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Response surface methodology for the evaluation of glucose-6-phosphate dehydrogenase enrichment process by soybean lecithin reversed micelles☆

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Abstract

Glucose-6-phosphate dehydrogenase (G6PD) present in *Saccahromyces cerevisiae* is an enzyme of the pentose pathway. An effective enrichment of this intracellular enzyme can be achieved with the reversed micellar methodology. In this work, this methodology was employed with soybean lecithin, a biocompatible surfactant. A factorial design was used to evaluate the influence of pH (A) and extraction runs (B) on the G6PD purification factor. After statistical analysis and process optimization, a mathematical model representing G6PD enrichment was obtained: $Y = 4.89 - 0.83A + 0.092B + 0.27AB - 1.37B2$ with an enzyme purification factor of about 5.2.

Keywords: Reversed micelles; Soybean lecithin; Response surface methodology; Glucose-6-phosphate dehydrogenase

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) belongs to one of the widest and most important classes of NAD-dependent enzymes. G6PD catalyzes dehydration of glucose-6-phosphate (G6P) into 6-phosphategluconic lactone. This enzyme uses $NADP^+$ as a hydrogen acceptor and catalyses the first step of the pentose phosphate pathway, which has three functions: to generate reducing equivalents in the NADPH form, to produce pentose phosphates, and to serve as a route of entry of pentose to the glucolytic pathway [1]. Moreover, G6PD is commonly used in biochemical and medical assays for measuring creatin-kinase and hexokinase activities, hexoses and ATP concentrations, etc. Although most animal tissues and microbial cells contain G6PD, yeasts are the main source of this enzyme [2], especially *Saccharomyces cerevisiae* (from baker's or beer's yeast) [3–5].

In this work, the enrichment of G6PD obtained from *S. Cerevisiae* cells was provided by the reverse micelles (RM) technique using soybean lecithin as a surfactant (in isooctane and hexanol). Reverse micelles are suitable for continuous extraction of specific proteins from an aqueous mixture, promoting enzyme concentration and purification. RM contains droplets of water stabilized within an organic solvent by a surfactant. The protein molecules often move from an original aqueous phase into these encapsulated water droplets [6]. Liquid–liquid extraction technique using RM enhances the efficiency of downstream processing, thus deserving more attention [7,8].

Problems can be solved by a series of designed experiments through statistical analysis. For each experiment exploring an experimental space, well-defined questions are raised, and simple statistical methods provide answers through mathematical models.

In this study the influence of the independent variables pH (A) and extraction runs (B) on G6PD purification factor and

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recovery was evaluated with a factorial design. This work also aimed at providing more technological support for application of the RM method in downstream processing. Response surface methodology was employed to optimize the purification process.

2. Experimental

2.1. Microorganism and cell disruption

Commercial *Saccharomyces cerevisiae* (baker's yeast) was purchased at a local market. The cells were washed with distilled water and resuspended in 50 mM Tris–HCl buffer (pH 7.5) containing 5.0 mM MgCl2, 10 mM β-mercaptoethanol, 2.0 mM amino caproic acid, 1.0 mM PMSF (phenylmethylsulfonyl fluoride) and 0.2 mM EDTA (ethylenediaminetetraacetic acid). This suspension was disrupted mechanically at 4 °C in a cooling jacketed mill containing glass beads 0.5 mm in diameter (proportion of 1:1 v/v).

The cell debris was removed by centrifugation in a Jouan Centrifuge (Mod. 1812, Saint-Herblain, France) for 30 min at 4 °C (8720 × *g*). The supernatant consisting in cell-free extract (CFE) was collected and stored at −10 °C. In addition to G6PD, several impurities (enzymes and proteins) were found in this CFE.

2.2. Liquid–liquid extraction

A 2^2 factorial design with a centered face and 3 repetitions at the center point was used to perform the liquid–liquid extraction [9]. The impurities were removed from the CFE (aqueous phase) by SL-reversed-micelles with hexanol (0.025 M) in isooctane using a procedure in cycles, with subsequent extractions [5]. For the CFE pH value to correspond to the ones defined by the experimental design, the original CFE (pH 7.5) was diluted with 0.5 M phosphate buffer. This dilution resulted in a pH adjustment to 5.3, 5.6 or 5.9 and in a final G6PD concentration of about 600 U L^{-1} (±50 U L^{-1}).

In each cycle of the extraction process (defined by the experimental design), the CFE, aqueous phase containing G6PD, was mixed with an equal volume of micellar phase (MP), SL in isooctane/hexanol. This mixture was agitated for 60 min in a shaker at 280 rpm to reach the equilibrium phase, and again was separated into two phases by centrifugation (Jouan Centrifuge Mod. 1812, Saint-Herblain, France) at 1.677 × *g*/10 min/25 °C.

The extraction results are reported in terms of purification factor (Pf) and G6PD content (specific activity) in the aqueous phase. The purification factor in the aqueous phase was determined by enzymatic activity and protein concentration.

2.3. G6PD activity and protein analysis

G6PD activity was measured through the continuous reduction of NADP at 30 °C in a spectrophotometer (Beckman 640 DU, USA) at 340 nm [10]. One G6PD unit was defined as the amount of enzyme catalysing the reduction of 1 μmol of NADP min^{-1} under the assay conditions.

Table 1
Matrix of 2^2 full factorial design with centered face and three repetitions at the central point used to evaluate the influence of pH and extraction runs (ER) on G6PD purification factor (Pf)

Assay	Coded variables	
	pH	ER
1	−1	−1
2	+1	−1
3	−1	+1
4	+1	+1
5	−1	0
6	+1	0
7	0	−1
8	0	+1
9, 10, 11	0	0

Protein concentration was determined by the method described by Lowry (1959) [11] using bovine serum albumin as a standard.

2.4. Factorial design

The G6PD purification factor, which is a dependent response variable of the experimental design, was measured as a function of pH (A) and extraction runs (B). For each one of the two factors, high and low set points (coded values: +1 and −1, respectively) were selected, and a 2^2 factorial design with a centered face and three repetitions at the central point was employed (Table 1). The extractions, represented by the 11 combinations, were made twice. The assays were conducted at random in order to minimize eventual systematic errors. A statistical examination of the results and a response surface study were carried out using the STATGRAPH 5.0 statistical program package.

2.5. Purification factor and G6PD recovery

Eq. (1) was used to evaluate the purification factor:

$$\mathrm{Pf} = \frac{\mathrm{Sa}_1}{\mathrm{Sa}_2} \tag{1}$$

where Sa_1 is the specific activity in the initial aqueous phase [U mg^{-1}], Sa_2 is the specific activity in the final aqueous phase [U mg^{-1}].

Eq. (2) was used to determine the specific activity (Sa):

$$\mathrm{Sa} = \frac{A}{P} \tag{2}$$

where Sa is the specific activity [U mg^{-1}], A the enzyme activity [U L^{-1}], P the protein concentration [mg L^{-1}].

Eq. (3) was used to determine the G6PD recovery (R):

$$R = \left(\frac{A_2 v_2}{A_1 v_1}\right) \times 100 \tag{3}$$

where A_1 is the G6PD activity before purification [U]; v_1 the volume of aqueous phase before purification [L]; A_2 the G6PD activity after purification [U]; v_2 the volume of aqueous phase after purification [L].

3. Results and discussion

A 2^2 full factorial design with a centered face and three repetitions at the central point was employed to optimize the G6PD enrichment by SL-reversed micelles [9]. The pH values and the extraction runs selected for this work were based on previous experiments in which the influence of several variables on SL-reversed micelles extraction was evaluated. In previous work, an enhanced purification factor was observed for pH values ranging from 5.3 to 5.9 [5]. Table 2 shows the values of the variables, the results of the purification factor (Pf) and the G6PD recovery obtained with the experimental design.

All the assays were carried out in duplicate.

As can be seen in Table 2, the G6PD purification factor values varied from 3.1 to 5.3. The highest values were observed in the assays employing the lowest pH value.

Although the extraction mechanism using zwitterionic surfactants has not yet been elucidated, it is well known that these surfactants are neutral compounds having positive and negative charges. In this way, the pH value would be limited by the enzyme stability, since the reversed micelles used in this work are not able to encapsulate the G6PD in their cores, due to a significant size-exclusion effect [5,12].

Different proteins (albumin of bovin serum, casein and papain) were previously investigated (unpublished results) in our laboratory, in experiments using SL-reverse micellar system. In all of them it was observed that the micelles diameter remained unaltered, irrespective of the conditions employed.

The reverse micelles extraction is governed by electrostatic and/or hydrophobic interaction. The kind of interaction between reverse micelles and biomolecules also depends on pH solution, since it can modify the biomolecules net-charge [6,13]. In this work, probably the net-charge of most proteins (considered as impurities) in the CFE allowed their removal by SL-reverse micelles by either electrostatic interaction or hydrophobic forces, or by both, simultaneously.

In this way, pH 5.3 favored the removal of impurities from the CFE obtained after disruption of *S. cerevisiae* cells.

Table 2
Variables, values, purification factor (Pf) and recovery of G6PD (R) using a 2^2-full factorial design with centered face and three repetitions at the central point

Assays	Variables		Pf	*R* (%)	
	pH	ER			
1	5.3	3	4.98	145.72	146.52
2	5.9	3	3.26	88.98	93.16
3	5.3	5	4.28	124.04	125.23
4	5.9	5	3.65	97.36	98.09
5	5.3	4	5.28	148.21	149.48
6	5.9	4	4.68	137.16	143.74
7	5.6	3	3.10	86.40	86.82
8	5.6	5	3.96	95.53	98.98
9	5.6	4	4.84	118.94	125.84
10	5.6	4	4.93	116.65	120.86
11	5.6	4	4.70	113.55	114.47

ER: extraction runs; error (±5%).

Table 3
Analysis of variance (ANOVA) for G6PD purification using the purification factor as a response variable

Source of variation	SQ	DF	MS	*F*-values	*p*-values
pH (A)	4.084	1	4.084	304.00	0.0033**
ER (B)	0.050	1	0.050	3.75	0.1923
AB	0.297	1	0.297	22.11	0.0424*
BB	4.953	1	4.953	368.73	0.0027**
Lack of fit	1.200	4	0.300	22.33	0.0478*
Pure Error	0.027	2	0.013		
Total	10.611	10			

SQ: sum of squares, DF: degree of freedom, MS: mean square; R^2: 0.89.
* 90%.
** 95%.

A biotechnological process is considered viable when the final recovery value is higher than 80%. The purification of biomolecules generally requires a series of "downstream processing" steps with different recovery values. Less than 80% recovery at the end of each step means an overall loss sufficiently high to make the process unviable [14]. In this work, the G6PD recovery values (Table 2) varied from 86.82% to 149.48%. These values are similar to those reported by Rangel-Yagui et al. [4].

From the absence of enzymatic activity after re-extraction, it can be concluded that the soybean lecithin system did not extract G6PD from CFE. Moreover, no change in the enzymatic activity was observed in the aqueous phase (CFE), while the total protein concentration decreased, implying that the contaminants were removed by the reverse micelles.

Table 3 shows the analysis of variance (ANOVA) including the significant factors for G6PD purification by SL-reversed micelles, with 25 mM hexanol in isooctane. In this study, instead of the enzyme recovery, it was the purification factor that was used as a response variable, since Pf informs the purification method resolution.

According to Table 3, variable A (pH) and the interaction between A and B (ER) had significant effects on the enzyme purification ($p < 0.05$). The model did not exhibit lack of fit at 10% confidence level for the range tested.

The parameters of the second-order model used to estimate the enzyme purification factor as a function of pH and ER were obtained from multiple regression analysis (Table 4).

The statistical significance of the quadratic model (Table 5) was revealed by the *F*-test (Fmodel > FTable = 5.05). The model does not show lack of fit and the determination coefficient ($R^2 = 0.89$) indicates that 89% of the variation in the evaluated response (Pf) can be explained by the model.

Table 4
Variables, coefficients, *t*-values and *p*-values of the 2^2-full factorial regression

Variables	Coefficients	*t*	*p*
Average	4.89	24.162	0.000*
pH (A)	−0.83	−4.469	0.0042*
ER (B)	0.092	0.497	0.6372
AB	0.27	1.205	0.2735
BB	−1.35	−4.922	0.0027*

* 90%.

Table 5
Analysis of variance of the model regression

Source of variation	SQ	DF	MS	F	p
Model	9.384	4	2.356	11.474	0.0056
Error	1.227	6	0.204		
Total	10.611	10			

R^2: 0.89, SQ: sum of square, DF: degree of freedom, MS: mean square.

The mathematical model representing the G6PD extraction process by SL-reversed micelles in the experimental region considered here can be expressed as Eq. (4):

$$y = 4.89 - 0.83A + 0.092B + 0.27AB - 1.37B^2 \quad (4)$$

where Y is the G6PD purification factor; A the pH and B the extraction runs.

The maximum G6PD purification factor (5.7) corresponded to the point defined by pH 5.3 ($A = -1$) and to 4.1 extraction runs ($B = 0.4$), in other words, four 60-min agitation periods and one 6-min agitation period. A detailed representation of the optimum value predicted from the results by the response surface model is presented in Fig. 1.

There are numerous reports on the purification of G6PD present in various species of animals, microorganisms and vegetables cells. Different methods have been used for purification of this enzyme, mainly the chromatographic methods, which are expensive. For example, some authors report the use of 2′,5′-ADP-Sepharose 4B and DEAE for purification of G6PD coming from bovine lens cells and kidney cortex cells, respectively. In the first case, the purification factor increased 8.4-fold, and, in the second, 1.7-fold [15,16].

In the second stage of this work, different volumetric ratios between AP and MP were tested, in order to find out their influence on the enzyme Pf (Table 6).

The volume of AP was fixed at 3 mL, whereas the volume of MP was changed to attain the following extraction conditions:

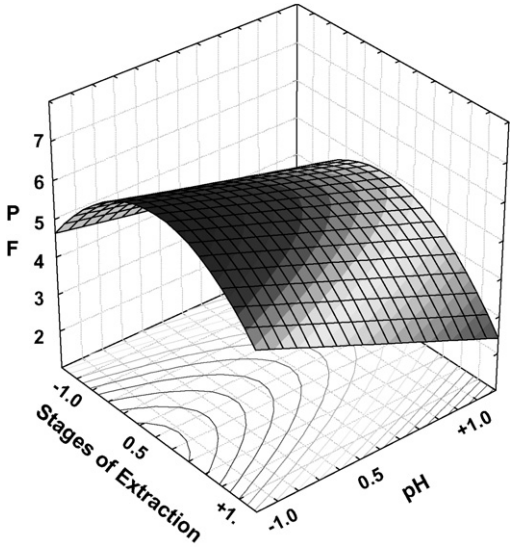

Fig. 1. Response surface and contour lines described by Eq. (4): interaction between A and B whose p-value was 0.0424 at 95% confidence level.

Table 6
G6PD activity, total proteins and recovery after purification by reversed micelles at different ratios between cell-free extract and micellar phase

Assay	AP–PM*	A (U L^{-1})	TP (mg L^{-1})	R (%)	Sa (U mg^{-1})
Control	1:1	5049.86**	21026.81**	–	0.24**
1	1:2	5632.93	15897.48	120.50	0.35
2	1:3	4419.61	16542.59	96.3	0.27
3	1:4	4215.20	15362.78	90.15	0.27
4	1:5	5137.64	17678.23	111.92	0.29
5	1:6	5090.47	17987.38	108.87	0.28

* AP–MP ratios; A: activity; TP: total protein; R: recovery; Sa = specific activity.
** Control, initial values.

1:1; 1:2; 1:3; 1:4 and 1:5 (v/v). Optimum conditions previously determined (Eq. (4)) were employed: 0.05 M LS-reversed micelles (with hexanol 25 mM in isooctane), pH 5.3 and 4.1 extraction runs. The results of G6PD activity, recovery values and total proteins obtained from different ratios are displayed in Table 6.

Table 6 shows that an increase in the MP ratio from 1:1 to 1:2 v/v (in relation to AP) resulted in an increase of 46% in G6PD specific activity and 11% in the volumetric activity. Raising the ratio between the phases to 1:3 v/v (Table 6) decreased the G6PD activity by 12% in comparison with the control (1:1), in spite of the decrease in the G6PD volumetric activity. This increase in specific activity was a consequence of the decrease in total protein content. The SL-reversed micellar system brought about the G6PD enrichment, but, as mentioned before, the system was not able to include this enzyme in the cores of the reverse micelles. The enzyme Pf increased due to both the extraction runs and the change of AP–MP ratio.

However, with the ratio of 1:4 v/v (Table 6), the G6PD specific activity did not change (in relation to 1:3 v/v), probably because the operational conditions were the same for all assays. Mass transfer is dependent on the agitation speed and is affected by the characteristics of the systems. For example, the density changed when the ratios between the phases were modified [17,18].

The G6PD specific activity increased with the increase in total protein removal. It was expected that the use of different AP–MP ratios would improve the G6PD recovery. Indeed, the recovery values increased by around 20% when the MP ratio was raised from 1:1 to 1:2 v/v, but stabilized as a consequence of the decrease in G6PD activity.

For an efficient use of different ratios between AP and MP, it is necessary to investigate further factors that could affect the mass transfer process and the enzyme stability, such as temperature, pH, time and type of agitation [18].

4. Conclusion

The SL-reversed micellar system was efficiently used for increasing the purification factor of G6PD through protein removal. This enzyme was obtained from *S. cerevisiae* cells, and the response surface methodology helped to understand the enzyme purification process. Extractions conducted under

optimal conditions, as indicated by the model, provided a purification factor of 5.2 (medium value). This result shows that the model was adequate to represent the process. The use of different ratios between micellar and aqueous phases in the micellar reversed extraction process provided an increase in the G6PD specific activity from 13% to 46%. This demonstrates that reversed micellar solutions are suitable for enzyme recovery, and for the upkeep of the enzyme activity, since the reagents do not denature the enzyme.

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