Full Length Research Paper

Application of Plackett–Burman experimental design to evaluate nutritional requirements for poly (γ-glutamic acid) production in batch fermentation by *Bacillus licheniformis* A13

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Abstract: The objective of this study was to use statistically based experimental design for the optimization of poly (γ-glutamic acid) production from *Bacillus licheniformis* A13. Nine components in the medium were screened for nutritional requirements. The parameters significantly affecting poly (γ–glutamic acid) production was found to be yeast extract and medium volume by using the Plackett–Burman experimental design. Analysis of variance exhibited a high coefficient of determination (R²) value of 0.98 and ensured that the polynomial model with the experimental data was a satisfactory one. The results showed that the final concentration of medium optimized with Plackett–Burman was (in g/l): glucose, 50; NH₄Cl, 3; yeast extract, 2; MgSO₄·7H₂O 0.8; NaCl 0.8; CaCl₂·2H₂O 0.00084; K₂HPO₄ 6.4; FeSO₄·4H₂O 0.006; and 0.1ml of trace element solution and culture volume 25 ml. Under optimized medium, the average γ–PGA yield reached 28.2 g/l after 72h of cultivation.

Keywords: poly (γ-glutamic acid), Plackett–Burman design, *Bacillus licheniformis* A13

Introduction

Poly-γ-glutamic acid (γ-PGA) is an anionic, watersoluble naturally occurring polyamide (Oppermann-Sanio and Steinbüchel 2002; Ashiuchi 2010). γ-PGA is completely biodegradable, edible and non-toxic to the human and environment (Yoon et al., 2000). Therefore, it has extensive prospects of applications in medicine, food, cosmetics, environmental protection, agriculture and other fields as a thickener, bitterness-relieving agent, drug carrier, cryoprotectant, humectant, curable biological adhesive (Buescher and Margaritis, 2007; Ben-Zur and Goldman 2007; Manocha and Margaritis 2008; Wang et al., 2008; Ikumi et al., 2008; Ashiuchi, 2010; Bajaj and Singhal, 2011). On the basis of the nutrient requirement, the γ-PGA producing bacteria are classified into two groups as glutamic acid dependant bacteria; require the addition of glutamate to the medium to stimulate γ-PGA production and glutamic acid independent bacteria; able to produce γ-PGA via de novo pathway in absence of glutamic acid (Bajaj and Singhal, 2011). In addition to glutamic acid, several other factors such as carbon and nitrogen sources, ionic strength, aeration, agitation and medium pH affected the productivity and quality of γ-PGA (Shih and Van, 2001; Bajaj and Singhal, 2011). A recent awareness of
biodegradable and sustainable properties of γ-PGA has stimulated the development of its fermentative production (Jeong et al., 2010; Zhang et al. 2012a, b). The production cost of any biotechnological process can be considerably reduced by optimization of the process (Sangkharak and Prasertsan, 2007). The use of multivariate experimental design technique is becoming increasingly widespread in applied biotechnology. Multivariate designs, which allow the simultaneous study of several control variables, are faster to implement and more cost effective than traditional univariate approaches (Montgomery, 1997; Nikel et al., 2005). The statistical method is a versatile technique for investigating multiple process variables because it makes the process easily optimized with fewer experimental trials (Bajaj et al., 2009). Several experimental design models could be employed to reduce the number of experiments under different conditions. If it is desired to screen a large number of factors, experimental designs for first-order models, such as the factorial design or Plackett–Burman design (Plackett and Burman, 1946), can be used. Application of statistical experimental methods to screen the significant medium components affecting γ-PGA production and to evaluate the optimal levels of the significant variables has been attempted (Berekaa et al., 2006; Bajaj and Singhal, 2009; Jeong et al., 2010). The objective of this study was to identify significant variables influencing γ-PGA production by B. licheniformis A13 using Plackett–Burman design.

Materials and methods

Microorganism

The bacterium employed in this study was isolated from a tannery effluent and identified as Bacillus licheniformis A13 on the basis of partial 16S rRNA sequence submitted to the Genbank under accession number AY 134872 (Ghozlan et al., 2006). It was maintained on LB-agar slant (g/L), yeast extract 5, tryptone 10, NaCl 10, agar, 15.

Inoculum preparation

A loopful of cells from a slant were transferred to 50mL of the seed medium dispensed into 250-ml Erlenmeyer flasks and incubated at 37°C and 200 rpm for 12h (optical density at 600nm = 1) , was used as the inoculum. The production medium was comprised of the following components (g/l) glucose, 20; MgSO\(_4\)·7H\(_2\)O 0.4 NaCl 0.4; CaCl\(_2\) 2H\(_2\)O 0.0084; K\(_2\)HPO\(_4\) 3.2; FeSO\(_4\)·4H\(_2\)O 0.06; and 1ml of trace element solution (NaMoO\(_4\); CuSO\(_4\); MnSO\(_4\); ZnSO\(_4\); CoCl\(_2\); H\(_3\)BO\(_4\) 1mM each); NH\(_4\)Cl (3g/L) was fixed throughout the study. Initial pH of the medium was adjusted to 6.5 by using 1N NaOH and/or 1N HCl. The medium was sterilized in an autoclave for 15 min at 121°C.

Fermentation conditions

Batch mode shake flask experiments were conducted in 250-mL Erlenmeyer flasks, containing 50mL of the sterile production medium at 37±2°C and 200 rpm for 72h. The production media were inoculated with 2% (v/v) of 12-h old inoculum. After the cultivation specified for each set of experiments, the culture broth was centrifuged at 12,000g for 20 min at 4°C and PGA was determined. All the experiments were carried out in duplicate.

PGA purification and quantification

PGA was purified by the ethanol precipitation method reported by Shih et al. (2002). After each set of fermentation was completed, cells were separated from the culture broth by centrifugation at 12000 rpm for 20min at 4°C. The culture supernatant containing PGA was poured into 4volumes of ice-cold ethanol with gentle stirring and kept at 4°C overnight. Precipitated γ-PGA was collected by centrifugation at 12000 rpm for 20 min at 4°C. Then crude γ-PGA was redissolved in deionized water and any insoluble contaminants were removed by centrifugation for 20 min at 12000 rpm at 4°C. After three consecutive ethanol precipitation steps, the resultant γ-PGA centrifuged at 4°C and finally dried at 70°C until its weight became constant.
Polyglutamic acid analyses

In order to analyze the produced polymer, the dried polymer was dissolved in 1ml of distilled water and hydrolyzed with an equal volume of 6 M HCl at 100°C overnight according to the method reported by (Kambourova et al., 2001).

Identification of the significant factors by Plackett–Burman design (PB)

The Plackett–Burman design, an efficient technique for medium-component optimization (Yong et al., 2011), was used to pick factors that significantly influenced poly γ (glutamic acid) production and insignificant ones were eliminated to obtain a smaller, more manageable set of factors. The Plackett–Burman experimental design (Plackett and Burman, 1946) based on the first-order model:

\[ Y = \beta_0 + \sum \beta_i x_i \]  

was used to screen the important variables that influence PGA production. Where \( Y \) is the response (PGA yield), \( \beta_0 \) is the model intercept and \( \beta_i \) is the linear coefficient, and \( x_i \) is the level of the independent variable. This model identifies the main parameters required for maximal PGA production. Total number of trials to be carried out according to the Plackett–Burman is \( n+1 \), where \( n \) is number of variables (medium components). Each variable is represented at two levels, high and low, which are denoted by (+1) and (−1), respectively. Table 1 lists the factors under investigation as well as the levels of each factor used in the experimental design with the symbol code, and actual level of the variables is shown in Table 2. Experimental error was estimated by calculating the variance among the dummy variables as follows:

\[ V_{eff} = \frac{\sum (Ed)^2}{n} \]  

where \( V_{eff} \) is the variance of the concentration effect, \( Ed \) is the concentration effect for the dummy variable and \( n \) is the number of dummy variables. The principal effects of each variable on PGA were estimated as the difference between both averages of measurements made at the higher level and at the lower level.

Table 1: Experimental variables at different levels used for the production of PGA by \( B. licheniformis \) A13 using Plackett–Burman design

<table>
<thead>
<tr>
<th>Variables</th>
<th>Units</th>
<th>Symbol code</th>
<th>Experimental values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>Glucose</td>
<td>g/l</td>
<td>G</td>
<td>30</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>g/l</td>
<td>Mg</td>
<td>0.04</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>g/l</td>
<td>Na</td>
<td>0.04</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>mg/l</td>
<td>Ca</td>
<td>0.084</td>
</tr>
<tr>
<td>Potassium Phosphate</td>
<td>g/l</td>
<td>K</td>
<td>0.32</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>mg/l</td>
<td>Fe</td>
<td>0.6</td>
</tr>
<tr>
<td>Trace element</td>
<td>ml</td>
<td>TE</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>g/l</td>
<td>Y</td>
<td>0</td>
</tr>
<tr>
<td>Medium volume</td>
<td>ml</td>
<td>V</td>
<td>25</td>
</tr>
</tbody>
</table>
Table 2: Twelve-trial Plackett–Burman design matrix for nine variables with coded values along with observed results for screening of significant factors affecting poly (γ-glutamic acid) production by *B. licheniformis* A13

<table>
<thead>
<tr>
<th>Run order</th>
<th>Experimental values</th>
<th>PGA (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>Mg</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>9</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>11</td>
<td>-1</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>-1</td>
<td>-1</td>
</tr>
</tbody>
</table>

D1 and D2 are independent dummy variables
The observed values of PGA were the mean values of duplicates.

Statistical analysis

Statistica® software (trial version 6.0, StatSoft, USA) was used for the experiment design and all statistical analyses. The variables with confidence levels above 95% were considered as influencing PGA production significantly.

Results and discussion

Screening of PGA producing strains

A total of 45 local isolates were screened for poly (γ-glutamic acid) production, by first selecting those with mucoid appearance on agar plates, then further in liquid broth. The production of γ–PGA was confirmed qualitatively by hydrolysis of polymer and detection of the monomers by TLC, and the result revealed that the glutamate is the major product (data not shown). Moreover the accumulation of polysaccharide in the broth was negligible according to the phenol–sulfuric acid method (Dubois et al., 1956), proving that the major precipitated product is only polyglutamic acid biopolymer. In particular by *B. licheniformis* A13 had good characteristics for γ–PGA production without the addition of glutamic acid so, it classified as a glutamate – independent PGA producer and hence selected for optimization. A few γ–PGA producing *Bacillus* strains have been reported as exogenous glutamic acid-independent type such as *B. licheniformis* A35 (Cheng et al., 1989), *Bacillus* sp.SAB-26 (Soliman et al., 2005), *B. amylobiquefaciens* LL3 (Cao et al., 2011) and *B.
subtilis C10 (Zhang et al., 2012b). The bacteria which do not require glutamic acid to produce γ–PGA are of great interest because of the lower cost and simplified process in industrial ferment or production systems (Cao et al., 2011).

**Evaluation of factors affecting γ-PGA production**

In this present study the statistical methodology, Plackett–Burman design is demonstrated to be effective and reliable in selecting the statistically significant factors. The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its ready applicability and validity (Bajaj et al., 2009; Yong et al. 2011).

**Screening of significant conditions for γ-PGA production using a Plackett–Burman design**

To evaluate the factors significantly affecting the production of γ-PGA by *Bacillus licheniformis* A13, Plackett–Burman experimental design was employed. The Plackett–Burman design for 12 trials with two levels of concentrations for nine different variables were carried out according to the experimental matrix as shown in **Table 2**, and γ PGA production was determined as a response. A large variation in γ-PGA from that mandated by the Plackett–Burman design experiments, the maximum PGA production (16 gL\(^{-1}\)) was achieved in trial number 6, while the minimum PGA production (2.56 gL\(^{-1}\)) was observed in trial number 3. Main effects of the examined variables on γ- PGA production were calculated and presented graphically in **Fig.1**. Main effects allow the determination of the effect of each constituent. A large contrast mean, either positive or negative, indicates that a factor has a large impact on titre; while a mean close to zero means that a factor has little or no effect. When the sign of the effect of the tested variable is positive, the influence of the variable on γ- PGA production is greater at a high level. And when negative, the effect of the variable is greater at a low level. As shown in **Fig.1**, it was found that all variables except trace elements, calcium chloride and culture volume within the test range had a positive effect on PGA production. The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. Some investigators find that confidence levels greater than 85 % are acceptable (Lu et al., 2011). In this study, variables with confidence levels above 95% (p<0.05) were considered significant. Based on the statistical analysis of confidence level of 9 variables (**Table 3**), yeast extract and medium volume had confidence levels above 95% and hence were considered the significant parameters influence PGA production. Yeast extract is the positive significant variable affecting γ–PGA production, while medium volume was the negative significant parameter affecting γ-PGA production. Whereas the other tested variables are insignificant.

![Fig.1. Effect of nutritional factors on poly (γ-glutamic acid) production by *Bacillus licheniformis* A13 based on Plackett–Burman design results.](attachment:Figure1.png)
Table 3 Statistical analysis of Plackett–Burman design results showing estimated effect, regression coefficient and corresponding t -values, P- values and confidence levels for each variable for PGA yield

<table>
<thead>
<tr>
<th>Variables</th>
<th>Effect</th>
<th>Coefficient</th>
<th>Standard error</th>
<th>t-value</th>
<th>P-value</th>
<th>Confidence level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-</td>
<td>2.550788</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.54</td>
<td>0.77167</td>
<td>0.670332</td>
<td>2.30234</td>
<td>0.148</td>
<td>85.2</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.86</td>
<td>0.42833</td>
<td>0.670332</td>
<td>1.27797</td>
<td>0.329</td>
<td>68.1</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1.13</td>
<td>0.565</td>
<td>0.670332</td>
<td>1.68573</td>
<td>0.234</td>
<td>76.6</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>-0.72</td>
<td>-0.36167</td>
<td>0.670332</td>
<td>-1.07907</td>
<td>0.393</td>
<td>60.7</td>
</tr>
<tr>
<td>Phosphate</td>
<td>2.6</td>
<td>1.31167</td>
<td>0.670332</td>
<td>3.91349</td>
<td>0.059</td>
<td>93</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>2.88</td>
<td>1.43833</td>
<td>0.670332</td>
<td>4.29141</td>
<td>0.050</td>
<td>95</td>
</tr>
<tr>
<td>Trace element</td>
<td>-0.59</td>
<td>-0.29833</td>
<td>0.670332</td>
<td>-0.89011</td>
<td>0.467</td>
<td>53.3</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>4.17</td>
<td>2.08500</td>
<td>0.670332</td>
<td>6.22080</td>
<td>0.025</td>
<td>97.5</td>
</tr>
<tr>
<td>Medium volume</td>
<td>3.54</td>
<td>-1.77167</td>
<td>0.670332</td>
<td>-5.28594</td>
<td>0.034</td>
<td>97</td>
</tr>
</tbody>
</table>

$R^2 = 98.25\%$; $\text{adj } R^2 = 90.36\%$

The goodness of fit of the model was checked by determination coefficient ($R^2$). In this case, the $R^2$ value was calculated to be 0.9825, indicated that 98.25% of the total variability in the response could be explained by this model and only 1.75% of the total variation was not explained. A regression model with $R^2$ closed to 1.0 is considered as having a very high correlation (Yong et al., 2011). Therefore, the present $R^2$ value reflected a very good fit between the observed and predicted responses, and implied that the model is reliable for predicting $\gamma$–PGA production. The value of the adjusted determination coefficient (Adj $R^2 = 0.9036$) confirmed the significance of the model as well. After applying the ANOVA statistical test, it was found that the first order models for $\gamma$–PGA production was satisfactory, the polynomial model equation was proposed to calculate the optimum levels of these variables for PGA yield can be written as:

$$y_{(\gamma-PGA)} = 2.550788 + 2.085 X_1 -1.77167 X_2$$

Where $X_1$, $X_2$ represents yeast extract, culture volume, respectively. One of the advantages of the statistical design is that it allows operators to rank the effect of different variables on the measured response independent on the nature of the variables as well as its sign.

Fig. 2 shows the ranking of factor estimates in a Pareto chart. The Pareto chart has been described as a useful tool for identifying the most important effects, display the magnitude of each variables and is a convenient way to view the results of a Plackett– Burman design (Haaland, 1989; Strobel and Sullivan, 1999). In this chart, the length of each bar on a standardized Pareto chart is proportional to the absolute
value of its associated regression coefficient or estimated effect. **Fig. 2** shows that K$_2$HPO$_4$ positively affects the γ-PGA production. It has been reported that the increase of γ-PGA biosynthesis attribute to effect of K$_2$HPO$_4$ concentration on the enzymes involved in γ-PGA biosynthesis (Jeong et al., 2010; Soliman et al. 2005). The positive effect of K$_2$HPO$_4$ could be attributed to the mechanism of γ-PGA synthetase in *B. licheniformis*, as proposed by Troy (1973) and Gardner and Troy (1979). In this mechanism, the activation of γ-PGA synthetase is accompanied by the cleavage of ATP into AMP, and consequently produces more γ-PGA. In addition, K$_2$HPO$_4$ is the main source of phosphate in the medium that promotes the activation of γ-PGA synthetase. **Fig. 2** indicates that effect of concentration of glucose on γ-PGA biosynthesis by *B. licheniformis* A13 is positive, increase of the initial glucose concentration led to the improvement of the γ-PGA production. This result is in accordance with the results reported for PGA production by *B. subtilis* R23 and *B. subtilis* ZJU-7 (Bajaj and Singhal, 2009; Chen et al., 2010).

<table>
<thead>
<tr>
<th>Yeast extract</th>
<th>Culture volume</th>
<th>FeSO$_4$</th>
<th>K$_2$HPO$_4$</th>
<th>Glucose</th>
<th>NaCl</th>
<th>MgSO$_4$</th>
<th>CaCl$_2$</th>
<th>Trace element</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6.220802</strong></td>
<td><strong>-5.26584</strong></td>
<td>4.291408</td>
<td>3.913466</td>
<td>2.302343</td>
<td>1.685753</td>
<td>1.277974</td>
<td>-1.07907</td>
<td><strong>-390107</strong></td>
</tr>
</tbody>
</table>

**Figure 2**: Pareto chart represent the estimates effect of variables in Plackett–Burman design on poly (γ-glutamic acid) production by *Bacillus licheniformis* A13.

It was observed that the selection of carbon source for PGA production is strain dependent. The glutamic acid independent bacteria such as *B. licheniformis* A35 usually used glucose as major carbon sources for PGA production (Cheng et al., 1989). Glucose and glycerol are reported to support PGA production in most of the strains. Ko and Gross (1998) suggested that PGA synthesis by *B. licheniformis* ATCC945A can occur by the conversion of glucose to acetyl-CoA and the TCA cycle intermediates which then forms L-glutamic acid. Previous studies have established that γ-PGA production by micro-organisms is substantially influenced not only by carbon and nitrogen sources, but also by minerals (Wei et al., 2010). Indeed, in line with these studies, our study showed that, increased NaCl, enhance γ-PGA production (**Fig 2**). This finding confirms previous works stating that the addition of NaCl in the culture medium increased γ-PGA production.
Bajaj and Singhal (2009) reported that sodium chloride is one of the most variables had significant effect on PGA production by *Bacillus subtilis* R23. Fig 2 showed that among the tested variables yeast extract had the highest significant effect on PGA production, as PGA production increased with increasing the yeast extract concentration. This is in line with previous findings reported that yeast extract (paste) is a suitable industrial nitrogen source for large-scale γ-PGA production by *B. subtilis* ZJU-7 (Chen et al., 2010). Jeong et al., (2010) reported that yeast extract is one of the most positive significant variables affecting γ-PGA synthesis by *Bacillus subtilis* RKY3. From the confidence level of variables, it is apparent that culture volume is the most negative significant variables affecting γ-PGA synthesis (Fig 2). Since PGA is an extracellular, high molecular weight polymer, the culture medium becomes highly viscous with polymer production. This increased viscosity is likely to decrease the volumetric oxygen mass transfer, leading to oxygen limitation. This can be worked out by increasing the available oxygen by decreasing the medium volume.

![Figure 3: Response surface and contour plots showing the relative effects of yeast extract and medium volume on γ-PGA production by Bacillus licheniformis A13.](image)

**Figure 3**: Response surface and contour plots showing the relative effects of yeast extract and medium volume on γ-PGA production by *Bacillus licheniformis* A13.

**Fig. 3** shows the surface plots showing the relative effect of the significant factors, yeast extract and culture volume on γ-PGA production. It was depicted that decrease in culture volume and increase yeast concentration increased γ-PGA production.

**Validation of the Model**

Validation of the model was carried out in shake flasks under conditions predicted by Plackett–Burman design. According to these results a medium of the following composition is predicted to be near optimum for the γ-PGA production (in g/l) glucose, 50; yeast extract, 2; NH₄Cl, 3; MgSO₄·7H₂O, 0.8; NaCl, 0.8;
CaCl$_2$.2H$_2$O 0.00084; K$_2$HPO$_4$ 6.4; FeSO$_4$.4H$_2$O 0.006; and 0.1ml of trace element solution and culture volume 25 ml. The yield of γ-PGA on this medium was 28.2g/l. This result presented about 5 fold increase in yield after 72h of cultivation, when compared to the results obtained in basal production medium (5.45g/l).

There are some striking similarities between our results and some literature data. The production of PGA increased significantly from 5.27 to 26.12g/l and from 7.64 to 25.38 g/l by B. licheniformis NCIM2324 and B. subtilis R23 respectively , when were cultivated in the optimal medium developed by using statistical optimization approach, as compared to basal medium (Bajaj and Singhal, 2009; Bajaj et al., 2009).

Conclusions

_Bacillus licheniformis_ A13 was characterized as a new exogenous glutamic acid-independent producer strain. The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its easy applicability, reliability and validity. In this study, it is evident that various process parameters like yeast extract and culture volume are the most significant factors influencing γ-PGA production. Under the optimized conditions, the maximum γ- PGA yield 28.2g/l was reached in batch fermentation culture. Therefore, Plackett–Burman design was proved to be effective method and useful tool for optimization of PGA production by _B. licheniformis_ A13.

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References


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