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STUDY ON FUNGAL EXOPOLYGALACTURONASE PRECIPITATION WITH ORGANIC SOLVENTS

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Abstract

Pectinases constitute a group of enzymes that act on pectic substances, acid polysaccharides present in the primary wall of plants. Pectinases are used in the food industry, in the processing of fruits, in pulp maceration, in the extraction and clarification of juices and in the wine industry. The juice extraction is carried out, generally, by the pressing and partial destruction of the pectin through the enzymatic treatment of the pulp. The application of pectinases in the extraction, purification and clarification of citric fruit juices, as agents facilitating the extraction operation, allowes an increase in the quantity of juice extracted and improves the fruit pigment extraction. An increase in the efficiency of essential oils extraction from citric fruits is also indicated as an advantage of the use of pectinases. The concentration processes of these enzymes obtained through fermentation by the solvent precipitation method with organic solvents, such as: ethanol, acetone, propanol and isopropanol, at temperatures of 0ºC, 4ºC, 10ºC and 20ºC and concentrations of 10%, 20%, 30%, 40% and 50% (v/v/), were studied and the best results were obtained with ethanol as the precipitation agent, mainly at temperatures of the 0ºC and 10ºC. The results obtained for the precipitation at 0°C, at an ethanol concentration of 20%, indicated an approximately 100% recovery of enzymatic activity which demonstrates the success of the method applied.

Key words: Pectin, extraction by precipitation , exopolygalacturonase.

I. INTRODUCTION

The use of enzymes in the industrial sector requires good quality preparations with costs compatible with the type of application. This includes the pectinolytic enzymes or pectinases.

The pectinolytic preparations are formulated to contain one or more types of enzymes, depending on the type of application, constituting an enzymatic cocktail (Peter, 1986). Among the organisms which produce fungal pectinases the following have been reported: *Aspergillus niger, Aspergillus oryzae* and *Aspergillus wentii* (Fogarty and Kelly, 1983). The former offers the advantage of being classified as GRAS ("Generally Recognized as Safe"), by the US Food and Drug Administration (Pariza and Foster, 1983).

For the concentration and extraction of the enzymes present in cultivation media, various physico-chemical processes are used, such as: filtration, sedimentation/flocculation, centrifugation, microfiltration (MF), ultrafiltration (UF), dialysis, reverse osmosis (RO), electrodialysis (ED) and precipitation.

Precipitation is the oldest method for the recovery and purification of proteins and enzymes of microbial, animal or vegetal origin, being used at the laboratory and industrial scales (Cohn *et al*, 1940). At larger scales, however, the precipitation of proteins is limited to five conventional methods (Oliveira, 1996), which may be described as: the use of salting-out to promote the hydrophobic interactions in the reduction of the protein hydration layer; organic solvents in order to reduce the dielectric constant of the medium increasing the intermolecular electrostatic interactions; non-ionic polymers which may exclude

the aqueous phase protein reducing the water quantity available for the protein solvation; polyelectrolytes to promote the binding with a protein molecule acting as a flocculation agent; metal salts capable of forming complexes; isoelectric precipitation to neutralize the global charge of the protein by ionization of the amino acid side chains through altering the medium pH.

Since the beginning of the 20th century, small chain alcohols, acetone, ethers and other organic solvents miscible with water have been used to precipitate proteins, however, the denaturation effects of the solvents were unknown. According to Wiseman (1991), the addition of organic solvents to aqueous solutions of proteins reduces their solubility on reducing the dielectric constant of the medium. With increasing quantities of organic solvents, the protein molecules interact more with other protein molecules than with water. The classic application of precipitation with solvents has been the cold-ethanol fractionation of blood plasma proteins, proposed by Cohn *et al*, (1940, 1946). Some advantages and disadvantages of the organic solvent used as the protein precipitant are described by Scopes (1994): (i) conformational changes may occur in the protein; (ii) the removal stages are of a drastic form; (iii) they may have negative hydrophobic interactions (iv) the density difference with water facilitates the decantation of the precipitate.

Many authors (Chan *et al.*, 1986; Rothstein, 1994; Bell *et al*,1983; Glatz, 1990; Scopes, 1994; Englard and Seifter, 1971) have described precipitation by organic solvents as being due to the reduction in the dielectric constant of the medium, through the addition of solvent, which increases the electrostatic forces of attraction between the protein molecules.

The precipitant solvents (Scopes, 1994) must be totally miscible in water, not react directly with the proteins and have a good precipitant effect. The most used solvents are: methanol, ethanol, acetone, n-propanol, isopropanol, dioxine, 2-methoxy ethanol and other alcohols: ethers and cetones. Their efficiency in the denaturation follows the following order: methanol< ethanol < propanol < butanol. The greatest denaturation effect of alcohols with aliphatic chains is a result of the increase in hydrophobic interactions with apolar groups of the protein and a weakening of the intraprotein interactions (Rothstein, 1994).

Some variables affect the process of protein precipitation with the risk of denaturation, such as: temperature; the most distant isoelectric point (pI) value and the pH of the operation. For proteins of equivalent hydrophobicity and isoelectric point, the greater the protein molecular weight the lower the percentage of organic solvent necessary to precipitate it, since the larger molecules aggregate more easily, because there is a greater probability for the charged areas to interacted with each other (Scopes, 1994).

In the precipitation with solvents, the proteins may undergo conformational changes, since the free energy of denaturation induced by ethanol, for example, is strongly related to the temperature, and at temperatures below 10°C the protein stabilizes in its native conformation. Different solvents result in different effects in the final conformation of proteins, which is also dependent on the temperature and on the solvent concentration (Schubert and Finn, 1981).

In this study, the concentration of the fungal pectinase exopolygalacturonase (exo-PG), obtained in a submersed process with *A. oryzae*, by precipitation with organic solvents (ethanol, acetone, n-propanol and isopropanol) was investigated.

II. MATERIAL AND METHODS

The microorganism utilized was the cell line CCT3940 of *A. oryzae*, donated by the Biotechnology group at IPT-USP (Brazil).

The conservation and preparation of the inocula, in solid medium, was carried out according to Maiorano (1982), with the following composition ($g.L^{-1}$): glycose, 25.0; glycerine, 25.0; yeast extract, 5.0; agar-agar, 20. The medium was distributed in culture tubes (8 mL/tube), sterilized at 121°C for 20 minutes and inclined for solidification.

The production of exopolygalacturonase by *A. oryzae*, followed the method of Malvessi and Silveira (2004): ($g.L^{-1}$): wheat bran, 40; citric pectins (Delaware, Brasil), 10; yeast extract (Sigma, USA), 0.5; $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 2.5; $MgSO_4$, 0.5; $FeSO_4.7H_2O$, $6.3x10^{-5}$; $ZnSO_4$; $6.2x10^{-5}$; $MnSO_4$, $1.0x10^{-6}$. The pH 4.0, with NaOH or H_2SO_4, distributed in 500 mL flasks, autoclaved for 20 minutes, at 121°C.

The preparation of the inocula was carried out in a laminar flow chamber, with a 0.9% solution of NaCl (p/v) in a sterilized tube with solid medium containing *A. oryzae* spores. The tubes were shaken to liberate the spores, forming a suspension whose concentration was determined by counting in a Neubauer chamber. The inoculation was prepared to obtain 1.0×10^5 spores $.L^{-1}$, of medium produced.

The cultivation media were inoculated, conserved and incubated in a reciprocal shaker, at 28°C at 180 rpm, for 96 hours. After the incubation, the flasks were weighed and kept under refrigeration for later processing.

The tests for the enzyme thermostability were carried out from 10°C to 50°C, with 10 mL of the enzymatic broth, for 3 hours in a thermostatic bath and the enzymatic activity quantified.

In the tests with the precipitation agents ethanol, acetone, n-propanol and isopropanol (Merck), concentrations of 10% to 50% (v/v) were used, at temperatures of 0°C, 4°C, 10°C and 20°C, for periods of 20 minutes, being centrifuged at 4000 rpm for 15 minutes and the supernatant and the precipitate separated with re-suspension to the initial volume in order to compare the enzymatic activities of the two phases.

In Fig. 1, the experimental sequence is represented schematically.

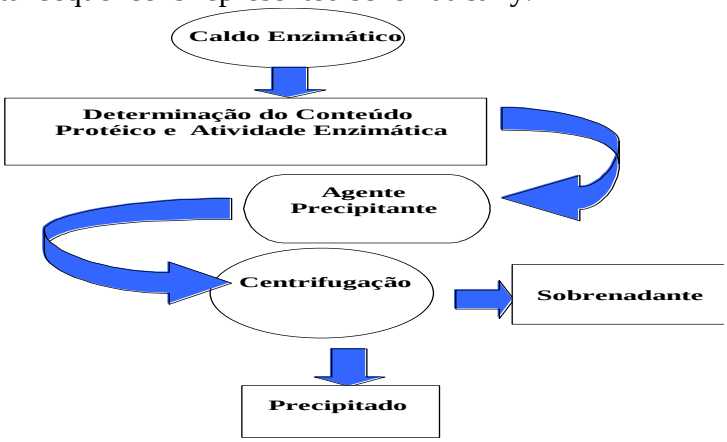


Figure 1 – Schematic representation of the system used in the tests on exo-PG precipitation with organic solvents

The production media of the enzymatic broth cultivated with *A. oryzae* were centrifuged at 9000 rpm for 10 minutes. The protein content was determined according Bradford (1976) and the exo-PG activity estimated as described by Malvessi and Silveira (2004).

III. RESULTS AND DISCUSSION

In stage 3 of the precipitation process (Fig. 1), initially, qualitative evaluations of the solvent capacity to promote enzyme precipitation were carried out, in the enzymatic broth, at temperatures of 0ºC, 4ºC, 10ºC and 20ºC.

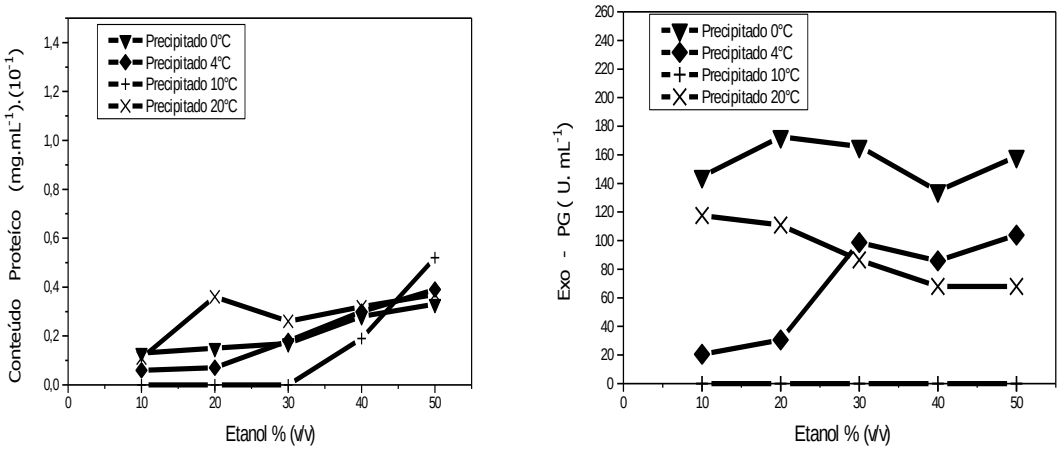
Table 2 – Precipitation of exo-PG with ethanol, acetone, n- propanol and isopropanol at 0ºC , 4ºC, 10ºC and 20ºC.

Concentration (% v/v)	**10**	**20**	**30**	**40**	**50**
0°C					

ethanol	⊗	⊗	⊗	⊗	⊗
acetone	⊗	⊗	⊗	⊗	⊗
n-propanol	Θ	⊗	⊗	⊗	⊗
isopropanol	Θ	⊗	⊗	⊗	⊗
4°C					
ethanol	⊗	⊗	⊗	⊗	⊗
acetone	Θ	Θ	Θ	Θ	⊗
n-propanol	Θ	Θ	Θ	⊗	⊗
isopropanol	Θ	Θ	⊗	⊗	⊗
10°C					
ethanol	Θ	Θ	Θ	⊗	⊗
acetone	⊗	⊗	Θ	Θ	Θ
n-propanol	⊗	⊗	⊗	⊗	⊗
isopropanol	Θ	Θ	⊗	⊗	⊗
20°C					
ethanol	⊗	⊗	⊗	⊗	⊗
acetone	⊗	⊗	⊗	⊗	⊗
n-propanol	⊗	⊗	⊗	⊗	⊗
isopropanol	⊗	⊗	⊗	⊗	⊗

⊗= a precipitate was formed Θ = no precipitate was formed

The solvent concentrations tested were 10%, 20%, 30%, 40% and 50% (v/v), the protein content and the enzymatic activity being evaluated as previously described.



(A) (B)

Figure 2 – Exo-PG precipitations as a function of ethanol concentration at 0ºC, 4ºC, 10ºC and 20ºC. (A) protein content; (B) enzymatic activity

In Fig. 2A, it can be observed that with respect to the protein content in the precipitate and the enzymatic activity the best results were obtained with ethanol at a temperature of 0°C. Rothstein (1994) described that in his studies, at a temperature of 0°C, or below, it can be guaranteed that protein denaturation does not occur, but he also stated that the greater denaturation effect provoked by the alcohols is a result of the increase in the hydrophobic interactions with apolar protein groups and a weakening of the intraprotein interactions of the alcohols with larger aliphatic chains. According to Van

Oss (1989), the use of low temperatures also inhibits strong electrostatic interactions between the proteins, which may lead to an irreversible aggregation between molecules, causing denaturation. However, it can be observed (Fig. 2B) that: i) from concentrations of 20% (v/v) of ethanol, at 0°C, there is no loss of enzymatic activity; ii) with 20% (v/v), at 4°C, it may be an important intersection for the use of ethanol, since it was observed that the activity in the precipitate is higher; iii) the enzymatic activity in the precipitate with ethanol at 10°C and 20°C is low, indicating that at temperatures above 10°C the denaturation of protein must occur.

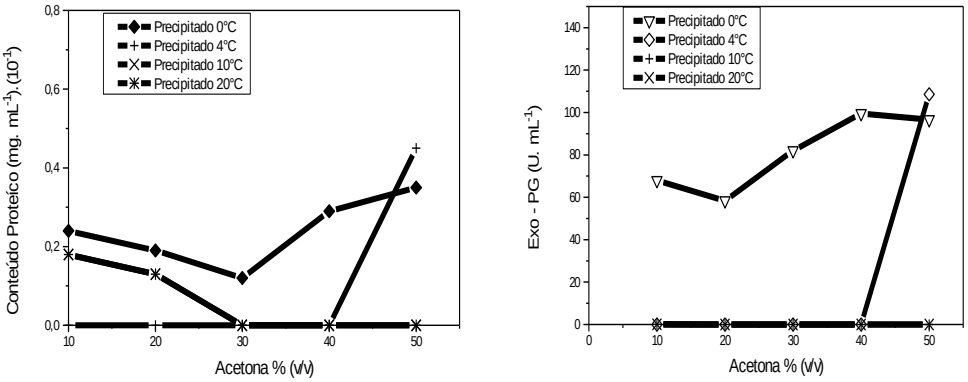
Lucarini (1998), in studies with amyloglycosides, in precipitation with ethanol at various temperatures and concentrations, obtained good results at a temperature of 5°C with 60% to 80% (v/v) of solvent.

For extractions of proteinases and α-amylases, Nakadai & Nasuno, 1989, with solid state cultures and *A. oryzae* 460, showed that the results using alcohols may be unstable as a function of concentration, pH and temperature. In the case of caseine, at pH 7.0, 44.5%, at 5°C and 31.3% at 22°C was recovered (Ladisch *et al.*, 1986).

Studies carried out by Pedruzzi *et al.* (2002) showed that the use of ethanol in the precipitation of endo-PG was efficient in the proportion of 1:2.3 (v/v).

Although precipitation occurred under different conditions, the maintenance of enzymatic activity of exo-PG after treatment was only observed at a temperature of 0°C (Fig. 2A). The acetone and solvents of smaller and linear chains, form part of the group of solvents most used in precipitations, since they are totally miscible with water, do not react with the proteins and have a good precipitant effect, with lower volumes than those used with ethanol (Scopes, 1994).

In Fig. 3, the results obtained in the precipitation of exo-PG with acetone at different temperatures and concentrations are shown.

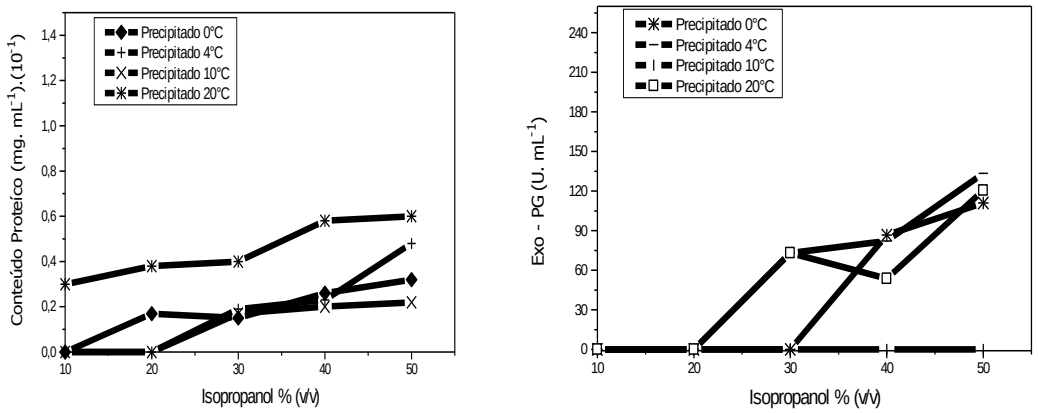


(A) (B)

Figure 3 –Precipitations of exo-PG as a function of acetone concentration at 0ºC, 4ºC, 10ºC and 20ºC. (A) protein content; (B) enzymatic activity.

Acetone is a precipitant for exo-PG (Tab. 1), however, according to Figs. 3A and 3B respectively, there is a loss of protein content and of enzymatic activity for all temperature ranges and concentrations tested.

Isopropoanol is a precipitation agent commonly used in systems of nucleotide precipitation (RNA and DNA) (Chomezynski, 1993). On analyzing the isopropanol results (Fig. 4) it can be observed that its behavior as an exo-PG precipitation agent is irregular and sensitive to temperature and concentration. Isopopanol, as with ethanol, has a better action at concentrations above 20% (v/v), while in the temperature interval of 0 to 4°C, according to Rothstein (1994), there is protein denaturation.

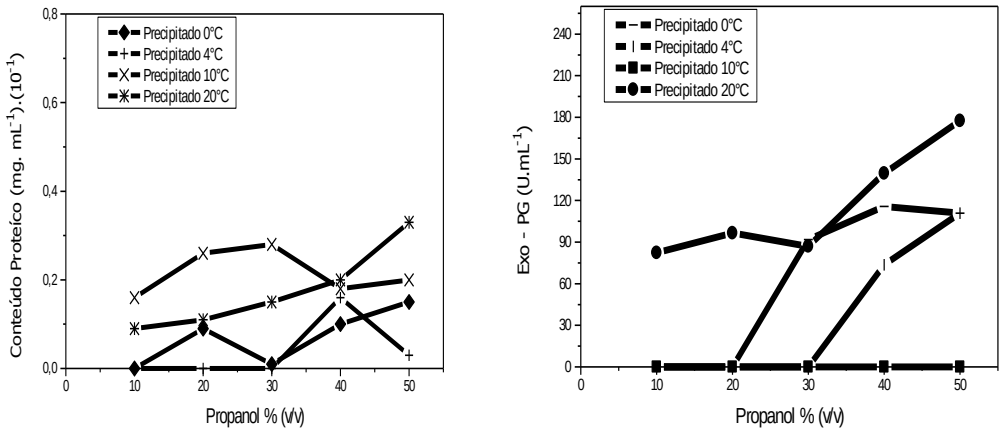


(A) (B)

Figure 4 – Precipitation of exo-PG as a function of isopropanol concentration at a 0ºC, 4ºC, 10ºC and 20ºC: (A) protein content; (B) enzymatic activity.

The branched alcohol chains are less denaturing and more miscible, indicating that it is the length of the aliphatic chain which leads to the greater denaturation. Thus, the order of the efficiency of denaturation is: methanol < ethanol <propanol <butanol. The exo-PG precipitated with isopropanol, Fig. 4B, shows the possibility for protein denaturation in relation to ethanol and n-propanol, as precipitants.

In Fig. 5, analyzing the behavior of n-propanol as an exo-PG precipitation agent, at temperatures of 0°C, 4°C, 10°C and 20°C, in relation to the enzymatic activity and the protein content it can be considered as an inadequate precipitant at the temperatures evaluated, since it also promotes the denaturation of protein at temperatures of 10ºC to 20ºC and at the concentrations tested (10 to 50% v/v).

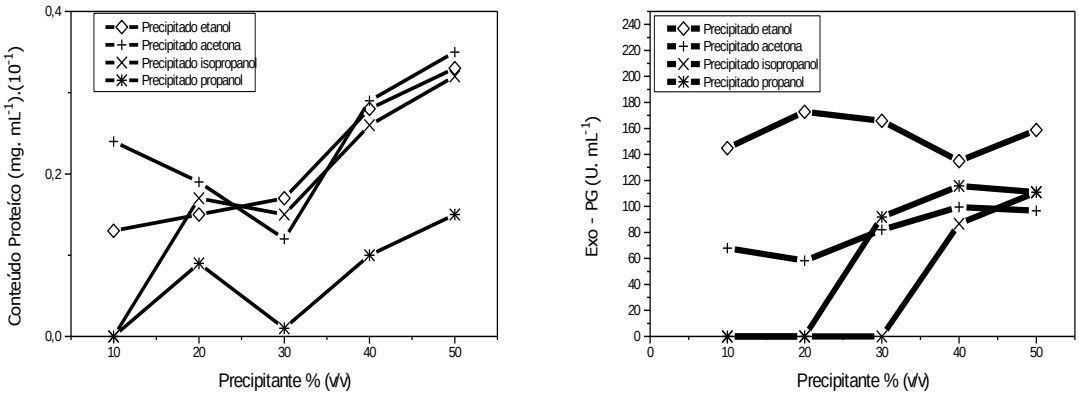


(A) (B)

Figure 5 – Precipitation of exo-PG as a function of n-propanol concentration at 0ºC, 4ºC, 10ºC and 20ºC: (A) protein content; (B) enzymatic activity.

In Fig. 6, it can be observed that all solvents, at a temperature of 0°C, present good behavior in relation to exo-PG precipitation, mainly at concentrations above 20% (v/v). In relation to the enzymatic activity (Fig. 6B), it can be observed that: i) the ethanol concentrates the enzymatic activity in the precipitate; ii) the acetone maintains the activity at this temperature; iii) propanol and isopropanol

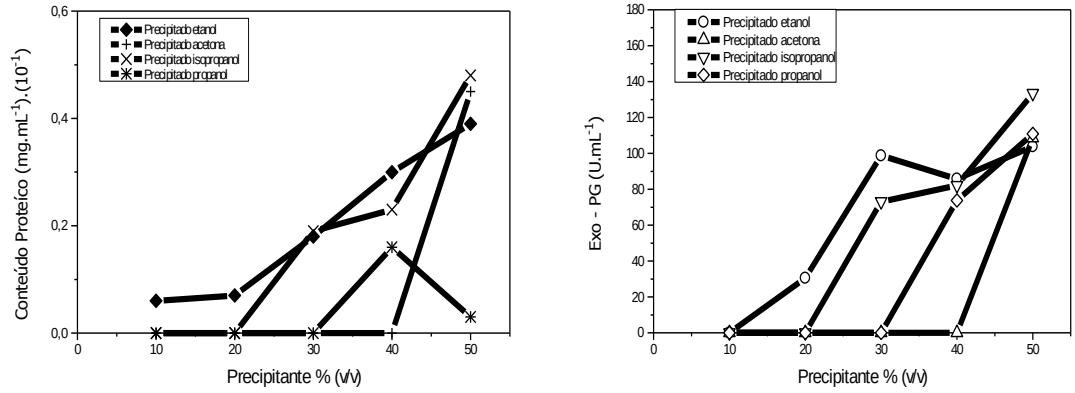
show a similar behavior, both in the precipitate and in the supernatant, not concentrating activity in the precipitate or in the supernatant.



(A) (B)

Figure 6 – Precipitation of exo-PG with organic solvent precipitants at different concentrations, at 0°C: (A) protein content; (B) enzymatic activity.

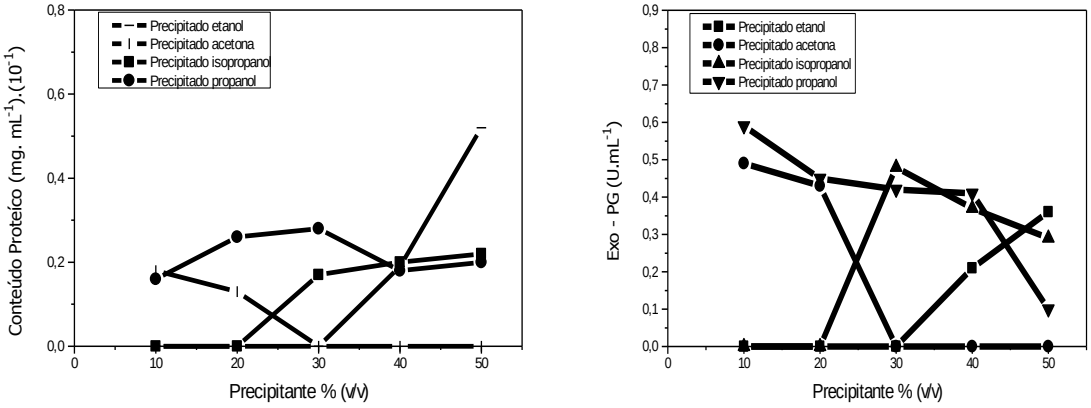
On analyzing Fig. 7, for a temperature of 4°C, it was found that: i) ethanol gave a low protein content increasing with precipitant concentration; ii) acetone, isopropanol and propanol, in relation to protein content, have similar behaviors not concentrating in the precipitate or the supernatant. In relation to enzymatic activity it can be stated that, at this temperature, ethanol concentrates activity in the precipitate, while acetone, isopropanol and propanol show a similar behavior.



(A) (B)

Figure 7 – Precipitation of exo-PG with organic solvent precipitants at different concentrations, at 4°C: (A) protein content; (B) enzymatic activity.

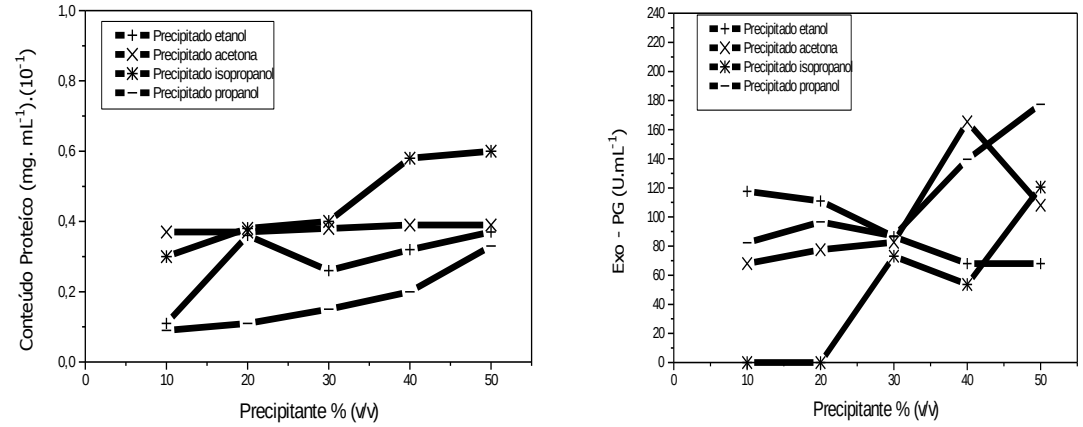
In Fig. 8, which gives the results obtained at a temperature of 10°C, it can be observed that: i) ethanol and acetone do not lead to significant results in relation to protein content or enzymatic activity; ii) propanol, in relation to the precipitation of proteins, is insufficient; iii) isopropanol does not show significant changes in protein content, it showing some positive results from 30% (v/v); iv) propanol, as with isopropanol, maintained a reasonable enzymatic activity from 30% (v/v).



(A) (B)

Figure 8 – Precipitation of exo-PG with organic solvent precipitates at different concentrations, at 10º C: (A) protein content; (B) enzymatic activity.

In Fig. 9, with data obtained at a temperature of 20°C, it can be observed that: i) ethanol is the precipitant which gives the best results for this temperature, but a loss of activity occurs from 20% (v/v); ii) acetone gives 50% (v/v) of protein content in the supernatant and 50% (v/v) in the precipitate; iii) propanol and isopropanol show a similar behavior, it can be observed that there is no concentration in the precipitate or in the supernatant, indicating that protein degradation had occurred.



(A) (B)

Figure 9 – Precipitation of exo-PG with organic solvent precipitants at different concentrations, at 20°C: (A) protein content; (B) enzymatic activity.

In general, it was found that the branched alcohol chains are less denaturing and more miscible, indicating that it is the length of the aliphatic chain which leads to the greater denaturation of proteins. The greater denaturation effect of alcohols with aliphatic chains is the result of increased hydrophobic interactions with apolar protein groups and a weakening of the intra-protein interactions (Martins *et al.*, 2002).

IV. CONCLUSIONS

The results obtained in this study allow the following conclusions to be drawn with respect to the use of a precipitation system in the concentration of exo-PG:

- from an analysis of the behavior of organic solvents as precipitation agents, it is possible to conclude that the most important factors in the experimental tests are the temperature and concentration of the precipitation agents used:
- an analysis of the protein content and enzymatic activity showed that lower temperatures, such as 0°C, led to better results in terms of the preservation of enzymatic activity;
- ethanol was the organic solvent which gave the best response, both in relation to the precipitation, and in the preservation of the enzymatic activity of exo-PG, at lower temperatures.

V. ACKNOWLEDGEMENTS

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