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Application of Plackett-Burman Design to Find Main Medium Components and Improve Yield of Diglyceride Synthesized by *A. niger* GZUF36 Lipase

Cui-Qin Li[1], Chao-Shuang Xia[2,3], La-Ping He[2,3,4]*, Lan-Xiang Li[2,4] and Huan-Jing Zhou[2,4]

[1]School of Chemistry and Chemical Engineering, Guizhou University, Guiyang 550025, China.
[2]Department of Food Science, College of Liquor and Food Engneering, Guizhou University, Guiyang 550025, China.
[3]Guizhou Province Key Laboratory of Fermentation Engineering and Biopharmacy, Guiyang 550025, China.
[4]Key Laboratory of Agricultural and Animal Products Store and Processing of Guizhou Province, Guiyang 550025, China.

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Main medium components that influence whole-cell lipase from *A. Niger* GZUF36 catalyzing synthesis of 1,3-diglyceride (1,3-DG) by glycerolysis of triglyceride (TG) are identified by Plackett-Burman (PB) design. The response index was yield of DG synthesized by the lipase-catalyzed glycerolysis. The selected factors for PB design included glucose, soybean meal, zinc sulfate, disodium hydrogen phosphate, calcium chloride, potassium chloride and potassium hydrogen phosphate. The results showed that the main medium constituents were glucose, soybean meal and potassium chloride. Other factors were not significant. On the base of PB design, the optimized medium was obtained, with which the yield of diglyceride (DG) was increased 0.78-fold compared with basal medium. The synthesized DG can be used as healthy lipid. This study would improve the further optimization steps on the bioprocess development tracks.

Key words: Plackett-Burman design, diglyceride, *A. niger* GZUF36, Main medium constituents.

Diglyceride (DG) is a natural component of edible oils. It exists in two region-isomeric forms, 1,3-DG and 1,2-DG with a natural isomeric ratio of approximately 3:7. DG oil, especially the 1,3-isomer, has positive effects on human health, namely, suppression of both postprandial serum triglycerides (TG) elevation and body fat accumulation[1] and can used as healthy lipid, if DG contained not less than 70% 1,3-isomer[1,2].

* To whom all correspondence should be addressed.
Tel/Fax: +86-0851-8298021;
E-mail: helaping@163.com

However, low DG content in natural lipid limits its application. So, chemical and enzymatic methods were developed to prepare diglycerides[3-7]. Compared with chemical method, enzymatic approaches to prepare DG take advantages of mild conditions, safe products, high regioselectivity and friendly to the environment[8]. Currently, no research is available on the preparation of DG using whole-cell lipase in non-aqueous medium.

In a previous study, considering whole-cell enzyme with no purification, thus decreasing the purification cost and simplifying the process, we screened a whole-cell lipase from a new isolated strain *A. niger* GZUF36 with synthesis of DG by

glycerolysis of TG. 1,3-DG content in total DG was up to 72.54% [9]. So, the DG synthesized by the whole-cell lipase can be as healthy lipid [2].

However, the yield of DG was low, only 15.33%, which could be deduced from the report[9]. The used medium for *A. niger* GZUF36 was re-screening medium. The yield can be improved by increasing the lipase amount, which can be attained by screening main medium constituent for *A. niger* GZUF36 via Plackett-Burman (PB) design[10]. That was the object of this work. The used basal medium was the re-screening medium[9].

MATERIALS AND METHODS

A. niger GZUF36 was screened by our laboratory, collected in China Center for Type Culture Collection. 1-Monoolein, 2-monoolein, 1,3-diolein, 1,2-diolein and triolein (purity > 99%) as standards were purchased from Sigma. Acetonitrile and hexne were chromatographically pure. All other chemicals used in this work were of analytical grade and commercially available. Seed culture medium contained (g/L) peptone 5, glucose 5, beef extract 3 and NaCl 5. Basal medium contained (g/L) soybean 20, corn syrup 20, K_2HPO_4 0.5 and $NaNO_3$ 0.5. Fermentation mediums were from PB design.

Seed culture: With inoculation loop picking two-ring spores of *A. niger* GZUF36 from slant tubes into 50-ml Erlenmeyer flask containing 10 ml seed medium with pH 7.0, which contained (g/L) peptone 5, glucose 5, beef extract 3 and NaCl 5. The seed culture in shake flasks was carried out at 30 °C and 180 r/m for 24 h.

Selection of main medium components by Plackett–Burman (PB) design: In our pre-trial of single-factor experiment based on basal medium, suitable carbon resource, nitrogen resource and inorganic salts were glucose, soybean, K_2HPO_4, Na_2HPO_4, $ZnSO_4$, $CaCl_2$ and KCl, respectively, which had distinct positive effects on the yield of DG. So they were chosen as the compositions of medium for PB design. The seven variables and four dummy variables were evaluated at two levels (level +1 and level -1, shown in Table 1) for PB design. The ingredients concentrations of medium are shown in Table 2 according to PB design, which conducted by Design-Expert software 8.06 (Stat-Ease, Inc., Minneapolis, USA).

Fermentation culture: Two percent 2.0 (v/v) seed culture was transferred into 250-ml erlenmeyer flask containing 50 ml medium from PB design. The initial pH was adjusted to pH 7.0. The fermentation culture in flasks was carried out at 30 °C and 180 r/min for 48 h.

Preparation of whole-cell lipase

After fermentation culture, the harvested cells were frozen dry, ground in liquid nitrogen into powder and used as whole cell lipase.

Glycerolysis reaction and HPLC analysis

Glycerolysis reaction was carried out to synthesize DG. After glycerolysis reaction, the organic phase was filtered with a 0.45 ìm microporous membrane for HPLC analysis of TG, 1,3-DG, 1,2-DG, and momoglycerides. Both of glycerolysis reaction condition and HPLC analysis were referred to the previous report[9].

Statistical analysis

All the experiments above were performed in triplicates. Data are means of three determinations, their relative standard deviation (RSD) all below 5%. Data were analyzed by Design-Expert version 8.06. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The application of statistically based experimental designs to optimize medium is an efficient approach to studying the effects of several factors and to improving product yields. PB statistical experimental design is a fraction of a two-level factorial design and allows the investigation of 'n-1' variables with at least 'n' experiments[10], which is extremely useful in finding importance of the factors affecting[11]. So, a total of seven medium components were analyzed with regard to their effects on DG production using a PB design.

The analysis of variance for results from Tables 2 using Design-Expert version 8.06 is shown in Table 3. The highest DG yield was 26.94% at run 9. It indicates that glucose (X_1), soybean meal (X_2) and potassium chloride (X_8) had significant effects on the yield of DG ($P < 0.05$), other factors had no significant effects ($P > 0.05$). The model F-value of 56.48 implies that the model is very significant because of the less than 0.08% chance that the 'Model F-Value' can occur due to noise. The Pred R-Squared of 0.9099 was in reasonable agreement

Table 1. Level of factors in the Plackett - Burman design

Variables	Factors	+1 level	-1 level
X_1	Glucose (g/L)	11.1	8.9
X_2	Soybean meal (g/L)	22.2	17.8
X_4	$ZnSO_4$ (g/L)	0.56	0.44
X_5	Na_2HPO_4 (g/L)	0.56	0.44
X_7	$CaCl_2$ (g/L)	0.56	0.44
X_8	KCl (g/L)	0.56	0.44
X_{10}	K_2HPO_4 (g/L)	0.56	0.44
X_3, X_6, X_9, X_{11}	Dummy variables	1	-1

Table 2. Plackett-Burman design with coded values, showing the experimental and predicted responses

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Yield of diglyceride
1	-1	1	-1	1	1	-1	1	1	1	-1	-1	17.59
2	-1	1	1	1	-1	-1	-1	1	-1	1	1	15.98
3	1	1	1	-1	-1	-1	1	-1	1	1	-1	12.1
4	1	-1	-1	-1	1	-1	1	1	-1	1	1	19.26
5	-1	1	1	-1	1	1	1	-1	-1	-1	1	19.25
6	1	-1	1	1	-1	1	1	1	-1	-1	-1	16.52
7	1	-1	1	1	1	-1	-1	-1	1	-1	1	18.9
8	-1	-1	1	-1	1	1	-1	1	1	1	-1	25.8
9	-1	-1	-1	1	-1	1	1	-1	1	1	1	26.94
10	1	1	-1	1	1	1	-1	-1	-1	1	-1	13.72
11	1	1	-1	-1	-1	1	-1	1	1	-1	1	10.43
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	25.87

Table 3. Analysis of variance for results of Plackett-Burman test

Source	Sum of squares	Degrees of freedom	Mean square	F-value	Pr>F	Significance
X_1	136.69	1	136.69	170.5	0.0168	significant
X_2	162.95	1	162.95	203.26	0.0152	significant
X_4	0.78	1	0.78	0.97	0.8490	
X_5	3.72	1	3.72	4.64	0.6192	
X_7	0.077	1	0.077	0.096	0.1566	
X_8	10.45	1	10.45	13.04	0.0269	significant
X_{10}	2.29	1	2.29	2.85	0.1068	
Model	316.96	7	45.28	56.48	0.0008	significant
R-Squared	0.99					
Adj R-Squared	0.9725					
Pred R-Squared	0.9099					
Adeq Precision	22.584					

Table 4 . Yield of DG catalyzed by whole-cell lipase from *A. niger* GZUF36 fermented with the optimized medium

No.	1	2	3	4	5	6	Mean	RSD
Yield of DG **(%)**	26.86	27.93	25.59	27.69	27.12	28.89	27.35	1.11
1,3-DG content in DG **(%)**	87	86	88	89	88	87	88	1.05

with the Adj R-Squared of 0.9725. Adeq Precision measures the signal to noise ratio. The ratio of 22.584 indicates an adequate signal because a ratio greater than 4 is desirable. This model can be used to navigate the design space.

So, after ignorance of the insignificant terms ($P > 0.05$), a modified first-order equation was employed to describe the yield of 1,3-DG in terms of coded factors as follows:

$$Y(\%) = 18.53 - 3.38X_1 - 3.69X_2 - 0.93X_8 \quad ..(1)$$

The equation 1 shows that high concentration of glucose could repress DG production, that is to say, high concentration of glucose inhibited the production of the whole-cell lipase from *A. niger* GZUF36. At the same time, we also observed that cell concentration was increased with the increase of glucose concentration. Shaeh and Zahran[12] have reported similar phenomenon, that, as the glucose concentration is increased there is a considerable reduction in synthesis of extracellular lipase by *P. fluorescens*. The reason could be that cell growth inhibited lipase production with the increase of glucose concentration, although it was suitable carbon resource according to our previous single-factor experiments. Similar phenomenon that high concentration of medium composition inhibited lipase production was found with soybean meal and potassium chloride.

According to the equation 1 and PB design, the optimized medium should contain (g/L): glucose 8.9, soybean meal 17.8, $ZnSO_4$ 0.56, Na_2HPO_4 0.56, $CaCl_2$ 0.56, K_2HPO_4 0.56 and KCl 0.44.

Then, the optimized medium was used to improve yield of DG catalyzed by whole-cell lipase from *A. niger* GZUF36. Fermentation culture was carried out with the optimized medium at 30 °C and 180 r/min for 48 h. Results are shown in Table 4

The yield of DG was 27.35% (Table 4), which was more than the highest yield in Table 2 (26.94%). Moreover, 1,3-DG content in DG was over 85%. So, the synthesized DG remained a healthy lipid[2]. That indicates that the test results from PB design are credible. According to previous report, the yield of DG was 15.33% with basal medium[9], namely basal medium in this work. So, the yield of DG catalyzed by the whole-cell lipase with the optimized medium from PB design was increased 0.78-fold.

CONCLUSION

The evaluation of the medium components and optimization of medium for *A. niger* GZUF36 to improve lipase yield was carried out by PB statistical design. The evaluation index was the yield of DG by the lipase-catalyzed glycerollysis of TG. The effect of seven medium components were studied and among them glucose, soybean and potassium chloride were found to be the significant variables for lipase production by *A. niger* GZUF36. Moreover, the optimized medium was obtained, with which the yield of DG was 27.35%. The yield of DG was increased 0.78 fold compared with using basal medium or re-screening medium[9]. This study provides the basis for further optimization using response surface optimization technique.

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