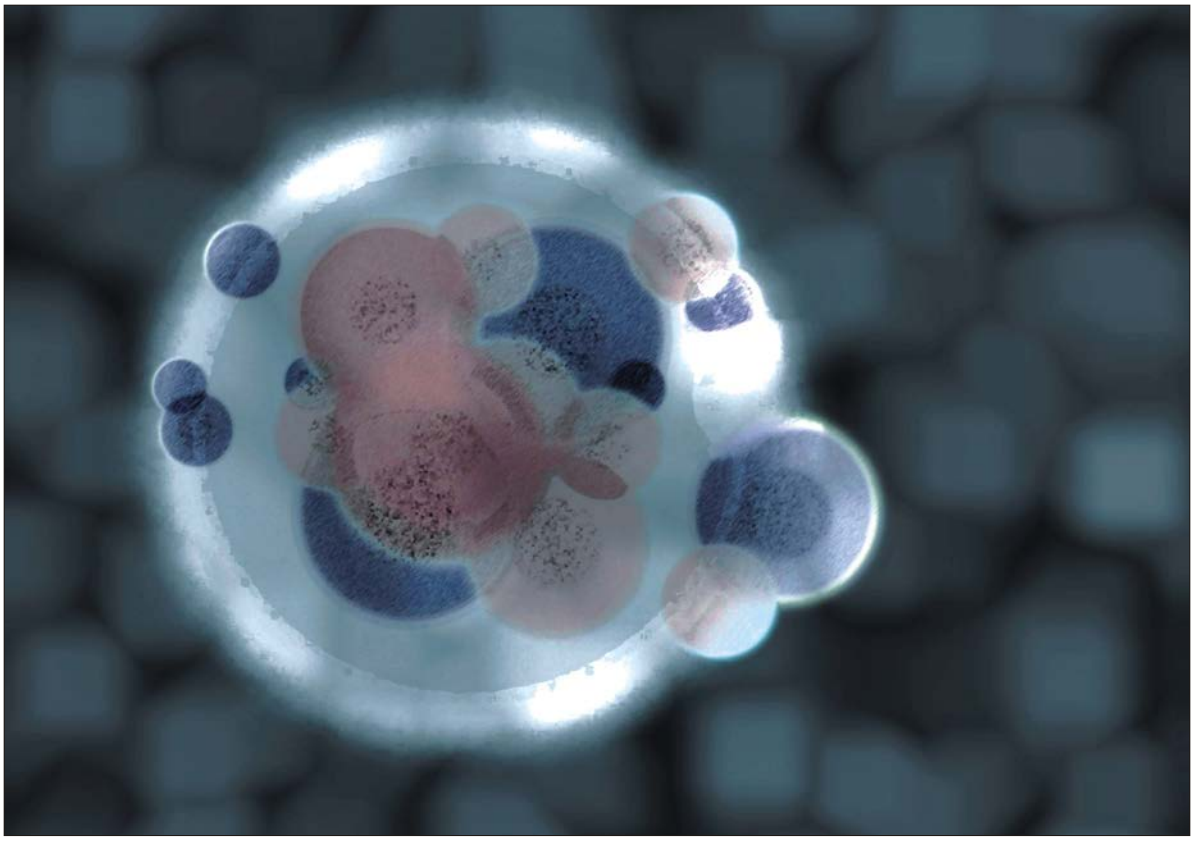

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Optimization of Culture Conditions for Production of Polyamide Biopolymer (Polyglutamate) by *Bacillus* sp. Strain-R

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Abstract: An optimization study of biopolymer production by locally isolated *Bacillus* sp. strain-R was carried out, where analysis indicated that it is a polyamide homopolymer consists mainly of glutamate. Preliminary experiment to address the most suitable carbon and nitrogen sources revealed that a production of 14.25 g L^{-1} of the polyamide (polyglutamate) was achieved in the presence of glucose and ammonium sulfate. To evaluate the effect of different culture conditions on the production of PGA, Plackett-Burman factorial design was carried out. Ten variables were examined for their significance on PGA production. Among those variables K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$ and glucose were found to be the most significant variables that encourage PGA production. The pre-optimized medium showed approximately three folds increase in PGA production.

Key words: Polyamide, polyglutamate, *Bacillus*, experimental design, optimization

INTRODUCTION

Polyglutamic acid (PGA) is one of the few naturally occurring polyamides which are not synthesized by ribosomal proteins (Oppermann-Sanio and Steinbüchel, 2002). The polymer is produced by several *Bacillus* sp. as an extracellular viscous polymer (Kubota *et al*., 1993; Kunioka and Goto, 1994; Ko and Gross, 1998). The PGA is completely biodegradable, water-soluble and non-toxic to human body (Yoon *et al*., 2000). The application of this biomaterial is both economical and environmental friendly. PGA has been suggested to be used as good candidate for water soluble biodegradable polymer in various biological applications including thickener, humicant, cosmetics and biological glue (Konno *et al*., 1988; Iwata *et al*., 1998; Otani *et al*., 1999; Cell Therapeutics, 2000). It is anticipated that PGA will be utilized not only in the areas of wastewater treatment but also drinking water processing and downstream processing in food and fermentation industry because of its harmless toward human and environment (Shih *et al*., 2001). For medical applications, the special chemical properties of the PGA polymers legislates it to fulfill several requirements: renders the drug water soluble, transport the drug to tumor sites and control release of drug over time as the polymer biodegrades (Sakurai *et al*., 1995; Kolar *et al*., 1985).

Recently various species of the genus Bacillus, e.g., *B. subtilis*, *B. licheniformis* and *B. anthracis* are known to be the most conspicuous bacteria producing PGA (Kunioka and Goto, 1994; Ko and Gross, 1998; Yoon *et al*., 2000; Giannos *et al*., 1990; Cromwick *et al*., 1996; Pötter *et al*., 2001; Hoppensack *et al*., 2003; Soliman *et al*., 2005). PGA was firstly discovered as an extracellular capsular polymer that serves as structural component in *Bacillus anthracis* and *Bacillus megaterium* and extensively studied (Hanby and Rydon, 1946; Thorne, 1956; Roelants and Goodman, 1968). In some bacterial species, e.g. *B. subtilis*, *B. licheniformis* and *Natrialba aegyptiaca*, PGA excreted into the fermentation to increase the survival of producing strains when exposed to environmental stresses (Gross, 1998; Hezayen *et al*., 2000). Culture conditions affecting PGA biosynthesis have been studied using various strains of *B. licheniformis* and *B. subtilis* in complex, ill-defined media and these studies have resulted in conflicting conclusions (Kambourova *et al*., 2001).

For a broad application, the cost of bio-products is one of the main factors determining the economics of a process. Reducing the costs of biopolymer production by optimizing the fermentation medium is the basic research for industrial application. The use of different statistical designs for medium optimization has been

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recently employed for many enzymes (Ghanem *et al.*, 2000; Francis *et al.*, 2003), antibiotics and metabolites. These statistical methods, as compared to the common ''one-factor-at-a-time'' method proved to be powerful and useful tools.

The objective of this study was to isolate, characterize and identify a PGA producing strain. Analysis of the PGA biopolymer by SDS-PAGE and amino acid analysis was recorded. Preliminary experiments have been carried out to address the most effective carbon and nitrogen sources in PGA production. To determine the significance among other physical and nutritional requirement, Plackett-Burman experimental design was applied.

MATERIALS AND METHODS

Microorganism: The bacterium used throughout this study was isolated from an agriculture farm in ABIS rectorate in Egypt and characterized by previous history of fertilizer's use. A1% soil suspension was prepared and preboilled for 15 min at 100°C for selective enrichment of spore-forming bacteria. A suitable dilution of the soil suspension was used to inoculate nutrient agar medium composed of (g L^{-1}): peptone; 5, beef extract; 3, NaCl; 2 and agar; 20. The produced colonies were subsequently plated out into the same medium for further purification. Screening of the purified colonies for polyamide (PGA) production was carried out by cultivation on basal mineral agar medium composed of (g L^{-1}): glucose; 20, K_2HPO_4; 14, KH_2PO_4; 6, $MgSO_4$; 0.2 and 2 mL of trace element solution ($FeSO_4.4H_2O$, $CaCl_2.2H_2O$, $MnSO_4.4H_2O$, $ZnCl_2$ 1 mM each), agar; 20 and supplemented with Na-glutamate (5 g L^{-1}) or ammonium sulfate (4 g L^{-1}) at 37°C. The exceptional viscous colony was chosen and maintained on nutrient agar slant. The bacterium was characterized morphologically and physiologically as described in Bergey's manual of systematic bacteriology (Sneath, 1986). For comparative study, a PGA producing *B. licheniformis* ATCC 9955 strain (kindly provided by Prof. A. Steinbüchel, Institut für Molekularbiologie und Biotechnologie, Universität Münster, Germany) was used.

Growth and PGA production: The bacterium was allowed to grow in 50 mL aliquot of nutrient broth dispensed in 250 mL Erlenmeyer flask and incubated at 37°C for 12 h at 120 rpm. One percent inoculum of the overnight culture was used to inoculate the basal salt production medium of the following composition (g L^{-1}): K_2HPO_4; 14, KH_2PO_4; 6, $MgSO_4$; 0.2, ammonium sulfate; 4 and 2 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. Seven different carbon sources were tested in one-factor-at-a-time experiments. The carbon sources used individually were as following (g L^{-1}): Glutamic acid 20, glucose; 20, sucrose; 20, fructose; 20, lactose; 20, glycerol; 20 mL or citric acid; 10. On the other hand, another set of flasks were prepared to test the effect of different nitrogen sources. The nitrogen sources tested individually were as following (g L^{-1}): Na-glutamate; 5, ammonium sulfate; 4, casein hydrolysate; 6.6, soyabean meal; 2.4, peptone; 6, yeast extract; 3.4, urea; 1.8, corn steep liquor; 28.4 or whey; 42.4 mL in presence of glucose as a carbon source. For each of the tested carbon or nitrogen source 50 mL of mineral medium in 250 mL Erlenmeyer flask was inoculated with 500 µl of the preculture. At the end of incubation period the growth was estimated as OD at 600_{nm} and PGA was determined in culture supernatants after clarifying cultures by centrifugation. For the recovery of polyglutamic acid, the most common technique was applied (Ashiuchi *et al.*, 1997; Soliman *et al.*, 2005).

Polyglutamic acid analyses: In order to analyze the produced polymer, the precipitated polymer material was hydrolyzed with 6 N HCl at 110°C for 24 h in a sealed and evacuated tube and the amino acid composition was determined with a Beckman system analyzer. For thin layer chromatography, the hydrolyzed polymer was neutralized with 6 M NaOH and chromatographed TLC cellulose plates against glutamate as authentic (Kambourova *et al.*, 2001). The degree of polymerization of PGA was analyzed by SDS-PAGE in 8% gel as described by Kambourova *et al.* (2001).

Fractional factorial design: Plackett-Burman experimental design (Plackett and Burman, 1946) was applied to investigate the significance of various medium components on PGA production. Ten culture variables were tested in two levels: –1 for low and +1 for high level based on Plackett-Burman matrix design, which is a fraction of a two-level factorial design and allows the investigation of n-1 variables in at least n-experiments. Table 2 represents the lower and higher levels of each variable. In this study the independent variables were screened in 14 combinations according to the matrix shown in Table 3. The main effect of each variable was calculated simply as the difference between the average of measurements made at high setting (+1) and the average of measurements observed at low setting (-1) of that factor.

Plackett-Burman experimental design is based on the first order model (Eq. 1):

$$Y = \beta_0 + \Sigma \beta_i x_i \quad (1)$$

Where Y is the predicted response, β_0, β_i are constant coefficients and x_i is the coded independent variables estimates or factors.

Analysis of data: The data of the PGA production was statistically analyzed. Essential Experimental Design free software (Steppan *et al.*, 2000) was used for data analysis and determination of coefficients. Factors having highest t-value and confidence level over 95% were considered to be highly significant concerning PGA production.

RESULTS

Isolation and characterization of *Bacillus* sp. strain-R: Screening program for the isolation of PGA producer from different agricultural localities, with previous history for the use of organic and inorganic fertilizers, in Egypt was carried out. For screening purposes, the isolated strains were plated on the basal medium supplemented either with ammonium sulfate or Na-glutamate as a sole nitrogen source and the visible viscous colonies were selected for further experiments. Among the isolated bacterial strains, the most potent biopolymer producing bacterium (strain-R) was isolated from ABIS rectorate by the use of basal medium supplemented with ammonium sulfate, indicating that the strain is a glutamate-independent polyamide producer. The polyamide (PGA) production was confirmed qualitatively by hydrolysis of the polymer and detection of the monomers on TLC as described in the material and methods (data not shown). The morphological and physiological characteristics of the isolate-R showed that it is rod shaped, gram positive endospore-forming (central) and thermotolerant bacterium, grow optimally at 37°C and capable of utilizing glucose, arabinose and mannitol producing acid. Furthermore, the isolate showed a capability to produce some hydrolytic enzymes such as protease, amylase, catalase and oxidase. In addition, the isolate failed to grow on salt concentrations above 5% NaCl. The results collectively indicated that the strain-R belongs to the genus Bacillus and nominated as *Bacillus* sp. strain-R.

PGA production by *Bacillus* sp. strain-R: The production of PGA by *Bacillus* sp. strain-R during cultivation on mineral salts medium supplemented with ammonium sulfate was closely investigated. In this concern, polyamide (Polyglutamate) production was determined and cell growth was monitored during cultivation every 24 h incubation for 96 h. Results in Fig. 1 show that the *Bacillus* sp. strain-R varied in its potentiality to produce PGA with varying incubation period. In general, maximum PGA production (14 g L^{-1}) was obtained after 96 h incubation. Moreover, a negligible

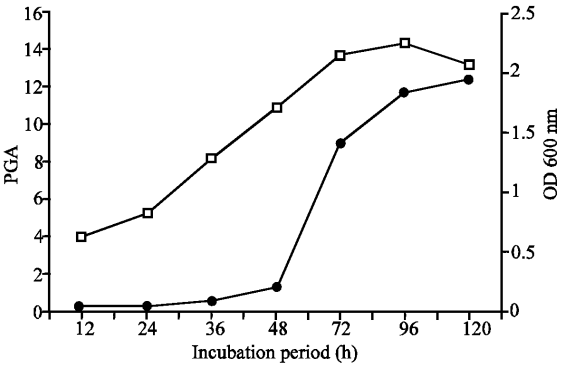

Fig. 1: Growth of *Bacillus* sp. strain-R (●) and polyglutamic acid (PGA) production (□) during different time intervals

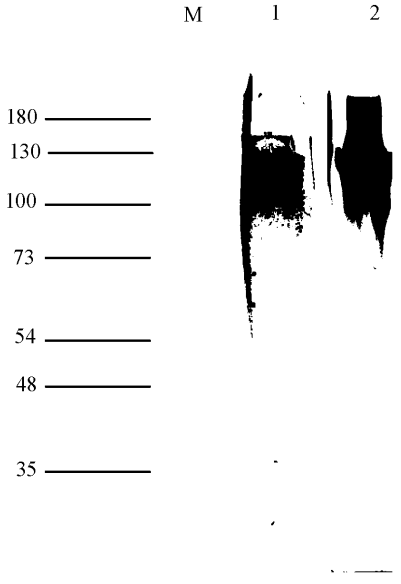

Fig. 2: Molecular size of extracellular polyglutamic acid (PGA) from *Bacillus licheniformis* ATCC9955 (lane 1) and *Bacillus* sp. strain-R (lane 2). PGA was visualized by methylene blue staining (Ashuichi *et al.*, 1998)

amount of polysaccharides was produced (data not shown), proving that the major precipitated product is only the polyglutamic acid polymer. TLC and SDS-PAGE analysis of precipitated PGA polymer was carried out. Running of the hydrolysed polymer on TLC indicated that the glutamate is the major product (data not shown). Furthermore, SDS-polyacrylamide electophoresis of the produced polymer, from both standard reference strain *B. licheniformis* ATCC 9955 and the testing strain *Bacillus* sp. strain-R, indicated that the strains produce polyglutamate biopolymer range from 80 to

180 kDa (Fig. 2). Indeed, amino acid analysis of the polymer hydrolysate indicated that the glutamic monomer is the dominant residue in the precipitated polymer (approximately 80%) indicating that the precipitated polymer material is polyglutamic acid biopolymer (data not shown).

Effects of carbon and nitrogen sources on the production of γ-PGA: A preliminary experiment was carried out to address the most effective carbon source. In this experiment, the effect of carbon source on production of PGA by bacterial strain-R was investigated. Four sugars (glucose, sucrose, fructose and lactose), two acids (citric acid and glutamic) or glycerol were used as carbon source. Results in Table 1 showed that glucose or glycerol increases the yield of PGA that was 14.2 and 12.6 g L^{-1}, respectively. Sucrose and fructose supported cell growth and led to moderate PGA production. Interestingly neither citric acid nor glutamic acid could support PGA production when used as carbon source.

The effect of different categories of nitrogen sources on PGA production was investigated. Two salts of nitrogen (ammonium sulfate, Na-glutamate), seven other complex nitrogen sources (casein hydrolysate, peptone, yeast extract, corn steep liquor, Soya bean meal or Whey) and Urea were tested as a sole nitrogen source during PGA production. Results presented in Table 1 indicated that ammonium sulfate and Urea supported the PGA produced after 4 days of incubation with a value of 14 and 12.4 g L^{-1}, respectively. Dramatic decrease in PGA production (approximately 70%) was recorded during the use of Na-glutamate as sole nitrogen source, indicating that the production of PGA by the strain-R is glutamate-independent. As shown in Table 1, complex natural carbon sources clearly enhance the cell growth with lower or moderate PGA productivity.

Evaluation of factors affecting PGA production: To determine the significant factors affecting PGA production, statistical experimental design approach was conducted. In such design, the identification of process factors is a crucial step in experimental design methodology. Preliminary results indicated that the most suitable carbon and nitrogen sources were glucose and ammonium sulfate, respectively. In order to evaluate factors affecting PGA production by *Bacillus* sp. strain-R, Plackett-Burman statistical design was employed (Plackett and Burman, 1946). Settings of the ten independent variables are shown in Table 2. The experiments were carried out according to the experimental matrix presented in Table 3, where PGA production and optical density were the measured responses. A wide variation in PGA production (2.4-41.2 g L^{-1}) was shown in Table 3, which

Table 1: Effect of different carbon and nitrogen sources on growth and production of PGA by *Bacillus* sp. strain-R

Source	Growth ($OD_{600\ nm}$)	PGA production (g L^{-1})
Carbon sources:		
Glucose	1.87	14.2
Sucrose	1.89	9.0
Fructose	1.83	8.7
Lactose	0.55	ND
Glycerol	2.1	12.6
Citric acid	0.06	ND
Glutamic acid	0.05	ND
Nitrogen source:		
Ammonium sulfate	2.20	14.0
Na-glutamate	2.44	4.3
Casein hydrolysate	2.6	9.0
Yeast extract	3.17	4.5
Urea	2.11	12.4
Peptone	3.75	7.1
Soya bean rueal	4.13	6.3
Corn steep liquor	3.46	9.8
Whey	1.90	7.1

OD: Optical Density, PGA: Polyglutamic Acid, ND: Not Detected

Table 2: Variables and their levels employed in Plackett-Burman design for screening of culture conditions affecting on PGA production by *Bacillus* sp. strain-R

Variable Code	Variable	Value -1	0	+1
X_1	Glucose (g L^{-1})	5	20	40
X_2	Glycerol (g L^{-1})	10	40	80
X_3	Ammonium sulfate (g L^{-1})	10	40	60
X_4	Ammonium chloride (g L^{-1})	3	12	24
X_5	Urea (g L^{-1})	2	8	16
X_6	K_2HPO_4 (g L^{-1})	2	8	16
X_7	KH_2PO_4 (g L^{-1})	2	8	16
X_8	$MgSO_4$ (g L^{-1})	0.1	0.4	0.8
X_9	Agitation (rpm)	50	120	150
X_{10}	Trace elements (mL L^{-1})	0.25	1	2

reflects the importance of medium optimization to attain high yield of the PGA. The main effects of the examined factors on PGA production were calculated and presented graphically in Fig. 3a. On analysis of regression coefficients and t-value (Table 4), $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, glycerol and glucose were the most significant factors increasing PGA production, whereas urea, magnesium sulfate and trace elements were the most significant factors decreasing PGA production.

On model level, the determination coefficient R^2 of the polynomial model was 0.97. The determination coefficient represents the quality of fitting the polynomial model, which can be represented as follows:

$$Y_{PGA} = 17.64 + 0.56X_1 + 0.73X_2 + 8.53X_3 + 1.73X_4 - 0.66X_5 + 5.03X_6 + 3.4X_7 - 1.7X_8 - 0.03X_9 - 0.9X_{10}$$

One of the advantages of the Plackett-Burman design is to rank the effect of different variables on the measured response independent on its nature (either nutritional or physical factor) or sign (whether contributes positively or negatively) as presented in

Table 3: Plackett-Burman experimental design for evaluation of factors affecting polyglutamic acid (PGA) production by *Bacillus* sp. strain-R

	Variable's code										
Trials	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	PGA (g L^{-1})
1	-1	1	-1	-1	-1	1	1	1	-1	1	13.6
2	0	0	0	0	0	0	0	0	0	0	15.6
3	1	-1	-1	-1	1	1	1	-1	1	1	17.2
4	0	0	0	0	0	0	0	0	0	0	13.8
5	1	-1	1	-1	-1	-1	1	1	1	-1	22
6	-1	1	1	1	-1	1	1	-1	1	-1	41.2
7	1	1	-1	1	-1	-1	-1	1	1	1	3.2
8	-1	1	1	-1	1	-1	-1	-1	1	1	15.8
9	1	-1	1	1	-1	1	-1	-1	-1	1	30.4
10	-1	-1	1	1	1	-1	1	1	-1	1	23.2
11	1	1	1	-1	1	1	-1	1	-1	-1	27.4
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	2.4
13	-1	-1	-1	1	1	1	-1	1	1	-1	9.2
14	1	1	-1	1	1	-1	1	-1	-1	-1	12

Variables coded as follows: X_1; glucose, X_2; glycerol, X_3; Amm sulfte, X_4; Amm chloride, X_5; Urea, X_6; K_2HPO_4, X_7; KH_2PO_4, X8; $MgSO_4$ $7H_2O$; X_9; agitation, X_{10}; trace elements, PGA: Polyglutamic Acid

Table 4: Statistical analysis of Plackett-Burman design showing coefficient values, t- and p-values for each variable

Variable	Coefficients	t-test	p-value	Confidence level (%)
Intercept	17.64			
Glucose (g L^{-1})	0.56	0.59	0.59	40.4
Glycerol (g L^{-1})	0.73	0.76	0.49	50.0
Ammonium sulfate (g L^{-1})	8.53	8.90	0.003	99.7
Ammonium chloride (g L^{-1})	1.73	1.80	0.16	83.2
Urea (g L^{-1})	-0.66	-0.69	0.53	46.3
K_2HPO_4 (g L^{-1})	5.03	5.25	0.01	98.6
KH_2PO_4 (g L^{-1})	3.4	3.54	0.03	96.2
$MgSO_4$ (g L^{-1})	-1.7	-1.77	0.17	82.6
Agitation (rpm)	-0.03	-0.03	0.97	2.5
Trace elements (mL L^{-1})	-0.9	-0.93	0.41	58.3

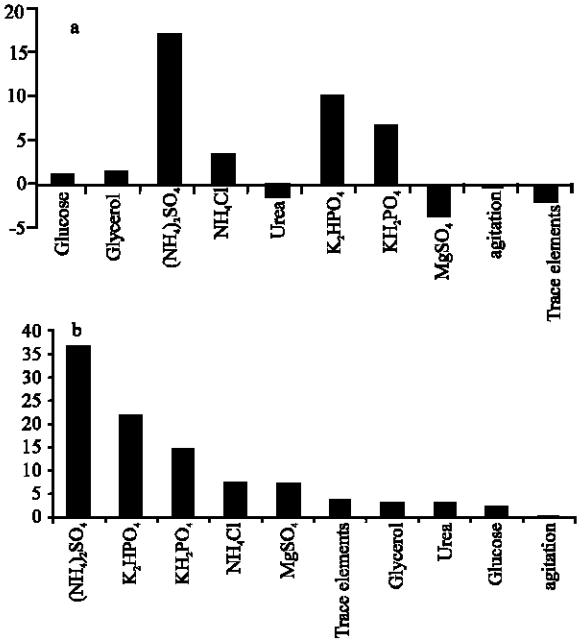

Fig. 3: Main effect of environmental and nutritional factors (a) and Pareto plot (b) for Plackett-Burman parameter estimates of PGA produced by *Bacillus* sp. strain-R

Fig. 3a. Interestingly, Fig. 3b shows the ranking of factor estimates in a Pareto chart. The Pareto chart displays the magnitude of each factor estimate and is a convenient way to view the results of Plackett-Burman design (1946).

DISCUSSION

In this study the production of a useful biodegradable polyamide biopolymer, polyglutamate (PGA), by *Bacillus* sp. strain-R was closely investigated. It was found that the PGA production and cell growth were synchronized and a maximum yield (14.2 g L^{-1}) was obtained after 96 h, which is in accordance to the finding of Shih *et al.* (2001). Ananlysis of the hydrolyzed biopolymer material by TLC against authentic from a positive control *Bacillus licheniformis* strain ATCC 9955 indicated that PGA is the main constituent of the material. Furtheremore, amino acid analysis of the polymer material showed that the glutamic acid constitute more than 80% of the polyamide. Kubota *et al.* (1993) reported that the PGA biopolymer have molecular weight ranging from 100 to over 1,000 kDa. SDS-PAGE electrophoresis indicated that the produced polymer is in the range of 80 to 180 kDa. This result is supported by the finding that the PGA from bacilli are usually produced as mixture with

molecular weights ranging from 10 to 1000 kDa, due to PGA depolymerases activity (Abe *et al*., 1997). Generally the final molecular weight of PGA is dependent on many factors and can decrease as fermentation time increases, owing to enzyme that catalizes the hydrolytic breakdown of PGA (Goto and Kunioka, 1992; King *et al*., 2000).

Given the industrial importance of Bacilli in the extracellular enzyme industry, we examined the synthesis of PGA in *Bacillus* sp. strain-R in more details. Preliminary one-factor-at-a-time experiments to determine the preferable carbon/nitogen nutrition for PGA production, which showed that glucose and ammonium sulfate are the most useful carbon and nitrogen sources, respectively. Other tested organic acids and peptone shown to be unfavourable for PGA production. Recently, the PGA producing bacteria were divided into two groups: glutamate-dependent and glutamate-independent producers (Kunioka and Goto, 1994; Ito *et al*., 1996). In the former, the PGA yield indeed increases upon addition of glutamate to the medium, but the bacteria can produce considerable PGA even in the absence of glutamate because of the operation of the *de novo* pathway of L-glutamate synthesis (Kunioka and Goto, 1994). Cheng *et al*. (1989) reported that *B. licheniformis* A35 is a glutamate independent bacteria where it uses glucose as a major carbon source. This finding concurs with results obtained in *Bacillus* sp. strain-R that was repressed on using glutamic acid as a nitrogen source. Therefore, it could be classified as glutamate independent PGA producer. A similar behaviour was recorded by Shih *et al*. (2001), whereas a *Bacillus subtilis* strain (chungkookjang) was shown to be glutamate-independent (Ashiuchi *et al*., 1998). In this study, the most promising carbon sources referring to PGA production were glucose and glycerol. This finding is in concordance with the results obtained by Du and his co-workers (Du *et al*., 2004) who reported an enhancement of PGA production on adding glycerol to the citric acid basal medium or glutamic\citric acid medium, respectively.

Fractional Factorial Design (FFD) is a kind of experimental design that enables researchers to evaluate the most significant factors affecting the process with the least number of trials. Plackett-Burman design is a FFD, which succeeds in ranking factors from different categories to enable better understanding of the medium effect. In the present study, ammonium sulfate was the most significant factor affecting the PGA yield (p-value = 0.003). Kambourova *et al*. (2001) reported a variable range of PGA production among a group of *B. licheniformis* on using ammonium sulfate as a nitrogen source, where *B. licheniformis* S173 produced maximum yield on using ammonium sulfate and casein hydrolysate as nitrogen sources. In addition, phosphate ion represented by K_2HPO_4 and KH_2PO_4 contributed positively showing high significant effect reflected by the p-value (0.01 and 0.03, respectively). The K_2HPO_4 and KH_2PO_4 positive significant effect can be attributed to the mechanism of PGA synthetase in *B. licheniformis* proposed by Troy (1973) and Gardner and Troy (1979). In this mechanism the PGA synthetase activation is accompanied by the cleavage of ATP into AMP and consequently produce more polyglutamate polymer. In this approach, K_2HPO_4 and KH_2PO_4 are the main source of phosphate in the medium that promotes the PGA synthetase activation. Besides, potassium phosphate salts possess the buffering effect that indirectly affects the pH of the medium.

In conclusion, *Bacillus* sp. Strain-R is shown to be a potent PGA producer. Ammonium sulfate, glucose and phosphate ions are the most significant nutritional factors affecting PGA production during batch fermentation. It is worthwhile to further optimize the significant variables determined in the present study to attain maximum PGA yield by applying response surface methodology or other suitable statistical designs.

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