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Xylanase Recovery

Effect of Extraction Conditions on the Aqueous Two-Phase System Using Experimental Design

SILGIA A. COSTA, ADALBERTO PESSOA JR,* AND INÊS C. ROBERTO

Department of Biotechnology/FAENQUIL, Rod. Itajubá-Lorena, Km 74,5. 12.600-000, Lorena/SP, Brazil

ABSTRACT

The partitioning of xylanase produced by *Penicillium janthinellum* in aqueous two-phase systems (ATPS) using poly(ethylene glycol) (PEG) and phosphate (K_2HPO_4/KH_2PO_4) was studied employing a statistical experimental design. The aim was to identify the key factors governing xylanase partitioning. The interactions of five factors (PEG concentration molecular weight, concentration of buffer K_2HPO_4/KH_2PO_4, pH, and NaCl concentration) and their main effects on the partition coefficient (K) were evaluated by means of a 2^5 full-factorial experimental design with four center points. The %PEG, %NaCl, and pH were the most important factors affecting the response variable (K). Response surface methodology (RSM) was adopted and an empirical second-order polynomial model was constructed on the basis of the results. The optimum partition conditions were pH 7.0, PEG = 8.83% and NaCl = 6.02%. Adequacy of the model for predicting optimum response value was tested under these conditions. The experimental xylanase partition coefficient (K) was 2.21, whereas its value predicted by the model was 2.33. These results indicate that the predicted model was adequate for the process. PEG molecular weight and phosphate concentration did not affect the xylanase partition coefficient.

Index Entries: Aqueous two-phase system; xylanase; *Penicillium janthinellum*; experimental design.

INTRODUCTION

Enzymes involved in lignocellulosic degradation *in situ* are of interest for their potential application to processes utilizing lignocellulosic sub-

* Author to whom all correspondence and reprint requests should be addressed.

strates. Xylanases and xylanase-producing microorganisms can potentially be applied to the production of hydrolysates from agro-industrial wastes *(1)*, to nutritional improvement of lignocellulosic feeds *(2)*, and to the processing of food *(3)*, agrofiber *(4)*, and pulp *(5)*. Efforts have been made by the pulp and paper industries to reduce the amount of chlorine needed for bleaching. Studies have been conducted on the effluent treatment and on the effectiveness of less toxic bleaching agents *(6)*. The residual lignin removal by enzymatic method is actually a very interesting research area *(7,8)*. According to Durán et al. *(9)*, xylanases produced by *Penicillium janthinellum* can be used for reducing the chlorine charge in *Eucalyptus*-pulp bleaching with a simultaneous gain in brightness. The xylanase production has been investigated *(10)*, but studies on downstream processes are still needed.

Extractive bioconversion using aqueous two-phase systems (ATPS) seems to be a very attractive method for the integration of fermentation and downstream processing of extracellular proteins *(11)*. In addition to forming a relatively mild environment suitable for cell and protein extraction, ATPS can be easily scaled up and continuously processed *(12–14)*.

The aim of this work was to identify the key factors governing xylanase partitioning using ATPS. Differences in enzyme partitioning can be ascribed to the interaction of the factors inherent in the system itself (such as choice of system components, polymer molecular weight, concentration of polymers and salts, ionic strength, and pH) with those of the target protein (such as hydrophobicity, charge, and molecular weight) *(12)*. The effects of variables like PEG molecular weight, PEG concentration, pH, and phosphate and NaCl concentration on partition coefficient (K) of xylanase were studied. Full factorial experimental designs were employed since they require a reduced number of experiments and help to identify important and interacting factors determining the enzyme partition *(15)*. Response surface methodology was used for optimizing xylanase partition coefficient (K) by ATPS and a statistical model correlating the variables was obtained.

MATERIALS AND METHODS

Microorganism and Cultivation

The microorganism *P. janthinellum*, isolated from decaying wood by Milagres *(16)*, was identified by the Biosystematic Research Center of Canada (Ottawa, Ontario, Canada) and deposited in their collection under the designation of CRC 87M-115. The strain was initially maintained in silica stocks and later on agar slants. The spore inocula were obtained after cultivation at 30°C for 5 d in medium containing 1% glucose, 0.1% yeast extract, 2% (v/v) concentrated salts solution based on Vogel's medium *(17)*, and 2% agar-agar. The final concentration of spores was 10^5 mL^{-1}. The

cultivation medium for enzyme production was composed of sugarcane bagasse hemicellulosic hydrolysate (800 g of dry milled bagasse mixed with 8 L 0.25% H_2SO_4 and autoclaved for 45 min at 121°C), supplemented with 2% (v/v) concentrated salts solution based on Vogel's medium and 0.1% yeast extract. The medium was then autoclaved for 15 min at 121°C. Shake-flask cultures were grown in Erlenmeyer flasks (125 mL) containing 25 mL of medium. Standard cultivation conditions were: temperature 30°C; initial pH 5.5 (uncontrolled); cultivation time 96 h. The xylanase activity after this time was 876 nanokatals/mL^{-1}.

Determination of Enzyme Activities

Xylanase activities were determined by incubating 0.5 mL of diluted culture filtrate with 0.5 mL of a "Birchwood" xylan suspension (10 g/L^{-1}) in 0.05 *M* phosphate buffer (pH 5.5) for 5 min at 50°C. The released reducing equivalents were measured by a colorimetric assay *(18)* using xylose solution as a standard reference. Activity units were expressed as micromoles of reducing equivalents released per min PEG concentration level influenced this activity. The three concentration levels tested—10, 22.5, and 35%—enhanced the enzyme activity by 2.6, 10, and 18%, respectively.

Preparation of Phase Systems

Phase systems were prepared from PEG, phosphate (KH_2PO_4/K_2HPO_4), and NaCl in solid form. Three milliliters of medium containing xylanase was added to the systems and deionized water was used to adjust the desired final concentrations of the components. By varying the proportion between KH_2PO_4 and K_2HPO_4, the pH of the system was adjusted. Centrifugation (2500*g* for 10 min) was used after thorough vortex-mixing of the system components; the phase volumes were measured using graduated centrifuge tubes. Samples of the top and bottom phases were then assayed for enzyme activity. During all partition experiments, the temperature was ~25°C.

Experimental Designs and Statistical Analysis

To quantify the partition coefficient (K), the fraction of enzymes present in the lower and upper phases after phase separation was used. This fraction (y), which is the response factor, was measured as a function of pH (X_1), PEG MW (X_2), PEG concentration (X_3), phosphate concentration (X_4), and NaCl concentration (X_5). For each of the five factors, high (coded value: +1) and low (coded value: −1) set points were selected (Table 1). ATPSs representing all 32 (2^5), set-point combinations were made, as well as an ATPS representing the center point in which the value of all factors was in between (coded value: 0). All factors were measured twice, whereas the center point was measured times.

Table 1
Factors and Levels in the Five-Factor, Three-Level Response Surface Design Used for Optimizing the Xylanase Partition Coefficient by ATPS

Run number	Factors	Inferior level (−1)	Superior level (+)	Center point (0)
X_1	pH	5.0	8.0	6.5
X_2	MW PEG	600	6000	4000
X_3	% PEG	10	35	22.5
X_4	%Phosphate	10	25	17.5
X_5	%NaCl	0	10	5

After statistical analysis (Tables 2 and 3) the optimization of xylanase partition coefficient (K) was achieved by three independent process variables using a 2^3-factorial experimental design with six star points (α = 1.41) and four replicates at the center point (Table 4), according to the method of Box et al. *(15)*. These independent variables (pH, %PEG, and %NaCl) acquired new values and are coded as X_1, X_3, and X_5 in the following equations:

$$X_1 = (\text{pH} - 7.4)/0.5 \quad (1)$$

$$X_3 = (\%\text{PEG} - 13.0)/5 \quad (2)$$

$$X_5 = (\%\text{NaCl} - 5.6)/0.3 \quad (3)$$

where pH, %PEG and %NaCl are true values and X_1, X_3, and X_5 are coded values.

A statistical examination of the results and a response surface study were carried out using the STATGRAPH 6.0 statistical program package. The polynomial model employed was of the form:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{1,2} X_1 X_2 + \beta_{1,3} X_1 X_3 + \beta_{2,3} X_2 X_3 + \beta_{1,1} X_1^2 + \beta_{2,2} X_2^2 + \beta_{3,3} X_3^2 \quad (4)$$

where:
β_0 = constant, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$ = cross product coefficients, β_1 β_2 β_3 = linear coefficients, X_1, X_2, X_3, = coded independent variables, and $\beta_{1,1}$, $\beta_{2,2}$, $\beta_{3,3}$ = quadratic coefficients.

Chemicals

Birchwood 4-*O*-methyl-β-D-glucoroxylan (90% xylose) was obtained from Sigma (St. Louis, MO), and poly(ethylene glycol) (PEG) from Merck (Darmstadt, Germany). All the other chemicals were of analytical grade.

Table 2
Xylanase Partition Coefficient (K) from 2^5-Full Factorial Design with Center Point under Different Treatments

Assay number	Assay sequence	Factors					K[a]
		X_1	X_2	X_3	X_4	X_5	
1	25	−1	−1	−1	−1	−1	0
2	36	+1	−1	−1	−1	−1	0
3	20	−1	+1	−1	−1	−1	0
4	28	+1	+1	−1	−1	−1	0.60
5	22	−1	−1	+1	−1	−1	0
6	4	+1	−1	+1	−1	−1	1.72
7	34	−1	+1	+1	−1	−1	0
8	23	+1	+1	+1	−1	−1	0.81
9	8	0	0	0	0	0	1.69
10	14	−1	−1	−1	+1	−1	0
11	26	+1	−1	−1	+1	−1	2.03
12	6	−1	+1	−1	+1	−1	1.16
13	3	+1	+1	−1	+1	−1	1.72
14	12	−1	−1	+1	+1	−1	0
15	19	+1	−1	+1	+1	−1	0.57
16	7	−1	+1	+1	+1	−1	0
17	35	+1	+1	+1	+1	−1	0
18	16	0	0	0	0	0	1.33
19	9	−1	−1	−1	−1	+1	0
20	30	+1	−1	−1	−1	+1	2.34
21	17	−1	+1	−1	−1	+1	1.42
22	15	+1	+1	−1	−1	+1	1.94
23	5	−1	−1	+1	−1	+1	0
24	21	+1	−1	+1	−1	+1	0
25	29	−1	+1	+1	−1	+1	0
26	1	+1	+1	+1	−1	+1	0
27	24	0	0	0	0	0	1.57
28	2	−1	−1	−1	+1	+1	0
29	33	+1	−1	−1	+1	+1	1.36
30	31	−1	+1	−1	+1	+1	2.11
31	11	+1	+1	−1	+1	+1	1.33
32	18	−1	−1	+1	+1	+1	0
33	13	+1	−1	+1	+1	+1	0
34	27	−1	+1	+1	+1	+1	0
35	10	+1	+1	+1	+1	+1	0
36	32	0	0	0	0	0	1.40

[a] $K = (K_1 + K_2)/2$ average partition coefficient.

Table 3
Estimated Effect, Standard Error and Student's t Test of 2^5 Factorial Design with Four Center Points

Variables	Estimated effects	Standard error	t Values
Average	0.713	±0.104	—
X_1	0.608	±0.224	2.71[a]
X_2	0.191	±0.224	0.86
X_3	−0.806	±0.224	3.36[a]
X_4	0.090	±0.224	0.41
X_5	0.118	±0.224	0.53
X_1X_2	−0.394	±0.224	1.76
X_1X_3	−0.221	±0.224	0.98
X_1X_4	−0.141	±0.224	0.63
X_1X_5	−0.187	±0.224	0.80
X_2X_3	−0.376	±0.224	1.68
X_2X_4	0.103	±0.224	0.46
X_2X_5	−0.195	±0.224	0.87
X_3X_4	−0.335	±0.224	1.50
X_3X_5	−0.505	±0.224	2.25[a]
X_4X_5	−0.203	±0.224	0.90

[a] Significant at the 5% level (t = 2.08012).

Table 4
Experimental Data for Xylanase Partition Coefficient (K) Under Different Treatments

Treatment	pH	%PEG	%NaCl	K
1	−1	−1	−1	2.10
2	+1	−1	−1	2.07
3	−1	+1	−1	1.75
4	+1	+1	−1	1.62
5	−1	−1	+1	2.32
6	+1	−1	+1	2.13
7	−1	+1	+1	2.14
8	+1	+1	+1	1.85
9	−1.41	0	0	2.10
10	1.41	0	0	2.27
11	0	−1.41	0	2.36
12	0	1.41	0	1.72
13	0	0	−1.41	2.27
14	0	0	1.41	2.30
15	0	0	0	2.27
16	0	0	0	2.08
17	0	0	0	2.07
18	0	0	0	2.11

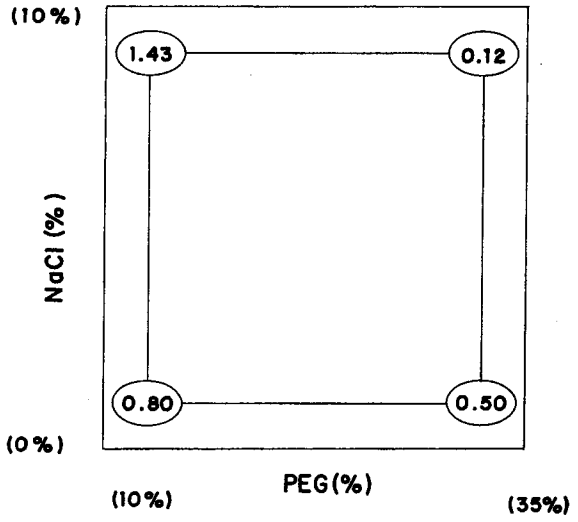

Fig. 1. Interpretation diagram of the interaction effects between %PEG and %NaCl in the 2^5 factorial design.

RESULTS AND DISCUSSION

The partition coefficients obtained after xylanase extraction by ATPS, according to the factorial design, are presented in Table 2. The factor levels were defined based on phase diagrams (MW PEG, % PEG, % phosphate and % NaCl factors) *(19)* and on the enzyme characteristics (pH factor) *(5)*. The individual effects of the experimental factors and their interactions on partition coefficient (K) are shown in Table 3. As can be seen the pH (X_1), %PEG (X_3) and the interactions between %PEG (X_3) and %NaCl (X_5) presented a significative influence on partition coefficient at the 5% level. The estimated effect for the pH (X_1) was positive indicating that from level -1 to $+1$ the K value augments as a function of pH. The effect of this variable is independent of the other factors since there was no significant interactions among them. On the other hand, the main effect of the %PEG (X_3) cannot be interpreted separately because its interaction with %NaCl (X_5) was significant at the 5% level. As can be seen in Fig. 1, an increase in the NaCl concentration from 0 to 10% provides an increase of 0.63 U in the K value. In this variation range, at 35% NaCl, a reduction of 0.38 U in the partition coefficient is observed. On the other hand, after increasing PEG concentration from 10 to 35%, without NaCl addition the K value decreases by 0.30 U. This variation (10 to 35%) causes a decrease of 1.31 Units in the K value. As a function of these results, further optimizing experiments were performed using a 2^3 orthogonal factorial design according to the Materials and Methods section (Table 4). The regression coefficients, t values and determination coefficients (R^2) for the full quadratic

Table 5
Regression Coefficients, t Values and R^2 of Quadractic Response Surface for K Values

Term of the model	Regression coefficients	Standard error	t Values
Interception	2.175	±0.044	49.489
Factor X_1	−0.066	±0.053	1.237
Factor X_3	−0.361	±0.053	6.704[a]
Factor X_5	0.157	±0.053	2.918[b]
X_1X_3	−0.05	±0.066	0.758
X_1X_5	−0.08	±0.066	1.214
X_3X_5	0.085	±0.066	1.289
X_1X_1	−0.075	±0.066	1.138
X_3X_3	−0.220	±0.066	3.337[a]
X_5X_5	0.025	±0.066	0.379

Standard error estimated from pure error with 3 df. (t = 3.18245).
[a] Significant at the 5% level.
[b] Significant at the 10% level.

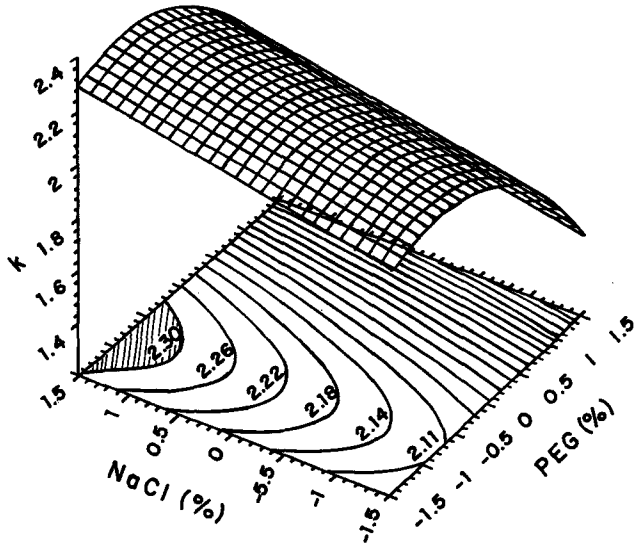

Fig. 2. Response surface of xylanase partition coefficient (K) as a function of %PEG, %NaCl, and pH.

response-surface models of K are shown in Table 5 and analysis of variance (Table 6). At the 5% probability, the pH value demonstrated no significant influence on the partition coefficient (K) as a main effect and as a second-order interaction effect, considering the new pH range (between 6.9 and 7.9). On the other hand, NaCl (%) presented a significant influence as a main effect, whereas PEG (%) also presented a second-order effect. Figure 2 illustrates the three-dimensional response surfaces and contour plots

Table 6
Analysis of Variance (ANOVA) for the Quadractic Model

Source of variations	Sum of squares	Degrees of freedom	Mean square	F Value	*P* value[a]
FactorX_3	0.3906	1	0.3906	31.06	0.0003
Factor X_5	0.0740	1	0.0740	5.88	0.0382
X_3^2	0.0968	1	0.0968	7.70	0.0216
Lack of fit	0.1286	5	0.0257	2.04	0.1656
Pure Error	0.1132	9	0.0126		
Total (Corr.)	0.8033	17			

$R^2 = 0.70$.
[a] $P < 0.05$.

Table 7
Regression Results: Observed Responses and Predicted Values

Observation number	Actual value (y_O)	Predicted value (y_P)	Residual ($y_O - y_P$)
1	2.10	2.15	−0.05
2	2.07	2.15	−0.08
3	1.75	1.79	−0.04
4	1.62	1.79	−0.17
5	2.32	2.31	0.01
6	2.13	2.31	−0.18
7	2.14	1.95	0.19
8	1.85	1.95	−0.10
9	2.10	2.16	−0.06
10	2.27	2.16	0.11
11	2.36	2.19	0.17
12	1.72	1.68	0.04
13	2.27	2.05	0.22
14	2.30	2.27	0.03
15	2.27	2.16	0.11
16	2.08	2.16	−0.08
17	2.07	2.16	−0.09
18	2.11	2.16	−0.05

showing the expected K values as a function of %NaCl and %PEG attained with equation 5.

$$y = 2.18 - 0.18X_3 + 0.08X_5 - 0.11X_3^2 \quad (5)$$

The mathematical model (Eq. 5) enables the maximal point calculation (K

= 2.33) for the coded values of −0.83 and +1.41 corresponding to 8.83% of PEG and 6.02% of NaCl, respectively.

Each of the actual K values (y_o) is compared with the values predicted from the model, y_p, in Table 7. The comparison of the residuals with the error variance S_e^2 (0.23) indicates that none of the individual residuals exceeds twice the square root of the residual variance. All of the above considerations indicate an excellent adequacy of the regression model *(20)*. After the optimum processing conditions were identified by the model derived by RSM, the xylanase partition was performed under the following conditions: PEG = 8.83%; pH 7.0 and NaCl = 6.02%. The experimental xylanase partition coefficient (K) was 2.21, whereas its value predicted by the model was 2.33. This experimental finding is in close agreement with the model prediction. Of the xylanase recovered, 80% remained in the top phase.

CONCLUSIONS

The partition behavior of *P. janthinellum* xylanase has been studied in aqueous two-phase systems. The response surface methodology (RSM) was useful in optimizing the xylanase partition coefficient and graphical analysis aided in locating optimum conditions. The %PEG, %NaCl, and pH were the most important factors affecting the response variable (K). The optimum partition conditions were selected at pH 7.0; PEG = 8.83% and NaCl = 6.02%. Adequacy of the model for predicting optimum response value was tested using these conditions. The experimental xylanase partition coefficient (K) was 2.21, whereas the value predicted by the model was 2.33. These results indicate that the model was adequate for the process. In the range of experimental conditions, MW PEG and phosphate concentration did not affect the xylanase partition coefficient. The results showed the feasibility of xylanase recovery by ATPS and the advantage of employing experimental design for determining optimal extraction conditions.

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REFERENCES

1. Eriksson, K. E. L., Blanchetter, R. A., Ander, P. (1990), in *Microbial and Enzymatic Degradation of Wood and Wood Components*, Springer-Verlag, New York.
2. Giovanozzi-Sermanni, G., Bertoni, G., Porri, A. (1989), in *Enzyme Systems for Lignocellulose Degradation*, (Coughlan, M. P. ed.) Elsevier New York, pp. 371–382.

3. Mutsaers, J. H. G. M. (1991), in *Xylans and Xylanases International Symposium*, Wageningen, The Netherlands, Novo Nordisk, p. 48.
4. Sharma, H. S. S. (1987), *Appl. Microbiol. Biotechnol.* **26,** 358–362.
5. Milagres, A. M. F. and Durán, N. (1992), *Prog. Biotechnol.* **7,** 539–545.
6. Parthasarathy, V. R. (1990), *Tappi J.* **73,** 243–247.
7. Senior, D. J., Hamilton, J., Bernier, R. L., and Dumanoir, J. R. (1992), *Tappi J.*, **11,** 125–130.
8. Allison, R. W., Clark, T. A., Wrathall, S. H. (1993), *APPITA* **46,** 269–273.
9. Durán, N., Milagres, A. M. F., Sposito, E., and Haun, M. (1995), in *ACS Symposium Series*. (Saddler, J. N. and Penner, N. H., eds.) American Chemical Society, San Diego, CA, pp. 332–338.
10. Palma, M. B., Milagres, A. M. F., Prata, A. M. R., Mancilha, I. M. (1996), *Proc. Biochem.* **31,** 141–145.
11. Zijlstra, G. M., Michielsen, M. J. F., Gooijer, C. D., Pol, L. A., and Tramper, J. (1996), *Biotechnol. Prog.* **12,** 363–370.
12. Schmidt, A. S., Ventom, A. M., and Asenjo, J. A. (1994), *Enzyme Microb. Technol.* **16,** 131–142.
13. Huddleston, J., Veide, A., Köhler, K., Flanagan, J., Enfors, S-O., and Lyddiatt, A. (1991), *TIBTEC* **9,** 381–388.
14. Kula, M-R., Kroner, K. H., and Hustedt, H. (1982), in *Advances in Biochemical Engineering*, Fiechter, A. ed., **24,** 73–118.
15. Box, G. E. P., Hunter, W. G., and Hunter, J. S. (1978), in *Statistics for Experiments*, John Wiley and Sons, New York.
16. Milagres, A. M. F. (1988), MSc. Thesis, Universidade Federal de Viçosa, Viçosa, Brazil.
17. Vogel, H. J. (1956), *Microbial Genet. Bull.* **13,** 42–43.
18. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
19. Albertsson, P. A. (1971), *Partitioning of Cell Particles and Macromolecules*, 2nd ed., Wiley Interscience, New York.
20. Sen, R. (1997), *J. Chem. Tech. Biotechnol.* **68,** 263–270.