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Optimisation of Lipase Extraction from Industrial Preparation using Aqueous Two Phase PEG6000/Phosphate System

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Abstract : Study of optimisation of lipase extraction using aqueous two-phase system containing PEG6000-phosphate system was initially carried out using industrial lipase preparation. The experiment was done by manipulating four parameters that mainly effect protein partition coefficient such as the composition of system components; PEG6000 and phosphate salts, the composition of an added salt, NaCl and the pH of the system. The Response Surface Methodology (RSM) had been applied to find the location of the most suitable system conditions for extracting lipase. It was demonstrated that at system compositions of 7.57% w/w PEG6000/ 12.01% w/w phosphate/ 3.0% w/w NaCl and pH of 7.37, the optimum conditions of predicted lipase recovery from the broth attained was 73.84%. The confirmation experiments were performed and the results of lipase extraction from *Aspergillus terreus* SUK-1 lipase have shown an increment of lipase recovery of 77% in the bottom phase and most proteins other than lipase were found to partition into the top phase. In the subsequent extraction experiments where higher volume of 100 times larger was applied, the result of partitioned lipase were not much different with the smaller scale and the lipase recovery increased 2% from the previous experiments.

Key words : Polyethylene glycol (PEG)-salt system, enzyme separation,Lipase, Optimisation experiment; Response surface methodology (RSM)

Introduction

Aqueous two-phase system (ATPS) has been identified as a good alternative separation method for initial recovery and isolation of highly sensitive bio-molecules (e.g. protein and enzyme). It is because this system offers a mild condition where both phases contain 85%-95% of water. The most common combinations of ATPS are polyethylene glycol (PEG)-dextran and polyethylene glycol (PEG)-salt (Walter *et al*., 1985; Albertsson, 1971). As compared to PEG-dextran, PEG-salt system has been extensively applied for large-scale extraction since the cost of system components, specifically salt solutions, is lower compared to Dextran. Therefore, PEG-salt system also has potential to offer inexpensive separation technique that would beneficial to economical downstream processing. In addition, PEG-salt system is ease to handle even in large-scale volume due to its lower viscosity than PEG-Dextran system. The application of using PEG-salt system for enzyme separation in the primer purification had been reported in previous studies (Schmidt *et al*., 1994;

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Costa *et al*., 2000; Zhi *et al*., 2005) PEG was more preferable among other polymer-salt systems formation due to the content of polyols present in the aqueous phase media that would help to stabilize the enzymes by reducing water content (Gupta *et al*., 2004). The mechanism governing the partition of bio-molecules is still not well understood. Generally, the partitioning behaviour is usually influenced by the system compositions including type, molecular weight, the concentration of phase-forming polymer and salt; the composition of neutral salt as the additional salt and pH system. Lipases or triacyl glycerol ester hydrolase are hydrolytic enzymes known as one of important group of biotechnologically relevant enzymes due to its wide application in industries (Schmidt *et al*., 1994; Sharma *et al*., 2001; Srinivas *et al*., 2001; Walker, 2002) and in catalyzing numbers of reactions (Pandey *et al*., 1999). The applications of lipase in industry have become more important and consequently the need to apply appropriate extraction method in the early stage of purification is in great demand in order to achieve more economical downstream processing. For that reason, a study to optimize selected ATPS systems containing PEG/phosphate for separation of lipase from industrial preparation were conducted by manipulating four selected variables such as concentration of PEG6000 and phosphate, concentration of additional salt (NaCl) and pH system. The best optimised ATPS parameters will be used in the subsequent experiment for lipase extraction using laboratory prepared raw supernatant from local isolate, *Aspergillus terreus* SUK-1. Furthermore, experiments of lipase extraction using large-scale optimum system, that was in100 times larger than the initial volume were conducted with the aim to study the behaviour of partition coefficient in two different volume phase systems.

Materials and Methods

Materials

Polyethylene glycols with molecular weight of 4000 and 6000 were purchased from Fluka (Germany). di-potassium hydrogen phosphate (K_2HPO_4) were purchased from Merck (Germany), while sodium di-hydrogen phosphate (NaH_2PO_4) were obtained from R&M Chemicals (U.K). p-nitrophenol were bought from Fluka (Switzerland). Bovine serum albumin (BSA), gum Arabic, Triton X-100, and p-nitrophenyl palmitate (pNPP) were supplied by Sigma (Germany). All chemicals were of analytical grade.

Lipases

Industrial lipase

Industrial lipase, Lipopan® F BG was purchased from Novozymes. The enzyme solution was prepared through diluting 1% of industrial enzyme in phosphate buffer solution. The enzyme solution was then filtered through Whatman filter paper no.1 and it was kept at 4°C for further partition experiments.

Preparation of raw lipase from local isolate

A local isolate, *Aspergillus terreus* SUK-1 obtained from Microbiology Laboratory, Faculty of Science & Technology, Universiti Kebangsaan Malaysia was used to produce raw lipases in this study. The culture was transferred on PDA agar slants monthly and stored at 4°C as a stock culture. The culture was grown on PDA agar plates at 37°C for 3 days until the brownish colour of spores appeared. The spore

suspension was prepared after the grown culture being added with sterile distilled water and filtered on muslin cloth and the solution of about 10^6 spores per ml was transfered into 100ml inoculm medium in 500ml Erlenmeyer flask as described by Gulati *et al*. (Saxena *et al*., 2003). Spore counting was done using haemacytometer according to Cameron (Gulati *et al.*, 1999) and it was done under microscope with magnification at ×400. The production medium was adjusted to pH 9.0 before inoculation of spore suspension was performed. The fermentation was run at 37±1°C for 7 days and samples were taken for every 24 hours. After that, the biomass was determined by dry weight technique. The filtered broth was kept at 4°C for further analysis.

Optimisation experiments of lipase partitioning in ATPS

All stock solutions of PEG4000 (50% w/w), PEG6000 (50% w/w) and NaCl (25% w/w) were prepared and they were stored at 4°C. Phosphate stock solution consists of a mixture of NaH_2PO_4 (40% w/w) and K_2HPO_4 (40% w/w) with weight ratio of NaH_2PO_4: K_2HPO_4 at 1:1.3 (for pH 6.0), 1:1.6 (for pH 7.0), 1:1.78 (for pH 7.37) and 1:3 (for pH 8.0). For partition experiments, the stock solutions were weighed accordingly to obtain the required total system and the amount of enzyme added into the prepared system was 10% of the total weight system. The solution were mixed thoroughly to equilibrate by using vortex mixer and for a complete two-phase formation, the prepared systems were centrifuged at 2500 g for 5 minutes at 25°C and finally the top and the bottom phase were withdrawn for sample analysis.

Experiments of lipase partitioning at optimum conditions

A selected extraction system at optimum conditions was scaled up to 1000g, which is 100 times higher than initial volume phase systems. The system with 10% enzyme solution was placed in tubular jacketed vessel that equipped with overhead stirrer. After 30 minutes of agitation, the system was allowed to settle at 25°C until the two phases were clearly formed.

Sample analysis

Lipase activity was measured by p-nitrophenyl palmitate (pNPP) assay (Maia *et al*., 2001). 1 unit activity (U) of lipase was defined as 1 μmol p-nitrophenol produced per minutes under the assay condition. The reaction involved was between the enzyme and the conversion of pNPP as substrate into p-nitrophenol (pNP) as the product. 10% of the enzyme assay was including 30mg pNPP in 10 ml of propan-2-ol and another 90% was tris-HCl buffer (50mM; pH:8.0) containing 400mg Triton X-100 and 100mg gum arabic were used. The mixture of 0.2 ml of enzyme solution and 1.8 ml assay solution were incubated at 37°C for the reaction to take place. The absorbance was measured spectrophotometrically at 410nm after 30 minutes reaction. The total protein content in samples was determined according to modified Lowry Method (Gulati *et al*., 2000) .

Calculations

The partitioning of enzyme or protein in aqueous two-phase system can be described by the value of partition coefficient, *k*. The calculation of partition coefficient of lipase activity and protein contaminants were given below in Eqs.(1) and (2), respectively.

Additionally, lipase specific activity was calculated according to Eqs.(3), while partition coefficient of lipase specific activity, kSA was determined according to Eqs.(4). The value of k_{SA} is estimated for evaluation of pure lipase partitioning behaviour in the aqueous two-phase system.

$$k_{\text{lipase}} = \frac{\text{lipase activity}_T \text{ (U/ml)}}{\text{lipase activity}_B \text{ (U/ml)}} \quad \text{.......(1)}$$

$$k_{\text{protein}} = \frac{\text{protein}_T \text{ (mg/ml)}}{\text{protein}_B \text{ (mg/ml)}} \quad \text{.......(2)}$$

$$SA_{\text{lipase}} = \frac{\text{lipase activity (U/ml)}}{\text{protein content (mg/ml)}} \quad \text{.......(3)}$$

$$k_{SA} = \frac{SA_{\text{lipase},T} \text{ (U/mg protein)}}{SA_{\text{lipase},B} \text{ (U/mg protein)}} \quad \text{.......(4)}$$

The percentage of yield (%Yield) and lipase purification factor (p.f) was calculated based on the phase where the enzyme was preferably partitioned as observed in our previous experiments. Both industrial and laboratory prepared lipases were found more preferred to partition into the bottom phase of PEG6000/phosphate system. The calculations of % yield, p.f and volume ratio were given by Eqs.(5), (6) and (7), respectively.

$$\%\text{Yield} = \frac{100}{1 + R_v . k_{\text{lipase}}} \quad \text{.......(5)}$$

$$\text{p.f} = \frac{SA_{\text{lipase},B}}{SA_{\text{lipase,initial}}} \quad \text{.......(6)}$$

where $SA_{\text{lipase,initial}}$ = lipase specific activity in the filtered raw broth (U/mg protein).

$$R_v = \frac{V_T}{V_B} \quad \text{.......(7)}$$

where V_T and V_B are volume of top and bottom phase, respectively.

Results and Discussion

Lipase production by *A.terrus* SUK-1

The growth profile of *A.terreus* SUK-1 fermentation and lipase production is shown in Figure 1. The production of lipase from *A.terreus* SUK-1 was found to be growth-associated with maximum production of 10.97 U/ml, where it occurred simultaneously with the growth of *A.terreus* SUK-1. This finding is in agreement with Gulati *et al.* (Schmidt *et al.*, 1994) that also been reported that lipase production was dependent on the growth of *Aspergillus terreus* RKS101. Furthermore, specific activity of lipase *A.terreus* SUK-1 was also measured and the progress of lipase specific activity during experiments was given in Figure 2. The highest specific activity of lipase was achieved at 48h of fermentation. The decreasing of lipase specific activity at 72h may be due to the presence of contaminant such as protease, which was also probably being produced during the period of fermentation process (Pandey *et al.*, 1999). In addition, the presence of oil residue that still remained in the culture media might be one of the key factors that affect the specific activity of lipase due to the enzymatic action.

Although the production of lipase from *A.terreus* SUK-1 was lower compared to *Aspergillus niger*, a renowned fungi in producing lipase, the highest production of lipase using this local strain was found comparable to previous studies that used another *Aspergillus sp*. The maximum shake-flask production of lipase by *A.terreus* SUK-1 in the experiment was

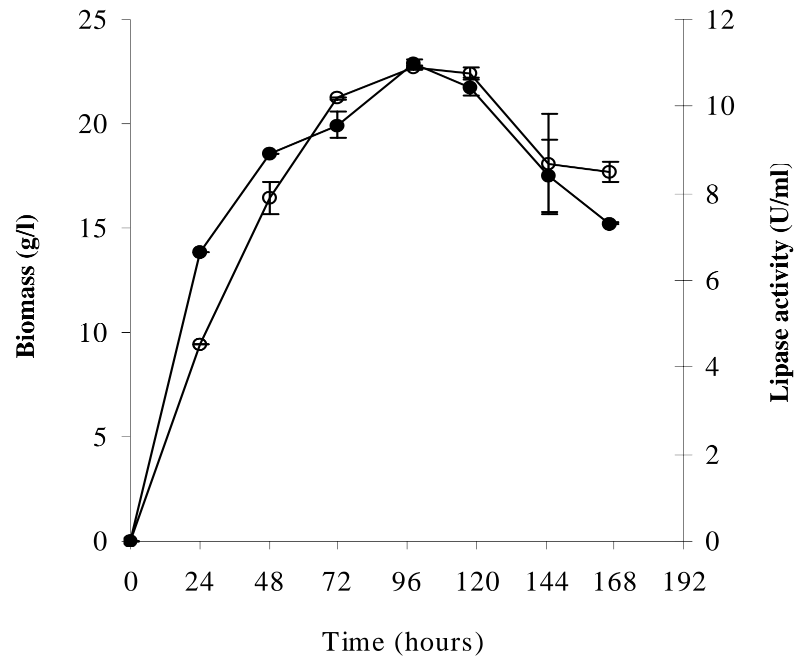
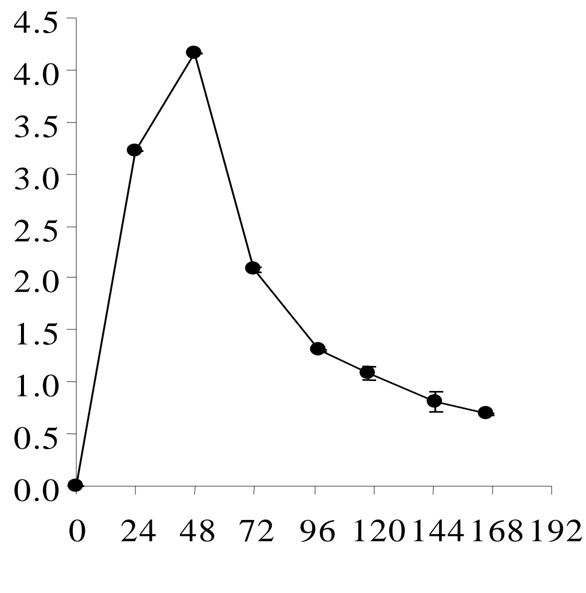


Figure 1 : Growth profile of *A.terreus* SUK-1 (ï) and lipase production (•)



Time (hours)

Figure 2 : Specific activity of lipase *A.terreus* SUK-1

higher 3.77 U/ml and 3.19 U/ml than the maximum lipase production of 7.2 U/ml by *A.carneus* (Schmidt *et al*., 1994) and of 7.78 U/ml by *A.terreus* RKS101 (Saxena *et al*., 2003), respectively.

Selection of suitable PEG6000-phosphate system for lipase separation using RSM

Response Surface Methodology (RSM) was used to obtain suitable phase compositions and pH system where the separation selectivity is achieved. List of parameters in coded and uncoded values is given in Table 1. In this experiment, face-centered design was applied and data from the results of conducted experiments are shown in Table 2.

Presented in Table 2, the highest yield of lipase obtained was 87.49% when the concentration of PEG6000 and pH system were at minimum while for the other 2 variables, concentration of phosphate and NaCl were located at maximum level. When the value of pH was kept closed to pH 8, which was at maximum level, the yield value of lipase observed were in range of 53-73% that shown the system could offer a better recovery for the enzyme within pH6 to pH8.

The quadratic model was also fitted to describe the relation of the response of results with the variables data. The regression coefficient obtained for quadratic model with statistical test results are shown in Table 3. The significant effect of *F*-test and *P*-value at 99% level of confidence with R^2 > 88% explained the quadratic model was adequate to represent the relation between response and variables. Additionally, the model was also sufficient to describe the optimum surface based on the non-significant effect obtained for 'lack of fit' test. Overlay contour plot of each response was then plotted to relocate a region of PEG6000/ phosphate composition in which lipase extraction with yield value more than 50% could be obtained simultaneously. Within this optimum region, composition of PEG6000/ phosphate system that is predicted could give the best lipase selectivity and lipase recovery could be determined using desirability function approach by Design Expert software package. The predictive optimum system and value of responses are given in Table 4. Location of selected system in optimum region is shown as indicated in Figure 3 by point (a).

Application of optimum PEG6000-phosphate system

The optimum system consists of 7.57% w/w PEG6000/ 12.01% w/w phosphate and 3.0% w/w added NaCl with pH 7.37 was then applied in extraction of lipase from *A.terreus* SUK-1 fermentation. The extraction experiments were conducted at 25°C and the partitioning results are given in

Table 1 : Actual and coded value for variables

Table 2 : Experimental design (in coded value) and results

Standard order	x_1	x_2	x_3	x_4	k_{lipase}	$k_{protein}$	k_{SA}	p.f	Yield value in bottom phase (%)
2^4 full factorial design with 4 center points									
1	-1	-1	-1	-1	0.87	1.37	0.63	1.09	65.39
2	1	-1	-1	-1	1.11	2.74	0.4	1.11	39.74
3	-1	1	-1	-1	0.97	2.1	0.46	1.35	71.76
4	1	1	-1	-1	1.99	4.85	0.41	2	34.4
5	-1	-1	1	-1	0.59	2.1	0.28	1.84	71.37
6	1	-1	1	-1	1.02	2.2	0.46	1.01	42.95
7	-1	1	1	-1	0.51	2.3	0.22	1.49	87.49
8	1	1	1	-1	0.97	2.94	0.33	1.31	49.66
9	-1	-1	-1	1	0.66	2.14	0.31	1.78	61.14
10	1	-1	-1	1	0.99	1.55	0.64	1.23	73.5
11	-1	1	-1	1	0.74	1.62	0.46	1.53	67.84
12	1	1	-1	1	1.11	2.38	0.47	1.07	50.63
13	-1	-1	1	1	1.03	3.86	0.27	2.29	62.98
14	1	-1	1	1	0.86	2.54	0.34	1.98	63.2
15	-1	1	1	1	1.13	2.86	0.39	1.75	59.36
16	1	1	1	1	1.09	2.05	0.29	1.23	53.34
17	0	0	0	0	1.64	1.53	1.07	0.76	45.46
18	0	0	0	0	1.47	1.54	0.95	0.92	45.99
19	0	0	0	0	1.46	1.76	0.83	1	50.6
20	0	0	0	0	1.31	2.1	0.62	1.22	55.75
Augmented 8 axial points with 2 center points									
21	-1	0	0	0	0.65	1.2	0.54	0.85	57.49
22	1	0	0	0	0.85	2.02	0.42	1.62	76.44
23	0	-1	0	0	1.03	1.98	0.52	1.11	60.38
24	0	1	0	0	0.86	2.2	0.39	1.33	60.26
25	0	0	-1	0	0.99	2.93	0.34	1.44	60.25
26	0	0	1	0	1.12	3.86	0.29	1.53	57.36
27	0	0	0	-1	1.6	2.35	0.68	0.95	50.1
28	0	0	0	1	1.75	2.99	0.58	1.02	41.69
29	0	0	0	0	1.14	2.2	0.52	1.29	56.9
30	0	0	0	0	1.41	2.04	0.69	0.87	53.89

Table 5. The average results obtained from duplicate experiments using the optimum parameter conditions indicated lipase *A.terreus* SUK-1 had similar partitioning behaviour with industrial lipase in which both enzymes had high affinity to partition into the bottom phase. This phenomenon could probably due to both lipases have the same value of isoelectric point as discussed by Menge and co-workers (Menge, 1992). They have reported lipases from two different sources could have identical partitioning behaviour in PEG-salt system due to the small difference of isoelectric point of both lipases. In addition, both industrial and prepared broth lipase are positive charged

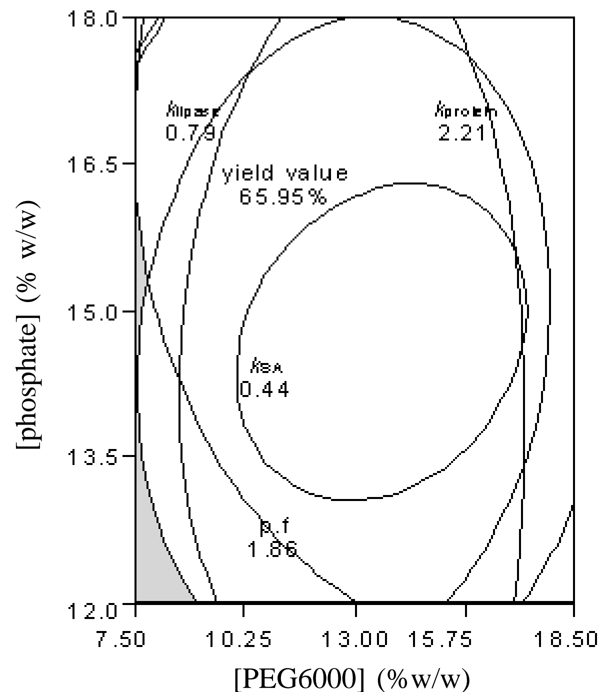


Figure 3 Overlay plot of response surfaces of k_{lipase}, $k_{protein}$, k_{SA}, yield value and purification factor

protein as in theory, protein with higher isoelectric point will have strong affinity to hydrophobic salt phase. Although, low purification factor of lipase is obtained from the experiment, yet it has potential to be improved in future by applying multi stages extraction or/and followed by another extraction method such as ultra filtration (Srinivas *et al*., 2001).

Lipase partitioning in 1kg optimum system

The partitioning of lipase *A.terreus* SUK-1 using the optimum parameter had also been attempted in 1000g system in order to study the effects of volume on lipase partition behaviour. Table 6 shows the comparison of the results between 10g and 1000g extraction system. Lipase partitioning behaviour in 10g optimum system was found to be the same as in 1000g optimum system as the yield value of lipase that achieved in both systems is in the range of 77-79%. However, the purification factor of lipase found in 1000g system is lower compared to 10g system. This is probably due to the entrapment of particles occurred at the interface of 1000g optimum system that would hinder the transfer of solutes between the two phases. As for 10g system, this problem was not obvious since a complete two phase is achieved after a short time centrifugation and thus minimised the probability of solutes entrapped at the interface.

Nevertheless, good recovery of lipase achieved in 1000g optimum system has proven that aqueous two-phase is capable to be scaled up and also the partitioned of lipase and protein contaminants does not change with different scale of extraction operation.

Table 3 : The regression coefficient of full quadratic model for responses in coded value with statistical test result

Regression coefficient	Responses				
	k_{lipase}	k_{protein}	k_{SA}	p.f	Yield value in bottom phase (%)
β_0	1.43	1.82	0.81	1	50.61
$\beta 1$	0.099	0.12	0.017	-0.16	-9.37
β_2	0.05	0.13	-0.017	-0.045	-0.86
β_3	-0.4	0.065	-0.057	0.091	0.7
β_4	3.89E-03	-0.12	0.012	0.089	2.01
β_1^2	-0.49	-0.64	-0.11	0.14	11.88
β_2^2	-0.29	-0.16	-0.14	0.12	5.24
β_3^2	-0.19	1.14	-0.27	0.39	3.72
β_4^2	0.44	0.42	0.04	-0.11	-8.89
β_{12}	0.1	0.15	0.051	-0.2	-3.21
β_{13}	-0.039	-0.35	0.021	-0.093	-0.4
β_{14}	-0.064	-0.43	0.027	-0.093	6.39
β_{23}	-0.021	-0.23	0.012	-0.13	1.4
β_{24}	0.02	-0.31	0.033	-0.17	-4.36
β_{34}	0.11	0.32	-6.88E-03	0.097	-4.02
***F*-value**	12.92*	6.87*	8.49*	5.98*	47.22*
***P*-value**	<0.0001	0.0014	0.0005	0.0026	<0.0001
Lack of fit test	0.8930°	0.2077°	0.8019°	0.8664°	0.5945°
$\mathbf{R^2}$	0.9427	0.8973	0.9153	0.8838	0.9836

** Significant, º Not significant*

Conclusion

Improved partition of lipase can be achieved by using PEG6000-phosphate system compared to PEG4000-phosphate system. By using Response Surface Methodology, the optimum PEG6000-phosphate system can be located at composition of 7.57% w/w PEG6000/ 12.01% w/w phosphate with addition of 3.0% w/w NaCl and pH system of 7.37. Lipases showed high affinity to bottom phase at the optimum condition while the other proteins were mainly partitioned into top phase. The lipase extraction of *A. terreus* by the optimum system gave 77% lipase recovery with 1.25 purification fold from the industrial broth in bottom phase. The scale-up of aqueous two-phase system could be possible to be carried out in aspect of volume to be done in a proportional manner. Moreover, by manipulating the system parameters, more lipase can be recovered and higher degree of purification can be achieved.

Table 4 : Comparison of lipase partitioning in 10g and 1000g PEG6000/phosphate system

Responses	Experimental value (10 g)	Experimental value (1000 g)
k_{lipase}	0.94	0.87
k_{protein}	1.84	3.07
k_{SA}	0.51	0.28
Yield value (%)	77.14	79.14
p.f	1.25	0.98

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