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Xylanase Recovery by Ethanol and Na_2SO_4 Precipitation

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ABSTRACT

Xylans are the major components of the hemicellulosic fraction of lignocellulosic biomass and their hydrolysis can be obtained using xylanases from *Penicillium janthinellum*. In this work, sugarcane bagasse hemicellulosic hydrolysate was used as the substrate for producing xylanase. The precipitation of these enzymes was studied using ethanol and Na_2SO_4 as precipitating agents. Ethanol precipitation experiments were performed batchwise in concentrations ranging from 10 to 80%, pH 4.0 to 7.0, at 4°C. The concentrations used in the precipitations with Na_2SO_4 were from 5 to 60% at pH 5.5 and 25°C. Solubility curves as a function of xylanase activity and total protein for both precipitating agents were made. According to the results, Na_2SO_4 is not appropriate for precipitating xylanases in this medium since at salt concentrations higher than 25%, the enzyme was denaturated and at this concentration less than 80% of the enzyme and total protein were precipitated. Because of differences in xylanase and total protein solubility, a fractionated precipitation using ethanol can be performed, since with 40% ethanol, 49% of the total protein was precipitated and more than 95% of the enzyme was kept in solution. On the other hand approx 100% of the xylanases were recovered by precipitation after adding 80% ethanol.

Index Entries: Xylanase; ethanol; precipitation; sodium sulfate.

INTRODUCTION

Xylans are the major components of the hemicellulosic fraction of lignocellulosic biomass and their hydrolysis can be obtained using xylanases *(1)*. The enzymatic complex is composed of endoxylanases that cleave internal xylosidic linkages on the xylan backbone and β-xylosidases that release xylosyl residues by endwise attack of xylooligosaccharides *(1,2)*. Efforts have been made in the pulp and paper industries to reduce

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the amount of chlorine used for bleaching. Studies have been conducted on the effluent treatment and the use of less toxic bleaching agents are under study *(3)*. The residual lignin removal by enzymatic method is a very active research area *(4,5)*. According to Durán et al. *(6)*, xylanases produced by *Penicillium janthinellum* can be used to reduce the chlorine charge in *Eucalyptus*-pulp bleaching with a simultaneous brightness gain. Xylanases have also been used in bread making *(7)*, clarification of beer and juice *(1)*, and partial xylan hydrolysis in animal feed *(2)*. According to Gattinger et al. *(8)*, xylanases are produced from processed or refined substrates such as sugars, cellulose, and xylan. All of these methods are expensive for industrial-scale production and to lower production costs, cheaper substrates must be employed. Sugarcane bagasse hemicellulosic hydrolysate, composed mainly of xylose oligomers, is a potential substrate. Optimal conditions for cultivation parameters (like agitation and aeration rates) have been investigated *(9)*. However, xylanase recovery from the cultivated medium using a scalable technique has to be studied.

Precipitation and recovery of protein precipitates by centrifugation are widely practiced in the biotechnological industry. Ethanol precipitation is a promising technique since it has been applied to other types of proteins on an industrial scale *(10,11)*. Because of their low dielectric constants (compared to water) organic solvents increase Coulombic attraction between protein molecules. Aggregates are formed until the particle size reaches macroscopic proportions and precipitation occurs. Ethanol is by far the most important of the solvents owing to its good physicochemical properties, like complete miscibility with water, good freezing-point depression, no explosive mixtures, high volatility, chemical inertness, low toxicity, and low cost (especially in Brazil). Protein precipitation by salting out is the oldest type of precipitation and is still used regularly on a laboratory scale. The mechanism is, nevertheless, not completely understood although it is clear that high-salt concentrations remove water associated with protein. The most commonly used salts are ammonium sulfate and sodium sulfate. Ammonium sulfate, although widely used, presents waste disposal problems because of the nitrogen content and corrosive properties. Sodium sulfase is an alternative to salt precipitation since it constitutes a simple means of recycling by reduction of the temperature and separation of the salt crystals. The present study was carried out to examine the xylanase and total protein precipitation using ethanol and sodium sulfate. Ethanol and salt precipitations are used in xylanase precipitations, but the experiments are performed exclusively for laboratory applications *(11–16)*.

MATERIALS AND METHODS

Preparation of Sugarcane Bagasse Acid Hydrolysate

In order to prepare the hydrolysate for cultivation, 800 g of dry-milled bagasse was mixed with 8 L sulfuric acid solution (0.25%) and autoclaved

for 45 min at 121°C. The liquid fraction was separated by filtration adjusted to pH 5.5 with NaOH.

Cultivation Medium and Enzyme Production

The isolation of *P. janthinellum* from the decaying wood was described by Milagres *(17)*. This microorganism was identified by the Biosystematic Research Center of Canada (Ottawa, Ontario) and deposited in their collection with the designation of CRC 87M-115. The strain was maintained in silica stocks and, by transfer, on agar slants. The fungus was cultivated at 30°C for 5 d in medium containing 1% glucose, 0.1% yeast extract, 2% (v/v) concentrated complete salts solution based on Vogel's medium (18), and 2% agar-agar. The medium was sterilized at 121°C for 15 min. The spore inocula were obtained by suspending spores in water and filtering through gauze into Erlenmeyer flasks. The final concentration of spores was 10^5/mL. The cultivation medium for enzyme production contained sugarcane bagasse hemicellulosic hydrolysate supplemented with 2% (v/v) concentrated salt solution based on Vogel's medium and 0.1% yeast extract. The medium was then autoclaved for 15 min at 121°C. The cultivation was carried out in Erlenmeyer flasks (125-mL) containing 25 mL of medium. Standard cultivation conditions were: temperature 30°C; initial pH 5.5 (uncontrolled); and 96 h of cultivation time. In general, xylanase produced by *P. janthinellum* in an aqueous solution is stable at a range between pH 4.0 and 8.0; it is rapidly inactivated below pH 4.0 and above pH 8.0 *(17)*. At temperatures above 30°C, the enzyme is also unstable.

Enzyme Activity and Protein Determination

Extracellular xylanase activities were determined by incubating 0.1 mL of diluted culture filtrate with 0.9 mL of a "Birchwood" xylan suspension (10 g/L) in 0.05 *M* phosphate buffer (pH 5.5) for 5 min at 50°C, according to Bailey et al. *(19)*. The released reducing equivalents were measured by a colorimetric assay *(20)* using xylose solution as a standard reference. Activity units were expressed as micromoles of reducing equivalents released per min at 50°C. The amount of total protein was determined according to the Coomassie blue method described by Bradford *(21)* using bovine serum albumin (BSA) as a protein concentration standard.

Protein and Enzyme Precipitation

The concentrations of Na_2SO_4 used in the precipitations were: 5, 15, 25, 40, and 60% (w/w). The calculated amount of salt in the solid form was added slowly to a 15-mL centrifuge tube containing culture medium. After the addition of salt, all of the tubes contained 5.0 g of the mixture, which was agitated for 15 s in a vortex at pH 5.5 at room temperature (25°C). The precipitate was collected by centrifugation (6000 *g*, for 20 min) and then dissolved in 0.05 *M* acetate buffer (pH 5.5) up to the initial mass (5.0 g).

The ethanol concentrations employed in the precipitation experiments were: 10, 20, 30, 40, 50, 60, 70, and 80%. The pH of the precipitation medium was adjusted to the desired value by adding 0.10 *M* acetate buffer (pH 4.0 and 5.5) or phosphate buffer (pH 7.0). The ethanol was slowly added to the medium under agitation (200 rpm) and the temperature was maintained at 4°C. After the addition of ethanol, the agitation was stopped for 30 min and the mixture was centrifuged (2000 *g* for 30 min, Centrifuge Jouan Mod. [Saint-Herblain, France] 1812) under refrigetation (4°C). The pellet was resuspended using 0.05 *M* acetate buffer (pH 5.5) at room temperature (~25°C). The total protein content was determined in triplicate: in the supernatant, in the pellet, and in the initial sample for both precipitations. Enzymatic activities were determined only in the resuspended pellet and in the initial sample since the ethanol and the Na_2SO_4 present in the supernate could interfere with the methodology.

Chemicals

Birchwood 4-*O*-methyl-D-glucoroxylan (90% xylose) were obtained from Sigma (St. Louis, MO). All of the other chemicals were of analytical grade.

RESULTS AND DISCUSSION

In large-scale enzyme precipitations, organic solvents were more successful than protein salting out *(22,23)*. The efficiencies of two compounds (ethanol and sodium sulfate) to recover *P. janthinellum* xylanase were studied. The cell-free filtrate of acid hemicellulose hydrolyzate culture was used as a crude enzyme mixture. The results shown in Figs. 1 and 2 indicate that recovered xylanase fractions ranged from 18 to 72% in the precipitations conducted with sodium sulfate, and from 0 to 100% in the precipitations with ethanol. In the precipitations performed with sodium sulfate, the maximal xylanase recovery (71.8%) was attained at 25% concentration (Fig. 1). At higher salt concentrations the enzyme redissolved in the supernate and the recovery yield decreased. The total protein precipitation curve showed a different behavior. The highest recovery level (68%) was observed at 40% salt concentration. A comparison between the behaviors of the curves for total protein and xylanase activity reveal that the only benefit derived from the precipitation studies is the concentration factor. A fractionation is not recommended in this case since a satisfactory increase in the purification factor cannot be achieved. As can be seen in Fig. 2, at 40% ethanol concentration, approx 30% of the total protein was precipitated, whereas 95% of the xylanase remained in the solution; however at 80% ethanol concentration, 100% of the xylanase precipitated and approx 30% of the total protein remained in the solution. These results suggest a fractionated precipitation when using ethanol as a precipitating agent. The fractionation can be performed in two steps; the first at 40% and the second

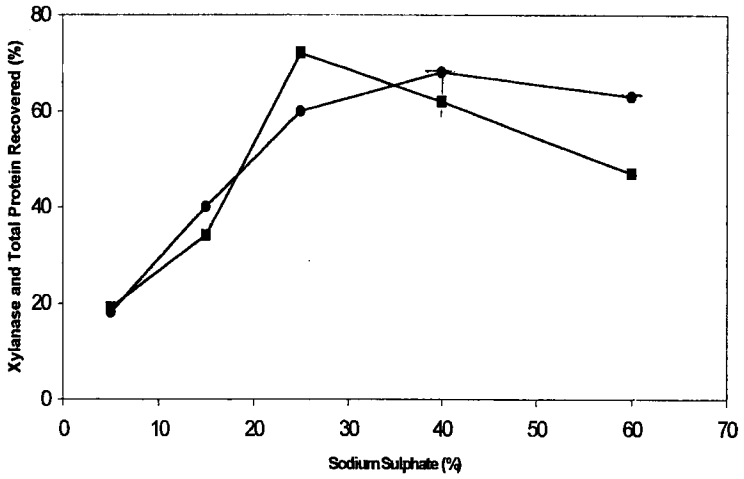

Fig. 1. Xylanase recovery (-■-) and total protein recovery (-●-) as a function of sodium sulfate concentration (%).

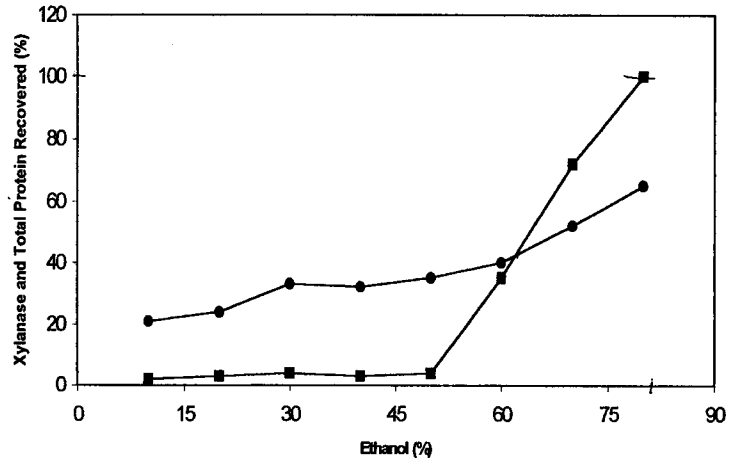

Fig. 2. Xylanase recovery (-■-) and total protein recovery (-●-) as a function of ethanol concentration (%).

one at 80% ethanol concentration. The purification factor can increase twofold.

CONCLUSIONS

The precipitation of xylanase from *P. janthinellum* using ethanol and Na_2SO_4 as precipitating agents was studied. Sodium sulfate proved to be inappropriate for enzyme purification, since the xylanase and total protein precipitations occurred similarly in all the concentrations tested. The ethanol precipitation showed promising results. A fractionated precipita-

tion is possible since at 40% ethanol, 30% of the total protein precipitates, whereas 95% of the enzyme keeps soluble. On the other hand, at 80% ethanol concentration, 30% of the total protein keeps soluble and 100% of the enzyme is precipitated. In this way the purification factor can increase twofold for the concentrated enzyme.

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