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Research Article

Influence of Different Solvents in the Recovery and Pre-Purification of Pectinases from *Aspergillus niger*

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Abstract: The present work aimed at studying the influence of different solvents (n-propyl alcohol, isopropyl alcohol and acetone) in the recovery and pre-purification of pectinases (exo-polygalacturonase-exo-PG, pectinmethylesterase-PME and pectin lyase-PMGL) from *Aspergillus niger*. The best results were obtained using isopropyl alcohol in the precipitated phase with purification factor and recovery of 2.1-fold and 20.5% for exo-PG, 7.9-fold and 77.8% for PME and 2.5-fold and 216% for PMGL enzyme. The studied method is promising in the recovery and pre-purification of pectinases enzymes.

Keywords: Pectin lyase, pectinmethylesterase, polygalacturonase, precipitation, purification, solvents

INTRODUCTION

The application of enzymes in biotechnological and industrial processes is fairly wide and diverse, covering areas such as food, textile and detergent industries (Gomes *et al*., 2011). The use of enzymes in industrial processes has been identified as a reducing factor on the energy required for obtaining certain manufactured products, improving quality, extractive processes yield and products stabilization.

Organic solvents precipitation is used to separate the solute in the solid conversion, which can be subsequently removed by solid/liquid separation. Due to its low dielectric constant (compared to water), the organic solvents increase the attraction between protein molecules. Aggregates are formed until the particles reach macroscopic proportions and then precipitate (Cortez and Pessoa Jr., 1999).

Precipitants that do not denature biological products, such as enzymes, may also be used, forming a precipitate that is normally more stable than the soluble material (Cortez and Pessoa Jr., 1999; Golunski *et al*., 2011; Soares *et al*., 2012). Most common precipitants involve salts, polyelectrolytes and organic solvents (Golunski *et al*., 2011; Soares *et al*., 2012; Valetti *et al*., 2012; Braia *et al*., 2013).

Organic solvents may also precipitate protein, reducing the solubility of proteins in their native state at low temperatures. However, as opposed to the salting-out salts, alcohols and other organic solvents destabilize protein that can be denatured at high concentrations or at high temperatures due to their favorable interactions with hydrophobic groups (Yoshikawa *et al*., 2012).

The main advantages of precipitation technique for pre-purification and concentration are the nonneed for equipment switching, easy scale-up and allowing the use of vast quantities of precipitants (Soares *et al*., 2012).

The present study reports the concentration and pre-purification of pectinase (exo-polygalacturonase-exo-PG, pectinmethylesterase-PME and pectin lyase-PMGL) produced from*Aspergillus niger* ATCC 9642 by precipitation with different organic solvents, n-propyl alcohol, isopropyl alcohol and acetone.

MATERIALS AND METHODS

Pectinase enzyme production: The complex-enzymes (exo-polygalacturonase, pectin methyl esterase and pectin lyase) were produced by the strain *Aspergillus niger* ATCC 9642 using submerged fermentation in a citrus pectin (32 g/L), L-asparagine (2 g/L), sulfate iron (1 g/L) and potassium phosphate (0.06 g/L) growth medium, at 30°C, initial pH 5.5, 180 rpm stirring and 27 h bioproduction in an orbital incubator (New

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Ethics), according to methodology described by Gomes *et al*. (2011). After bioproduction, the fermented medium was filtered (Whatman n°. 1), yielding the crude enzyme extract, which was stored in polyethylene containers, vacuum closed and stored at -20°C.

Precipitation of enzymes with acetone, isopropyl and n-propyl: Preliminary tests were carried out for the precipitation of exo-PG using acetone (Vetec, Rio de Janeiro, Brazil), isopropyl alcohol (iPA) (Quimex, São Paulo, Brazil) and *n*-propyl alcohol (nPA) (Synth, São Paulo, Brazil) at 56.2% and 67% concentrations, added to the enzyme extract aliquotusing flow rates from 3.0- to 20 mL/min. Control samples were prepared replacing the enzyme extract with distilled water. Enzyme activity was determined both in the precipitate and supernatant phases. The results were analyzed based on the Purification Factors (PF) and recovery yields (R) of enzymes.

Based on the preliminary tests results, a 2^2 full factorial design was performed using isopropyl alcohol concentration levels of 10, 22, 50, 78 and 90 % (v/v) and 0.1, 3.0, 10, 17 and 20 mL/min flow rates. The enzyme extract volume for each test was 10 mL and kept in an ice bath at 4°C, under slow stirring. After adding the solvent, the samples were left to stand for 1 h for complete precipitation and centrifuged (centrifuge MPW-351R) at 2150×g for 15 min at 4°C. Control samples were prepared using distilled water instead of sample. The runs were performed in duplicates. Enzyme activity (exo-PG, PME and PMGL) was determined both in the precipitate and supernatant phases, for purification factorand enzymes recovery calculation.

Exo-Polygalacturonase (exo-PG) activity: Polygalacturonase (exo-PG) activity was determined by measuring the release of reducing groups using the dinitrosalicylic acid method (DNS), as originally proposed by Miller (1959), with some modifications.

The substrate solution consisted of 0.5% citrus pectin (Sigma) in a sodium acetate buffer at pH 5.5. A 1000 µL aliquot of this solution was incubated at 37°C for 15 min for temperature equilibriumand then, 500 µL of enzyme extract were added to the substrate solution. After 6 min, 1 mL DNS solution was added to the mixture and the vial boiled for 5 min. The mixture was then cooled in an ice bath and 8.0 mL of 50 mM sodium and potassium tartrate was added for color stabilization. The absorbance was measured at 540 nm (Spectrophotometer-Beckman Coulter, DU640). For specific activity determination (U/mg), the enzyme activity (U/mL) was divided by protein content (mg/mL), which was quantified by method from Bradford (1976) with bovine serum albumin (Sigma A3294) as standard. One exo-polygalacturonase activity unit is the amount of enzyme needed to release 1 µmol of galacturonic acid per minute (U = µmol/min), according to a standard curve established with D-galacturonic acid (FlukaChemica) as a reducing sugar.

Pectin Methyl Esterase (PME) activity: Pectinmethyl esterase activity was determined by method proposed by Hultin *et al*. (1966). A 30 mL substrate solution aliquot (1% citrus pectin solution in 0.2 mol/L sodium chloride), had its pH adjusted to 7.0 with 0.01 mol/L sodium hydroxide in a water bath at 20°C. Then, 1 mL of enzyme extract was added to the substrate and the solution was titrated with 0.01 mol/L sodium hydroxide for 10 min, keeping the reaction mixture at pH 7.0. For specific activity determination (U/mg) the enzyme activity (U/mL) was divided by protein content (mg/mL), which was quantified as per methodology by Bradford (1976) with bovine serum albumin (Sigma A3294) as standard. One PME unit was defined as the amount of enzyme capable to catalyze pectin demethylation corresponding to the consumption of 1 µmol of NaOH/min/mL, under assay conditions.

Pectin lyase (PL or PMGL) activity: Pectin lyase activity was determined as described by Ayers *et al*. (1966). A5 mL substrate solution aliquot (1% citrus pectin solution in Tris-HCl buffer at pH 8.5) was mixed with 1 mL of 0.01 mol/L $CaCl_2$, 1 mL enzyme extract and 3 mL distilled water. The reaction medium was incubated for 2 h at 30°C. After that, 0.6 mL of 9% $ZnSO_4.7H_2O$ and 0.6 mL of 0.5 mol/L NaOH were added to the mixture, centrifuged at 3000×g for 10 min at 5°C. The supernatant was collected and 3 mL of 0.04 mol/L thiobarbituric acid, 1.5 mL of 0.1 mol/L HCl and 0.5 mL of distilled water were added before. The mixture was boiled for 30 min and then cooled in an ice bath. The absorbance was measured in a spectrophotometer (Beckman Coulter, DU640 model) at 550 nm. For specific activity determination (U/mg), the enzyme activity (U/mL) was divided by protein content (mg/mL), which was quantified according to methodology by Bradford (1976) using bovine serum albumin (Sigma A3294) as standard. One enzyme activity unit was defined as the enzyme amount that causes a change of 0.01 units in absorbance at 550 nm, under assay conditions.

Polyacrylamide gel electrophoresis (SDS-PAGE): Polyacrylamide gel electrophoresis of samples with higher PF was carried out, following method proposed by Laemmli (1970). Stacking gel was removed after the run and the gel resolution was stained with silver nitrate for approximately 24 h until perfect bands visualization. Subsequently, the gel was placed in a acetic acid solution to stop the development.

Purification Factor: The Purification Factor (PF) was calculated using Eq. (1) (Ooi *et al*., 2009; Antov and Omorjan, 2009; Mehrnoush *et al*., 2011), is a measure for purification systems monitoring.

Table 1: Exo-PG enzyme Purification Factor (PF) and Recovery (R) after precipitation with solvents

				Precipitate		Supernatant	
Run	Solvent	Concentration (%)	Flow rate (mL/min)	PF*	R* (%)	PF*	R* (%)
1	Acetone	56.2	10	0.1	2.5	0.6	20.1
2	Acetone	67	3.0	1.4	55.6	0.4	21.2
3	Acetone	67	17	1.4	54.4	0.2	13.6
4	nPA	56.2	10	0	0.5	0	30.7
5	nPA	56.2	20	0	4.9	1.8	33.2
6	nPA	67	3.0	0	33.4	0	32.1
7	nPA	67	17	0	50.0	0	40.6
8	nPA	67	20	0	9.6	0.5	7.7
9	iPA	56.2	10	0	0	0.3	11.9
10	iPA	56.2	20	0	0.7	2.0	24.0
11	iPA	67	3.0	20.0	56.6	0	21.8
12	iPA	67	17	12.7	40.0	0	18.2
13	iPA	67	20	0	36.1	0.6	16.8

*Three replications average

$$FP = \frac{A_f}{A_i} \quad (1)$$

where,

A_f = Specific enzyme activity of step (U/mg)

A_i = Initial specific activity of crude extract (U/mg) (crude extract before equilibrium phase (SAB) or precipitation)

Enzyme recovery: Enzyme recovery (R) was calculated by Eq. (2) (Ooi *et al*., 2009; Antov and Omorjan, 2009; Mehrnoush *et al*., 2011):

$$R = \frac{A_f \times V_f}{A_i \times V_i} \times 100 \quad (2)$$

where,

A_f = Total activity of enzyme extract phase (U/mL)

Ai = Total activity of crude extract in the diet (U/mL)

Vi = Initial volume of crude extract added in mL

Vf = Phase volume in mL

Statistical treatment: Purification factor and recovery results for pectinase were statistically treated by the experimental design methodology. The models were validated by Analysis of Variance (ANOVA). For comparison of sample averages significant differences, ANOVA was carried out followed by averages comparison using Tukey test at 95% for confidence level.

RESULTS AND DISCUSSION

The results for exo-PG precipitation with solvents are shown in Table 1 for the precipitate and supernatant phases. It is noted that the enzyme is mostly recovered in the precipitate. However, under some conditions (1, 4, 5, 8, 9 and 10), the enzyme was recovered in the supernatant, indicating lesser ability for protein precipitation under these conditions. The balances for enzymatic activity recovered in both phases show a great activity loss in all cases, probably due to enzyme instability in the solvents. Maximum total recoveries (precipitate and supernatant) were obtained in runs 2, 3, 7 and 11. However, the PF for those conditions were very low or even null, except in run 11 that was carried out with slow addition of 67% isopropyl alcohol (v/v), yielding 57 % recovery and 20 PF in the precipitate. Another noteworthy condition was run 12, which was also carried out with 67 % isopropyl alcohol (v/v) at a 17 mL/min flow rate, yielding 40 % recovery and 12.7 PF. This result suggests that, enzyme recovery and purification factor, are very sensitive to the addition rate of the solvent. Isopropyl alcohol demonstrated to be the best precipitating agent since it leads to lower activity losses and good PF.

The results obtained using ethanol precipitation were still lower than those obtained with other solvents. The low recoveries obtained with solvents may be related to enzyme denaturation when exposed to solvents, as demonstrated by other studies (Cortez and Pessoa Jr., 1999; Ooi *et al*., 2009).

Based on the results listed in Table 1, an experimental design was carried out to investigate the effects of alcohol concentration and its addition rate to the sample on PF and R. The results for the precipitated and supernatant phase are presented in Table 2 and 3, respectively. The best PF (2.1) for exo-PG was achieved in run 6. The divergence from results in Table 1 maybe attributed to the difference in the enzyme extract batch. This shows that precipitation results are very susceptible to small changes in the extract composition. It was observed that in the studied range, quadratic reaction concentration and flow rate showed significant negative effects ($p<0.10$) compared to purification factor, as seen in the Pareto chart (Fig. 1a) for the exo-PG enzyme precipitated phase.

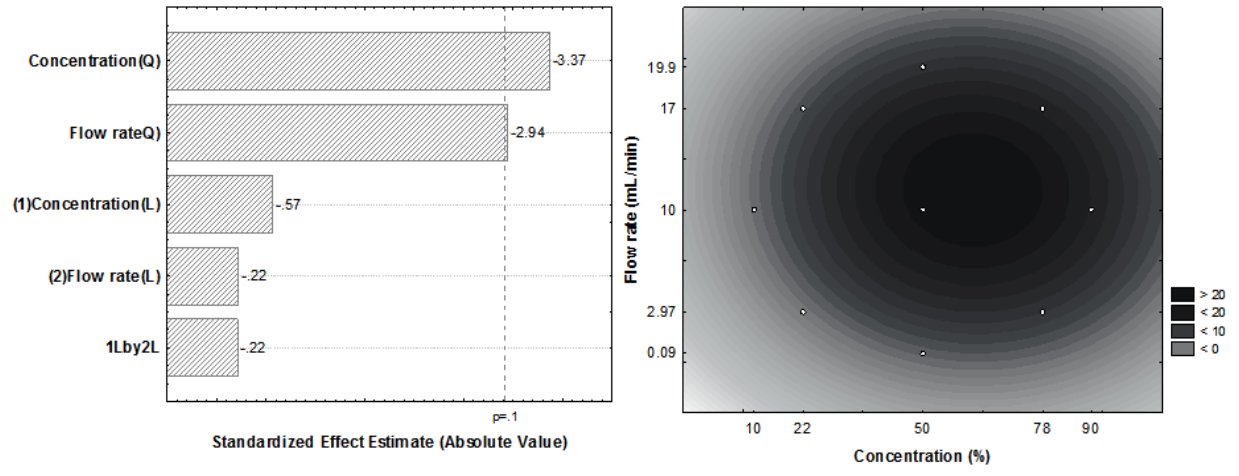
The encoded second-order statistical model is valid for R of exo-PG enzyme precipitated phase as the F calculated value was1.51 times greater than the F tabulated one $_{and}$ a 0.91 regression coefficient. Eq. (3) shows the encoded recovery planning model:

Table 2: Matrix of 2^2 full factorial design (coded and real values) for pectinase concentration and purification by precipitation with isopropyl (iPA) and responses in terms of purification factor and recovery on the precipitated phase

	Independent variables		Exo-PG		PME		PMGL	
Run	Concentration (%)	Flow rate (mL/min)	PF	R (%)	PF	R (%)	PF	R (%)
1	-1 (22)	-1 (3.0)	0	0	0	0	0	0
2	+1 (78)	-1 (3.0)	0.16	8.8	0	0	0	0
3	-1 (22)	+1 (17)	0.01	0.89	0.3	22,2	2.5	216
4	+1 (78)	+1 (17)	0.28	8.4	1.5	44.4	0	0
5	-1.41 (10)	0 (10)	0	1.8	0	11.1	0	118
6	+1.41 (90)	0 (10)	2.1	20.5	7.7	77.8	2.3	73.4
7	0 (50)	-1.41 (0.1)	0.01	1.7	0.1	22.2	0.83	133
8	0 (50)	+1.41 (20)	0.29	18.0	0.5	33.3	0.95	59.8
9	0 (50)	0 (10)	0.95	21.8	2.4	55.6	1.2	93.3
10	0 (50)	0 (10)	0.95	21.2	2.7	44.4	1.2	71.3
11	0 (50)	0 (10)	1.2	19.5	3.2	33.3	1.2	81.0

Table 3: Matrix of 2^2 full factorial design (coded and real values) for pectinase concentration and purification by precipitation with isopropyl (iPA) and responses in terms of purification factor and recovery on the supernatant phase

	Independent variables		Exo-PG		PME		PMGL	
Run	Concentration (%)	Flow rate (mL/min)	PF	R (%)	PF	R (%)	PF	R (%)
1	-1 (22)	-1 (3.0)	0.98	50.9	0	0	2.4	124
2	+1 (78)	-1 (3.0)	0.09	6.0	0	0	0.04	2.7
3	-1 (22)	+1 (17)	0.25	35.3	0.24	33.3	0.80	116
4	+1 (78)	+1 (17)	0.04	2.8	0	0	2.0	142
5	-1.41 (10)	0 (10)	0.12	14.9	0.69	83.3	0	0
6	+1.41 (90)	0 (10)	0.16	5.6	1.4	50.0	1.9	66.3
7	0 (50)	-1.41 (0.1)	0.09	23.0	0	0	0.47	156
8	0 (50)	+1.41 (20)	0.24	14.5	0.46	27.8	4.1	246
9	0 (50)	0 (10)	1.0	50.8	0.57	16.6	1.8	271
10	0 (50)	0 (10)	1.1	50.4	0.49	22.2	1.8	271
11	0 (50)	0 (10)	1.1	45.1	0.33	22.2	2.1	248



(a) (b)

Fig. 1: Pareto chart; (a): with the estimated effects (absolute value) for the purification factor and contour curve; (b): for the recovery obtained with 2^2 full factorial design for exo-PG enzyme precipitated phase using isopropyl alcohol

$$R = 20.85 + 5.36 \times (C) - 6.37 \times (C)^2 + 2.95 \times (V) - 7.014 \times (V)^2 \quad (3)$$

where,

R = Recovery
C = Alcohol concentration
V = Reaction flow rate

The model allowed the contourcurve construction (Fig. 1b); demonstrating optimizing the exo-PG precipitated phase recovery using isopropyl alcohol in the region close to 10 mL/min flow rate and 50% isopropyl alcohol concentration.

Analysis of variance validated the encoded second-order model Eq. (4), which describes the PF for the

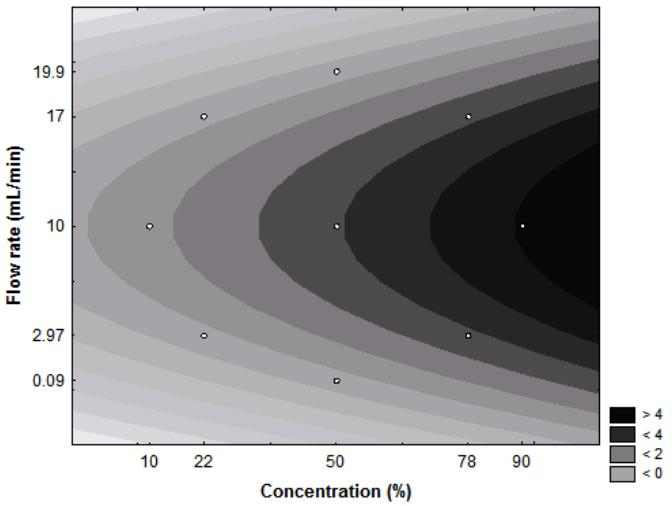


Fig. 2: Response surface: purification factor for PME enzyme precipitated phase using isopropyl alcohol

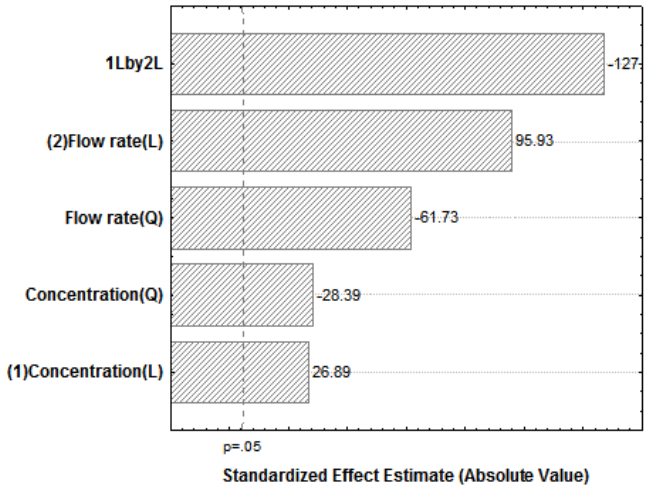


Fig. 3: Pareto chart with the estimated effects (absolute value) obtained with 2^2 full factorial design for the precipitated phase using isopropyl alcohol in comparison to the purification factor for PMGL enzyme

PME enzyme precipitated phase as a function of the variables tested within the studied range and the F calculated value was 1.67 times greater than the F tabulated one, with a 0.81 regression coefficient. It was possible to construct a contour curve for R as described in Fig. 2, showing that alcohol at concentrations above 78% and a 10 mL/min flow rate presented a maximized PF region (~ 7.86):

$$PF = 2.77 + 1.54 \times (C) - 1.65 \times (V)^2 \quad (4)$$

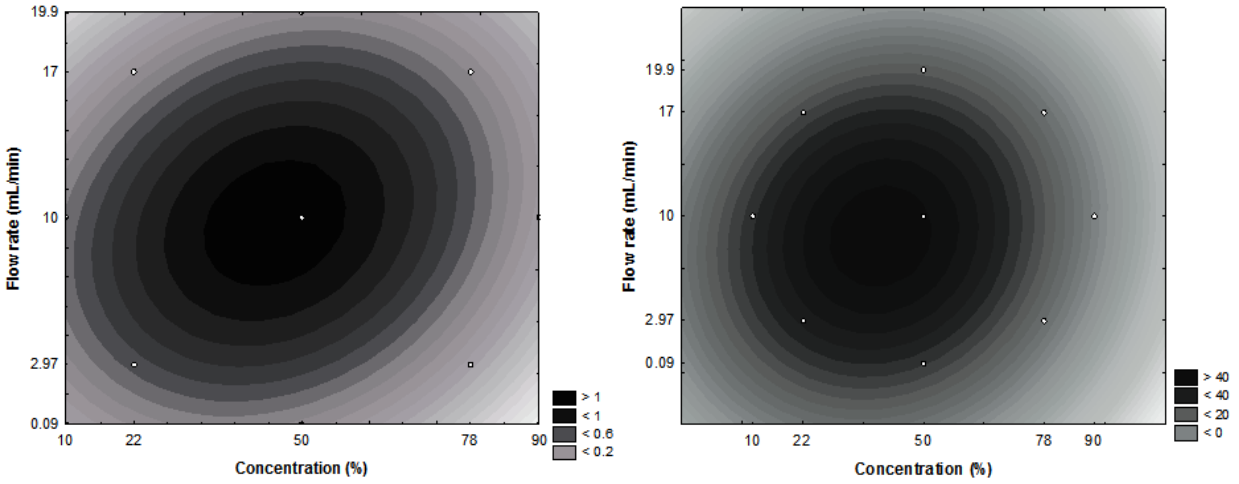
where,
PF = Purification factor
C = Alcohol concentration
V = Reaction flow rate

The R for PME enzyme precipitated phase of the planning study did not present any significant effect.

In terms ofPF for PMGL enzyme precipitated phase, all variables showed significant effects, as seen in Fig. 3. However, the precipitated phase value had no significant effect in the studied groups.

Table 3 shows the matrix of 2^2 full factorial design, with the (actual) coded values of the precipitation test with isopropyl and responses in terms of Purification Factor (PF) and Recovery (R) for exo-PG, PME, PMGL enzymes and the supernatant phase.

The encoded second-order model Eq. (5) was validated by analysis of variance, which describes the PF for exo-PG enzyme supernatant phase as a function of the variables, within the range studied. The F calculated value was 1.37 times greater than the F tabulated one, with a 0.90 correlation coefficient, which also enabled the construction of a contour curve (Fig. 4a). The nonsignificant effects were added to the lack of fit to perform the analysis of variance. Figure 4a



(a) (b)

Fig. 4: Contour curve; (a): for the purification factor and contour curve; (b): for the recovery obtained with 2^2 full factorial design for exo-PG enzyme supernatant phase using isopropyl alcohol

shows that the use of close to 50% ethanol concentrations and a 10 mL/min flow rate presents the maximum PF (1.12):

$$FP = 1.06 - 0.131 \times (C) - 0.416 \times (C)^2 - 0.403 \times (V)^2 + 0.17 \times (C) \times (V) \quad (5)$$

where,
PF = Purification factor
C = Alcohol concentration
V = Reaction flow rate

Equation 6 shows the encoded second-order model that describes the R for exo-PG enzyme supernatant phase as a function of the variables tested within the studied range. The model was validated by analysis of variance, as the F calculated value was 1.85 times greater than the F tabulated one, with a 0.88 correlation coefficient, the nonsignificant effects were added to a lack of fit. Thus, allowing the construction of a contour curve (Fig. 4b) for R, which demonstrates the R maximization (~ 50.45%), in the region close to 50% alcohol and 10 mL/min flowrate:

$$R = 48.75 - 11.36 \times (C) - 16.97 \times (C)^2 - 12.73 \times (V)^2 \quad (6)$$

where,
R = Recovery
C = Alcohol concentration
V = Reaction flow rate

Figure 5a and 5b present Pareto charts with PF and R estimated effects for PME enzyme supernatant phase, respectively. Only the mean flow had a significant quadratic negative effect ($p<0.05$), showing that PF tends to decrease when the flow is increased. For R, all independent variables had a significant effect (Fig. 5b) and the best results obtained for PF and R (Table 3), were 1.41 and 50%, respectively.

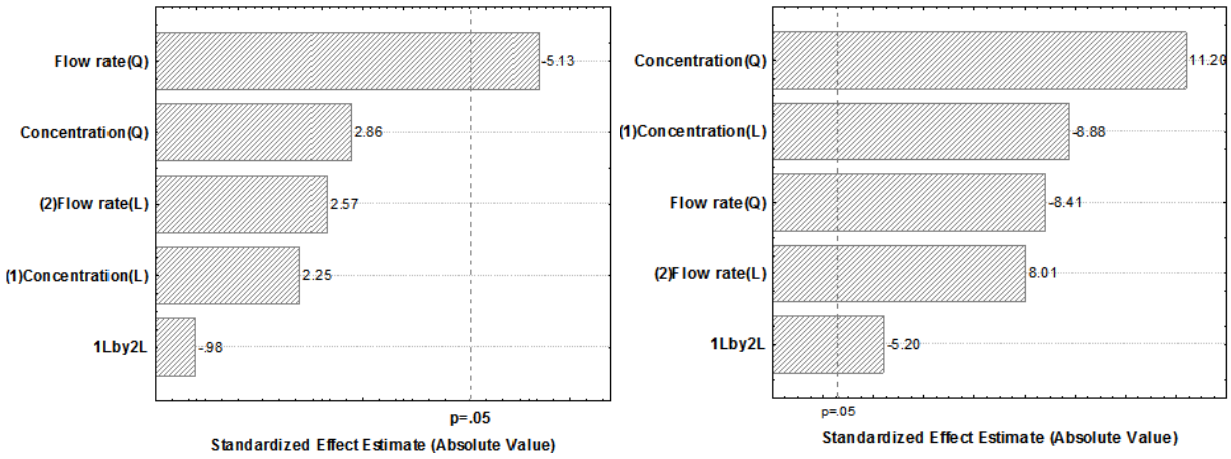
Figure 6a shows the Pareto chart for the PF for PMGL enzyme supernatant phase. The reaction flow rate and interaction between concentration and flow rate had significant positive effects ($p<0.05$) in the dependent PF variable. The best results for PF and R for PMGL enzyme supernatant phase was obtained in run 8 (Table 3), with PF and R of 4.12 and 246.07%, respectively.

Equation 7 shows the encoded second-order model for R for PMGL enzyme supernatant phase obtained with the variables tested, within the studied range. The model was validated by analysis of variance, as the F calculated value was 5.62 times greater than the F calculated one, with a 0.97 correlation coefficient. Nonsignificant factors were added to the lack of fit for Analysis of Variance (ANOVA). Thus, it allowed the construction of a contour curve (Fig. 6b) for R, which demonstrates maximizing in the region close to 50% alcohol and to a 10 mL/min flow rate:

$$R = 263.29 - 120.81 \times (C)^2 + 32.255 \times (V) - 36.387 \times (V)^2 + 36.94 \times (C) \times (V) \quad (7)$$

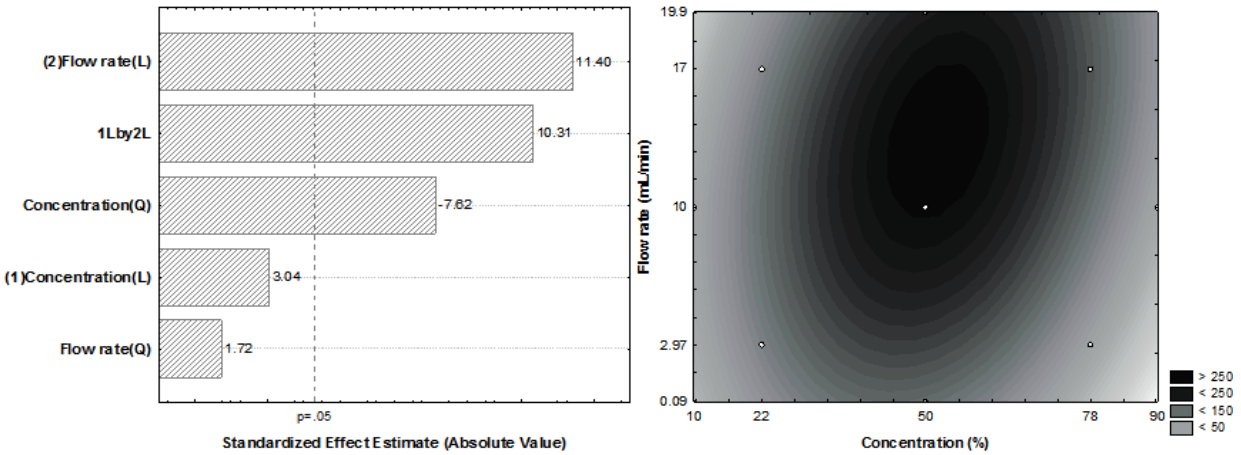
where,
R = Recovery
C = Alcohol concentration
V = Reaction flow rate

The SDS-PAGE of different fractions of the enzyme extract after precipitation with 50% isopropyl



(a) (b)

Fig. 5: Pareto chart; (a): with the estimated effects (absolute value) for the purification factor and Pareto chart; (b): with the estimated effects (absolute value) for the recovery obtained with 2^2 full factorial design for PME enzyme supernatant phase using isopropyl alcohol



(a) (b)

Fig. 6: Pareto chart; (a): with the estimated effects (absolute value) for the purification factor and contour curve; (b): for the recovery obtained with 2^2 full factorial design for PMGL enzyme supernatant phase using isopropyl alcohol

alcohol and 10 mL/min flow rate is shown in Fig. 7. A small variation in the protein profile can be obtained when the crude extract (line 2) is compared to the treated extract. Kant *et al*. (2013) studied the purification of polygalacturonase from *Aspergillus niger* MTCC 3323 with 60% ethanol precipitation by gel-filtration chromatography, from which SDS-PAGE analysis showed molecular mass of 69 and 34 kDa, close to the results found in this study (70 and 60 kDa).

CONCLUSION

The best results for purification factor and recovery of pectinase enzymes in the precipitated phase were 2.08 times and 20.53% for the exo-polygalacturonase enzyme and 7.86 times and 77.78% for pectin methylesterase, both using a 90% isopropyl alcohol concentration and a 10 mL/min flow rate feed. For pectin lyase enzyme, the best results were 2.54 times for PF and 216.44% for recovery at 22% alcohol and 17 mL/min flow rate. In the supernatant, it was possible to obtain a purification factor and recovery of 1.12 and 50.45% for the exo-polygalacturonase enzyme with 50% isopropyl alcohol and 1.41 and 50% for the pectinmethylesterase, at a 90% alcohol concentration and 4.12 and 246.07% for pectin lyase in 50% concentration, all with a10 mL/min flow rate, which is the maximum region for purification factor and recovery. The method studied was effective in the purification of pectinases (exo-polygalacturonase, pectinmethylesterase and pectin lyase).

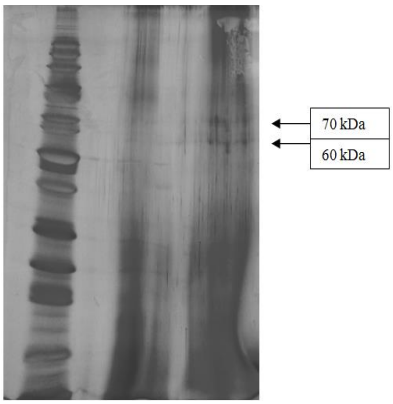


Fig. 7: SDS-PAGE of different fractions of enzyme extract. From left to right: Line 1: Molecular weight marker (from top to bottom) 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15, and 10 kDa. Line 2: Crude enzyme extracts (*Aspergillus niger*). Line 3: Enzyme extracts after precipitation with 50 % isopropyl alcohol at 10 mL/min flow rate

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