

Partial Purification, Stability Analysis, and Preservation of Xylanase from Xylanolytic Alkalophylic Bacteria

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Abstract

A xylanase, which produces xylose from oat spelt xylans, was isolated from the culture medium of xylanolytic alkalophylic bacteria mutant. The enzyme was purified by ammonium sulphate with level 30, 40, 50, 60, 70, 80, and 90%. The purify of the final preparation was demonstrated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The molecular masses of the purified xylanase were 137.61 and 165.34 kDa. Result of ammonium sulphate saturation with the highest activity was used as standart for saturation for enzyme production and preservation, using corn, tapioca, soy bean meal and gaplek flour as carriers. Addition of 60% ammonium sulphate showed the highest xylanase activity (62.03 U/g), and produced 89.40% enzyme recovery. Tapioca, as a carrier, produced the highest xylanase activity.

Key words: preservation, purification, stability analysis, xylanase.

Introduction

The plant cell wall is a complex composite of structural polysaccharides that represents the most abundant source of organic molecules in the biosphere. The annual recycling of 1011 tons of plant structural polysaccharides is an important biological process that is integral to the carbon cycle (Taylor *et al.*, 2006). The catabolic breakdown of hemicellulose thus represents a critical step in the recycling of carbon in nature and has been targeted as a subject of intense research with respect to renewable energy resources. β -1,4-linked xylopyranose is the principal component of plant cell wall hemicellulose, which represents the second largest reservoir of fixed carbon in the biosphere (Dodd *et al.*, 2009). Hemicelluloses are a complex heteropolymer made up of glucuronoxylans, arabinoxylans,

glucomannans, arabinogalactans and galactomannans (Singh *et al.*, 2007). Xylan is the most abundant renewable polysaccharide after cellulose (Gupta and Kar, 2009) and as a major component of hemicellulose contributing 15-30% of the total dry weight in angiosperm an 7-12% in gymnosperm. The backbone of xylan consists of β -1,4-xylopyranosyl residues (Singh *et al.*, 2007), depending on the plant source, can be variably substituted by side chains of arabinosyl, glucuronosyl, ethylglucuronosyl, acetyl, feruloyl and p-coumaroyl residues (Pastor *et al.*, 2007). Xylanases are glycosidase, which hydrolyze the endo- β -1,4-xylopyranosyl linkages and degrade xylan into xylooligosaccharides. They are the key enzymes for xylan degradation and differ in their specificities toward the xylan polymer (Pastor *et al.*, 2007). Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes. Endo-1,4- β -D-xylanase (EC.3.2.1.8) randomly cleaves the xylan back bone at β -1,4-xylopyranosyl linkages and releases xyooligosaccharides while β -D-xylosidase (EC.3.2.1.37) cleave the

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terminal β -1,4-xylosidic linkage and releases xylose monomers from the reducing end of xylooligosaccharides and xylobiose (Singh *et al.*, 2007; Dodd and Cann, 2009).

There is an increasing trend towards using enzymes for catalyzing biotransformations (Dalal *et al.*, 2007). Due to their biotechnology application in various industrial processes, xylanase from microorganism have attracted increasing attention in the last decade. Their applications include biopulping wood and pulp bleaching, wastewater treatment, treating animal feed to increase digestibility, processing food to increase clarification, pretreatment of forage crop converting lignocellulosic substances into feedstocks and fuels, improve cell wall maceration for the production of plant protoplasts. Purification has become a necessity as it leads to reduction in bulk, concentration enrichment, removal of specific impurities (e.g. toxins from therapeutic products), prevention of catalysis other than the type desired (as with enzymes), prevention of catalyst poisoning (as with enzymes), recommended product specification (e.g. pharmacopocia requirement), enhancement of protein stability and reduction of protein degradation (e.g. by proteolysis). Most of the purification methods, which are used in laboratory research, can be scaled up to industrial processes. Such methods are filtration, centrifugation, microfiltration, ultrafiltration, diafiltration, precipitation, ion-exchange chromatography and gel filtration. Ammonium sulphate precipitation is an efficient method for removal of lower molecular mass xylanases (Kuhar *et al.*, 2007)

Selected strain from the xylanolytic alkaliphilic mutan has been shown to be producer of an active xylan degradation enzyme. Previously, we have isolated the xylanolytic alkaliphilic bacteria from the crabs and mutation them by ethyl methanesulfonate. The present article reports the purification and preservation of xylanase obtained from liquid state culture of xylanolytic mutan when grown on medium

containing xylans from oat spelt and xylose as substrates.

Materials and Methods

Microorganism and culture conditions

The xylanolytic mutan used in this investigation were obtained from crabs (*Eriocheir sinensis*) from previous study as reported by Hanim *et al.* (2013). For xylanase production, the bacterium was grown on a medium containing oat spelt xylan 1% and xylose 0.02% as carbon source, supplemented with $(\text{NH}_4)_2\text{SO}_4$, 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; NaCl, 2 g; K_2HPO_4 , 7 g; resazurin, 0.1%; yeast extract, 1 g; cysteine HCl, 0.5% and dH_2O , 1.000 ml (Omelianski (1902) cit. Skinner (1971) at pH 9.5. The cultivation was carried out for 5 days at 35°C.

Xylanase assay

Xylanase activity was assayed by measuring the amount of reducing sugar liberated from enzymatic hydrolysis of soluble oat spelt xylan. Briefly, assays containing 0.4 ml of 50 mM sodium acetate buffer (pH 6.0) with 0.2 ml of 4% soluble oat spelt xylan, and 0.2 ml of enzyme preparation were incubated at 50°C for 20 min (Ruiz - Arribas *et al.*, 1995), after which the amount of reducing sugar was measured by the Nelson-Somogyi method with D-xylose as the standard (Plummer, 1978). Substrate and enzyme controls were always used. All assays were performed in duplicate. One unit of activity is defined as the amount of xylanase needed to liberate 1 μmol of D-xylose per min under these assay condition (Ruiz-Arribas *et al.*, 1995).

Protein measurement

The protein concentration of the enzyme preparation was measured by Lowry method (Plummer, 1978), with bovine serum albumin as the standard.

Enzyme purification

Xylanase was purified from 1.5 l of a culture supernatant. The cell-free culture supernatant, as a crude enzyme, was

extracted by centrifugation (14,000 × g for 20 min, 5°C), and divided into 7 tubes (each 50 ml). Enzyme was partially purified by ammonium sulphate (30-90% saturation) precipitation. After incubating overnight at 4°C, the precipitate was discarded by centrifugation (3,000 × g for 20 min, 5°C), and followed by dialysis (dialysis tubing 22 kDa-molecular weight-cutoff membranes) that performed against 10 mM sodium acetate buffer (pH 6.0). The dialysate was determined xylanase activity. The level of ammonium sulphate that gave the highest activity was carried out to enzyme production, and then it was measured the molecular mass and preserved with various carriers.

Molecular mass estimation

The molecular mass of xylanase was estimated by 12% SDS-PAGE. Proteins were stained with Coomassie brilliant blue, and PageRuler Unstained Protein Ladder was used as molecular mass marker.

pH and thermal stability analysis

For pH stability determination, partially purified enzymes were incubated in buffers of varying pH (sodium acetate buffer for pH 3.0-6.0, sodium phosphate buffer for pH 6.5-7.5) at room temperature and for 1h. For thermal stability characterization, various temperature (30-60°C) were used at sodium acetate buffer pH 6 for 1h. The residual xylanase activity was assayed under standard conditions.

Enzyme preservation

Xylanase was preserved with various carriers (tapioca, corn, gapek and soy bean meal), and freeze-dried. Each treatments were determined for xylanase activity.

Statistical methods

Treatments were arranged in a one way design, with the main factors being kinds of carrier (tapioca, corn, gapek and soy bean). The data in the main study were analyzed as a one way arrangement. The differences of mean value were analyzed by Duncan's new multiple range test (Rosner, 1990).

Results

Enzyme purification

The xylanase was tested for the effect of level of ammonium sulphate on the activity (Figure 1). Precipitation with 60% of ammonium sulphate was optimum level that result the highest activity (62.03 U/g). After the enzyme was precipitated with 50% of ammonium sulphate, 38% of the activity was retained. Thirty seven percent of the activity remained after the enzyme was precipitated with 70% of ammonium sulphate. Rapid inactivation of enzyme activity was observed below and above those level.

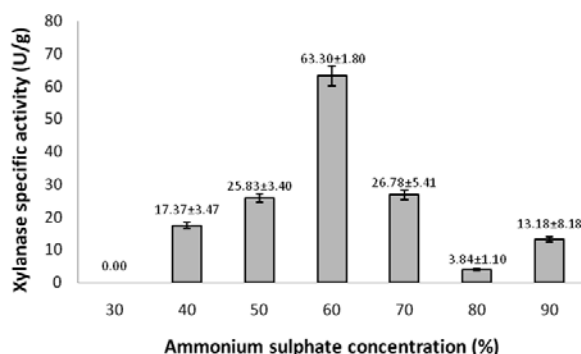


Figure 1. The effect of ammonium sulphate precipitation on enzyme activity (U/g)

The xylanase was purified with 60% of ammonium sulphate to apparent homogeneity. Two protein bands were seen after Coomassie brilliant blue staining, and

Table 1. Purification chart of xylanase from xylanolytic alkalophylic bacteria

Purification Step	Total protein (g)	Total activity (U)	Specific activity (U/g)	Purification (fold)	Recovery (%)
Culture supernatant	0.290	1.18	19.66	1.00	100.00
Ammonium sulphate (60%) fraction	0.045	1.05	62.03	3.15	89.40

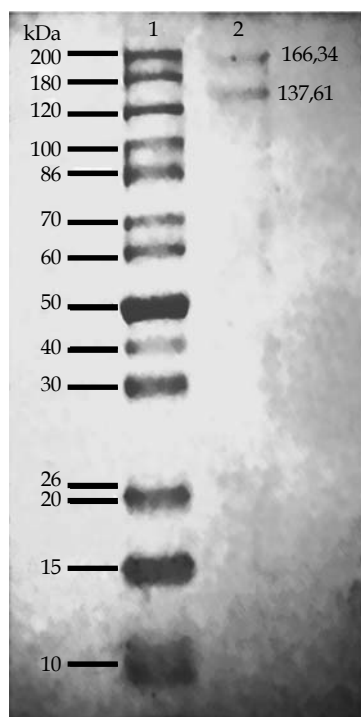


Figure 2. SDS-PAGE analysis of xylanase from alkaliphilic xylanolytic bacteria. Gels were loaded with molecular mass marker (lane 1), and protein after precipitation with 60% of ammonium sulphate (lane 2)

had mass of 137.61 and 165.34 kDa on SDS-PAGE (Figure 2). Specific activity (62.03 U/g) of the purified protein was higher than that crude enzyme (19.66 U/g), as presented in Table 1. It increased 3.15-fold during this step, and resulting 89.40% of enzyme recovery. This purification procedure led to the recovery of 0.045 g of xylanase protein per liter of culture with a specific xylanase activity of 62.03 U/g of protein.

Stability analysis of enzyme

Analysis of the effect of pH and temperature on the hydrolytic activity of

xylanase on oat spelt xylan showed similar pH and temperature profiles (Figure 3 and 4). Analysis of the influence of pH on enzyme stability showed that while the enzyme retained more than 80% of its initial activity after incubation at room temperature for 1h in buffers ranging from pH 3.0 to 7.5, the enzyme was less stable under these conditions, losing more than 50% of its initial activity in buffers at pH lower than 3.5 or higher than 7.0. Thermostability assays showed that the enzyme remained highly stable at 35 to 40°C after 1h of incubation at pH 6.0, while it lost 30% of its initial activity after incubation more than 40°C.

Enzyme preservation

The purified enzyme was tested for the effect of kind of carriers on the activity. The activity of this enzyme was highest

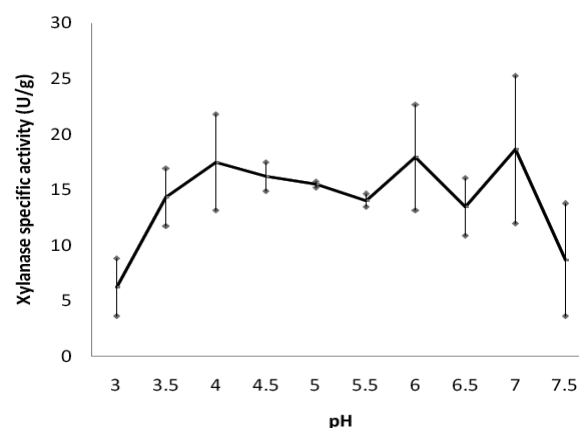


Figure 3. Influence of the pH on the activity of partially purified xylanase. For the pH profile, the enzyme activity was measured at room temperature in 50 mM sodium phosphate and sodium acetate buffer adjusted to the correct pH. Values are the means of results of duplicate experiments. Each point represents the mean of standard deviation (SD) (indicated by error bar) for two different enzymes.

Table 2. The effect of various carriers on xylanase activity

Type of carriers	Xylanase activity (U/g)
Corn	46.80 ^{ab} ± 0.01
Tapioca	60.30 ^b ± 3.27
Gaplek	35.09 ^a ± 10.41
Soy bean meal	28.30 ^a ± 3.18

^{a,b} significantly different (P<0.01)

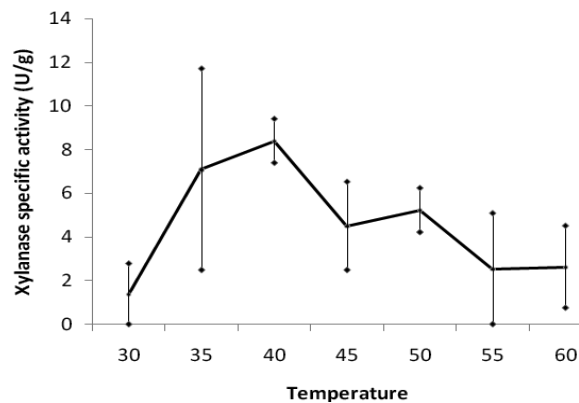


Figure 4. Influence of the temperature on the activity of partially purified xylanase. For the temperature profile, the enzyme activity was measured in 50 mM sodium acetate buffer (pH 6.0) at different temperatures. Values are the means of results of duplicate experiments. Each point represents the mean of standard deviation (SD) (indicated by error bar) for two different enzymes.

with tapioca as carrier (60.30 U/g) ($P < 0.01$), as presented in Table 2. Corn, gapelek and soy bean meal as carriers gave less xylanase activity, those were lower 22.39, 41.81, and 53.07% respectively than that tapioca.

Discussion

The solubility of a globular protein in an aqueous solvent is influenced by four main factors: salt concentration, pH, the organic content of the solvent and the temperature (Palmer, 1991). Ammonium sulphate precipitation is an efficient method of lower mass xylanases. The material needed for the method is cheap, and finds much use in industrial purifications. Ammonium sulphate precipitation led to several fold purification such as 1.2 to 11-fold (Kuhar *et al.*, 2007). Partially purified xylanase in the culture supernatant of *Streptomyces galbus* NR by salting out at 40-60 and 60-80% ammonium sulphate saturation which led to the purification of 9.63-fold and 68.80% recovery (Kansoh and Nagieb, 2004). In this study, 60% ammonium sulphate saturation led to the purification 3.15-fold and 89.40% recovery. In the enzyme purification steps, addition of a small amount of neutral salt to a solution increased the solubility of a

protein. The added ions changes ionization of amino acid side chains and can also interfere with interactions between protein molecules, the overall effect being to increase interactions between solute and solvent. At very high salt concentration, the abundance of interactions between the added ions and water decreases the possibilities for protein-water interactions, often resulting in the protein being precipitated from solution (Palmer, 1991).

Xylanase secreted from xylanolytic alkalophilic bacteria had large molecular weight. Most xylanases are often found as monomeric protein with a molecular weight ranging from 11-80 kDa (Sunna *et al.*, 1996). But xylanases of molecular weight as high as 537 kDa (Matte and Forsberg, 1992), 350 kDa (Shao *et al.*, 1995), 340 kDa (Sa-Pereira *et al.*, 2000), 212-253 kDa (Anthony *et al.*, 2003), 150 kDa (Sunna *et al.*, 1996), 150 kDa (Khanna and Gauri, 1993), 140 kDa (Ito *et al.*, 2003) 131 kDa (Touhy *et al.*, 1993), 124.136 kDa (Cazemier *et al.*, 1999) have been also reported.

After the enzyme has been recovered and concentrated, it is usually formulated in order to meet the product stability specifications suited to the application and handling practices employed by the end-user. Food-grade ingredients are required for food and feed applications. For solid products, the enzyme concentrate or formulated product, if suitable, can be granulated or dried in a manner that provides a solid product that meets health and safety requirements, such as low dusting (Clarkson *et al.*, 2001). Most feed enzymes are supplied as liquid formulations at the end-user level, as these are convenient to use. It is mostly dry enzyme premixes that are being sold to the feed manufacturer. However, solid formulations can provide some significant advantages, such as enhanced stability, delayed or controlled release and protection against deactivation during harsh applications (Kuhar *et al.*, 2007).

It could be concluded that the molecular masses of the xylanase were 137.61 and

165.34 kDa. Addition of 60% ammonium sulphate showed the highest xylanase activity (62.03 U/g) and 89.40% enzyme recovery. Tapioca, as a carrier, had the highest xylanase activity.

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