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ETHANOL PRECIPITATION OF GLYCOSYL HYDROLASES PRODUCED BY *Trichoderma harzianum* P49P11

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Abstract - This study aimed to concentrate glycosyl hydrolases produced by *Trichoderma harzianum* P49P11 by ethanol precipitation. The variables tested besides ethanol concentration were temperature and pH. The precipitation with 90% (v/v) ethanol at pH 5.0 recovered more than 98% of the xylanase activity, regardless of the temperature (5.0, 15.0, or 25.0 °C). The maximum recovery of cellulase activity as FPase was 77% by precipitation carried out at this same pH and ethanol concentration but at 5.0 °C. Therefore, ethanol precipitation can be considered to be an efficient technique for xylanase concentration and, to a certain extent, also for the cellulase complex.
Keywords: *Trichoderma harzianum*; Glycosyl hydrolases; Ethanol precipitation.

INTRODUCTION

Lignocellulosic biomass is a renewable raw material with great potential as a source of clean energy. To compete with fossil fuels, it has to be adequately used. However, a key challenge is the development of efficient technologies to convert biomass components into different types of fuel (Ptasinski *et al*., 2007).

The cellulose in biomass can be hydrolyzed chemically, with dilute or concentrated acid, or through an enzymatic treatment to produce glucose, which is the main source of carbon in the production of second-generation ethanol (2G ethanol). The enzymatic route occurs under mild conditions, which allows for greater reaction control to minimize the degradation of biopolymers. Also, lignin is not solubilized, and the carbohydrate structures are well preserved. On the other hand, chemical hydrolysis using concentrated or dilute acid, generates toxic substances such as furfural and 5-hydroxymethylfurfural and phenolics from lignin degradation (Badger, 2002; Brodeur *et al*., 2011).

The enzymatic hydrolysis of the lignocellulosic material results from the cooperative action of several glycosyl hydrolases. These include endo-β-1,4-glucanases (EG, E.C. 3.2.1.4), cellobiohydrolases or exoglucanases (CBH, E.C. 3.2.1.91) and β-glycosidases (E.C.3.2.1.21), which act on the cellulosic fraction, and a set of hemicellulases, such as xylanases, β-xylosidases, glucuronidases, acetyl esterases, galactomannanases and glycomannanases, which act on the hemicellulosic fraction (Canilha *et al*., 2012). Many efforts to increase the efficiency of the enzymatic hydrolysis have focused on preparing adequate proportions and highly concentrated enzyme mixtures to maximize the synergistic effect of the enzyme complex, in addition to minimum dilution of

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the hydrolysis mixture and the ease of storage and transportation of the biocatalysts (Queiroz *et al*., 2001).

Regarding 2G ethanol production, some reports have anticipated that *on-site* production of the enzyme cocktail in an adjacent production plant could be the best option to enable the enzymatic technology because lignocellulosic biomass can be used as the main carbon source (Barta *et al*., 2010; Piovesan *et al*., 2013). Therefore, primary recovery to concentrate the enzyme cocktail is a necessary step to obtain an acceptable protein concentration for the hydrolysis reaction, especially regarding the efforts to develop reactions with high loads of solids to increase the productivity of the hydrolysis reactor.

Filamentous fungi are the most efficient organisms for producing extracellular cellulases. Some of them have been extensively studied and utilized in industrial applications, such as in the food industry (coffee processing and the drying of beans) (Kasai *et al*., 2006), agribusiness, animal feed, paper production, the textile industry, waste treatment, detergents, and the chemical industry (Wonganu *et al*., 2008). Therefore, the study of different cellulases is not restricted to the current and growing biofuels market; it also extends to other industries that make use of enzymatic processing.

A new species of filamentous fungus, *Trichoderma harzianum*, isolated in the Amazon, has shown the potential to hydrolyze biomass in ways similar to the *Trichoderma reesei* fungus (Delabona *et al*., 2012), which is one of the most studied and utilized fungi for the hydrolysis of biomass. *T. harzianum* was studied and used for phytopathogenic biocontrol, but recently it has been studied as a hydrolysis agent for the production of 2G ethanol (Thrane *et al*., 1997).

Different studies have shown that strains of *T. harzianum* grown in an environment with wheat bran as the carbon source produce enzyme activities that hydrolyze different types of carbohydrates (Medeiros *et al*., 2002; Silveira *et al*., 1997; Ruegger and Tauk-Tornisielo, 2004). This fungus, as well as the well studied *T. reesei*, has a genetic capability to produce a variety of cellulases and has demonstrated great efficiency in deconstructing and converting lignocellulosic materials (Liberato *et al*., 2012; Maeda *et al*., 2011; Colussi *et al*., 2011).

The IOC-3844 *T. harzianum* strain has been widely studied in the last years as a potential producer of enzyme complexes. In terms of the volumetric yield and specific activity of the enzymes, this strain has proved to be able to produce large amounts of cellulases, even when compared with the standard industrial *T. reesei* RUT C30 strain. It was reported that the IOC-3844 strain produces larger quantities of FPAse, endoglucanase, and β-glycosidase than the reference fungus (Castro *et al*., 2010). Other studies showed that the high β-glycosidase and xylanase activities of *T. harzianum* IOC 3844 prevent the accumulation of inhibitors. This improves the enzymatic hydrolysis yields because the increase in the degree of synergism depends mainly on the enzyme concentrations (Nobuyuki *et al*., 2011).

Besides the importance to concentrate the individual activities in the cocktail, it is well known that protein molecules in the precipitated state have high stability because of their restricted mobility, and this is the ideal state for storage and transportation (Schmid, 2011).

Precipitation is considered to be a simple unit operation. It is widely used to concentrate biomolecules, mainly proteins, in aqueous solutions (Queiroz *et al*., 2001). Specifically, precipitation with ethanol has low capital costs and low operating costs. The precipitating agent can be recycled by simple distillation after the liquid and precipitated phases separation (an easy procedure for the case of *on-site* cellulase production), thereby reducing the environmental impact of the effluent.

Given the importance of the concentration of enzymatic cocktails in the context of a sugarcane biorefinery aiming at 2G ethanol production by the enzymatic hydrolysis route, the objective of this study was to evaluate the possibility of concentrating the glycosyl hydrolase activities from the fermentation broth of *T. harzianum* P49P11 strain using ethanol.

EXPERIMENTAL PART

Production of Fermented Product of *Trichoderma harzianum* P49P11

The production of the fungal enzyme cocktail was performed by using the *T. harzianum* P49P11 strain, following the protocol established in the Brazilian Bioethanol Science and Technology Laboratory (CTBE), Campinas – SP, Brazil. This strain was isolated from the Amazon forest reserve of Embrapa, Belém – PA, Brazil and grown on potato dextrose agar plates (Difco^{TM}, USA) at 29 C for 7 days (Delabona *et al*., 2012).

The procedure consisted of two phases carried out at constant temperature and culture pH (29 °C and pH 5.0). The first phase (pre-culture) was conducted in an Erlenmeyer flask with 1 L of culture medium containing 10 g of celuflock, 10 g of glucose, 1.0 mL of Tween 80, 1.0 g of peptone and 50 mL of salt solution. This composition of the pre-culture medium was adapted from Mandels and Webber (1969).

Agitation was applied for 72 hours at 150 rpm using orbital agitators (Inova 44, New Brunswick Scientific, USA).

Next, in the second phase, the 1 L of the pre-culture was aseptically transferred to a 20 L (total volume) Bioflo bioreactor (Eppendorf, Germany) containing 10 L of medium. In the reactor, the propagation medium consisted of vegetal peptone medium 1.5 g/L, Tween 80 1.5 mL/L, salt solution 150 mL/L, 10 g/L of pre-treated sugarcane bagasse, and sucrose in a 3:1 ratio as the carbon source. The pretreated sugarcane bagasse was prepared by the steam explosion technique followed by de-lignification with NaOH (Rocha *et al*., 2012).

During culture, 1.0 mL samples of the fermented broth were taken to determine the total protein concentration and enzymatic activity. At the end of the culture, 72 h, the raw fermented product was centrifuged at 6,600 g (Centrifuge Sorvall RC 6+, Thermo Scientific, USA) for 20 min to remove the cellular biomass and the residual bagasse. The liquid phase was filtered with Whatman no. 1 paper to clarify the culture supernatant, and it was sampled in 2 L volumes in plastic bottles and stored at -20 °C for further use in the precipitation experiments. For the precipitation tests, aliquots of 15 mL were thawed and then centrifuged at 4,800 g for 10 min (5408R centrifuge, Eppendorf, Germany) before use.

Analytical Methods

Determination of Protein Concentration

Protein concentration in solution was determined by a method based on Bradford (1979) with BSA (bovine serum albumin) as the standard protein. The method was adapted to micro-plates by using Coomassie Brilliant Blue reagent previously diluted 1:4 in water. Absorption measurements at 595 nm were conducted using a Spectramax model M5 microplate reader (Molecular Devices, USA).

Enzyme Activity Assays

The determination of β-glycosidase activity was performed through the quantification of p-nitrophenol (Sigma-Aldrich, USA), which is released after the hydrolysis of p-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich, USA) under the assay conditions: 50 mmol.L^{-1} sodium citrate buffer, pH 4.8, for 10 min at 50 °C (Nexus Thermocycler, Eppendorf, Germany). The calculation of units of enzymatic activity (UA) was based on the amount of enzyme required to catalyze the release of 1 μmol of product (p-nitrophenol) per minute at 50 °C (Zhang *et al*., 2009).

The endoglucanase and xylanase activities were determined by the method of Bailey and Poutanen (1989). The substrates were 0.5% (m/v) carboxymethylcellulose and 0.5% (m/v) xylan, respectively. A colorimetric test with 3,5-dinitrosalicylic acid (Miller, 1959) and standard curves of glucose and xylose for the endoglucanase and xylanase assays, respectively, were used to quantify the concentrations of reducing sugar released. Xylanase activity measurement is related to the quantification of reducing sugar released by the action of mainly three enzymes, endo-1,4-β-xylanase (E.C. 3.2.1.8), endo-1,3-β-xylanase (E. C. 3.2.1.32) and xylan 1,4-β-xylosidase (3.2.1.37).

The cellulase activity test on filter paper (FPAse) was performed as described by Ghose (1987) with a 10x workload scale reduction.

Precipitation Tests

The desired volume of fermented broth was distributed in 2 mL Eppendorf tubes and kept at a predefined temperature in a thermostatic bath with 0.1 °C precision (TE-2000, Tecnal, Brazil). Next, absolute ethanol (Sigma-Aldrich, USA) at the desired precipitation temperature was added dropwise to the tubes until the final desired concentration was reached. After the samples were homogenized by inversion, they remained in the bath without agitation for predefined periods (6 h in the initial precipitation tests and 0 to 6 h in the precipitation kinetics). Then, the precipitates were separated by centrifugation at the precipitation temperature, at 9,000 g for 10 min. The precipitates were dissolved in 0.05 mol/L sodium citrate buffer, pH 4.8. All of the precipitations were performed in triplicate.

RESULTS AND DISCUSSION

Determination of the Effective Ethanol Concentration Range for Protein Precipitation

Two preliminary precipitation tests were performed at 5 °C and pH 5.0. The first test, conducted at a concentration of 60% (v/v) ethanol (percentage of the ideal final volume of the mixture of ethanol and fermented broth), was used to determine the aging time (the period between the end of ethanol addition and the beginning of the centrifugation step) to be used in subsequent experiments. The tested aging times were 0, 3, 6, and 16 h, and the 6 h time was considered sufficient to achieve equilibrium (data not shown). Farinas *et al*. (2011) determined similar equilibrium time for the kinetics of xylanases

precipitation with ethanol from *Aspergillus niger* fermentation broth. The second preliminary precipitation test, with a 6 h aging time, was used to define a relatively narrow ethanol final concentration range to be studied in further detail (Figure 1).

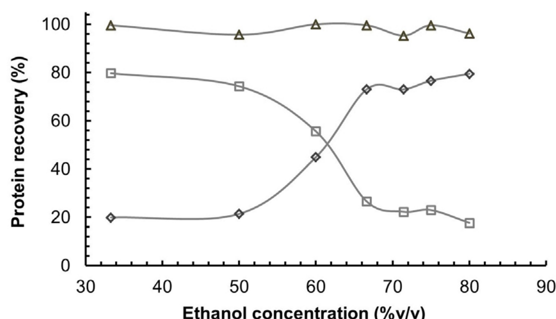

Figure 1: Ethanol precipitation of proteins in the fermented broth of *T. harzianum* P49P11 at 5 °C, pH 5.0 and 6 h aging time. Δ: Total recovery; ◊: precipitate; □: supernatant.

Precipitation increased as increasing amounts of ethanol were added to the fermented broth, and the amount of protein in the supernatant decreased concurrently. The quantification of precipitation was satisfactory in terms of mass balances for all tests. There was a significant increase in protein recovery in the precipitate for ethanol concentrations between 50% and 66% (v/v). However, it was considered appropriate to test higher ethanol concentrations because there was the possibility of some glycosyl hydrolases being present at very low concentrations that would affect mass recovery only slightly, but could provide a significant amount of activity. Therefore, the ethanol concentrations studied were 60%, 75%, and 90% (v/v).

Protein Precipitation and Enzyme Activity Partitioning in the Precipitated Phase at Different Conditions

Precipitation studies with ethanol were performed by using the OFAT (one-factor-at-a-time) method to test the effects of pH, temperature, and ethanol concentration. The tested pH values were 5.0 (fermentation pH), 6.5 and 8.0, covering a range from slightly acidic to slightly basic. The temperature varied from 5 °C (relatively low temperature to minimize protein denaturation) to 25 °C (close to room temperature, practical for industry because it minimizes heating and cooling expenses).

The study evaluated the recovery of the activities of xylanase, endoglucanase, β-glycosidase and FPAse in the precipitate phase, and the corresponding protein mass balances were determined. The enzyme activities of the supernatant phases are not shown because an evaluation of the experimental methodology revealed that ethanol has a strong interference on the activity tests at concentrations higher than 9% (v/v) ethanol. Because of the high sensitivity to ethanol in the enzyme assays, the option of diluting the samples was not considered because enzyme activity could not be detected in the highly diluted samples.

Protein Mass Balance and Recovery in the Precipitate Phase

As shown in the protein mass balances in Table 1, it was possible to achieve a recovery close to 100% for almost all of the precipitation conditions, except for ethanol concentrations of 60% (v/v). In general, the solubility of proteins in the presence of ethanol was higher at high temperatures, and this trend was more evident at ethanol concentrations of 75% and 90% (v/v) (Figure 2).

Table 1: Protein mass balances for the precipitations with ethanol as a function of the pH of *T. harzianum* fermented broth, temperature and ethanol concentration (6 h aging time). Standard deviations of triplicate experiments are shown.

pH of the fermented broth	Temperature (°C)	Ethanol % (v/v)	Protein mass of the precipitate phase (%)	Protein mass of the supernatant phase (%)	Total recovery (%)
		60	36.4 ± 4.9	57.6 ± 3.7	94.0
	5.0	75	81.7 ± 1.1	15.1 ± 2.7	96.8
		90	94.2 ± 2.4	10.6 ± 4.2	105
		60	31.2 ± 4.7	73.3 ± 1.4	104
5.0	**15.0**	75	76.1 ± 5.4	10.6 ± 3.3	86.7
		90	85.5 ± 1.9	13.7 ± 6.1	99.2
		60	36.3 ± 4.5	48.7 ± 1.7	85.0
	25.0	75	63.7± 1.8	20.6± 6.9	84.3
		90	85.2 ± 2.2	13.5± 5.1	98.7
		60	30.0 ± 2.7	60.0 ± 7.4	90.0
	5.0	75	80.1 ± 3.2	19.1 ± 3.3	99.2
		90	95.7 ± 3.6	6.50 ± 0.5	102
		60	34.9 ± 7.9	53.8 ± 7.3	88.7
6.5	**15.0**	75	84.0 ± 16	16.5 ± 7.1	100
		90	90.1 ± 14	10.8 ± 1.8	101
		60	34.4 ± 1.0	51.4 ± 9.4	85.8
	25.0	75	59.3 ± 4.6	34.8 ± 4.0	94.1
		90	71.4 ± 2.4	21.5 ± 9.7	92.9
		60	36.4 ± 2.3	44.3 ± 5.4	80.7
	5.0	75	75.2 ± 1.9	23.9 ± 5.9	99.1
		90	93.9 ± 1.9	6.70 ± 2.2	101
		60	33.7 ± 10	50.7 ± 1.9	84.4
8.0	**15.0**	75	58.7 ± 13	46.6 ± 4.6	105
		90	83.7 ± 1.1	12.8 ± 3.2	96.5
		60	25.4 ± 1.3	48.7 ± 5.2	74.1
	25.0	75	51.2 ± 4.8	43.4 ± 6.8	94.6
		90	73.0 ± 10	27.3 ± 2.6	100

The average protein concentration of the clarified fermented broth was 1.08 mg/mL.

Therefore, retrograde solubility behavior (solubility reduction due to temperature increase), a usual phenomenon in proteins, was not observed (Christopher *et al*., 1998).

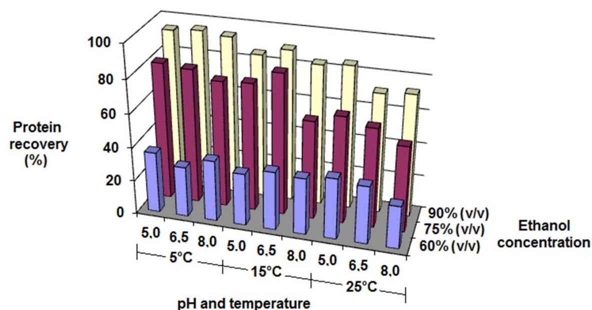

Figure 2: Recovery of proteins of the fermented broth of *T. harzianum* P49P11 precipitated by ethanol as a function of pH, temperature, and ethanol final concentration. Aging time, 6 h.

Figure 2 shows that there was a low recovery of precipitated protein for the fermented products with pH 8.0 at 15 °C and 25 °C, primarily when 75% and 90% ethanol were used.

Partitioning of the Glycosyl Hydrolase Activities in the Precipitate Phase

Figure 3 shows the recoveries of the glycosyl hydrolase activities in each precipitate phase as a function of the precipitation conditions. The xylanase recovery profile was characterized by a high activity recovery (close to 100%) in the precipitates formed by using 90% ethanol at all tested temperatures and pH values. According to the studies by Tan *et al*. (1985) and Wong and Saddler (1992), the pI values of the main xylanases of *T. harzianum* are very close each other (9.4, 9.5 and 8.5) and for molecules with mass very close to each other too (20, 29 and 22 kDa xylanases, respectively). Considering that the behavior of the xylanases for each ethanol concentration showed no remarkable changes in terms of activity recoveries at different pH values, this effect may result from the similarity of the xylanase molecules in this fermented broth. Further studies estimated that the xylanases of *T. harzianum* constitute approximately 25% of the total extracellular protein and that they have an activity ratio three times higher than the FPAse in the raw preparation (Serpa, 2012).

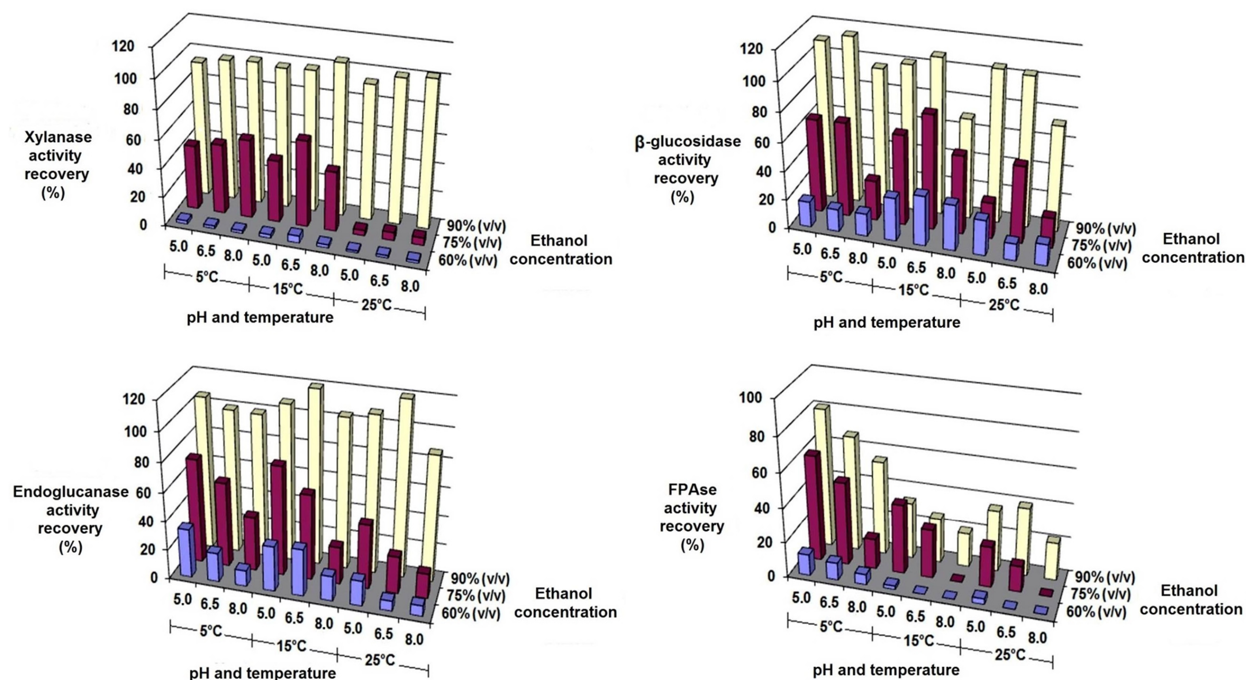

Figure 3: Recovery of glycosyl hydrolase activities in the fermented broth of *T. harzianum* P49P11 in the precipitate phases obtained with ethanol as a function of pH, temperature, and ethanol final concentration. Aging time, 6 h.

Regarding the β-glycosidase enzyme, nearly complete recovery of activity was obtained with 90% (v/v) ethanol; this was independent of precipitation temperature, although pH 8.0 conditions produced lower recovery values. In the cases of 60% and 75% (v/v) ethanol, no well-defined trend was observed, except for consistently lower recoveries at 25 °C.

The recovery profile of the endoglucanase activity was affected mainly by the pH of the fermented product, except for precipitates formed with 90% ethanol, for which there was almost complete recovery irrespective of pH and temperature. In the cases of 60% and 75% (v/v) ethanol, the pH increase resulted in lower recovery of the enzyme.

In addition to the partitioning of specific glycosyl hydrolases, this study also determined the recovery profile of the cellulolytic complex activity through measurements of the FPAse activity, which represents the activities of several enzymes, mainly the β-glycosidases, endoglucanases and exoglucanases. A high recovery of the FPAse activity, 77%, was only possible at 5 °C, pH 5.0 and 90% (v/v) ethanol. These activity recoveries of xylanases and cellulases can be considered high compared to previous studies. Tan and coworkers (1987) recovered 63% of xylanase activity by precipitation with 80% ethanol (v/v) at 4 °C for 15 min, whereas those authors recovered 70% of this activity from enzymes produced by *T. harzianum* E8 by using a 39% (v/v) saturated solution of ammonium sulfate. In subsequent studies, Avelino *et al*. (1999) and Silva *et al*. (2010) performed precipitations with ammonium sulfate and ammonium carbamate, respectively, with hydroxypropylmethylcellulose as a coprecipitant in both cases, to precipitate enzymes from a commercial enzyme cocktail of *T. reesei*. The ammonium sulfate recovered 78% of the FPAse activity, whereas the precipitations with ammonium carbamate recovered only 61% of the activity. However, the study performed by Sidhu and coworkers (1985) utilizing a fermented broth of *T. harzianum* RIFAI obtained a higher FPAse activity recovery. In that case, 93% of the FPAse activity was recovered by using saturated ammonium sulfate solution (80% v/v).

Figure 3 also shows the strong influence of pH on the recovery of cellulases. Lower recoveries were obtained at higher pH values, and this effect is similar to the one observed for endoglucanase, most likely because of repulsion between the positively charged protein molecules (Sashi *et al*., 2012). The reported pI values of the endoglucanases produced by the *Trichoderma* genus are between 5.1 and 6.2, and the pI of the exoglucanase (CBHI) of *T. harzianum* IOC 3844 is 5.2 (Colussi *et al*., 2011). Therefore, for a pH increase from 6.5 to 8.0, the endoglucanase and exoglucanase molecules gradually increase their positive charge.

The analysis of the temperature increase during the protein aggregation process in the mixtures containing 60% and 75% ethanol showed that the recovery activities of all enzymes of interest were lower for precipitation at 25 °C. This can be explained by two factors: higher protein solubility at higher temperatures and an increase in protein denaturation by the organic solvent due to increased temperature. However, the activity recoveries of the precipitations at 25 °C with 90% ethanol (v/v) did not show a considerable reduction compared to precipitations conducted at lower temperatures. One explanation for this fact may be fast nucleation kinetics, because a high ethanol concentration results in a high supersaturation of protein and consequently a high nucleation rate. The very fast kinetics ensures almost instant removal of the enzyme from the liquid phase, thereby impeding the enzyme-ethanol interaction that potentially causes protein denaturation (Schubert and Finn, 1981; Yoshikawa *et al*., 2012). To confirm this kinetic effect, a new precipitation kinetic study was conducted under the conditions that provided the highest recoveries.

Precipitation Kinetics as a Function of Ethanol Concentration at 5 °C and pH 5.0

This new study describing the kinetics as a function of ethanol concentration at 5 °C and pH 5.0, for aging times of 0 to 6 h, resulted in instant recoveries of both the precipitated protein (Figure 4a) and of the cellulase partition for all the three studied activities, as shown by the FPAse activity data (Figure 4b). The only observed discrepancy was the lower recovery of total protein and consequently the lower FPAse activity as a function of aging time obtained with 80% (v/v) ethanol.

Since instantaneous precipitation was observed, the protein recoveries obtained with no aging was compared with the recoveries of the two activities most efficiently precipitated: xylanase and cellulase (Figure 5).

The recoveries of both proteins and their hydrolytic activities increased with increasing ethanol concentration. With 90% ethanol, the recovery of the xylanase activity was 100%, and the recovery of the FPAse activity was almost 80%, as already shown.

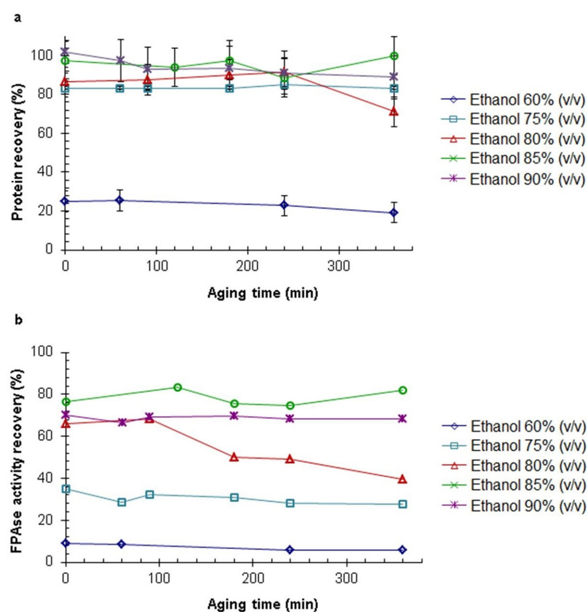

Figure 4: Kinetics of protein and cellulase precipitation from the fermented broth of *T. harzianum* P49P11 at 5 °C and pH 5.0, as a function of final ethanol concentration. Error bars are standard deviations of triplicate experiments.

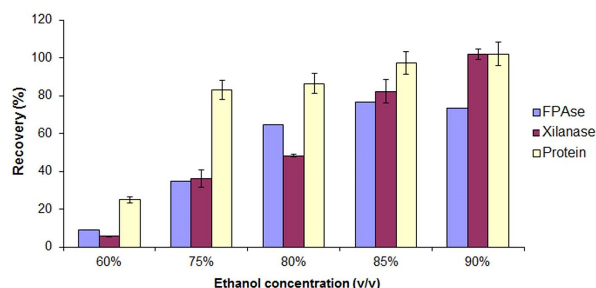

Figure 5: Comparison of the recoveries of total protein and xylanase and cellulase activities of the fermented broth of *T. harzianum* P49P11 precipitated at 5 °C and pH 5.0, with zero aging time. Error bars are standard deviations of triplicate experiments.

CONCLUSION

Based on the results of this study, it can be concluded that the addition of 90% ethanol to the fermented broth of *T. harzianum* at 5 °C causes instantaneous and complete precipitation of proteins. This guarantees the complete recovery of the xylanase activity and a 77% recovery of the cellulase activity. Therefore, ethanol was shown to be useful as a concentrating agent with good potential for use in the 2G ethanol industry, mainly because of its desirable characteristics as a biorefinery product and easy recyclability by distillation. Ethanol was able to provide optimal xylanase recovery and substantial cellulase recovery.

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