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Journal of Chromatography B, 858 (2007) 227–233
JOURNAL OF CHROMATOGRAPHY B
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Continuous extraction of α- and β-amylases from *Zea mays* malt in a PEG4000/$CaCl_2$ ATPS

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Received 3 April 2007; accepted 30 August 2007
Available online 8 September 2007

Abstract

In the present work, α- and β-amylase enzymes from *Zea mays* malt were recovered by continuous extraction in a PEG/$CaCl_2$ aqueous two-phase system (ATPS). The influences of the flux rate (R_Q), free area of vane (A_{free}) and vane rotation (R_V) on enzyme recovery were studied by optimization using response surface methodology (RSM). The protein content and enzyme activity were measured from time to time in the extract and refined fluxes. RSM curves showed a squared dependence of recovery index with the R_Q, A_{free} and R_V. The best system for recovering the maize malt enzymes was with low vane rotation and flux rate and high free area of vane. α- and β-amylases were purified 130-fold in the salt-rich phase.
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Keywords: Continuous extraction; α- and β-amylases; PEG/$CaCl_2$ ATPS; *Zea mays* malt; Maize seeds

1. Introduction

Aqueous liquid–liquid two-phase systems are formed when two polymers are dissolved together above certain concentrations. The most characteristic feature of these systems is that both phases are aqueous (water content 85–99%), allowing partition of biomacromolecules and cellular particles of diverse origin under non-denaturing conditions. Several pairs of polymers can be used to form aqueous two-phase systems. Many polymers form two-phase liquid–liquid systems when combined with suitable salts (e.g. phosphates or sulphates) [1,2].

The use of aqueous two-phase systems in biotechnology basically exploits this varying distribution of biomaterials between the phases. These systems can be buffered and are suitable for carrying out bioconversions. The phase polymers have also been shown to have a stabilizing influence on biocatalysts; the latter are, in a way, temporarily immobilized within liquid droplets. The different areas in which two-phase systems have shown potential include extractive fermentations, purification of biomolecules, cells, membranes and organelles, and biological binding assays. However, most of the systems reported so far, showed an increase in purified components in the scale-up of the processes. Nevertheless, there has been a serious attempt to employ low-cost phase components, for example, in purification of proteins on a large scale [2,3].

During recent years, the well-documented phenomenon of separation of an aqueous solution of two different water-soluble polymers into individual phases has shown widespread potential in biotechnology. A number of polymers and salts have been employed for the preparation of these bi-phase systems. The most commonly used systems have been those of poly(ethylene glycol) (PEG)/NaCl, PEG/phosphate and PEG/dextran. The molecular weight of the polymers used plays an important role in determining the characteristics of the phase system [3].

The partition coefficients of hydrolytic enzymes, α- and β-amylases and glucoamylase, of high purity, have been determined in PEG/dextran [4,5], PEG/$MgSO_4$ [6] and PEG/$CaCl_2$ [7] batch aqueous two-phase systems (ATPS). The influence of polymer, salt and initial enzyme concentration on partition

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doi:10.1016/j.jchromb.2007.08.042

was investigated in these studies. The experimental and correlated partition coefficient by method Flory–Huggins equation into PEG/dextran and PEG/$MgSO_4$ were measured between 0.04 and 0.97. Santana [7] had obtained a partition coefficient of fivefold for α- and β-amylases from maize malt into PEG/$CaCl_2$ ATPS, and Gu and Glantz [8] had obtained a partition coefficient of threefold for corn protein into PEG/NaCl ATPS. Both sets of authors were working with the logarithmic equation of the partition coefficient reported by Albertsson. Continuous extraction had been used in chromatographic purification of proteins as this process increases the partition of proteins into the target compared with the batch ATPS process [9,10].

Methods of conventional extraction such as spray columns [11,12] and no-conventional as pulsed plates column [11,12–15], agitated plates columns [16] and pulsed flower columns [17–21] can be conveniently applied to aqueous two-phase systems. The liquid–liquid continuous extraction using a micro-column had been applied to purify the biotechnological products for shows advantages: both phases are aqueous, low superficial tension and allowing partition of biomacromolecules and cellular particles of diverse origin under non-denaturing conditions and their reagents show low price [1,2].

The cytochrome *b*5 from *Escherichia coli* [15,20] and xylanase from *Penicillium janthinellum* [19], tripsin enzyme [18] and other protein [11,12,17,18]. The effect of dispersed phase velocity, system composition, discs rotation speed, ionic strong and disc pulse quantity on the protein mass transfer coefficients or column hydrodynamics had been studied [11,13–19] and had been modeling [9,10].

The enzymes α-amylase (EC 3.2.1.1, α-1,4-glucan-4-glucanohydrolase) and β-amylase (EC 3.2.1.2 1,4-α-D-glucan maltohydrolase), are exoenzymes that degrade starch to produce glucose, maltose and boundary dextrins, mostly utilized in food industries, at pH values of 4.8 and 5.4 and at temperatures of 75 and 55 °C, respectively; with molecular mass about 50 kDa [7,21–23]. According to Sigma [24], the price of amylase enzymes is currently about US$ 1500/g of purified material. Thus, the recovery of these enzymes from maize malt will aggregate price the maize culture. The recovery of α- and β-amylase enzymes from maize (*Zea mays*) malt by an aqueous two-phase PEG/$CaCl_2$ system at pH 5 has been studied in the present work, with malt/$CaCl_2$ and PEG solutions operating in continuous flux in the liquid–liquid extraction micro-column, at 22 ± 2 °C and 1 atm. Vane agitation, solvent/concentrate flux rate and vane free area were used in factorial planning for studying the influence on the purification factor by response surface methodology (RSM).

2. Materials and methods

2.1. Materials

2.1.1. Chemical reagents

Chemicals: polyethylene glycol (molecular weight 4000 Da) was provided by Sigma (Switzerland). Ultrapure $CaCl_2$ was from Vetec (Brazil). Hydrolytic enzymes α- and β-amylases from maize malt were supplied by Embrapa (Brazil). Milli-Q-quality distilled water was used.

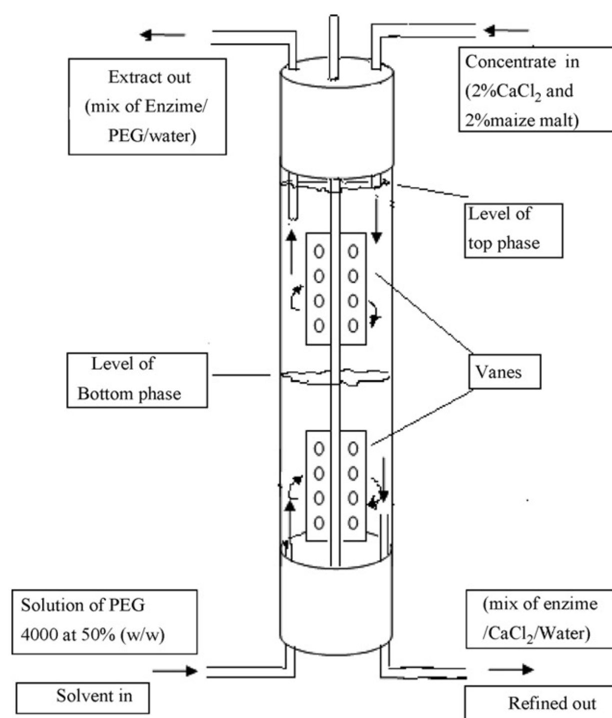

Fig. 1. Scheme of liquid–liquid extraction micro-column used in amylase partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS.

2.1.2. Equipment

UV–vis spectrophotometer (Model B582, Micronal, Brazil) and liquid–liquid extraction micro-column (manufactured by DEQ-UFS, Brazil; see Fig. 1), it was made of glass with 31.9 cm of height and 39.8 mm of inner diameter, and the vanes had 40 mm × 20 mm × 5 mm of size.

2.1.3. Preparation of concentrate flux

The hydrolytic enzymes α- and β-amylase were obtained by germination of maize seed (maize malt) according to Santana [7] and Biazus et al. [23] provided by Embrapa from Sergipe, Brazil. Maize malt (2%) was added to 2% $CaCl_2$ solution at pH 5 (in 0.015 M phosphate buffer solution) and used in concentrate flux (or salt-rich flux).

2.1.4. Preparation of solvent flux

A solution of PEG4000 of 50% (w/w) of concentration at pH 5 (in 0.015 M phosphate buffer solution) was prepared and used in solvent flux (or PEG-rich). The tie-line length composition used was of 25.26% (w/w) of PEG4000 and 1.82% (w/w) of $CaCl_2$ and the phases compositions were of 45.75% (w/w) of PEG4000 and 0.04 (w/w) of CaCl2 in top phase and 3.58% (w/w) of PEG4000 and 4.62 (w/w) of $CaCl_2$ in bottom phase compositions, according to Santana [7].

2.2. Methods

2.2.1. Amylase partition

The solution contained 2% malt and 2% $CaCl_2$ was loaded to the top of the micro-column as concentrate flux and the 50% PEG solution was loaded to the bottom of the micro-column as solvent flux. The system was mixed with an agitator and the initial operation time was noted. The extract was loaded out in column top and the refined was loaded out in column bottom. The solvent and concentrate fluxes were varied between 3 and 11 mL/min and the fluxes of extract and refined were similar to the fluxes of solvent and concentrate, respectively. The vane agitation was varied among 64.65 and 135.4 rpm and the free area of vanes were varied among 0 and 18% (area/area). Total protein concentration (C) was determined from time to time in samples of PEG-rich (or extract) and salt-rich (or refined) fluxes by the Bradford method [25]. Extraction curves were plotted using the rate between the total protein concentration changing with time (C) and the initial total protein concentration (C_0); these curves were made to show the mass transfer between the phases during the extraction process and to determine the time for that the process will be in standing state [11–16]. All assays of amylases extraction were made at 22 ± 2 °C and 1 atm. The enzyme activity was measured by the Woulghnuch modified method (SKB), according to Reguly [21] and electrophoresis was carried out according to Toledo et al. [26]. The purification factor (FP) was obtained by

$$FP = \frac{SA_{phase}}{SA_{crude}} \tag{1.1}$$

where SA_{phase} is the specific activity (SKB/mg of protein) in the evaluated phase and SA_{crude} is the specific activity in *Z. mays* malt (SKB/mg of protein).

2.2.2. Experimental design

An orthogonal experimental design was used in the optimization of partitioning of hydrolytic enzymes in continuous extraction by modified star methods. Factorial planning 2^{3-1} was used with three factors: vane rotation, R_V (x_1), flux rate, R_Q (x_2) and free area of vane, A_{free} (x_3), and one response (y), the purification factor, FP. The assays are shown in Table 1; the level changed as follows: −1, 0, +1, and $\alpha = \pm 1.414$, with three repetitions in the center point [27]:

$$x_1 = \frac{R_{Vj} - 100}{25} \tag{2.1}$$

$$x_2 = \frac{R_{Qj} - (3/4)}{1/4} \tag{2.2}$$

and

$$x_3 = \frac{A_{freek} - 9}{9} \tag{2.3}$$

where R_Q is the rate between solvent and concentrate fluxes.

The evaluation of the empiric models was performed by variance analysis (ANOVA) and optimization by response surface methodology (RSM) in the program Statistics for Windows 5.0 [27]. The planning matrix used to obtain the optimal condition for recovery of amylases from maize malt by PEG/$CaCl_2$ ATPS in the continuous extraction process is shown in Table 1.

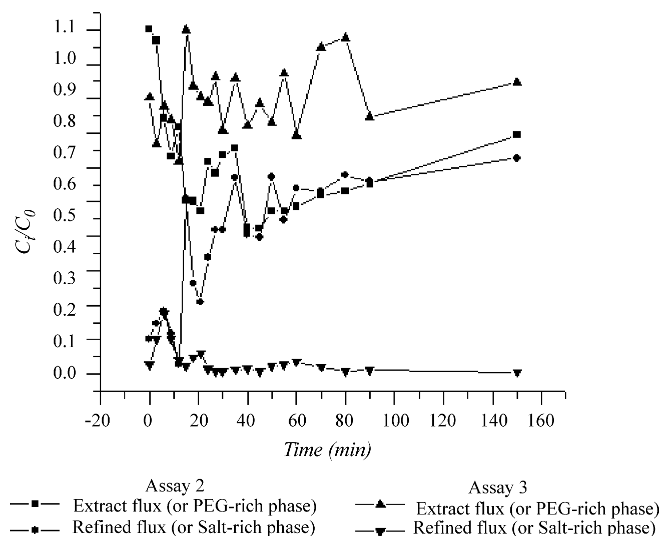

Fig. 2. Extraction curves for the amylase partition from maize malt by continuous PEG4000/CaCl2 ATPS, at O% of free area of vanes.

3. Results and discussion

Figs. 2–7 show the continuous extraction curves for α- and β-amylase enzymes from maize (*Z. mays*) malt by PEG/$CaCl_2$ ATPS into a micro-column with agitation of vanes, these figures show the protein transfers between the bottom and top phases and time for the process is in standing state. It is possible to note that the protein is concentrated into the PEG-rich phase (top phase) and shows that PEG has good affinity for biomolecules in maize malt. Due to the high molecular weight or the high surface area of the protein and, the hydrophobic properties of PEG phase systems and of proteins are the dominant factors [1–3,10–16,21,28].

The figures are organized by a similar vane free area. In assays 1, 3, 5, 6, 7 and 8 the systems are operating in

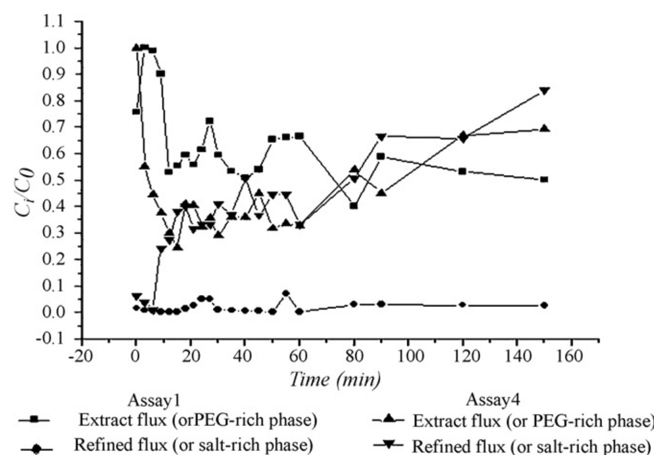

Fig. 3. Extraction curves for the amylase partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS, at 18% of free area of vanes.

Table 1
Planning matrix used to optimize, via response surface methodology (RSM), the amylases partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS

Assay	x_1	x_2	x_3	R_V (rpm)	R_Q	A_{free} (%)	FP_{Top}	FP_{Bottom}
1	−1	−1	1	75	1:2	18	4.5770	132.659
2	1	−1	−1	125	1:2	0	1.1193	1.3872
3	−1	1	−1	75	1:1	0	3.7862	58.174
4	1	1	1	125	1:1	18	1.4424	1.2298
5	0	0	0	100	3:4	9	5.6208	4.1499
6	0	0	0	100	3:4	9	1.5587	21.3632
7	0	0	0	100	3:4	9	8.9299	16.3516
8	−1.414	0	0	64.65	3:4	9	20.2427	45.0082
9	1.414	0	0	135.4	3:4	9	7.2011	10.0066
10	0	−1.414	0	100	3.2:8	9	2.8718	51.1253
11	0	1.414	0	100	11:10	9	2.1953	3.7853

Where x_1, x_2 and x_3 are the coded variables for the vane rotation (R_V), the flow rate (R_Q) and the free area of vanes (A_{free}), and FP_{Top} and FP_{Bottom} are the purification factor in top and bottom phases, respectively.

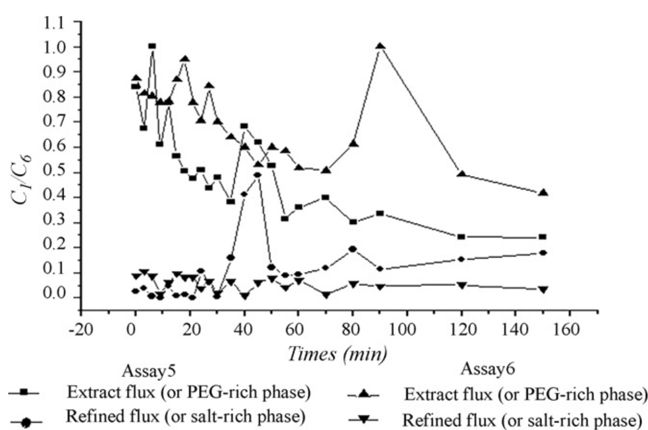

Fig. 4. Extraction curves for the amylase partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS, at 9% of free area of vanes.

plug flow regime, while mass transfer rates are not constant. Between 20 and 40 min of system operation, the extraction curves tend to a constant value, determining the time for completing of the continuous extraction process of the enzymes from the maize malt, as the mass transfer rate is constant. After this time, storage of the products is possible as the protein concentration is constant, and maize enzyme manufacturing in industrial scale operations is possible [9,11,12,16].

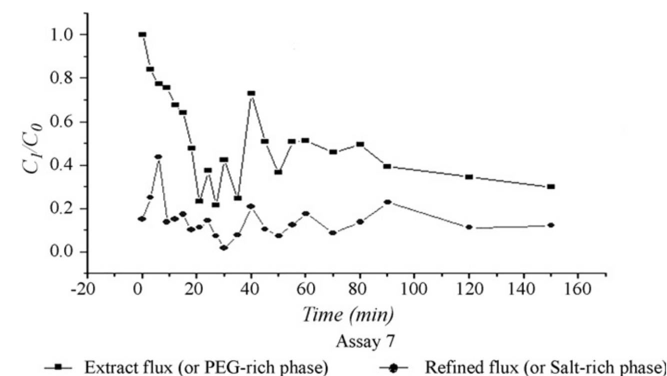

Fig. 5. Extraction curves for the amylase partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS, at 9% of free area of vanes.

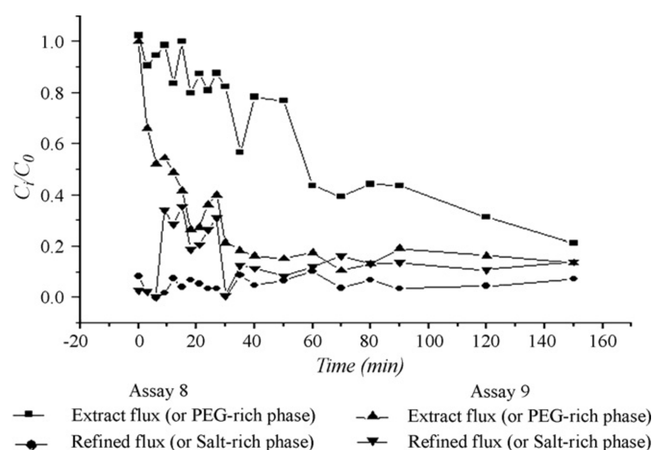

Fig. 6. Extraction curves for the amylase partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS, at 9% of free area of vanes.

In assays 2, 4, 9, 10 and 11, the protein concentrations in both phases are similar due to high agitation applied to the system by back-mixing, which promotes a high mass transfer rate. Standing state occurs between 15 and 30 min.

Table 1 shows the results and planning matrix used for optimization of recovery of amylases from maize malt by

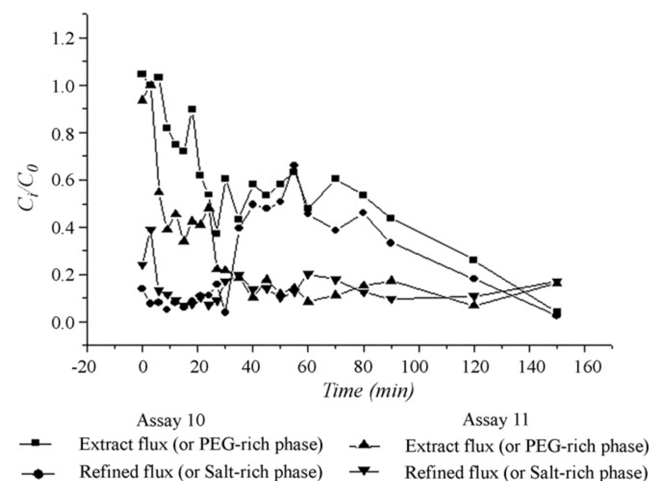

Fig. 7. Extraction curves for the amylase partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS, at 9% of free area of vanes.

Table 2
Variance analysis (ANOVA) of the best empiric model used to optimize, via response surface methodology (RSM), the amylases partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS

Source	Square sum	Freedom degree	Mean square	F_{cal}	F_{tab}
Regression	15645.518	7	2235.074		
Residual	164.980	3	54.993	40.643	8.89
Fitting fault	8.215	1	8.215		
Error	156.765	2	78.383	0.105	18.51
Total	15645.518	10			
Explaining variance (%)				100.000	
Maximum explaining variance (%)				98.998	
Multiple correlation (R^2)				1.0000	

Where F_{cal} and F_{tab} is the calculated and tabled Fisher tests, respectively.

PEG/$CaCl_2$ ATPS in the continuous extraction process. Continuous extraction of α- and β-amylase from maize malt produced two products with good amylase activity, one in the PEG-rich flux and the other in the salt-rich flux. It is perceived that α- and β-amylase enzymes are present in both phases.

In the salt-rich phase (refined), the FP value was higher than the FP value in the PEG-rich phase (extract), so in the refined phase, there is higher affinity for α- and β-amylase enzymes from maize malt. This occurs because of the presence of Ca^{2+} in the salt-rich phase, which has the power to increase the activity of α-amylase enzymes [8,22–24]. The electrical potential effect between the phases is very important for amylase separation, it increased the purification factor (FP) in 130-fold in the salt-rich flux [1–3,19,21,29]. This FP value is higher than the FP value obtained by Furuya et al. [4,5], Blasquez et al. [6] Gu and Glatz [8] and Santana [7], showing that continuous PEG/$CaCl_2$ ATPS is the best system for amylase recovery. α And β-amylases PF values were more than founds by Nirmala and Muralikrishna [22] using CPC, CCC and fractional precipitation methods, by Demirkan et al. [30] using gel chromatography and CCC methods and by Aguilar et al. [29] and Nguyen et al. [31] gel chromatography and fractional precipitation methods. The resolution of this continuous extraction is compared to affinity chromatography [23,32].

Fitting the data indicates that the best model for showing the influence of vane rotation (x_1), flux rate (x_2) and free area of vane (x_3) on purification was the squared model Eq. (3.1). Table 2 shows the ANOVA results for evaluating the model fitting. The multiple correlation (R^2) was about 1.0, and the variances were about 100%, and this shows that there is good fitting of the model [27]. F_{cal1}/F_{tab1} and F_{tab2}/F_{calc2} were increased more than four times and show that the model is statistically significant, predictive and has a good fit [20,27]:

$$FP_{Bottom} = 13.9549 - 12.7003x_1 - 17.7003x_2 + 18.5819x_3 + \cdots + 6.7783x_1^2 + 6.7522x_2^2 + 20.8771x_3^2 - 34.6772x_2x_3 \quad (3.1)$$

Figs. 8–10 show the RSM curves for the continuous extraction optimization. The influences of vane rotation and flux rate on purification factor (FP) are analyzed in Fig. 8, which shows an increase in FP with decreases in vane rotation and flux rate. Fig. 9 shows the influence of free area of vane and vane rotation on FP value. This shows that FP increases with the decrease in

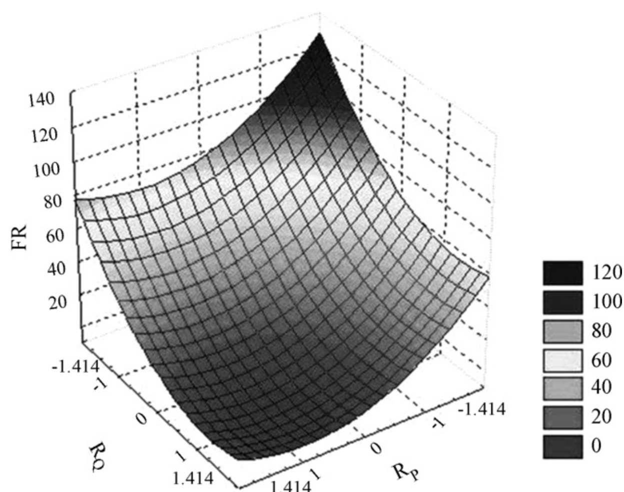

Fig. 8. Response surface for evaluation of the effects of pallets rotation and flux rate, on the amylases partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS. Where R_Q is the flow rate, R_P the vane rotation and FP is the purification factor.

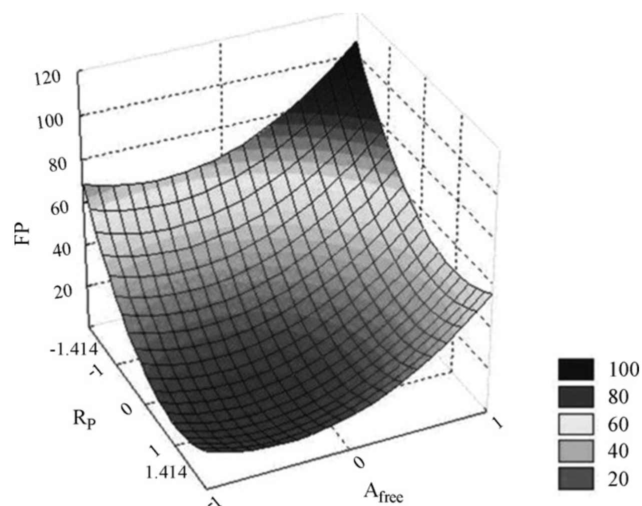

Fig. 9. Response surface for evaluation of the effects of pallets rotation and free area of pallets, on the amylases partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS. Where A_{free} is the free area of vanes, R_P the vane rotation and FP is the purification factor.

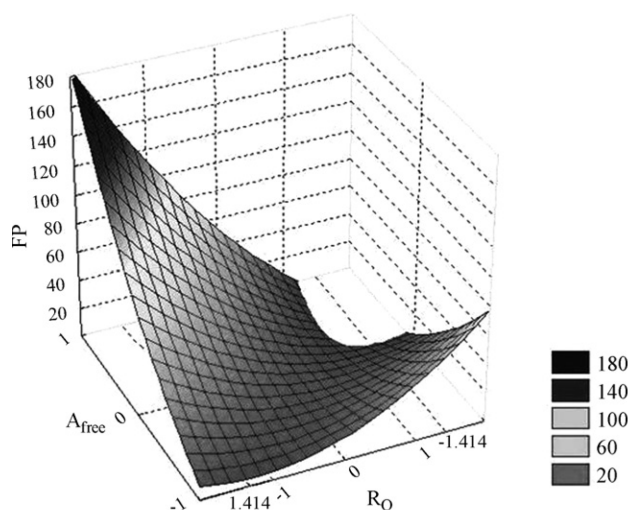

Fig. 10. Response surface for evaluation of the effects of free area of pallets and flux rate, on the amylases partition from maize malt by continuous PEG4000/$CaCl_2$ ATPS. Where A_{free} is the free area of vanes, R_Q the flow rate and FP is the purification factor.

pellet agitation and increase in pellet free area. The influence of flux rate and free area of vane on FP are analyzed in Fig. 10, which shows an increase in FP with the decrease in flux rate and increase in free area of vane.

According to Hansson [33] and Rod [34], with the increase in system agitation there is an increase in the mass transfer between phases, however, high agitation increases the surface area of droplets, it provokes a body-rigid behaviour and reduces the mass transfer rate. It is known that the extract phase is rich in PEG4000 and from Table 1, it can be seen that this phase has a lower affinity for α- and β-amylase enzymes, so increasing the flux rate increases the PEG concentration and reduces the FP value [9,16]. Thus, it has been confirmed that with lower vane rotation and flux rate and higher free area of the vane, the purification factor (FP) of the salt-rich flux is maximized.

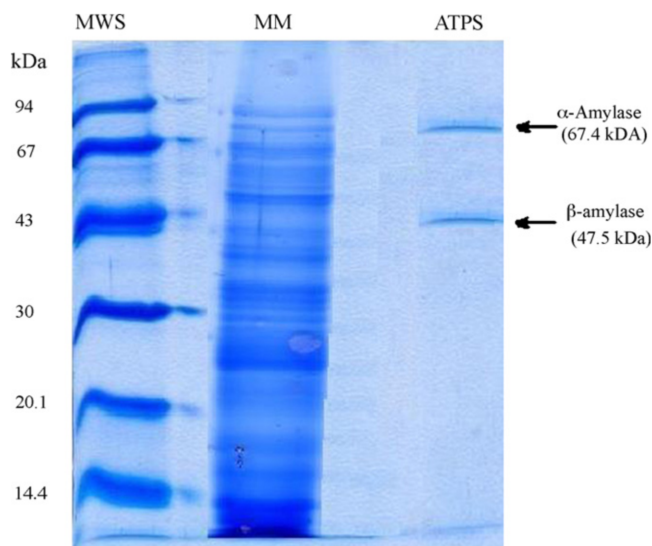

Fig. 11. Molecular weight determination by SDS-PAGE. MWS is molecular weight standard, it is compound of following proteins: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa), MM is proteins of maize malt in concentrate sample and ATPS is amylase enzymes from maize malt obtained of continuous PEG4000/$CaCl_2$ ATPS.

After the continuous ATPS process, the bottom phase sample of assay 1 (75 rpm, 1:1 of flow rate and 18% free area of vane) was analyzed by SDS-PAGE for determination of enzyme molecular weights and degree of purification (Fig. 11). The analysis of this figure shows that the eluted material is pure (two proteins were captured from maize malt sample (MM)) and their molecular weights were 67.4 and 47.5 kDa, respectively, due to α- and β-amylase, because, according to Forgaty and Kelly [10] and Reguly [21], α-amylase molecular weight is between 50 and 120 kDa and β-amylase molecular weight is between 20 and 50 kDa. Some authors have reported the molecular weights of α-amylase from different sources: from *A. suum* (83 kDa), *H. dromedarii* (106 kDa) [35], from *B. stearothermophilus* (59 kDa) [36] and *L. manihotivoras* (135 kDa) [30] and from *A. niger* (116 kDa) [5].

4. Conclusion

Extraction curves show that the micro-column operated by plug-flow or back-mixing. In the PEG-rich and salt-rich fluxes, there were good enzymatic activities. Process optimization showed that the purification factor was maximized with low vane rotation and flux rate and high free area of vane. The purification factor was about 130 times, more than has been reported in the current literatures and shows that the continuous PEG4000/$CaCl_2$ ATPS is the best system for α- and β-amylase recovery. Thus it is possible to increase the value of the maize culture by enzyme production and partition the enzymes by continuous PEG4000/$CaCl_2$ ATPS in a liquid–liquid extraction micro-column.

Nomenclature

A_{free}	vane free area
C	total protein concentration (mg/L)
C_0	initial total protein concentration (mg/L)
F_{Calc}	calculated F-test
F_{Tab}	tabled F-test
FP	purification factor
R_P	vane rotation (rpm)
R_Q	flux rate
R^2	multiple correlation
SE_{crude}	malt specific activity (SKB/mg)
SE_{phase}	specific activity of analyzed phase (SKB/mg)
x_1, x_2, x_3	coded factors

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