Aiming to wide range of industrial applications and establishing method for changing PHAs compositions as well as PHAs' overproduction, many researchers have been conducted molecular biology and protein engineering of PhaC synthases, phbA, phbCand phbB genes (Amara *et al*., 2001; 2002; Taguchi, 2001, 2002; Segura *et al*., 2003). The optimization method of Plackett-Burman, that still one of the most promising approaches was recommended by Page and Cornish (1993). Recently, Diaz-Barrera and Soto (2010) proposed new experimental strategies based on integration between molecular and bioengineering techniques to enhance the productivity of PHP from *A. vinelandii.*

This study aimed to evaluate the potentiality of *Azotobacter vinelandii*, isolated from soil samples collected from Tanta, Gharbia Governorate, Egypt for PHB production. The advantages of using *A. vinelandii* in PHB production is the easy attainment of this bacterium from soil and fresh water. The most economic parameters, which gave the highest amount of the biopolymer PHB, were undertaken using Plackett-Burman and Box-Behnken designs, in addition to statistical analyses.

Materials and Methods

Microorganisms used

Thirty isolates belonging to the genus *Azotobacter* were isolated previously from cultivated and non-cultivated soil samples, collected from different localities at Tanta, Gharbia Governorate, Egypt. The isolates were purified on nitrogen free medium described by Vancura and Mancura (1960). The pure isolates were characterized and the most PHB promising *Azotobacter vinelandii* has been used in this study and identified according to the methods described in Tchan (1984) and by sequencing its16SrDNA genes and comparing the sequences with a data base library using the BLASTN (Altshul *et al*., 1997) program, a subprogram of Basic Local Alignment Search Tool (BLAST), through the network service of the National Center for Biotechnology Information (NCBI) against nonredundant sequences. The sequences obtained were aligned with *A. vinelandii* 16SrRNA sequences available in the GenBank using the EasyAlign (Miranda, 2002) program that employs the MALIGN algorithm (Wheeler and Gladstein, 1998)

Medium and environmental conditions used

The following enrichment N2-Free medium after Vancura and Mancura (1960) with modification was used for PHB production. It contained the following components as g/l: Sucrose 20; K_2HPO_4 0.65; KH_2PO_4 0.16; NaCl 0.2; $MgSO_4.7\ H_2O$ 0.2; $CaCO_3$ 2; $FeSO_4$ 0.005; $NaBO_4$ 0.005; and bacto agar (Law and Slepecky, 1961). The medium constituents and the environmental conditions have been randomized according to Plackett-Burman (1946) design (+1,-1); temperature (37,30) ^oC, pH (7,5), sucrose (30,5) g/l, NaNO3 (2,0.1) g/l, KH2PO4 (1,0) g/l, NaCl (0.5,0) g/l, MgSO4 (0.5,0) g/l, FeSO4 (0.005,0) g/l, shaking (250,200) rpm.

Experimental designs

Plackett-Burman

Eighteen experiments containing +1 and -1 values following Plackett-Burman

design have been conducted as in table 1 to optimize ten variables represented at high and low levels, which are donated by +1 and -1. All the eighteen experiments were conducted using 250 ml Erlenmeyer flasks containing 100 ml media. The media and environmental conditions have been randomized using Plackett-Burman design (+1,-1). The mean of +1 experiment has been calculated using the following formula: $(\sum+1)/\ n_{(+1)}$. The mean of -1 experiment has been calculated using the following formula: $(\sum-1)/\ n_{[-1]}$. The main effect of each factor has been calculated from the following formula:

$$\textbf{Main effect} = \sum(+1)/n_{[+1]} - \sum(-1)/n_{[-1]}.$$

The main effect show whether the variable(s) amount is effective positively or negatively on PHB production.

The results of the Plackett-Burman design experiments were applied to linear multiple regression analysis using Microsoft Excel 2002. The statistical analysis of the experimental results in Table 1 has been summarized in Table 3. The variables whose confidence levels % were ≥ than 90% were considered to be significantly affected on the PHB. Variables with confidence level% less than 90% till 70% were considered effective (Stowe and Mayer, 1966).

Generating 1st order Model

The model created from the analysis of Plackett-Burman experimental design using multiple regression analysis is based on the 1st order-model $Y= \beta_0 +\sum \beta_i$ Xi13, Where Y is the predicted response, β_0 model intercept, β_i variables linear coefficient. ANOVA test was generated for each response to determine the relationship between the variables at the 90% or higher confidence level.

Extraction, purification and determination of PHB

The assay was performed spectrophotometrically (PerkinElmer-.UV/VIS Spectrometer Lambda) to determine PHB as crotonic acid. The PHB amount was determined by calculating the amount of crotonic acid derived from the standard curve and calculated as gm PHB/gm cells. *Azotobacter* cultures were grown and inoculated with single colony using tooth peak to 100 ml of modified liquid medium of Vancura and Mancura (1960) in 250 ml Erlenmeyer flasks and incubated in an orbital shaker at different shaking rates for 6 days of incubation. Bacterial cells were separated with centrifugation at 4000 rpm for 15 min and were dried at 40°C for 24 h. The dry weight of the pellets was determined. Bacterial cell walls were lysed by adding sodium hypochlorite, mixing and incubating at 60°C for 1 h. Supernatant was obtained by centrifugation and transferred to a Soxhlet system. Cell lipids and other molecules were treated with 5 ml 96% ethanol and acetone. PHB was extracted by hot chloroform (adding 10 ml chloroform in a water bath). Then chloroform was evaporated to obtain PHB crystals. By adding 10 ml of 98% sulfuric acid at 60ºC for 1 h, PHB crystals were converted into crotonic acid. The absorbance of the solution was measured at 235 nm in a UV spectrophotometer (Perkin-Elmer Lambada EZ201-UVvis) against sulfuric acid as blank. The amounts of PHB per gram dry weight of bacterial cells were determined using a standard curve of PHB according to Kuniko *et al.,* (1988).

Result and Discussion

Taxonomic classification and identification of *A. vinelandii*

The morphological, physiological and biochemical characters of the most PHB active isolate (data not shown) were found to be similar with those cited in Bergey s Manual for Systematic Bacteriology for the genus *Azotobacter*. The isolate was also subjected to molecular identification and its DNA was isolated as pure unique band.

The sample of 16S ribosomal DNA contained approximately 1500 base pairs in length (Fig. 1) that was amplified by PCR, and partially sequenced as the following:

CGCGGTAATACGAAGGGTGCAAGT AATCGGAATTACTGGGCGTAAACGC GCTAGGTGGTTCGGCAAGTTGGATG TGAAAGCCCCGGG CTACCTGGGAAC-CGCATCCAAAACT ACTGGGCTAG A G T A C G G T A G A GGGTGGTGGAATTTCCTGTGTAGCG GTGAAATGCGTAGATATAGGAAGGAACA CC GTGGCGAAGGCGGCC ACCTGGACC GATACTGAC ACTGAGGTGCGAAAG CGTG GGA GCAAA CAGGATTAGAT ACCCTGGTAGTCCA CAGGACCC TG-GTAGTCCA CGCCGTAAA CGATGT C G A C T A G C C G T T G G G CTCCTTGAGAGCTTAGTGGCGCAGC T AACGCATTAAG TCGAACCGC-C T G G G G A G T A C G G C C GCAAGGTTAAAACTCAATGAATTGA CGGGGGCCCG ACAAGCGGTGGAGCT-GTG GTTTAATTCGAAGCCAACGCGA

Sequence was then compared to the public database of National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). Analysis of the sequences data revealed that query sequence showed greatest homology with *A. vinelandii* (95%). Thus identification of the selected and most potent isolate was confirmed to be *A. vinelandii*.

Variables affecting PHB production by *A. vinelandii*

Increasing the productivity of PHB using Plackett and Burman design

The results obtained in Table1 indicated that there was a wide variation in PHB production from (0 to 0.88 PHB g/g D. wt.), using Plackett and Burman experiments that highlighted the importance of optimizing culture variables in attaining higher PHB production rate. The experiment showed the maximum PHB production rate was at the following conditions: temperature at 37°C, pH 7, sucrose 30 g/l, $NaNO_3$ 2 g/l, KH_2PO_4 1 g/l, NaCl 0 g/l, $MgSO_4$ 0.5 g/l, $FeSO_4$ 0.005 g/l, $CaCl_2$ 0.06 g/l, $NaMoO_4$ 0.05 and shaking rate at 250 rpm. From the results obtained in Table 2 by regression analysis of Plackett and Burman design, it was found that non of the variables has a p=0.05 significant effect on the PHB production which indicate the need for conducting a further optimization. The R-Squared statistic indicates that the model as fitted explains 60.6577% of the variability in PHB. The adjusted R-squared statistic is 16.3977%. The standard error of the estimate shows the standard deviation of the residuals to be 0.229216. The mean absolute error of 0.116353 is the average value of the residuals. The Durbin-Watson statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in your data file. Since the P-value is greater than 0.05, there is no indication of serial autocorrelation in the residuals at the 95.0% confidence level.

Table.1 Plackett-Burman design for PHB optimization

	Experiment No.	Temperature	pH	sucrose	$NaNO_3$	KH_2PO_4	NaCl	$MgSO_4$	$FeSO_4$ **	$NaMoO_4$ **	Shaking	PHB gm/gm cells
Plackett-Burman Experiments	1	-1 (30)	1(7)	1(30)	1(2)	1(1)	1(0.5)	1(0.5)	1(0.005)	1(0.05)	-1(200)	0.199
	2	-1(30)	-1(5)	1(30)	-1(0.1)	1(1)	1(0.5)	1(0.5)	-1(0)	-1(0)	1 (250)	0.000
	3	-1(30)	1(7)	-1(5)	1(2)	-1(0)	1(0.5)	1(0.5)	1(0.005)	1(0.05)	-1 (200)	0.047
	4	1(37)	1(7)	1(30)	1(2)	1(1)	-1(0)	1(0.5)	1(0.005)	1(0.05)	1 (250)	0.880
	5	1(37)	-1(5)	1(30)	-1(0.1)	1(1)	1(0.5)	-1(0)	1(0.005)	1(0.05)	1 (250)	0.600
	6	1(37)	-1(5)	-1(5)	1(2)	-1(0)	1(0.5)	1(0.5)	-1(0)	-1(0)	1 (250)	0.500
	7	1(37)	-1(5)	-1(5)	1(2)	1(1)	-1(0)	1(0.5)	1(0.005)	1(0.05)	-1 (200)	0.083
	8	-1(30)	-1(5)	-1(5)	-1(0.1)	1(1)	1(0.5)	-1(0)	1(0.005)	1(0.05)	1 (250)	0.042
Plackett-Burman Experiments	9	1(37)	1(7)	-1(5)	-1(0.1)	-1(0)	1(0.5)	1(0.5)	-1(0)	-1(0)	1 (250)	0.000
	10	1(37)	-1(5)	1(30)	-1(0.1)	-1(0)	-1(0)	1(0.5)	1(0.005)	1(0.05)	-1 (200)	0.042
	11	-1(30)	-1(5)	-1(5)	-1(0.1)	-1(0)	-1(0)	-1(0)	1(0.005)	1(0.05)	1 (250)	0.000
	12	-1(30)	1(7)	-1(5)	1(2)	-1(0)	-1(0)	-1(0)	-1(0)	-1(0)	-1 (200)	0.000
	13	-1(30)	1(7)	1(30)	-1(0.1)	1(1)	-1(0)	-1(0)	-1(0)	-1(0)	-1 (200)	0.097
	14	1(37)	1(7)	1(30)	-1(0.1)	-1(0)	1(0.5)	-1(0)	-1(0)	-1(0)	-1 (200)	0.02
	15	-1(30)	-1(5)	1(30)	1(2)	-1(0)	-1(0)	1(0.5)	-1(0)	-1(0)	-1 (200)	0.000
	16	-1(30)	1(7)	-1(5)	1(2)	1(1)	-1(0)	-1(0)	1(0.005)	1(0.05)	-1 (200)	0.050
	17	1(37)	1(7)	1(30)	1(2)	1(1)	1(0.5)	-1(0)	-1(0)	-1(0)	1 (250)	0.060
	18	1(37)	-1(5)	-1(5)	-1(0.1)	-1(0)	-1(0)	-1(0)	-1(0)	-1(0)	1 (250)	0.010

** variables have been used as one unit [one variable] during the regression analysis

Table.2 The main effect of the variables

Variables	Main effect Main effect = $\sum(+1)/n_{(+1)}-\sum(-1)/n_{(-1)}$.
Temperature	1.76
pH	0.076
Sucrose	1.166
$NaNO_3$	1.008
KH_2PO_4	1.392
NaCl	0.306
$MgSO_4$	0.872
$NaMoO_4$ and $FeSO_4$	1.256
Shaking rate	1.554

Table.3 Multiple regression analysis of the data in Table 1

Parameters	Estimate	P value	Std Error	t Stat	Confidence %
Constant	0.143	0.027	0.054	2.704	97.312
Temperature	0.068	0.292	0.060	1.129	70.85
pH	0.032	0.643	0.066	0.482	35.72
sucrose	0.091	0.211	0.067	1.359	78.88
$NaNO_3$	0.091	0.227	0.069	1.308	77.27
KH_2PO	0.000	0.998	0.071	0.003	0.21
NaCl	-0.031	0.631	0.062	-0.500	36.93
$MgSO_4$	0.013	0.853	0.066	0.191	14.96
$FeSO_4$	0.091	0.188	0.063	1.438	81.16
Shaking rate	0.136	0.100	0.073	1.862	90.04

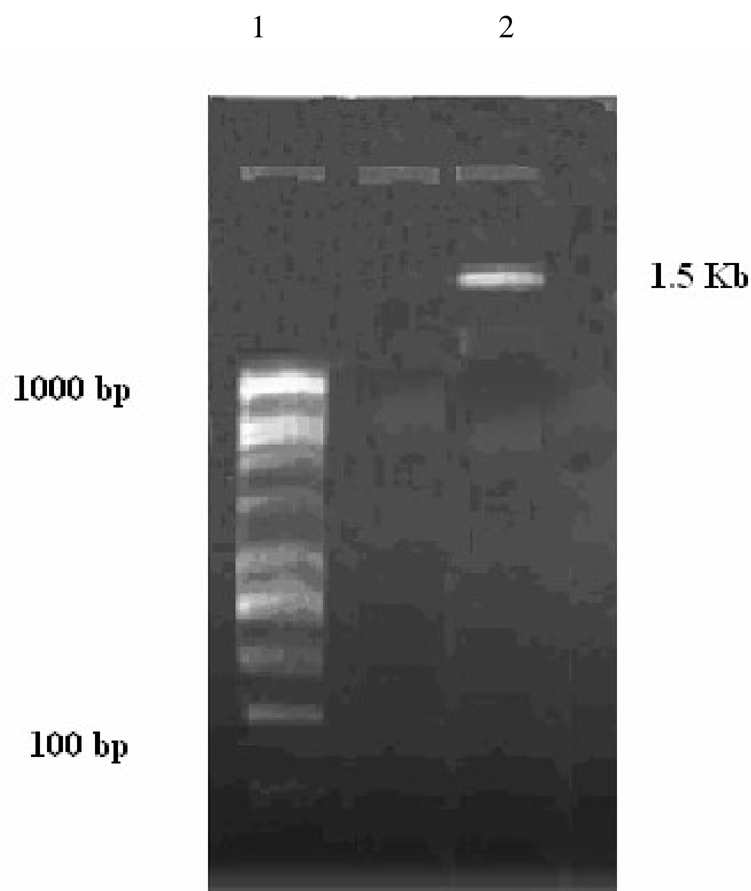
Table.4 Summary of the regression results

Summary	
R-squared	60.65 persent
R^2 adjusted	16.39
log 1 residual autocorrelation	0.385
Standard Error	0.229
Durbin-Watson statistic	1.219 (p=0.053)
Mean absolute error	0.116

Table. 5 ANOVA test

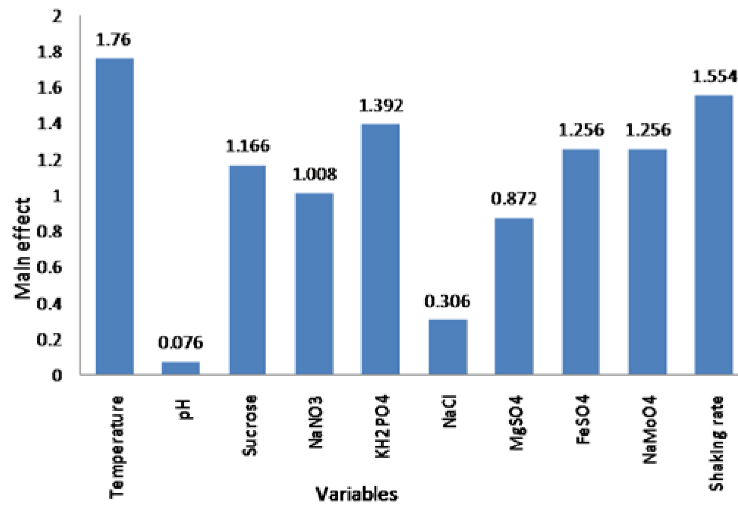
ANOVA					
Source	SS	MS	F	P. Value	df
Regression	0.640	0.072	1.37	0.3338	9
Residual	0.408	0.052			7
Total	1.063				16

Fig.1 Amplification of 16S rDNA PCR product from ***A***. *vinelandii* .



Lane (1) marker DNA 1kb and lane (2) 16s rDNA PCR product with mw (~1.5 kb).

Fig. 2 Main effect o diferent variables used in this study



Optimization of the most significant factors for PHB production using Box-Behnken design

Box-Behnken design was used to optimize the most significant factors affecting PHB production obtained from Plackett and Burman. The results obtained in Table 4 showed the optimum levels of the most significant factors are KH_2PO_4, pH and agitation rate. The results were analyzed by the second order regression equation provided the levels of PHB production. From the results, it was found that PHB content increased from 0.199 (PHB g/g dry weight) using Plackett and Burman design to 0.7 PHB g/g dry weight using Box-Behnken at the following conditions: sucrose 30, g/l; $NaNO_3$, 2 g/l; KH_2PO_4, 2 g/l; NaCl, 0.5 g/l; $MgSO_4$, 0.5 g/l; $FeSO_4$, 0.05 g/l; $CaCl_2$, 0.06 g/l; $NaMoO_4$, 0.005; shaking rate at 225 rpm, pH 7 and 30^oC. The most effective variable [data not shown] was the shaking rate with confidence % 88.87, followed by each of $FeSO_4$, temperature and sucrose with confidence levels 80.30,76.41 and 75.84, respectively. Since the P-value in the ANOVA (Table 5) is greater or equal to 0.10, there is no statistically significant relationship between the variables at the 90% or higher confidence level. From the results in Fig. 2, temperature, KH_2PO_4, $NaNO_3$, $MgSO_4$, $FeSO_4$, shaking, $CaCl_2$ and $NaMoO_4$ showed positive effect on PHB production by *A. vinelandii*. Three factors showed negative effect on PHB production represented by NaCl, pH and sucrose. However temperature, $NaNO_3$, KH_2PO_4, $FeSO_4$ and $MgSO_4$ are the most effective variables as shown in Fig. 3.

Production of microbial Polyhydroxybutyrate (PHB) is a promising biotechnological area. Optimization the production of biotechnological products is an aim for many industrial and research sectors (Plackett and Burman, 1946; Amara *et al*., 2001, 2002; Taguchi *et al*., 2001, 2002). Basic strategies for economic PHB production based on consideration of good PHB producers and good productivity, as well as PHB recovery affecting overall economics. In spite of great deal of efforts directed towards commercializing PHA, the wide use of PHB remains rare because of its high production cost. One reason for this unfavorable cost for commercial use is that the methods used must select good PHB producers to increase the concentration of PHB, develop the fermentation process, and enhance PHB recovery/purification system. The ideal way to enable PHB to compete with conventional petrochemical plastic is to combine the following methods simultaneously; increase the absolute concentration of PHB per host cell, increase packing of PHB granules into the host, achieve high glucose conversion rate and release the PHB outside the cell to simplify recovery/purification process (Lae *et al*., 2005).

In this study, Plackett and Burman design was used to investigate significant factors affecting PHB production. Experimental design, as a powerful tool for optimizing different complicated conditions was used to investigate the principal factors for PHB production, and identification of medium components that play a significant role in cell growth and PHB production, as well as their concentrations. Three environmental factors represented in temperature, pH and shaking rate as well as six nutritional factors included sucrose, $NaNO_3$, KH_2PO_4, NaCl, $MgSO_4$ and $FeSO_4$ were randomized in eighteen experiments using Plackett-Burman design (1946). The results showed different

responses based on the variation on environmental factors and different nutritional conditions. The variables are heterogeneous as proved by the T-test analysis of variance (ANOVA) where the F significant was > 0.05 (0.3338). This proved the presence of conflict between different factors used in this study. This conflict has been perfectly solved by randomizing the different factors. Plackett-Burman design proved to be a powerful tool for optimizing biotechnological products. However, the study presented here succeeded in producing 88.87% PHB, regarding to the cell dry weight. The variation on the amount of PHB calculated as gm PHB/gm cells proved that Plackett-Burman design is a versatile tool for optimizing complicated conditions. In this context, PHB content in *Azotobacter vinelandii* cells varied from 0.0 to 0.88 g PHB/g dry cell weight (DCW). This mean that the method used herein is successful methods for increasing PHB content. In order to optimize the most significant factors obtained from Plackett and Burman design, Box-Behnken design was applied and maximum PHB production were obtained using 2 g/l KH_2PO_4, shaking rate at 225 rpm and pH 7. In this respect, Gu *et al*. (2005) reported that response surface methodology provides important information regarding the optimum level of each variable along with its interactions with other variables and their effects on product yield. To validate the exact optimum values of KH_2PO_4, pH and agitation rate and their interactions, statistical designs were used and the range between the optimum points were selected. Therefore, Box-Behnken design was focused on the interaction between these three factors while other factors were effectively insignificant for PHB production. In this connection, optimization for PHB in *Rhizobium* *meliloti* was reported by taking variables such as sucrose, urea, inoculums size and K_2HPO_4 (Lakshman *et al*., 2004). Similar methods were used by (Khanna and Srivastava, 2005; Lorrungruang *et al*., 2006). They optimized the concentrations of KH_2PO_4, Na_2HPO_4, $MgSO_4.7H_2O$ and fructose for PHB production in *Wautersia eutropha* using central composite design.

Thus, the highest PHB yield (0.88 g PHB/g DCW) using *A. vinelandii* occurred in medium containing NaCl, 0; sucrose, 30 g/l; KH_2PO_4, 1 g/l; $MgSO_4$, 0.5 g/l; FeSO4, 0.05 g/l; $NaMoO_4$, 0.005 g/l; $CaCl_2$, 0.06 g/l; pH 7 and shaking rate at 225 rpm. Thus, the before mentioned constituents and conditions were promising for PHB production. In this connection, Pal *et al*. (1998) reported that PHB accumulation was enhanced when growth was restricted due to unavailability of phosphorus. Under nitrogen deficiency a rise in PHB pool up to 9.5% (w/w of dry cells) was observed. Lugg *et al*. (2008) observed that enterobacteria of natural habitat accumulate 50% PHB of CDW, in medium containing excess carbon source and a deficiency in nitrogen. However, Lee *et al*. (2001) and Li *et al*. (2009) reported that PHB is a NADPH-dependent pathway and increased under high intracellular concentrations of NADPH or high ratio of NADPH/NADP. The possible explanation for the rise in PHB pool under supplementation of glucose, fructose, maltose and ethanol is due to boost in growth. The possible explanation for this rise could be the availability of plenty of precursors i.e., acetate for biosynthesis of PHB, as reported in many bacteria (Zinn *et al*., 2001; Galino *et al*., 2007). In some cases, these sugars may be used to enhance cell growth and, in turn, the volumetric productivity as recommended by Quillaguaman *et al*. (2008) for complex nitrogen sources.

The spectrophotometer assay of the polymer that extracted from *A. vinelandii* and the standard (DL-β-hydroxybutyric acid) were analyzed after sulphuric acid digestion and measured at 235 nm which depicted highest degree of similarity with the spectrum of crotonic acid in accordance with Law and Slepecky (1961). Thus, the complete matching of the spectra of the acid-digested sample and the standard with the spectrum of crotonic acid demonstrated the presence of PHB in *A. vinelandii*.

Selecting effective variables is very important in experimental design to reduce the number of the experiments and the number of designs. Not only Plackett-Burman design is able to optimize the nutritional constituents, but also the environmental factors. On the basis of the data obtained in the present study, *A. vinelandii* is capable of PHB accumulation up to 88.89% of dry cell weight. Thus, it can be selected as a candidate for PHB production and commercialization. Molecular studies are needed to increase more PHB content in *Azotobacter* cells. Such studies will be the focus of the nearest future.

References

Alderet, J.E., D.W. Karl and Park C.H. 1993. Production of poly(hydroxybutyrate), homopolymer and copolymer from ethanol and propanol in a fed-batch culture. Biotechnol. Progress 9: 520-25.

Aldor, I.S. and Keasling J.D. 2003. Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates. Curr. Opinion Biotechnol.14: 475-483.

Alias, Z. and Tan K.P.I. 2005. Isolation of palm oil utilizing, polyhydroxyalkanoate (PHA)-producing bacteria by an enrichment technique. Biores. Technol. 96: 1229-1234.

Altshul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and Lipman D.L. 1997. Gapped BLAST and PSI-BLAST A new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Amara A. A. 2008. Polyhydroyalkanoates: from Basic Research and Molecular Biology to Application. IIUM Engineer. J. 9 (1): 37-73.

Amara, A.A. and Rehm B. H. A. 2001. Steinbuchel: Biopolymer overproduction by new mutants using simple methods for selection. In: DAAD-Bioforum-Berlin-Grenzenlos forschen, DAAD, Biotechnologische Methode. Pp. 231-239.

Amara, A.A., A. Steinbuchel and Rehm B.H. 2002. *In vivo* evolution of the *Aeromonas punctata* polyhydroxyalkanoate (PHA) synthase: isolation and characterization of modified PHA synthases with enhanced activity. Appl. Microbiol. Biotechnol. 59 (4-5): 477-482.

Anderson, A. J. and Dawes E.A. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54: 450-472.

Apostolis, A.K., Y. Xu, R. Wang and Webb C. 2006. Polyhydroxybutyrate production from a novel feedstock derived from a wheat-based biorefinery. Enzyme Micro. Technol. 40: 1035-1044.

Ashby, R.D., D.K. Solaiman and Foglia T.A. 2002 The synthesis of short- and medium-chain-length poly [hydroxyalkanoates] mixtures from glucose or alkanoic acid-grown

Pseudomonas oleovorans. J. Indian Microbiol. Biotechnol. 28:147-153.

Bormann, E.J., M. Leihner, M. Roth, B. Beer and Metzner K. 1998. Production of poly-hydroxybutyrate by *Ralstonia eutropha* from protein hydrolysates. Appl. Microbiol. Biotechnol. 50: 604-607.

Burns, K.L., C.D. Oldham, J.R. Thompson, M. Lubarsky and May S.W. 2007. Analysis of the in vitro biocatalytic production of poly- β -hydroxybutyric acid. Enzyme. Micro. Technol. 41(5): 591-599.

Byrom, D. 1987. Polymer synthesis by micro-organisms: technology and economics. Trends. Biotechnol. 5: 246-250.

Chaijamrus, S. and Udpuay N. 2008. Production and characterization of polyhydroxybutyrate from molasses and corn steep liquor produced by *Bacillus megatirium* ATCC 6748. Enginerr. Inter. CIGR E J; Manuscript FP 07 030.; X: 1-12.

Chen, G.Q. and Page W. 1997. Production of poly- β -hydroxybutyrate by *Azotobacter vinelandii* in a two-stage fermentation process. Biotechnol.Techniq. 11: 347-350.

Chen, D., Y. Han and Gu. Z. 2006. Application of statistical methodology to the optimization of fermentative medium for carotenoids by *Rhodobacter sphaeroides.* Process Biochem. 41: 1773-1778.

Diaz-Barrera, A. and Soto E. 2010. Biotechnological uses of *Azotobacter vinelandi*: Current state, limits and prospects. African. J. Biotechnol. 9 (33): 5240-5250.

Doi, Y. 1990. Microbial Polyesters. VCH, New York.

Doi, Y and Steinbüchel A. 2001. Steinbüchel. Biopolymer:polyesters II-properties and chemical synthesis. Wiley-VCH.

Galindo. E., C. Pena, C. Nunez, D. Segura and Espin G. 2007. Molecular and bio engineering strategies to improve alginate and polydydroxyalkanoate production by *Azotobacter vinelandii*. Micro. Cell Factory. 6 : 7.

Gouda, M.K., A. E. Swellam and Omar S. H. 2001. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbonand nitrogen source. Microbiol.Res. 156: 201-207.

Grothe, E. and Chistri Y. 2000. Poly (β hydroxybutyric acid) thermoplastic production by *Alcaligenes latus*: behavior of fed-batch cultures. Bioprocess Engineer. 22: 441-449.

Gu, X.B., Z.M. Zheng, H.Q. Yu, J. Wang, F.L. Liang and Liu R.L. 2005. Optimization of medium constituents for a novel lipopeptide production by *Bacillus subtilis* MO-01 by a response surface method. Process Biochem. 40: 3196-3201.

Khanna, S. and Srivastava A.K. 2005. Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha.* Process Biochem. 40: 2173-2182.

Kishk, S.S. 2009. Production of biopolymer from selected *Azotobacter* species. M. Sc. Thesis, Tanta University, Tanta, Egypt.

Kothuis, B. and Schelleman F. 1998. Environmental economic comparison of biotechnology with traditional alternativesIn: Meesters, K.H.P. (Ed). Production of poly-(3-hydroxyalkanoates) from waste streams. Delft, Tudelft Press. p 4.

Kuniko, M., Y. Nakamura and Doi Y. 1988. New bacterial copolyesters produced in *Alcaligenes eutrophus* from organic acids. Polymer Commun. 29: 174-176.

Lae, J., H.P. Ki, C.K. Kug and Hyo K.P. 2005. Spontaneous liberation of

intracellular polyhydroxybutyrate granules in *Escherichia coli.* Res. Microbiol. 156: 865-873.

Law, J.H. and Slepecky R.A. 1961. Assay of poly-β-hydroxybutyric acid. J Bacteriol. 82: 33-36.

Lee, S.Y and Chang H.N. 1995. Production of poly (hydroxyalkanoic acid). Adv. Biochem. Engineer. Biotechnol. 52: 27-58.

Lee, S.Y. and Choi J. 1998. Effect of fermentation of performance on economics of poly (3-hydroxybutyrate) production by *Alcaligenes latus*. Polymer Degrad. Stability. 59: 387-393.

Lee, S.Y., A.P.J. Middelberg and Lee Y.K. 1997. Poly (3-hydroxybutyrate) production from whey using recombinant *E. coli*. Biotechnol. Lett. 19:1033-1035.

Lenz, R.W. and Marchessault R. H. 2005. Bacterial polyesters: biosynthesis, biodegradable plastics and biotechnology. Biomacromole. 6**:** 1-8.

Lakshman, K., N.K. Rastogi and Shamala T.R. 2004. Simultaneous and comparative assessment of parent and mutant strain of *Rhizobium meliloti* for nutrient limitation and enhanced polyhydroxyalkanoate (PHA) production using optimization studies. Process Biochem. 39: 1977-1983.

Li, R., H. Zhang and Qi Q. 2007. The production of polyhydroxyalkanoates in recombinant *Escherichia coli.* Biores. Technol. 98: 2313-2320.

Li, Z.J., L. Cai, Q. Wu and Chen G.Q. 2009. Overexpression of NAD kinase in recombinant Escherichia coli harboring the phbCAB operon improves poly (3-hydroxybutyrate) production. Appl. Microbiol. Biotechnol. 83: 939-947.

Lorrungruang, C., J. Martthong, K. Sasaki and Noparatnaraporn N. 2006. Selection of photosynthetic bacterium *Rhodobacter sphaeroides* 14F for polyhydroxyalkanoate production with two-stage aerobic dark condition. J. Biosci. Bioengineer. 102: 128-131.

Lugg, H., R.L. Sammonas, P.M. Marquis, C.J. Hewitt, P. Yong and Paterson-Beedle *et al*. 2008. Polyhydroxybutyrate accumulation by a *Serratia* sp. Biotechnol. Lett. 30 (3): 481-491.

Miranda, V.F.O. 2002. Easy Align, versão 1.0. Universidade Estadual Paulista, Rio Claro, Brasilian Curr. Microbiol. 50 (4):190-195.

Nur Yuksekdag, Z., B. Aslim, N. Beyatli and Mercan Y. 2004. Effect of carbon and nitrogen sources and incubation times on Poly-beta-Hydroxybutyrate synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12. African. J. Biotechnol. 3 (1): 63-66.

Page, W.J. 1989. Production of Poly-beta-Hydroxybutyrate by *Azotobacter vinelandii* during growth on molasses and other complex carbon source. Appl. Microbiol. Biotechnol. 31 (4): 329-333.

Page, W.J. and Knosp O. 1989. Hyperproduction of Poly-beta-Hydroxybutyrate during Exponential Growth of *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. 55: 1334-1339.

Page, W. J. and Cornish A. 1993. Growth of *Azotobacter vinelandii* UWD in fish peptone medium and simplified extraction of Poly-β-Hydroxybutyrate. Appl. Environ. Microbiol. 59 (12): 4236-4244.

Plackett, P. and Burman J.P. 1946. The design of optimum multifactorial experiments. Biometrika 37: 305-325.

Quagliano, J.C., P. Alegre and Miyazaki S.S. 1994. Isolation and characterization of *Azotobacter* sp. for

the production of poly-β-hydroxyalkanoates. Rev.Argentinian Microbiol. 26**:** 21-27.

Quillaguaman, J., D. Van-Thuoc, H. Guzman, D. Guzman, J. Martin, A. Everest and Hattikaul R. 2008. Poly (3-Hydroxybutyrate) production by *Halomonas boliviensis* in fed batch culture. Appl. Microbiol. Biotechnol. 56 (3-4): 315-325.

Segura, D., T. Cruz and Espin G. 2003. Encystment and alkylresorinol production by *Azotobacter vinelandii* strains impaired in poly-beta-hydroxybutyrate synthesis. Arch. Microbiol.179 (6): 437-443.

Sims, R. 2003. Climate Change solutions from Biomass, Bioenergy and Biomaterials. Agricul. Eng. Internat. CIGR Journal of Scientific Research Development; Invited Overview; Vol. V., September.

Song, C., L. Zhao, S. Ono, C. Shimasaki and Inoue M. 2001. Production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from cotton seed oil and valeric acid in batch culture of *Ralstonia* sp. strain JC-64. Applied Biochem. Biotechnol. 94: 169-178.

Steinbüchel, A. 1995. Use of biosynthetic, biodegradable thermoplastics and elastomers from renewable resources. JMS Pure Appl. Chem. 32: 653-660.

Stowe, R.A. and Mayer R.P. 1966.Efficient screening of process variables. Industrial Engineer. Chem. 58: 36-40.

Suzuki, D.V., J.M. Carter, M.F.A. Rodrigues, E.S. Silva and Maiorano A.E. 2008. Purification of hydroxybutyrate produced by *Burkholderia oepacia* IPT64 through a chemical and enzymatic route. World. J. Microbiol.24(6): 771-775.

Taguchi, S., A. Maehara, K. Takase, M. Nakahara, H. Nakamura and Doi Y. 2001. Analysis of mutational effects of a polyhydroxybutyrate (PHB) polymerase on bacterial PHB accumulation using an in vivo assay system. FEMS Microbiol. Lett. 198 (1): 65-71.

Taguchi, S., H. Nakamura, T. Hiraishi, I. Yamato and Doi Y. 2002. *In vitro* evolution of a polyhydroxybutyrate synthase by intragenic suppression-type mutagenesis. J. Biochem. 131(6): 801-806.

Tchan, Y.T. 1984. Azotobacteraceae. In: Krieg, N. R. and J. G. Holt (Ed.). Bergey's Manual for Systematic Bacteriology, 8th Ed., Vol. 1. Williams and Wilkins Co., Baltimore. Pp. 219-234.

Vancura, V. and Mancura J. 1960. Indole derivative in *Azotobacter* culture. Folia Microbiologica 5: 293-297.

Wheeler, W., D. Gladstein and Malign V. 1998. American Museum of Natural History, New York, USA. Brazilian. J. Microbiol. 38 (1):159-165.

Yamane, T. 1993. Yield of poly-D-3-hydroxybutyrate from various carbon sources: a theoretical study. Biotechnol. Bioengineer. 41: 165-170.

Yu, H.H., Y. Shi, Y. Zhang, S. Tang and Shen Z. 2002. Effect of Vitreoscilla hemoglobin biosynthesis in *Escherichia coli* on production of poly (betahydroxybutyrate) and fermentative parameters. FEMS Microbiol. Lett. 214: 223-227.