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Intensification of *endo*-1,4-Xylanase Extraction by Coupling Microextractors and Aqueous Two-Phase System

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Abstract: The extraction of xylanase was performed using an aqueous two-phase system (ATPS) based on polyethylene glycol (PEG1540) and various salts. Preliminary studies in a batch extractor showed that the highest extraction efficiency, E = 79.63 ± 5.21%, and purification factor, PF = 1.26 ± 0.25, were obtained with sodium citrate dihydrate-H_2O-PEG1540-based ATPS for an extraction time of 10 min. The process was optimized using the experimental Box-Behnken design at three levels with three factors: extraction time (t), xylanase concentration (γ), and mass fraction of PEG in the ATPS (w_{PEG}). Under optimal process conditions (γ = 0.3 mg/mL, w_{PEG} = 0.21 w/w, and t = 15 min), E = 99.13 ± 1.20% and PF = 6.49 ± 0.05 were achieved. In order to intensify the process, the extraction was performed continuously in microextractors at optimal process conditions. The influence of residence time, different feeding strategies, and channel diameter on extraction efficiency and purification factor was further examined. Similar results were obtained in the microextractor for a residence time of τ = 1.03 min (E = 99.59 ± 1.22% and PF = 6.61 ± 0.07) as in the experiment carried out under optimal conditions in the batch extractor. In addition, a batch extractor and a continuous microextractor were used for the extraction of raw xylanase produced by *Thermomyces lanuginosus* on solid supports.

Keywords: xylanase; extraction; aqueous two-phase system; optimization; batch extraction; continuous microextractor

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1. Introduction

endo-1,4-xylanase, E.C.3.2.1.8. or xylanase for short, belongs to a group of hydrolases characterized by the cleavage of chemical bonds such as C-O, C-N, and C-C. It is an extracellular enzyme produced by microorganisms such as bacteria, fungi, and yeasts [1]. Xylanases are used in many industries, such as the food industry, where it is used to improve the quality of dough, in the extraction of coffee, and the production of beer and juices, but especially in the pulp and paper industry, where it is used as a bleaching agent [2]. The use of xylanase in the pulp and paper industry reduces the need to use chlorine, which significantly reduces the negative impact of the process on the environment [3]. In the production of animal feed, it is used in the pretreatment of plants to improve the digestibility of food, which leads to better energy utilization, and the unused residues of such food are easier to compost [4].

Xylanases can be produced by fermentation in liquid media or by fermentation on solid supports. The choice of method will depend on the type of microorganisms used in the production process. During the cultivation of microorganisms, either by fermentation in a liquid medium or by fermentation on solid supports, various enzymes are produced simultaneously. For this reason, it is necessary to purify a raw enzyme mixture. The most commonly used methods for enzyme purification are salt precipitation (isolation), followed by dialysis and chromatography (exclusion, ion exchange, affinity chromatography). Alternatively, if the volume of the media containing the desirable enzyme is large, the application of these methods will be expensive [5]. In addition, conventional multi-step purification techniques are time-consuming, which increases the overall cost of the process and results in protein losses at each step. Due to that, extraction has attracted much attention in the industry due to its high selectivity and efficiency, the possibility of continuous operation, the ease of scaling, the elimination of unnecessary sample pretreatment, and its relatively low cost [6,7].

Aqueous two-phase systems (ATPS) are increasingly used for the extraction of proteins [8–14], enzymes [15], viruses [16], antibodies [17], polysaccharides [18], and nucleic acids [19] because their use does not disturb the structure of these components or their activities, and the process is effective and inexpensive. In addition, the advantages of this method compared with other methods of enzyme purification are the short duration of the process, the low price of the extraction agent, the low energy consumption, the high utilization, the high capacity of the extraction process, and the relatively simple scale-up of the process [20,21]. ATPS are formed by mixing two different polymers or a polymer and an inorganic salt at specific concentrations. In a system consisting of polymer and salt, both components must be present at concentrations above the critical value, because then spontaneous phase separation occurs, resulting in two phases in equilibrium, where the concentration of the salt is higher in the bottom phase and the upper phase is rich in the polymer. Both phases usually contain water (in a proportion of 65–90%), and phase separation in ATPS is influenced by several factors, such as the concentration and molecular weight of the polymer, the concentration and composition of salts, pH, and temperature. Due to their high water content, they are considered safe and environmentally friendly. Moreover, this type of extraction is carried out under mild conditions, which allows the biomolecules to maintain their original conformation and structure. The systems are extremely flexible, i.e., changing the concentration of individual components can significantly affect the change in ATPS properties [22–24].

Up to now, xylanase has been successfully purified by applying ATPS [5,25–28]. According to Taddia et al. [27], one of the current challenges in biotechnology is the development of new protocols that would enable the purification of macromolecules in fewer steps. One of the possible directions is the application of continuous systems, and more specifically, microcontinuous systems. Microextractors have been successfully applied in several extraction processes [29,30], including those based on ATPS [30–34]. Enhanced mass and energy transfer, easier process control, lower feed consumption, and negligible diffusion limitations are just some of the microextractor properties that could be used for the intensification of extraction.

The aim of this work was to investigate the use of microextractors in the purification of xylanase. As a first step, ATPS systems based on polyethylene glycol (PEG1540) and various salts (sodium sulphate, sodium citrate dihydrate, sodium formate, sodium potassium tartrate tetrahydrate, and ammonium sulphate) were tested in a batch extractor with the aim of selecting the best system in which the highest extraction efficiency and purification factor of xylanase will be achieved. After the best system was selected, the additional optimization of the xylanase extraction was carried out using a three-level experimental Box-Behnken design with three factors analyzing the influence of extraction time (t), xylanase concentration (γ), and mass fraction of polymer in the ATPS (w_{PEG}) on the extraction efficiency and purification factor. The process was then intensified in a microextractor at optimal process conditions, where the influence of residence time, different feeding

strategies, and channel diameter on the extraction efficiency and purification factor were investigated. As a final step, a batch extractor and a continuous microextractor were used for the extraction of the raw xylanase produced by the solid-state fermentation of *Thermomyces lanuginosus*.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

3,5-dinitrosalicylic acid and Commassie Brilliant Blue G-250 were purchased from Fluka (Buchs, Switzerland). Ammonium sulphate and potassium-sodium-tartrate-tetrahydrate were purchased from Kemika (Zagreb, Croatia). Arabinoxylan was purchased from Megayzme (Wicklow, Ireland). Copper(II) sulphate and sodium hydroxide were purchased from Gram Mol (Zagreb, Croatia). D(+)-xylose and citric acid were purchased from VWR Chemicals (Radnor, PA, USA). Commercial xylanase from *Trichoderma longibrachiatum* (V.A. = 1000 U/g) was purchased from Biocat (Troy, VA, USA). Folin-Ciocalteu reagent, bovine serum albumin, and sodium formate were purchased from Sigma-Aldrich (Wien, Austria). Sodium citrate dihydrate was purchased from T.T.T. Doo (Zagreb, Croatia). Sodium carbonate and orthophosphoric acid were purchased from Merk (Darmstadt, Germany). Polyethilenglicol (MW 1540) was purchased from Acros Organics (Waltham, MA, USA). The chemicals were of analytical grade and were used without further purification.

2.1.2. Raw Enzyme

The crude enzyme was produced at Josip Juraj Strossmayer University of Osijek, Faculty of Food Technology Osijek, by the cultivation of *Thermomyces lanuginosus* on barley husk in solid-state fermentation conditions (m_s = 30 g, $n_{\text{mycelial discs}}$ = 5, $\phi_{\text{mycelial discs}}$ = 1 cm, w_{H2O} = 70%, t = 8 days, T = 45 °C). 80 g of the fermented sample was extracted in 650 mL of the phosphate buffer (pH 6.0, 0.1 mol/L) during 60 min at 450 rpm to obtain a xylanase-rich extract (V.A.$_{\text{xylanase}}$ = 32.51 ± 1.22 U/mL, S.A. = 0.12 ± 0.01 U/mg).

2.2. Methods

2.2.1. Analytical Methods

The xylanase activity was determined according to the DNS method [5]. Protein concentration was determined according to the Lowry method [35], using BSA as a standard. To overcome the possible interference by the phase-forming components in the analyses, systems of identical composition without enzymes were used as blanks [5].

2.2.2. Batch Extraction of Xylanase

Sodium sulphate, sodium citrate dihydrate, sodium formate, potassium sodium tartrate tetrahydrate, and ammonium sulphate (γ = 0.5 g/mL) were used in ATPS preparation. The concentration of PEG1540 was set at 0.5 g/mL. ATPS was prepared by mixing selected salt, PEG1540, and enzyme (γ = 0.1 mg/mL) dissolved in 0.05 mol/L Na-citrate buffer pH 5.3 in the amounts calculated from the phase diagrams [36–38] and presented in Table 1. The final weight of the ATPS was adjusted to 5 g by the addition of 0.05 mol/L Na-citrate buffer at pH 5.3. The phase components were added in the following order: water, PEG, salt, and enzyme solution.

Table 1. ATPS component volumes.

Salt-H_2O-PEG1540 Salt	$V_{\text{PEG solution}}$, mL	$pH_{\text{PEG solution}}$, -	$V_{\text{salt solution}}$, mL	$pH_{\text{salt solution}}$, -	V_{buffer}, mL	$V_{\text{xylanase solution}}$, mL
sodium sulphate	1.965	7.36	1.598	6.56	0.938	0.500
sodium citrate dihydrate	2.080	7.36	2.079	8.99	0.341	0.500
sodium formate	2.730	7.36	3.053	8.84	0	0.500
potassium sodium tartrate tetrahydrate	2.319	7.36	2.350	8.33	0	0.500
ammonium sulphate	2.040	7.36	1.694	5.01	0.766	0.500

The obtained systems were homogenized on a shaker (ThermoMixer C, Eppendorf, Hamburg, Germany) for 10 min at 400 rpm and a temperature of 25 °C (Figure 1A). After homogenization, the mixture was centrifuged (Universal 320 R, Hettich, Buford, GA, USA) for 10 min at 4000 rpm and 25 °C. The volume of the obtained layers was determined in graduated glass tubes, and the layers were separated from each other using a needle to avoid phase mixing. The bottom phase, the raffinate phase, was rich in salt while the upper phase, the extract phase, was rich in polymer. Before determining the protein concentration in the extract phase, the extract phase was treated with 1:6 (v/v) chloroform [39] to remove PEG1540 in order to negate its interference with the protein assay. After the addition of the chloroform, the samples were homogenized and centrifuged at 4000 rpm and 25 °C for 10 min, after which the protein concentration and enzyme activity were determined.

From the values obtained, the partition coefficient, K (Equation (1)), extraction efficiency, E (Equation (2)), and purification factor, PF (Equation (3)) were calculated.

$$K = \frac{\gamma_E}{\gamma_R} \tag{1}$$

$$E = \frac{\gamma_E}{\gamma_0} \cdot 100\% \tag{2}$$

$$PF = \frac{\text{S.A.}_{\text{xylanase,E}}}{\text{S.A.}_{\text{xylanase,0}}} \tag{3}$$

where E denotes extract phase, R raffinate phase, 0 initial enzyme solution, and S.A. is specific xylanase activity.

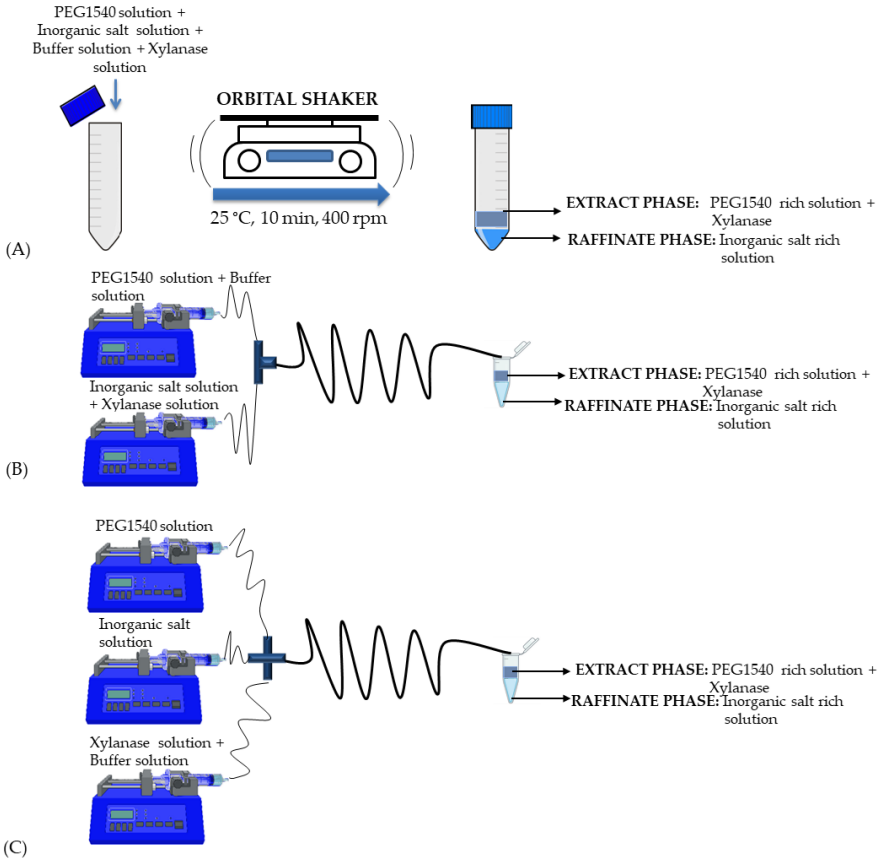

Figure 1. Schemes of the experimental set-ups used for xylanase extraction in a (**A**) batch, (**B**) microextractor with two inlets, and (**C**) microextractor with three inlets.

Optimization of Extraction

By applying the Box-Behnken experimental design at three levels (−1, 0, 1) with three factors using Statistica 13.0 (Tibco Software Inc., Palo Alto, CA, USA), an experimental plan with a total of 17 experiments (Table 2) was created to optimize the influence of extraction time (t), xylanase concentration (γ), and PEG mass fraction in an ATPS (w_{PEG}) on the xylanase extraction efficiency. Experimental data were fitted to the second-order polynomial equation (Equation (4)):

$$Z = \beta_0 + \sum_{i=1}^{3} \beta_i \cdot X_i + \sum_{i=1}^{3} \beta_{ii} \cdot X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} \cdot X_i \cdot X_j \quad (4)$$

where Z is the predicted response, β_0, β_i, $\beta_{ii,}$ and β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms, and X_i and X_j are the independent variables. The response surface analysis was performed using Statistica 13.0 (Tibco Software Inc., Palo Alto, CA, USA).

Table 2. The influence of ATPS system composition on xylanase purification.

Salt-H_2O-PEG1540 / **Salt**	***E*, %**	***K*, -**	***PF*, -**
ammonium sulphate	43.72 ± 10.23	0.78 ± 0.29	1.31 ± 0.19
sodium sulphate		No phase formation	
potassium sodium tartrate tetrahydrate	60.12 ± 8.71	1.01 ± 0.36	1.36 ± 0.09
sodium citrate dihydrate	79.63 ± 5.21	3.91 ± 0.26	1.26 ± 0.25
sodium formate		No phase formation	

2.2.3. Continuous Extraction in a Microextractor

The xylanase extraction was performed in a PTFE (polytetrafluoroethylene) microextractor device with different channel diameters (500 and 1000 µm, V = 273.15 µL) equipped with two "T"-shaped (Figure 1B) or three "ψ"-shaped inlets (Figure 1C). When a microextractor with two "T"-shaped inlets was used, two syringe pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA) equipped with high-pressure stainless-steel syringes (8 mL, Harvard Apparatus, Holliston, MA, USA) were used to feed in solutions (one syringe pump for PEG1540 solution and buffer solution mixture and a second for the mixture of enzyme dissolved in buffer and selected inorganic salt solution). In experiments where the microextractor was equipped with three "ψ"-shaped inlets, three syringe pumps were used to feed in solutions (one syringe pump for the inorganic salt solution, a second for PEG1540 solution, and a third for enzyme dissolved in buffer). Total flow rates investigated in this research were in the range of q = 20–1000 µL/min, which corresponded to a residence time of τ = 0.2–9 min. At the outlet of the microextractor, approximately 1–2 mL of samples were collected in a plastic tube. Samples were centrifuged (10 min, 4000 rpm, 25 °C). The volume of obtained layers was determined, and the layers were separated from each other using a needle to avoid phase mixing. Before determining the protein concentration in the extract phase, the phase was treated with 1:6 (v/v) chloroform to remove PEG1540 [39] and negate its interference with the protein assay. After the addition of the chloroform, the samples were homogenized and centrifuged at 4000 rpm and 25 °C for 10 min, after which the protein concentration and enzyme activity were determined.

2.2.4. Mathematical Modelling of the Xylanase Extraction in a Microextractor

Xylanase extraction in a microextractor was described following the model proposed by Jurinjak Tušek et al. [34]. The process was described with a 2D model, including convection in the flow direction (x) and diffusion in two directions (x and y). The mathematical model for steady-state conditions in the microextractor was composed of dimensionless partial differential equations for xylanase concentrations in raffinate (salt-

rich) and extract (PEG-rich) phases and corresponding boundary and initial conditions (Equations (5) and (6)):

- Xylanase concentration in raffinate phase (R):

$$\begin{array}{ll} v\cdot\frac{\partial\gamma_{\text{xylanase,R}}}{\partial\xi}=\frac{D_{\text{xylanase/R}}}{W}\cdot\left(\frac{\partial^2\gamma_{\text{xylanase,R}}}{\partial\xi^2}+\frac{\partial^2\gamma_{\text{xylanase,R}}}{\partial\psi^2}\right) & \\ \gamma_{\text{xylanase,R}}\left(0,\psi\right)=0 & 0\le\psi\le1 \\ \frac{\partial\gamma_{\text{xylanase,R}}\left(\frac{L}{W},\psi\right)}{\partial\xi}=0 & 0\le\psi\le1 \\ \gamma_{\text{xylanase,R}}\left(\xi,0\right)=K_P\cdot\gamma_{\text{xylanase,E}}\left(\xi,0\right) & 0\le\xi\le\frac{L}{W} \\ \frac{\partial\gamma_{\text{xylanase,R}}\left(\xi,1\right)}{\partial\psi}=0 & 0\le\xi\le\frac{L}{W} \end{array} \quad (5)$$

- Xylanase concentration in extract phase (E):

$$\begin{array}{ll} v\cdot\frac{\partial\gamma_{\text{xylanase,E}}}{\partial\xi}=\frac{D_{\text{xylanase/E}}}{W}\cdot\left(\frac{\partial^2\gamma_{\text{xylanase,E}}}{\partial\xi^2}+\frac{\partial^2\gamma_{\text{xylanase,E}}}{\partial\psi^2}\right) & \\ \gamma_{\text{xylanase,E}}(0,\psi)=0 & -1\le\psi\le0 \\ \frac{\partial\gamma_{\text{xylanase,E}}\left(\frac{L}{W},\psi\right)}{\partial\xi}=0 & -1\le\psi\le0 \\ \gamma_{\text{xylanase,E}}\left(\xi,0\right)=\frac{D_{\text{xylanase/R}}}{D_{\text{xylanase/E}}}\cdot\frac{\partial\gamma_{\text{xylanase,R}}\left(\xi,0\right)}{\partial\psi} & 0\le\xi\le\frac{L}{W} \\ \frac{\partial\gamma_{\text{xylanase,E}}\left(\xi,-1\right)}{\partial\psi}=0 & 0\le\xi\le\frac{L}{W} \end{array} \quad (6)$$

where v represents linear velocity, ξ and ψ represent independent dimensionless variables $\xi = x/W$, $\psi = y/W$, x and y are coordinated in the length (L) and microchannel width ($2W$). $D_{\text{xylanase/R}} = 9.328\cdot10^{-10}$ m^2/s and $D_{\text{xylanase/E}} = 2.426\cdot10^{-10}$ m^2/s are diffusion coefficients for xylanase in salt and PEG phases. The molecular diffusion coefficients for polyphenols were calculated using the Young empirical correlation ([40], Equation (7)):

$$D_{\text{xylanase/R(E)}}=\frac{8.34\cdot10^{-8}\cdot T}{\eta_{\text{R(E)}}\cdot M_{\text{xylanase}}^{1/3}} \quad (7)$$

where M_{xylanase} is molar mass of xylanase and η is water dynamic viscosity of a prepared solution of salt (PEG). A system of partial differential equations was solved by using the 2D finite differences method. Partial derivatives were discretized on the static equidistant grid. Mathematica 10.0 (Wolfram Research, Champaign, IL, USA) codes were used for simulations.

3. Results and Discussion

3.1. Selection of the ATPS for Xylanase Extraction

Considering that ATPS can be formed by mixing the polymer with inorganic salt, the influence of ATPS composition on xylanase extraction efficiency was first investigated. An ATPS system based on polyethylene glycol (PEG1540) and various salts, including sodium sulphate, sodium citrate dihydrate, sodium formate, potassium sodium tartrate tetrahydrate, and ammonium sulphate, was tested. The PEG1540 was selected based on the results obtained by Taddia et al. [27], where the authors compared several different PEGs (600, 1450, 2000, and 46,000 g/L) during the extraction process and observed that the system based on PEG1450 was practically free of other contaminating proteins and contained only xylanase after extraction. Additionally, the authors observed that, by increasing PEG molecular mass and consequently increasing the polymer hydrophobic character, the molecules migrate gradually to the bottom salt phase, so PEG with a lower molecular mass is more preferable. The same results were reported in several different research papers, where bromelain [41], protease [42], and invertase [43] were extracted using PEG. The higher partition coefficients were observed when PEG with a lower molar mass was used. According to Da Silva et al. [44], the increase in the polymer chain length also leads to the reduction of the free volume of the extract phase, which induces protein exclusion. The salts were selected based on literature [36–38] available data and phase diagrams. Phase

diagrams were used to determine the concentrations of the components required to form two phases and the critical point at which there would be an even distribution of the volumes of the phases in ATPS. As a result, the two phases (polymer phase and salt phase) would be in equilibrium, with the salt concentration being higher in the raffinate phase while the upper phase is rich in polymer. The concentration of salt was also kept in the lower concentration range (γ = 0.5 g/mL) to avoid the salting-out effect.

As can be seen from Table 2, the highest xylanase extraction efficiency (E = 79.63 ± 5.21%) was obtained with the ATPS system of sodium citrate dihydrate-H_2O-PEG1540. The second-best performance was obtained with potassium sodium tartrate tetrahydrate salt-H_2O-PEG1540, followed by ammonium sulfate-H_2O-PEG1540 salt. It was also found that there was no phase separation for the sodium sulfate-H_2O-PEG1540 system, so this system was not investigated further. There were also problems with the sodium formate-H_2O-PEG1540 system, as it was not possible to achieve a working mass of 5 g (Table 1). In addition to extraction efficiency, xylanase purification factors were also determined for the two best systems. In the sodium citrate dihydrate-H_2O-PEG1540 system, the *PF* was 1.26 ± 0.25, and in the potassium sodium tartrate tetrahydrate-H_2O-PEG1540 system, it was 1.36 ± 0.09. Considering the results obtained, the ATPS sodium citrate dihydrate-H_2O-PEG1540 system was used for further research. Additionally, another benefit of the selected system based on sodium citrate is due to the biodegradability of the citrate anion. This makes this system more sustainable because recovery is high and the cost is low [27].

The pH values of the extract and raffinate phases for phase-forming ATPS were also measured after the extraction process. For the ammonium sulphate-H_2O-PEG1540 ATPS, the pH of the raffinate phase was 5.12 and the extract phase was 6.21; for the sodium citrate dihydrate-H_2O-PEG1540 ATPS, the pH of the raffinate phase was 8.22 and the extract phase was 7.99; and for the potassium sodium tartrate tetrahydrate-H_2O-PEG1540 ATPS, the pH of the raffinate phase was 6.40 and the extract phase was 5.99. According to the literature [45], the two-phase region is extended with increasing pH. For this reason, these systems are more suitable when working with lower salt concentrations, as was the case in this study. The results obtained are also in favor of the ATPS sodium citrate dihydrate-H_2O-PEG1540.

Comparing the obtained data with the available literature, the extraction efficiency and purification factors obtained in this research were lower. When the xylanase from *Polyporus squamosus* was purified using the PEG/sulphate system, 4.8 purification folds and 97.37% yield were achieved [46]. A purification factor of 5.54 with 98.7% yield was obtained when xylanase from the thermostable fungi *Paecilomyces thermophile* was purified with PEG/sulphate [47]. Garai et al. [39] used an ATPS system based on PEG/phosphate in order to extract alkaline fungal xylanase. After optimization, a purification factor of 3.03 and an 88.10% yield were attained. Poornima et al. [48] applied the PEG6000/dipotassium hydrogen phosphate system for partition of xylanase produced by *Streptomyces geysiriensis* and obtained a 94% yield and a 3.65 purification factor. Based on all that was mentioned, it was evident that the ATPS selected in this investigation needed additional optimization.

3.2. Extraction Optimization

According to Rahimpour et al. [5], in order to find the optimal ATPS for successful protein extraction, many tests need to be performed. The large number can be reduced by using an experimental design methodology that explores the influence of system parameters on protein partitioning between phases. To achieve higher extraction efficiency, the present process was optimized using a three-factor Box-Behnken design. The Statistica 13.0 program package was used to design an experimental plan with a total of 17 experiments (Table 3), which were carried out to investigate the influence of extraction time, xylanase concentration, and mass fraction of PEG in the ATPS on xylanase extraction. The results obtained are shown in Table 3.

Table 3. Experiment plan according to the Box-Behnken design obtained in the Statistica 13.0 software package and experimentally observed extraction efficiencies and RSM model predicted extraction efficiencies.

Exp.	t, min	$\gamma_{xylanase}$, mg/mL	w_{PEG}, w/w	$E_{observed}$, %	$E_{predicted}$, %
1	5	0.10	0.21	95.318 ± 0.848	96.461
2	15	0.10	0.21	91.227 ± 0.811	78.988
3	5	0.30	0.21	106.515 ± 8.088	101.102
4	15	0.30	0.21	103.364 ± 7.874	96.461
5	5	0.20	0.20	85.500 ± 6.447	82.128
6	15	0.20	0.20	88.568 ± 6.678	94.737
7	5	0.20	0.22	86.523 ± 6.524	94.573
8	15	0.20	0.22	102.886 ± 7.758	103.839
9	10	0.10	0.20	72.906 ± 8.145	80.429
10	10	0.30	0.20	97.724 ± 3.214	91.029
11	10	0.10	0.22	96.517 ± 4.541	103.268
12	10	0.30	0.22	104.509 ± 7.065	103.786
13	10	0.20	0.21	89.318 ± 6.735	86.513
14	10	0.20	0.21	88.568 ± 6.679	85.373
15	10	0.20	0.21	88.600 ± 6.681	86.870
16	10	0.20	0.21	87.046 ± 6.563	86.119
17	10	0.20	0.21	82.432 ± 6.216	88.069

Statistical analysis performed using the Levenberg-Marquardt algorithm implemented in the Statistica 13.0 software package was used to analyze the results of the experiments performed according to the Box-Behnken design. By analyzing the results, the values of the model coefficients that depend on the process parameters (X_1 time, X_2 enzyme concentration, and X_3 mass fraction of PEG) and the process reaction (xylanase extraction efficiency) were determined. To describe the influence of selected process variables on xylanase extraction efficiency in an ATPS, a second-degree polynomial was chosen to describe the linear and quadratic influence of each process condition and their interdependence on extraction efficiency. In addition to the determination of the value of the influence coefficient, a statistical analysis of the values obtained by the experiment and those obtained by the simulation of the model was performed to test the validity of the obtained model and determine whether it could be used to determine the optimal conditions for the implementation of the process.

In order to determine the influence of all process variables as well as their mutual influence, the p-value was chosen as a criterion. Concato and Hartigan [49] stated in their work that a value of $p < 0.05$ indicates a significant influence of the analyzed process variables or interactions and that these variables cannot be ignored in further analysis, while values of $p > 0.05$ indicate that the values of the analyzed variable or interaction are not significant and can be ignored. Table 4 shows the obtained analysis of the influence of each process parameter and their mutual interactions on the efficiency of xylanase enzyme extraction. The value β_n in Table 4 refers to the linear term, and $\beta_n{}^2$ refers to the quadratic term.

As can be seen, time (β_2) and enzyme concentration (β_3) have a significant effect on increasing the xylanase extraction efficiency. In addition to the individual process parameters, the interaction between the enzyme concentration and the percentage of PEG also influences the extraction efficiency. Table 3 also shows that the time and the interaction between the protein concentration and the mass fraction of PEG have a negative influence on extraction efficiency. A negative value of influence indicates that the process variable has a negative effect on the extraction efficiency, i.e., its further increase lowers the extraction efficiency. The most significant positive influence on the xylanase extraction efficiency is the enzyme concentration, while the most negative influence on the extraction is the interaction between the enzyme concentration and mass fraction of PEG.

Table 4. RSM model for description of xylanase extraction efficiency.

Parameter	Influence	*p*-Value
$\boldsymbol{\beta_0}$	**94.69**	**0.000000**
β_1 (t)	3.44	0.388259
$\boldsymbol{\beta_2}$ (t^2)	**−9.49**	**0.007820**
$\boldsymbol{\beta_3}$ (γ_{xylanase})	**15.21**	**0.048380**
β_4 ($\gamma_{\text{xylanase}}{}^2$)	1.45	0.787007
β_5 (w_{PEG})	−1.44	0.827753
β_6 ($w_{\text{PEG}}{}^2$)	10.78	0.074961
β_7 ($t \cdot \gamma_{\text{xylanase}}$)	0.27	0.960227
β_8 ($t \cdot w_{\text{PEG}}$)	7.33	0.208460
$\boldsymbol{\beta_9}$ **($\gamma_{\text{xylanase}} \cdot w_{\text{PEG}}$)**	**−13.49**	**0.038038**

The comparison of the values predicted by the mathematical model and those obtained by the experimental measurements is presented in Table 3. The deviation (R^2) between the values was calculated to be 0.88244, which means that the mathematical model satisfactorily describes the experimental results and can be used with sufficient reliability to simulate the process of xylanase extraction in an ATPS based on sodium citrate dihydrate.

Despite the satisfactory R^2 value, the reliability of the model was also tested by a residual analysis, which gives a better insight into the values of the proposed mathematical model for the tested system. The residual analysis is based on a comparison of the experimentally determined values of the process parameters with the values obtained by simulating the mathematical model of the process. The results of the residual analysis are shown in Figure 2. Figure 2A shows that the assumption of normality is satisfied since almost all values are in the same direction. Figure 2B shows a bell-shaped histogram with a small lack of symmetry, which is an additional confirmation that the values are well distributed. By comparing the residual values and the predicted values, the correctness of the model is confirmed when no pattern is observed in the image (Figure 2C), which is an indication that the model fits the data well. The last analysis to be performed is the analysis that checks whether the sequence of experimental runs affects the data, i.e., the residual and the experimental data are compared (Figure 2D). The results obtained indicate that the degree of randomization is appropriate and that the order of the trials did not affect the data. Based on the obtained analysis, it can be concluded that the proposed process model is acceptable and can be safely used for further optimization of the process.

The optimal conditions of the enzyme extraction process (extraction time, enzyme concentration, and mass fraction of PEG) were determined using simulations of the developed RSM model of the process in the Statistica software package. The estimated optimal conditions represent the local maximum for the selected range of input variables. Figure 3 illustrates 3D response surface plots showing interaction effects of process variables (time, enzyme concentration, and mass fraction of PEG) on the extraction efficiency using ATPS based on sodium citrate dihydrate. According to the RSM model, the optimal extraction conditions for the enzyme extraction process in an ATPS based on sodium citrate dihydrate are as follows: t = 15 min, γ_{xylanase} = 0.3 mg/mL, and w_{PEG} = 0.21 w/w.

After determining the optimal processing conditions, the enzyme extraction procedure was repeated. The extraction efficiency under optimal conditions was E = 99% ± 1.20%, K = 20.86 ± 3.89, and PF = 6.49 ± 0.053, which was a significant increase compared with the process before optimization (E = 79.63 ± 5.21%, PF = 1.26 ± 0.25, K = 3.91 ± 0.26). Better results were also achieved compared with the literature data [39,46–48].

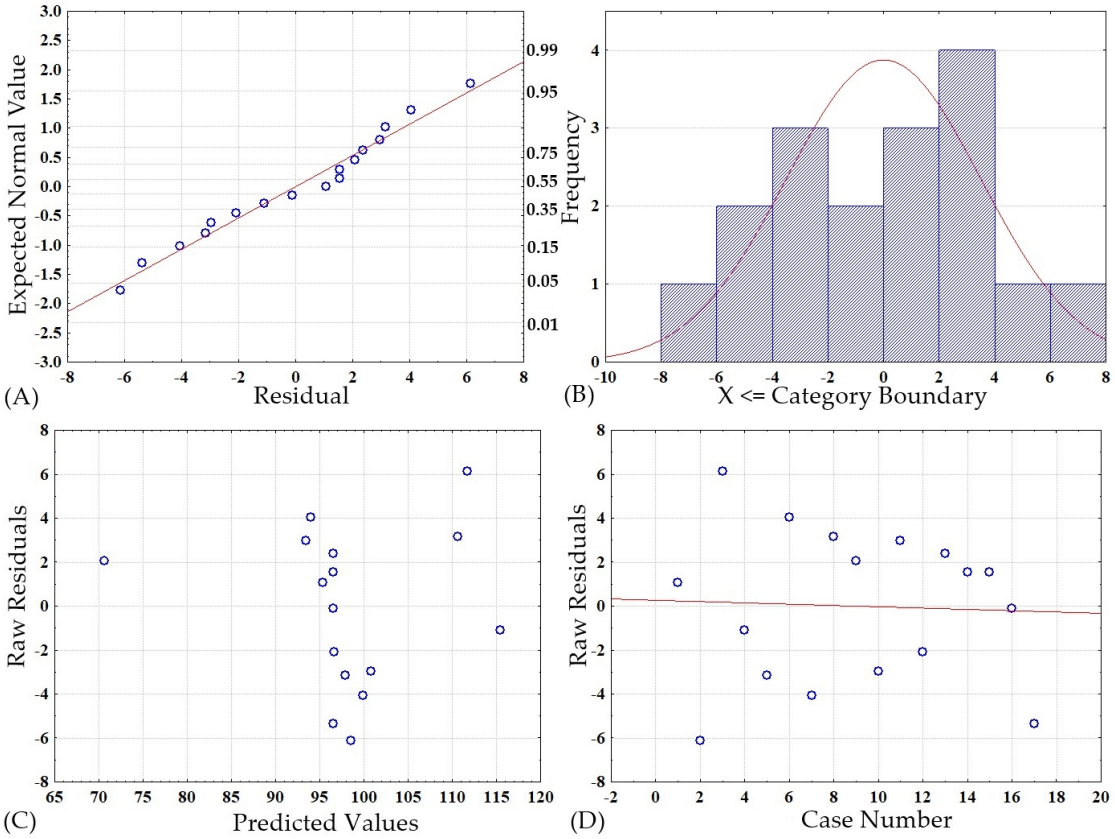

Figure 2. Residue analysis for xylanase extraction performed in an aqueous two-phase system based on sodium citrate dihydrate to obtain the highest possible extraction efficiency value: (**A**) residue plot, (**B**) histogram, (**C**) ratio of predicted value to residue values, and (**D**) comparison of experimental and residue values.

After the optimization, the most common next step is the scale-up of the process [5]. According to Moteshafi et al. [28], by scaling up ATPS, the loss of enzyme activity will be insignificant. Alternatively, scaling down also displays benefits. As mentioned in the introduction, microextractors have been successfully applied in several extraction processes [29,30], including those based on ATPS [31,32]. By applying microextractors, enhanced mass and energy transfer could be obtained.

3.3. Extraction Intensification in a Microextractor

After optimization, extraction was performed in three different microextractors under optimal process conditions, with additional investigation of the influence of residence time, different feeding strategies, and channel diameter on extraction efficiency and purification factor. Microextractors are of particular interest for the purification of proteins since the probability of denaturation of selected proteins is minimal due to the laminar flow prevailing in such systems during extraction [50].

Before the extraction process was carried out in the microextractor, the volume flow ratio of each inlet stream was determined. The volume flow ratio had to be adjusted in order to obtain a phase volume ratio of 1:1 in the sample collected at the outlet of the microextractor since the same ratio was obtained in the macroextractor. It was found that the volume ratio of PEG1540, buffer, enzyme, and inorganic salt at the outlet of the microextractor was 1:1, with a volume flow ratio of PEG1540 solution, buffer, enzyme solution, and inorganic salt solution of 1:1.17. In experiments using a microextractor with three inlets, the volume flow ratio of PEG1540 solution:inorganic salt solution:enzyme dissolved in buffer was 1:1.17:0.18. These ratios were used in further experiments.

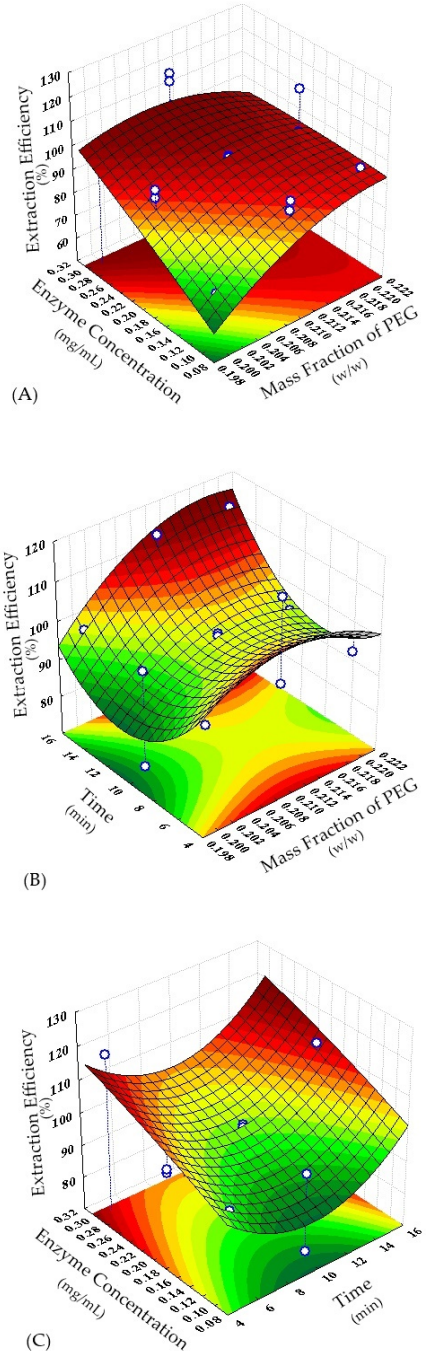

Figure 3. A 3D response surface plot showing the interaction effects of process variables (time, enzyme concentration, and mass fraction of PEG) on the extraction efficiency using ATPS with sodium citrate dihydrate. (**A**) enzyme concentration and mass fraction of PEG, (**B**) time and mass fraction of PEG, and (**C**) enzyme concentration and time.

As mentioned above, in the experiments carried out in the microextractors, the influence of the residence time, different feeding strategies, and the diameter of the channels on the efficiency of extraction were studied. The results obtained are shown in Figure 4. It can be seen that by conducting experiments in a microextractor, a significant intensification of the process was achieved compared with experiments in a batch extractor. Specifically, the extraction efficiency of 99.59 $\pm$ 1.22% and the purification factor of 6.61 $\pm$ 0.07 were obtained at a residence time of τ = 1.03 min for a microextractor with two inlets and a diameter of 500 µm (Figure 4A). When the diameter of the microchannel was increased to 1000 µm, an

extraction efficiency of 86.48 ± 2.56% and a purification factor of 6.52 ± 0.17 were achieved for the same residence time (Figure 4B). As the channel width increased, the extraction efficiency decreased due to the decrease in the surface-to-volume ratio and the increase in the diffusion pathway. In the next experiment, the inlet strategy was changed, with each component (enzyme dissolved in buffer, PEG1540 solution, and inorganic salt solution) being fed separately to avoid pretreatment of the sample before extraction. As can be seen in Figure 4C, the same results were obtained as in the experiment using two inlet strategies (Figure 4B). Based on the obtained results, it can be concluded that microextractors are more effective than batch extractors for xylanase extraction with ATPS.

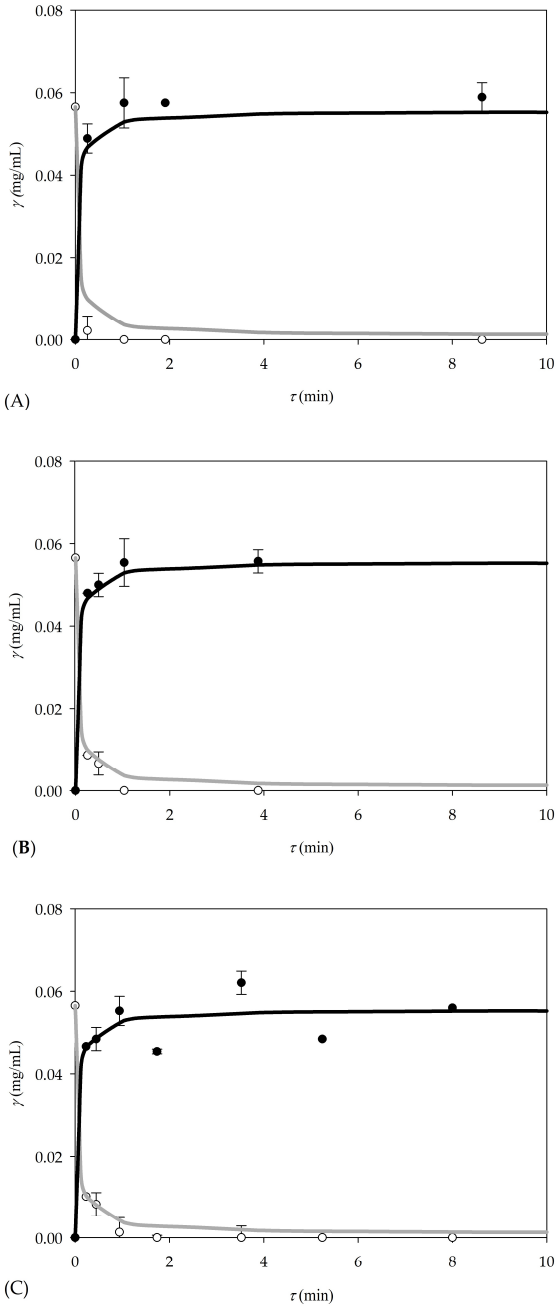

Figure 4. Effect of residence time on xylanase concentration in extract and raffinate phases for microextractors with two inlets and channel diameters of 500 µm (**A**) and 1000 µm (**B**) and for microextractors with three inlets and channel diameters of 1000 µm (**C**) (● extract phase, ○ raffinate phase, — mathematical model).

Based on the experiments performed and considering that there is a negligible time difference to achieve the same extraction efficiency, a microextractor with a channel diameter of 1000 μm was chosen. A microextractor with a larger diameter was chosen to reduce the possibility of clogging the microchannel. During the extraction of the desired enzyme from real samples, there are many impurities in the raw enzyme that could potentially clog the microchannel.

3.4. Extraction of Raw Xylanases Produced by Solid-State Fermentation of Thermomyces Lanuginosus

Finally, the developed process was applied to the extraction of xylanase from raw extract produced by solid-state fermentation of *Thermomyces lanuginosus*. The extraction was performed in a batch extractor and a microextractor under defined optimal conditions. An extraction efficiency of 73.21% ± 3.89% and a purification factor of 18.33 ± 2.15 were obtained in a batch extractor, proving that the developed process was efficient for purifying the xylanase from the raw sample. The extraction efficiency was slightly lower than the one obtained for the model solution, most likely due to the presence of various substances in this extract, such as carbohydrates and other enzymes.

Continuous extraction of the raw xylanase extract was also performed using a microextractor with a channel diameter of 1000 μm and two inlets. Figure 5 shows the results of testing the effect of residence time on xylanase concentration in the extract and raffinate phases. At a residence time of τ = 0.49 min, E = 58.99 ± 5.69% and K = 3.22 ± 0.77 were obtained, which did not change significantly with increasing residence time.

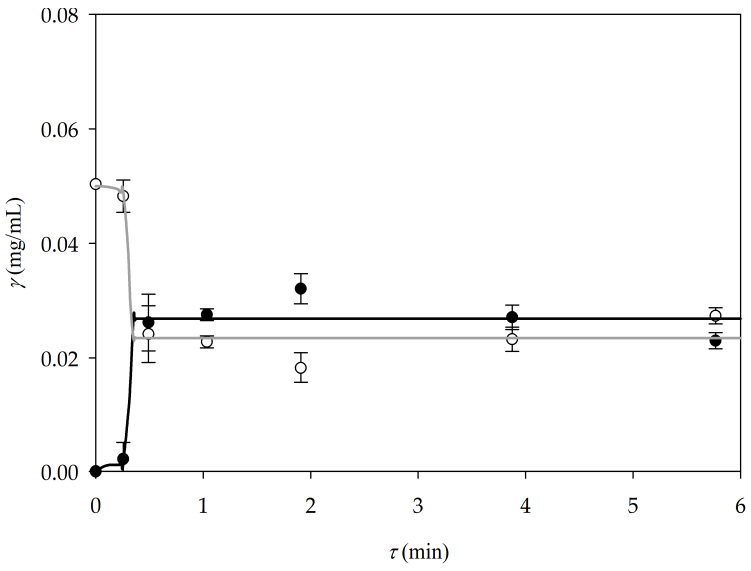

Figure 5. Effect of residence time on the mass concentration of xylanase in the raffinate and extract phases (● extract phase, ○ raffinate phase, — mathematical model).

Regardless of the slightly lower efficiency obtained in the microextractor experiments with the raw enzyme extract, the results of these experiments are promising and open up room for further research and improvement of the xylanase extraction process in the microsystem, especially when it is considered that the purification factor achieved in the microextractor was 136.83 ± 9.31. In addition, when comparing the enzyme obtained after purification ($V.A._{xylanase}$ = 383.02 ± 30.58 U/mL, S.A. = 16.65 ± 1.33 U/mg) with the raw enzyme extract ($V.A._{xylanase}$ = 32.51 ± 1.22 U/mL, S.A. = 0.12 ± 0.01 U/mg), a significant improvement in enzyme activity was obtained.

4. Conclusions

The ATPS sodium citrate dihydrate-H_2O-PEG1540 proved to be the most effective system for xylanase purification by extraction. During the optimization process, among the

analyzed variables, time and enzyme concentration were found to have a significant effect on increasing the xylanase extraction efficiency. After optimization, the extraction efficiency and purification factors were E = 99.13% ± 1.20%, K = 20.86 ± 3.89, and PF = 6.49 ± 0.053, which was a significant increase compared with the performance of the process before optimization (E = 79.63 ± 5.21%, PF = 1.26 ± 0.25, K = 3.91 ± 0.26). The process was additionally intensified by carrying out continuous extraction in different microsystems. Consequently, practically the same extraction efficiency (E = 99.59 ± 1.22%) and purification factor (PF = 6.61 ± 0.07) were obtained in significantly less time (τ = 1.03 min) compared with the experiment conducted in a batch extractor (t = 15 min). Finally, continuous extraction in the microsystem was applied to raw xylanase produced by solid-state fermentation, resulting in E = 58.99% ± 5.69%, K = 3.22 ± 0.77, and PF = 136.83 ± 9.31 for a residence time of τ = 0.49 min. The obtained results clearly indicate that the process is suitable for the effective purification of raw xylanase produced by solid-state fermentation.

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