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ALKALINE CELLULASE FREE XYLANASE FROM *BACILLUS* SP. ASX42: PROPERTIES, PURIFICATION AND ITS EFFECT ON SEED GERMINATION

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ABSTRACT

Bacillus sp. ASX42 isolated from soil samples of Lake Van shores, Turkey, and the strain producing cellulose-free xylanase enzyme with an optimum pH 9.0, at 60ºC. The molecular weight of the enzyme was estimated as 66 kDa on SDS PAGE analysis. Thermal stability of enzyme was detected average 68.7% at 4-60ºC and 60% at 65-95ºC while pH stability was observed about 90% between pH 3.5-13.0 for 15 min. As $HgCl_2$ presented a strong inhibitor activity, Cobalt (132%) and Manganese (130%) showed a stimulatory effect on xylanase activity. The remaining activity was found to be 77%, 89%, and 92% in the presence of EDTA, 1,10-Phenanthroline monohydrate and β-mercaptoethanol, respectively. In this study, some known purification materials were compared for effectiveness. According to the results, cellulase-free xylanase ASX42 has shown a stimulatory effect on germination of *Anagyris foedita* and *Ceratonia siliqua* seeds. It has also produced a whitening effect on wastepaper pulp.

Keywords: Alkaline, *Bacillus* sp., Cellulase-free xylanase, Germination, Purification

INTRODUCTION

Xylanase (EC. 3.2.1.8) is one of the important enzymes acting on xylan which is one of the major components of plant cell walls (**Sá-Pereira *et al*., 2002; Lama *et al*., 2004; Baramee *et al*., 2020**). The xylan is a branched heteropolysaccharide composed of β-1,4 linked xylopyranosyl units backbone with substitution of arabinosyl, glucuronyl and acetyl residues (**Shallom and Shoham 2003; Li *et al*., 2020**). Several enzymes are necessary for complete degradation of xylan, including β-1,4 xylanase (EC 3.2.1.8), arabinofuranosidase (EC 3.2.1.55), β- glucuronidase (3.2.1.131), xylan esterase (EC 3.1.1.6), and xylosidase (EC 3.2.1.37) (**Sharma and Chand, 2012; Baramee *et al*., 2020**). Among these enzymes, β-1,4 endoxylanase plays a key role in the hydrolysis of the xylan backbone into smaller parts (**Wong *et al*., 1988; Dhillon *et al*., 2000; Baramee *et al*., 2020**). Recently, xylanase enzymes become the focus of interest for the second most abundant polymer xylan. So, bioconversion of lignocellulosic material, agro-wastes fuel, digestibility of animal feed, wine-brewery, and juices industry, extraction of plant oils, degumming of plant fibers are the potential applications of xylanases (**Wong *et al*., 1988; Eriksson 1990; Dhiman *et al*., 2008; Baramee *et al*., 2020**). It has also attained the most prominent attention in the paper-pulp industries (**Bajpai, 1999; Li *et al*., 2020**). Xylanase enzyme is especially preferred in paper pulp bleaching which reduces the use of chlorine compounds. This elemental chlorine is the cause of producing carcinogenic, toxic, and persistent material especially for watercourses (**Ali and Sreekrishnan, 2001**). But, the use of enzymes in pulp industries requires both cellulase-free xylanases which are not reducing the pulp quality and active at high temperature and alkaline pH (**Viikari *et al*., 1994; Li *et al*., 2020**). Therefore, microbial xylanases are preferred against plant or animal sources because of their easier and low cost industrial production and structural stability (**Asha Poorna and Prema, 2006; Li *et al*., 2020**).

A large number of microorganisms producing xylanases such as bacteria, yeast and fungi have been reported (**Goulart *et a.*, 2005; Sridevi and Singara Charya, 2011; Sharma *et al*., 2012; Baramee *et al*., 2020**). Among the microbial xylanases, cellulase-free xylanases are important due to the co-secretion of cellulases of which may affect the pulp quality adversely (**Cristov *et al*., 1999; Li *et al*., 2020**). Thus, cellulase-free xylanase producing bacteria isolation is one of the successful management to overcome this problem. Although the *Bacillus* is an industrially important source for extracellular enzyme production, purification steps are still necessary for their enzymes. There are many reports on literature using different methods such as gel filtration (**Blanco *et al*., 1995; Hakamada *et al*., 1997; Dhillon *et al*., 2000; Tseng *et al*., 2002; Lama *et al*., 2004; Li *et al*., 2020**), ion-exchange (**Gerber *et al*., 1997; Hakamada *et al*.,1997; Breccia *et al*., 1998; Saha, 2002; Wejse *et al*., 2003**) chromatographic application following ethanol (**Aygan and Arikan., 2009**), ammonium sulfate (**Hakamada *et a*l., 1997; Wejse *et al*., 2003**) or ultrafiltration (**Cardoso and Filho., 2003**) for xylanase purification. However, the success rate of these methods is different due to the effectiveness of the methods. For industrial applications, the simplest and cheapest way is always preferable and sustainable. **Tachaapaikoon *et al* (2006) and Prakash *et al* (2011**) have been tested corn husk, which is an abundant agricultural waste material, for xylanase purification. In this study, we report the isolation of *Bacillus* sp. ASX42 producing alkaliphilic cellulase-free xylanase and the comparison of some known purification methods along with the enzymic characterization of cellulase-free xylanase as well as assessing the enzyme's usability potential in the ambient conditions. The aim of our study is to obtain and purify alkaline and cellulase-free xylanase enzyme from *Bacillus* sp. ASX42 strain and investigate its industrial usability.

MATERIALS AND METHODS

Organism and culturing

Bacillus sp. ASX42 was isolated from coastal soil samples of lake Van, which is the largest lake in Turkey and also one of the largest endorheic lakes in the world. Gram-positive spore forming bacteria, *Bacillus* sp. was selected by pasteurizing soil samples at 80°C for 10 min (**Ozcan *et al*., 2010**). Total of 235 *Bacillus* sp. were isolated and screened for xylanase production on agar plates (pH 9.0) containing ($g.L^{-1}$) peptone (Sigma-Aldrich, Germany) 5, yeast extract 1 (Millipore, Germany), Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$, Sigma-Aldrich, Germany) 0.2, Calcium chloride dihydrate ($CaCl_2.2H_2O$, Sigma-Aldrich, Germany) 0.1, Potassium phosphate dibasic (K_2HPO_4, Sigma-Aldrich, Germany) 1, Sodium chloride (NaCl, Sigma-Aldrich, Germany) 5, xylan (Oat Spelt, 4-O-Methyl-D-glucurono-D-xylan, Sigma-Aldrich, Germany) 5, agar (Sigma-Aldrich,

Germany) 15 (**Voget *et al*., 2006**). The xylanase producing strains were also tested on agar plates for cellulase production by replacing the xylan with CMC (carboxymethyl cellulose, Sigma-Aldrich, Germany). The strains showing the highest xylanolytic ratio (R/r) were chosen for enzyme production (**Bernhardsdotter *et al*, 2005**). The identification of the strain was accomplished on the basis of morphological and biochemical characteristics (**Ratanakhanokchai *et al*., 1999**).

Enzyme production

The selected strain, *Bacillus* sp. ASX42, was cultured in the medium containing oat spelt xylan (Sigma-Aldrich, Germany) (**Voget *et al*., 2006**) at 30°C with initial pH of 9.0 in a shaking incubator (200 rpm) for 24 h. After centrifugation (Hettich Mikro 22R, Germany) (4020 *g*) for 30 min at 4°C, the supernatant was used as the crude enzyme and divided into three parts. Additionally, inoculum size, carbon sources (maltose (Sigma-Aldrich, Germany), xylose (Sigma-Aldrich, Germany), dextrose (Sigma-Aldrich, Germany), lactose (Sigma-Aldrich, Germany), glucose (Sigma-Aldrich, Germany), sucrose (Sigma-Aldrich, Germany), soluble starch substrate (Sigma-Aldrich, Germany), and nitrogen sources urea (Merck, Germany), ammoniumnitrate (NH_4NO_3, Sigma-Aldrich, Germany), ammonium chloride (NH_4Cl, Sigma-Aldrich), ammonium sulfate ($(NH4)_2SO_4$, Sigma-Aldrich, Germany), sodium nitrate ($NaNO_3$, Sigma-Aldrich, Germany), beef extract (Sigma-Aldrich, Germany), casein (Sigma-Aldrich), tryptone (Sigma-Aldrich, Germany), and some agricultural products were also examined for voluminous enzyme production. Effect of inoculum size for enzyme production was determined by inoculating the culture flask with 5, 10, 15, 20 and 30% of overnight culture arranged to McFarland 1 turbidity standard (approx. $3.0x10^8$ cell /mL) with sterile saline water.

The effect of different carbon sources on xylanase production was assayed by cultivating the *Bacillus* sp. ASX42 in media having one of the substrates (1%) such as starch (Sigma-Aldrich, Germany), sucrose (Sigma-Aldrich, Germany), glucose (Sigma-Aldrich, Germany), lactose (Sigma-Aldrich, Germany), dextrose (Sigma-Aldrich, Germany), maltose (Sigma-Aldrich, Germany). Agro-industrial biomass such as oat bran, barley bran, wheat bran, and wheat straw, were substituted with xylan and studied the enzyme production performance. The agricultural substances were ground in a waring blender and sieved to 60-100 mesh size before adding to the media.

Enzyme purification

Three parts of the crude enzyme were taken to determine and compare the efficiency of purification methods. The first part of the crude enzyme was subjected to Sephadex G-100 column (Sigma-Aldrich, G100120, Germany), Toyopearl HW-40 (Tosoh Bioscie, Japanese), DEAE-Sepharose (Sigma-Aldrich, DFF100, Germany) with ethanol (Sigma-Aldrich, Germany) and ammonium sulfate (Sigma-Aldrich, Germany) precipitation. The second part of the crude enzyme was tested for purification with an agricultural waste product, corn husk (**Tachaapaikoon *et al.* 2006**).

For ethanol precipitation, previously chilled ethanol (-20°C) was added to the clear supernatant with continuous stirring at +4°C, up to the final concentration of 75%, and the solution was left at -20°C overnight. The precipitate was removed by centrifugation at 4020 *g* and +4°C, for 30 min. On the other hand, ammonium sulfate crystals were added to the cell free supernatant to make fractionated saturation (50-80%) at +4°C. Six hours later, each of the precipitates was recovered by centrifugation and then resuspended in 100 mM at pH 7.6 (**Tigue *et al.*, 1995**). Subsequently, they were dialyzed in dialysis tubes (Sigma D9777, MWCO> 14 000 Da, Germany) against the same buffer for 24 hours with several changing. After dialysis, the fractions showing enzymatic activity were gathered together.

For column purification, Toyopearl HW-40 (Tosoh Bioscie, Japanese) and Sephadex G-100 (Sigma-Aldrich, G100120, Germany) (1× 30 cm) were equilibrated with 100 mM phosphate buffer (Sigma-Aldrich, Germany) at pH 7.6. The enzyme suspension (1.5 mL) was eluted at a flow rate of 14 mL/h using the same buffer at room temperature. On the other hand, DEAE-Sepharose CL-6B (Sigma-Aldrich, DFF100, Germany) column (1x15 cm) was also equilibrated with phosphate buffer (pH 8.0, Sigma-Aldrich, Germany). After the enzyme samples were applied to the column, 40 mL of phosphate buffer (pH 8.0, Sigma-Aldrich, Germany) was added at a flow rate of 20 mL/h to remove the unbound proteins. The bound proteins were then eluted discontinuously with 0.1 to 2 M NaCl (Sigma-Aldrich, Germany) dissolved in phosphate buffer (pH 8.0, Sigma-Aldrich, Germany). After dialysis of the elutes against the phosphate buffer (pH 7.6, Sigma-Aldrich, Germany) the fractions showing enzymatic activity (0.1 to 0.5 M of NaCl) were also gathered together.

Another purification method (**Tachaapaikoon *et al*., 2006**) to test is the use of corn husk which contains insoluble xylan on the basis of adsorption and desorption of the enzyme. First of all, washed fresh corn husk was cut into small pieces and dried overnight at 50°C. They were then ground in a waring blender and sieved to 60-100 mesh size. Subsequently, the powder was washed with distilled water and dried at 50°C. Before starting to purification processes, adsorption and desorption assays were also accomplished to determine the best pH values at which the enzyme adsorb and desorb from the insoluble xylan. Firstly, the crude enzyme (supernatant) was concentrated using Amicon Ultra-15 Filter Units tubes (Merck Millipore, Germany) and 250 µL of the enzyme was mixed with 1 mL of 50 mg corn husk powder prepared in different pH buffers (7.0-13.0). After incubation of the mixture at 4°C for 30 min with intermittently shaking by hand, it was centrifuged and the supernatant was used for enzymatic activity. The lowest activity showing that meant the highest adsorption accomplished buffer. For determination of the desorption buffer, phosphate buffer (pH: 7.0, Sigma-Aldrich, Germany), 0.1% and 0.5% of SDS (Merck, Germany), 10% and 50% of Urea (Merck, Germany) buffers were used. After desorption processes, the mixture was centrifuged and the supernatant was used for enzymatic activity determination. The highest activity obtained with buffer used was chosen for desorption buffer.

Enzyme assay

Enzyme activity was assayed by using Miller's method (DNS) (**Miller, 1959**). The reaction mixture was composed of 400 µL of xylan (Sigma-Aldrich, Germany) (1% v/v) and 100 µL of enzyme solution. After 30 min incubation at 60°C, the reaction was ended with the addition of 500 µL of 3,5-dinitrosalicylic acid (Sigma-Aldrich, Germany). Then absorbance was measured at 550 nm in a spectrophotometer (Perkin Elmer Lambda EZ 150, USA). One unit of enzyme activity was defined as 1 µmol of reducing sugar liberated from oat spelt xylan (Sigma-Aldrich, Germany) per min under the standard assay conditions (60°C, pH 9.0). Additionally, the enzyme was tested for substrate specificity with oat spelt xylan (Sigma-Aldrich), beechwood xylan (Sigma-Aldrich), soluble starch (Sigma-Aldrich, Germany), CMC (Sigma-Aldrich, Germany), avicel (Micro-crystalline Cellulose, Sigma-Aldrich, Germany), and 4-nitrophenyl β-D-xylopyranoside (Sigma-Aldrich, Germany).

Effects of pH, temperature and some chemicals on enzyme activity and enzyme stability

Temperature and pH effects on enzyme activity were investigated at different temperatures ranged from 4 to 95°C and at pH 7.0 to 12.0 for 30 min. The following buffers were chosen for the reactions:100 mM Na-phosphate (pH:7.0, Sigma-Aldrich, Germany), 100 mM Tris-HCl buffer (pH: 8.0-9.0, Sigma-Aldrich, Germany), 100 mM Glycine-NaOH (pH:10.0, Sigma-Aldrich, Germany) and 100 mM Borax-NaOH (pH: 11.0-12.0, Sigma-Aldrich, Germany). As for the stability experiment, the enzyme was pre-incubated at different temperatures and pH buffers for 15 and 30 min, and the remaining activity was determined under the standard enzyme assay conditions. Additionally, substrate specificity, substrate concentration, and chemicals' effects on enzyme activity were assayed. For determination of substrate specificity of the cellulase-free xylanase enzyme, 1% of CMC (Sigma-Aldrich), Avicel (Sigma-Aldrich, Germany), 4-nitrophenyl β-D xylopyranoside (Sigma-Aldrich, Germany), soluble starch (Sigma-Aldrich, Germany), oat spelt xylan (Sigma-Aldrich, Germany) and beechwood xylan (Sigma-Aldrich, Germany) were used under the standard enzyme conditions.

The best substrate concentration for enzymatic activity was determined by using 0.1, 0.5, 1.0, and 2.0% of substrate in optimum pH buffer (9.0) and incubating at optimum temperature (60°C). On the other hand, the effects of metal ions $MnCl_2$ (Merck, Germany), $HgCl_2$ (Merck, Germany), $CaCl_2$ (Merck, Germany), $MgCl_2$ (Merck, Germany), $FeCl_3$ (Merck, Germany), $NiCl_2$ (Merck, Germany), $CuCl_2$ (Merck, Germany), KCl (Merck, Germany), 1,10-Phenanthrolin monohydrate (Merck, Germany), Na_2SO_3 (Merck, Germany), $ZnCl_2$ (Merck, Germany), $CoCl_2$ (Merck, Germany), $BaCl_2$ (Merck, Germany), Pb_2Cl_2 (Merck, Germany), chelating agents (EDTA (Merck, Germany), ß-merkaptoetanol (Merck, Germany), and inhibitors (Triton-X 114 (Merck, Germany), Sodium Azide (Merck, Germany), SDS (Sigma-Aldrich, Germany), PMSF (Merck, Germany), Urea (Sigma-Aldrich, Germany) on xylanolytic activity were studied by pre-incubating the enzyme in the presence of test materials with a final concentration as 5 and 10 mM; except SDS (1%; 3%), and 2-Mercaptoethanol (5 mM). Then the relative remaining activity was determined according to the standard enzyme assay condition. The activity in the absence of any additives was taken as control (100%).

PAGE analysis

Efficiency of the purification methods and molecular weight of the enzyme samples were determined with electrophoresis that was prepared as homogenized 10% acrylamide gel. After electrophoresis, the gels were subjected to Coomassie Blue R 250 dye (Merck, Germany) and silver staining (Merck, Germany) (**Bollag et al 1996; Rabilloud, 1999**). Zymogram analysis was performed by incubating the xylan (1%, Sigma-Aldrich, Germany) containing gel in 0.1% Congo red solution (Merck, Germany) and subsequently 1 M NaCl solution (Sigma-Aldrich, Germany) (**Aygan and Arikan, 2009**).

End product analysis

The end products of the ASX42 cellulase-free xylanase were analysed on silica gel 60 (GF254) neutral plate (Merck, Germany) thinlayer chromatography. The development of the products was carried out with Chloroform-Acetic acid-distilled water (Merck, Germany) (6:7:1, v/v/v), and spots were visualized by baking the

plates in an oven at 160°C for 30 min after spraying aniline (Merck) (1% v/v), diphenilamine (Merck, Germany) (1% w/v), orthophosphoric acid (Merck, Germany) (10% v/v) mixture prepared in acetone (Merck, Germany) (**Singh *et al.*, 2004**).

Effect of ASX42 cellulase free xylanase on germination of some seeds and pulp bleaching

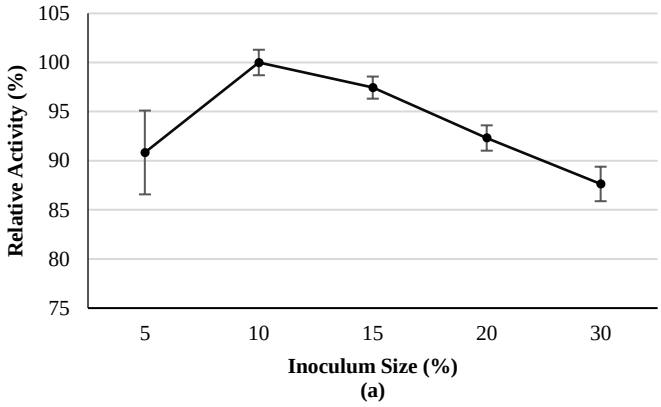
Ceratonia siliqua L. (Carop tree) and *Anagyris foedita* L. (Stinking Bean Trefoil) seeds were chosen for germination studies to determine the assistive effect of cellulase-free xylanase enzyme. The experimental design were arranged as three replicates of seeds per flask. First of all, the seeds in each group were disinfected in 3% sodium hypochlorite solution (Merck, Germany) for 5 min and then rinsed with sterile distilled water several times. They were then placed in a sterile flask, mixed with enzyme suspensions (1.44 U mL^{-1}) and incubated in a shaking incubator (50 rpm) at 60°C for 6 to 24 hours. After incubation period, the seeds were rinsed again with sterile distilled water and transfered to the petri dishes containing a sterile filter paper saturated with distilled water. Finally, all groups were left to germinate upto 14 days at 25°C.

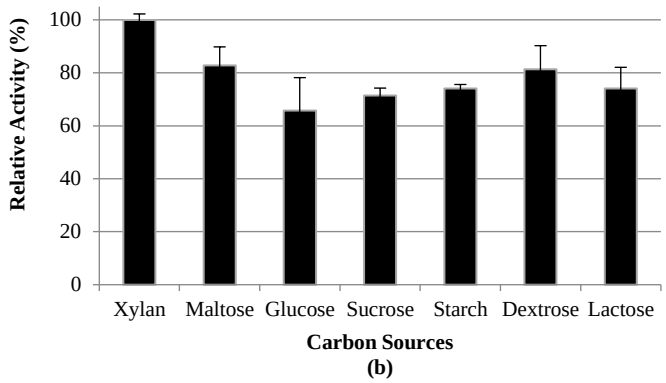
For the enzymatic treatment of waste paper, the pulp was prepared from office paper (30%), newspaper (40%) and carton boxes (40%). The fiber freeing process was accomplished according to the ISO 5267-1 method using Schopper-Riegler tester with 0.4% consistency. The enzyme was added to the pulp as 1.44 U/g and stirred for 1 h at 60ºC and pH 9.0. After completing the pulping process (50±5 ºSR) in Hollander beater, 70±3 g/m^2 of papers were produced in Rapid Köthen RK-21. Finally, physical and optical parametres of the papers were measured according to TAPPI T 402 om-88 after 24 h in a conditioned lab with 23±1 ºC and 65±1% relative humidity.

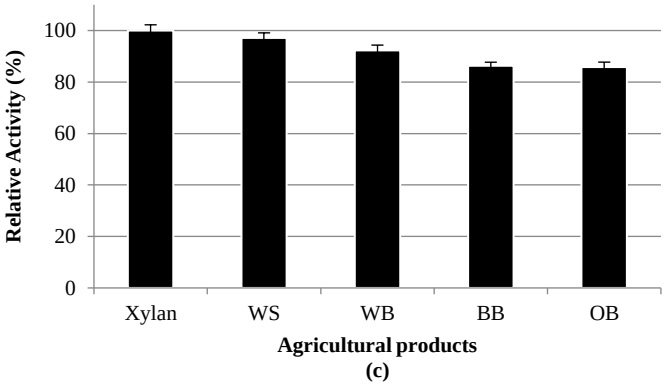
RESULTS

Microorganism and enzyme production

The strain (ASX42) producing extracellular xylanase was identified as *Bacillus* sp. on the basis of gram staining, cell morphology, motility, spore formation, and aerobic growth potential. The enzyme synthesis was occurred at temperatures between 20 to 45°C and pH 5.0- 10.0 while the maximum xylanolytic activity (r/R) was at 30°C and pH 9.0. Effect of inoculum size for enzyme production was determined by inoculating the culture flask with 5, 10, 15, 20, and 30% of overnight culture arranged to McFarland 1 turbidity standard (approx. $3.0x10^8$ cell /mL) with sterile saline water. The inoculum size at 10% was found to be optimum for enzyme production (Fig. 1a). Although the inoculum size of 15% has found to be productive the enzyme production was steadily reduced as the inoculum size increased. Similar findings with different microorganisms have been reported for maximum production at 10% (**Babu and Satyanarayana, 1995; Asha Poorna and Prema, 2007**). Among the carbon sources tested in this study, xylan was the best one for xylanase production for *Bacillus* sp. ASX42 (Fig.1b). Wheat straw was gound to be the best substrate after xylan which could be a cost-effective biomass for bulk production of the enzyme, compared to expensive xylan (Fig. 1c). Subsequently, the best substrate (straw) concentration was also determined for maximum enzyme production. The media containing 1% of ground straw was the highest enzyme producing set of *Bacillus* sp. ASX42 culture (Fig. 1d). Among the nitrogen sources tested, there was no sample that acting to increase the enzyme production (data not shown here).







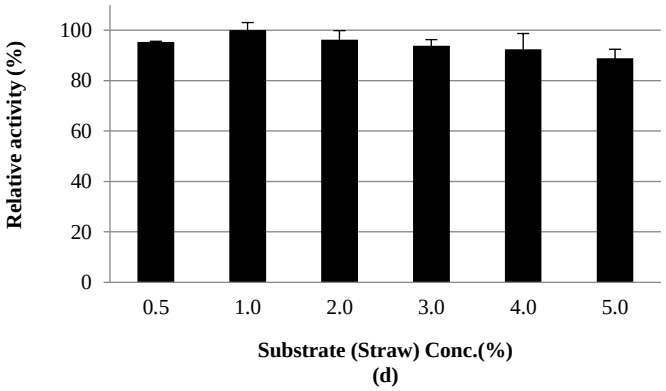


Figure 1 The Effect of inoculum size (a), carbon sources (b), agricultural products (c), substrate concentration (d) on enzyme production (Xylan: Oat spelt xylan, WS: Wheat straw, WB: Wheat bran, BB: Barley bran, OB: Oat bran)

Enzyme properties

The enzyme cellulase-free-xylanase ASX42 showed a relative activity over 61% between pH 7.0 and 10.0 with maximum activity at pH 9.0 (Fig. 2). Although the maximum activity was observed at 60°C, the enzyme revealed an activity over 60% between 35-75°C (Fig. 3). The remaining activity experiments showed that the enzyme stable for only 30 min up to 55°C with activity around 40% (Fig. 4). The influence of pH on the stability of the enzyme determined by incubating it at optimum temperature for 15 and 30 min. The enzyme was almost stable for 15 min up top pH 9.0, but a decreased remaining activity was obtained for 30 min incubation for all pH values except pH 9.0 (Fig. 5). Substrate specificity of the enzyme was carried out with oat spelt xylan, beech wood xylan, avicel, CMC, starch and 4-nitrophenyl β-D-xylopyranoside. There was no enzymatic activity observed against the substrate tested apart from xylan (OSX: Oat spelt xylan, BWX: Beech wood xylan) (Fig. 6a). In terms of substrate concentration, 1% of the substrate was enough for the best enzyme reaction (Fig. 6b).

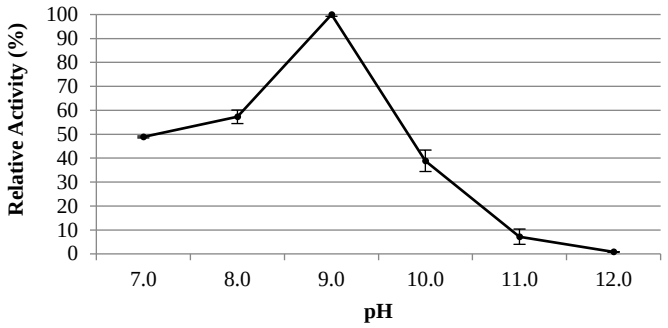


Figure 2 Effect of pH on the activity of ***Bacillus*** sp. ASX42 cellulase-free xylanase

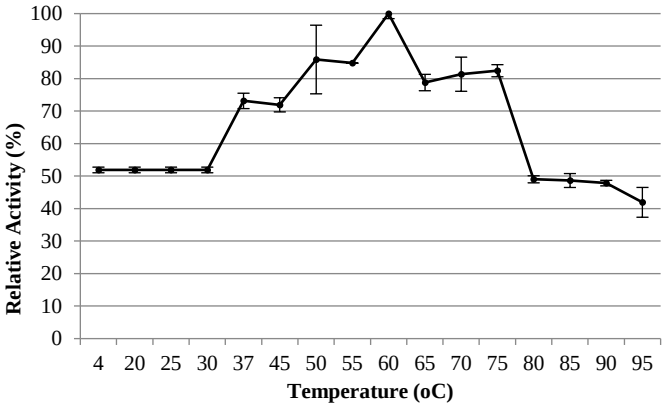


Figure 3 Effect of temperature on *Bacillus* sp. ASX42 cellulase-free xylanase enzyme activity

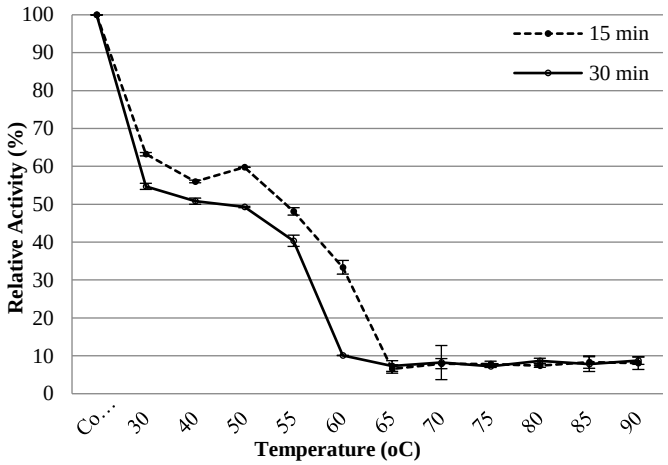


Figure 4 Effect of temperature on the stability of ASX42 cellulase-free xylanase enzyme

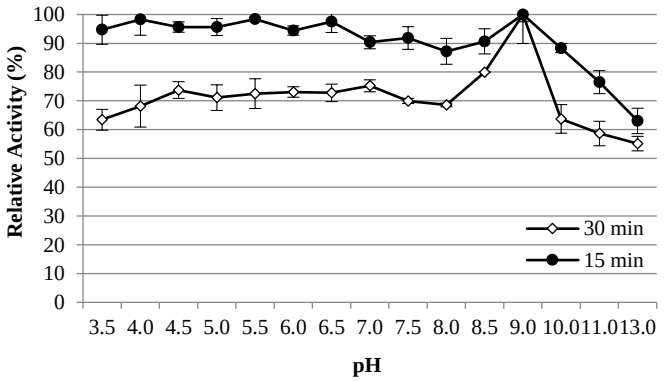
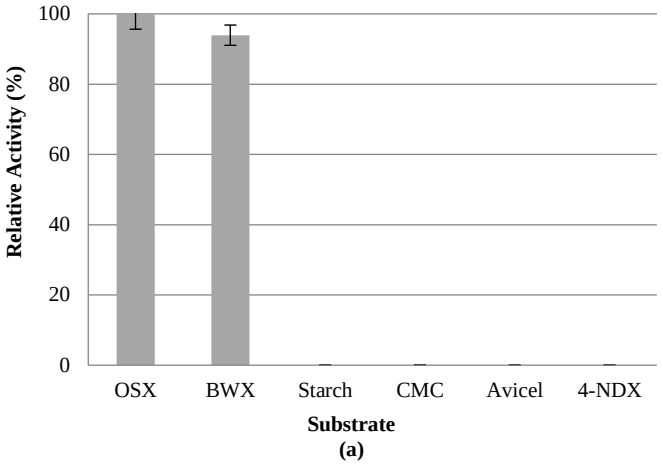


Figure 5 Effect of pH on the stability of ASX42 cellulase-free xylanase enzyme



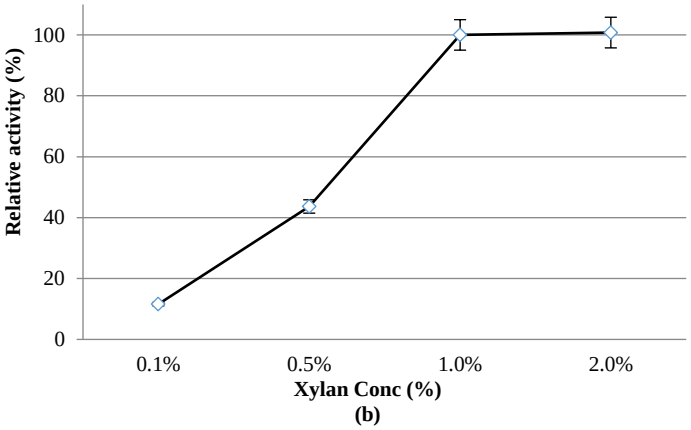
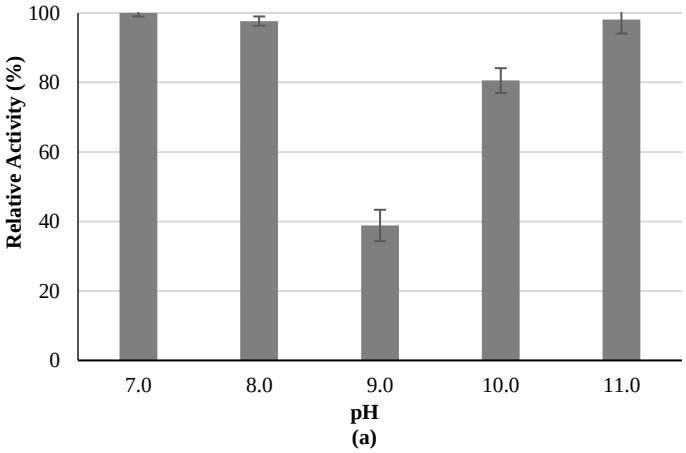


Figure 6 Substrate specifity of ASX42 (a) and the effect of substrate concentration on enzyme activity (b) (**OSX**: Oat spelt xylan, **BWX**: Beech wood xylan, **CMC**: Carboxymethyl cellulose, **4-NDX**: 4-Nitrophenyl β-D-xylopyranoside)

Enzyme purification

The lowest activity was obtained at pH 9.0 which means the enzyme stick together with corn husk powder. For desorption process, phosphate buffer (pH: 7.0), 0.1% and 0.5% of SDS, 10% and 50% of Urea buffers were used. Phosphate buffer (pH: 7.0) produced the highest enzymatic activity (Fig.7a, b) which was prefered reasonably as a choice of desorption buffer. According to SDS-PAGE analysis with silver staining, among the column purification, DEAE-sepharose was the most efficient method compared to other column methods (Fig.10). But the yield of enzyme purified was negotiable. The results also showed that no need to use a precipitating agent like chilled ethanol or ammonium sulfate.



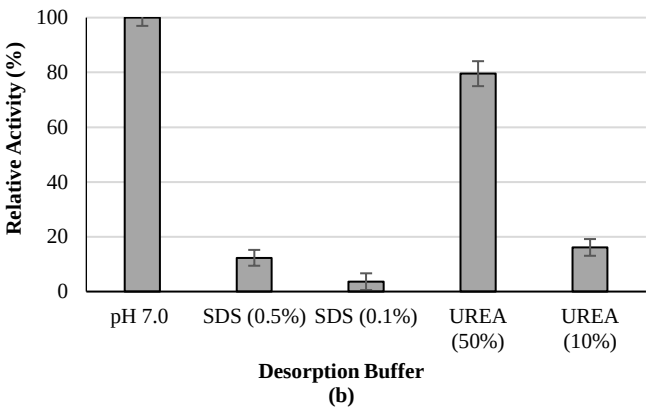


Figure 7 Adsorption pH of cellulase-free xylanase ASX42 (a) to and desorbtion of cellulase-free xylanase ASX42 from corn husk powder (b)

Effect of some metal ions and chemicals on activity

The enzyme was incubated at 60°C for 15 min at different concentrations (5 mM and 10 mM) of substances prior to standard enzyme assay. The activity detected was expressed as residual activity. The activity was strongly inhibited in the presence of $HgCl_2$. Increasing concentrations of metal ions had a stimulating effect on enzyme activity (Fig.8).

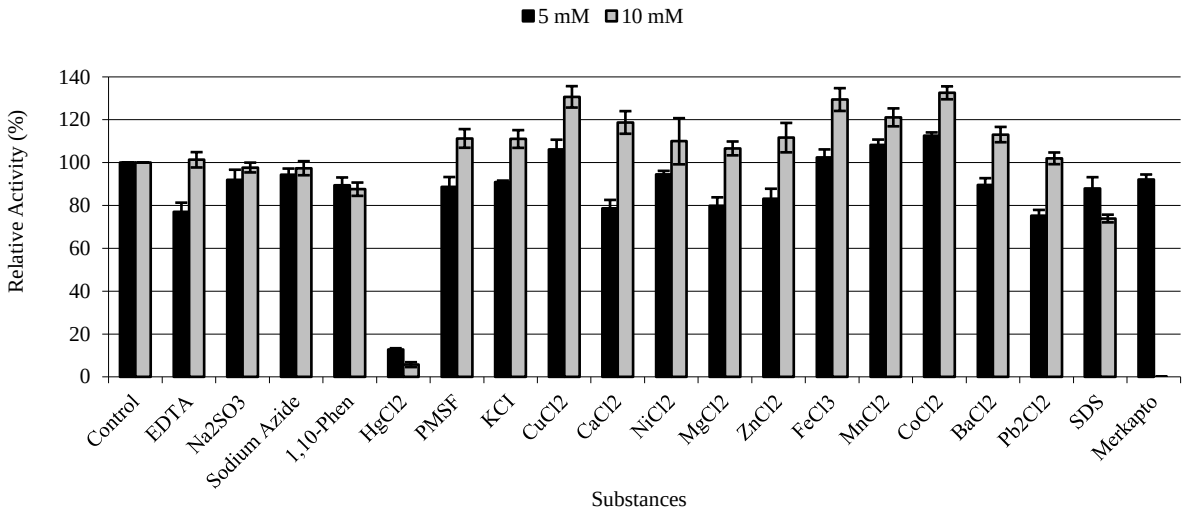


Figure 8 Effect of different metal ions and chemicals on enzyme activity of cellulase-free xylanase ASX42

End product analysis of enzyme activity

After 2 hours of incubation of enzyme and substrate (0.5%) at 60°C, xylose and xylooligosaccharides were formed. This analysis also confirmed that the enzyme ASX42 is a cellulase-free xylanase (Fig.9).

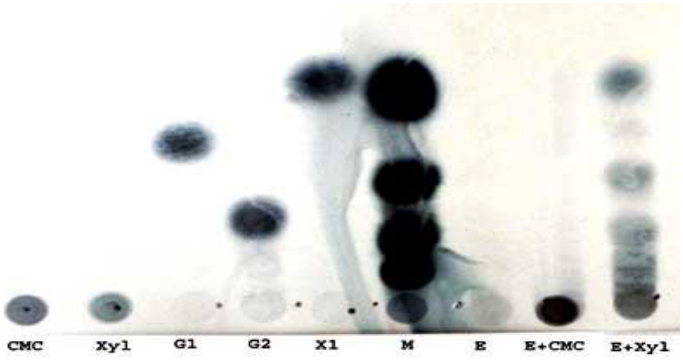


Figure 9 Thin layer chromatography of end products of the enzyme from *Bacillus* sp. ASX42. **CMC**: Carboxymethylcellulose, **Xyl**: Xylan, **G1**: Clucose **G2**: Maltose, **X1**: Xylose, **M**: Marker (1,4-β-D-Xylotetraose, 1,4-β-D-Xylotriose, 1,4-β-D-Xylobiose, β-D-Xylose) (Sigma), **E**: Enzyme (cellulase-free xylanase ASX42), **E+CMC**: Enyzme and CMC, **E+Xyl**: Enzyme ASX42 and xylan.

PAGE and zymogram analysis

SDS-PAGE analysis (**Bollag *et al.*, 1996**) was performed for the determination of purification efficiency and molecular mass estimation. After electrophoresis, the protein bands were visualized either Coomassie Brillant Blue R-250 (**Bollag *et al.*, 1996**) or silver staining (**Rabilloud, 1999**). Activity staining was accomplished by cutting the gel, including substrate (**Aygan and Arikan, 2009**), into two pieces, one was processed for protein staining and the other was subjected to renaturation and staining with Congo Red. According to the results, the enzyme was estimated as 66 kDa (Fig. 11).

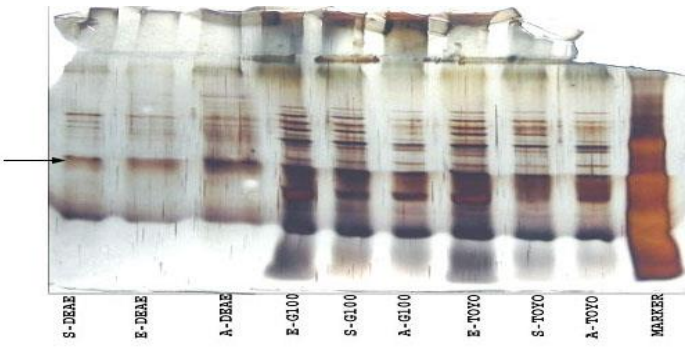


Figure 10 Comparison of purification methods for ASX42 cellulase-free xylanase with silver staining (**S**: Supernatant (Previously concentrated in Amicon Ultra-15 Filter tubes by centrifugation at 4020 g and applied to DEAE, G100 and Toyopearl; **E**: Supernatant was precipitated with previously chilled ethanol and applied to column; **A**: Supernatant was precipitated with ammonium sulfate and applied to column.

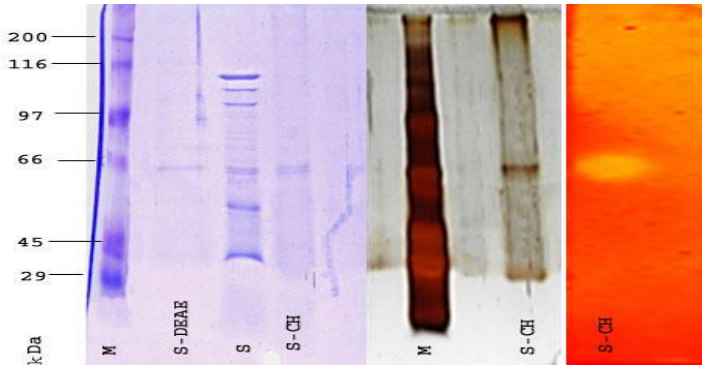


Figure 11 SDS PAGE Molecular weight determination and zymogram analysis of ASX42 cellulase-free xylanase (**M**: Marker-Sigma SDS6H2; **S**: Supernatant (Previously concentrated in Amicon Ultra-15 Filter tubes by centrifugation at 4020 *g*; **S-DEAE**: Concentrated supernatant applied to DEAE-sepharose column, **S-CH**: Concentrated supernatant applied to corn husk purification)

Effect of seed germination and pulp bleaching

A correlation has been observed with incubation time and germination of the seeds (Tab 1). But enzyme treatment has improved the final germination of seeds. Germination was increased with the pretreatment incubation period for both plant seeds. The enzyme action on germination was confirmed with sugar determination from pre-treatment liquid (data not shown here). As for the effect of the enzyme on physical and optical parametres of pulp from waste-paper, there was a visible increase in whiteness although increase in yellowness (Fig. 12). This could be due to unpurified enzyme application. Enzyme application has also decreased the physical quality of the paper (Tab 2).

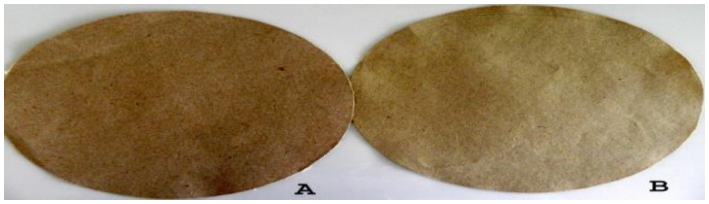


Figure 12 Pre-bleeaching process of Pulp from Waste-paper with ASX42 cellulase-free xylanase. A) Untreated B) Treated with ASX42 cellulase-free xylanase.

Table 1 ASX42 Cellulase-free xylanase activity on seed germination.

Incubation Time	**6 hrs**		**12 hrs**		**24 hrs**	
Treatment	**Dw**	**AS42**	**Dw**	**ASX42**	**Dw**	**ASX42**
***Anagyris foedita* (%)**	0	20	10	30	20	50
***Ceratonia siliqua* (%)**	30	50	40	60	80	100

Legend: Dw: Distilled water, ASX42: Cellulase-free xylanase (1.44 U mL^{-1}),(%): Germination percentage

Table 2 Physical and Optical Parametres of Pulp from Waste-paper

Physical and Optical Parametres of Pulp from Waste-paper	**Control**	**ASX42 xylanase**
ISO Whiteness (%)	44.21	45.91
ISO Brightness (%)	36.87	35.08
Yellowness (ASTM-E313)	23.47	32.31
ISO Opacity (%)	99.59	99.58
Breaking lenght (km)	29.53	27.14
Burst index (kPa.m^2 g^{-1})	1.50	1.8
Tear index (mN.m^2 g^{-1})	1.54	1.32

Legend:ASX42: Cellulase-free xylanase (1.44 U mL^{-1})

DISCUSSION

Bacillus sp. ASX42 presented a mesophilic growth between 20 to 45°C and pH 4.0-12.0, enzymatic production capacity was limited to pH 6.0 to 11.0 like *Bacillus* sp. C14 and JB-99 (**Johnvesly and Naik, 2001; Aygan and Arikan, 2009; Li *et al*., 2020**). In terms of optimal growth (pH 9.0), the strain is called alkaliphilic. For enzyme production, the inoculum size, 10%, has found to be productive but the enzyme production was steadily reduced as the inoculum size increased. Similar findings with different microorganisms have been reported for maximum production at 10% (**Babu and Satyanarayana, 1995; Asha Poorna and Prema, 2007**). For bulk production of the enzyme, wheat straw could be preferred for cost-effective production (Fig.2b). The molecular weight of ASX42 enzyme was estimated to be around 66 kDa. Different xylanases from various *Bacillus* sp. with different molecular weights have been reported by many researchers. **Li., (2009)** reported xylanases between 40 and 70 kDa while **Li *et al*., (2004), Yin *et al*., (2010), Monishe *et al*., (2009), Sanghi *et al*., (2010), Shrinivas *et al*., (2010), and Li *et al*., 2020** detected xylanase enzymes lower than 40 kDa.

The alkaline cellulase-free xylanase showned optimal activity at pH 9.0 (Fig. 5), which was similar to **Khandeparkar and Bhosle (2006); Gessesse (1998); Nakamura *et al*., (1993); Mamo *et al*., (2006); Prakash *et al*., (2008), Shrinivas *et al*., (2010), Li *et al*., (2020).** The pH stability of ASX42 enzyme was also similar to **Yin *et al*., (2010); Shrinivas *et al*., (2010) and Li *et al*., (2020)** findings with 100% stability for 15 min. The enzyme, cellulase-free xylanase ASX42, was tested between 4 to 95°C and average of 68% activity was detected with a maximum at 60°C (Fig. 3). **Antanopoulos *et al*., (2001), Chadha *et al*., (2004)**, was also reported their xylanase enzyme with 60°C optimum. This kind of enzyme having a temperature optimum like ASX42 is preferred for paper pulp and detergent industries. The stimulatory effect of increased metal ions on the xylanase enzyme was also reported by **Gessesse (1998), Liu *et al*., (1998), and Li *et al*., (2020).** On the other hand, **Khasin *et al*. (1993)** reported a weak inhibition with Fe^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+} Cu^{2+} Al^{3+}. As 1% of SDS inhibited cellulase-free xylanase enzyme by 13%, 3% of SDS inhibited the activity by 27%, which indicates that affected hydrophobic aminoacids amounts are increased with SDS concentrations. Although metallo enzymes are inhibited with EDTA, this cellulase-free xylanase ASX42 remains unaffected in the presence of EDTA. As **Li *et al*., (2009)** reported an inhibitory effect of EDTA on their cellulase-free xylanases, **Prakash *et al*., (2011), Sanghi *et al*., (2010), Dutta *et al*., (2007), Khandeparkar and Bhosle (2006) and Lama *et al*., (2004)** reported similar findings to ASX42. Although a mild inhibition was observed with 1,10-Phenantroline, a slight inhibition was detected with sodium azide and Na_2SO_3. Stimulatory effect of PMSF (10mM) on enzyme activity (111%) could be due to its protective action on serine and cysteine residues in the catalytic domain of the enzyme. The outer surface of the seeds of *Ceratonia siliqua L.* and *Anagyris pheodita L.* has a very hard and impermeable shell. The most important factor preventing seed germination is this impermeable layer. This hard shell prevents the seed from taking water and significantly affects germination **(Batlle and Tous, (1997); Carvalho and Nakagawa, (2000)**. For this reason, it has been reported that subjecting the seeds to different pre-treatments (such as water and hot water soaking, sulfuric acid and gibberellic acid (GA3)) before planting has a positive effect on germination **(Ortiz *et al*., (1995); Martins-Louçao *et al*., (1996); Piotto and Di Noi, (2003); also El-Shatnawi and Eriifej (2001)**. **Tsakaldimi and Ganatsas (2002)** reported that *Ceratonia siliqua L. (Carop tree) seed* has a very hard structure and therefore has a physical dormancy. Unlike the enzyme application to the seeds of *Ceratonia siliqua L.* (*Carop tree*) in this study, **Yıldız (1995); Balkıç et al. (2017); Pérez-García (2009); Gübbük *et al*., (2008)** applied 5 different pre-treatments to carob seeds and in their study, they reported that the combinations they used different pretreatment for seeds has low efficiency in germination yield. Similarly **Martins-Louçao *et al*., (1996); Batlle and Tous, (1997); Eler (1988); Alpacar (1988); Köse (2000); Eler (2002); Avşar (2004); Wycoff (1961); Benson (1976); Meınes (1965)**, reported that different pre-treatment combinations were tried for Juniperus communis L. seeds, which have germination problems due to their shell thickness, but their germination yield was low. Contrary to the literature data given above, the method of abrading the seed coats with enzymes used in this study has been found to be very effective in breaking the dormancy that occurs in the seed due to the shell thickness of the seeds. In addition, the method used in this study, contrary to the literature data given above, shortened the germination time, increased germination efficiency and accelerated seed germination, without the need for costly preliminary steps for germination, and showed its usability in the forest industry and industrial branches such as landscaping (**Xiuting *et al*., 2011; Beg *et al*., 2001)**. According to the literature data, it was determined that the enzyme and seed coat abrasion method used in this study was used for the first time in the literature. In this study, we have also tested corn husk for cost-effective method for purification

of xylanase enzyme over some traditional methods and the data confirmed that corn husk is able to produce beneficial yield (Fig.11).

CONCLUSION

Bulk production of industrial enzymes still requires bioprocessing and the majority of the enzymes produced are of microbial origin. Although there are many advantages of microbial production of enzymes, it still needs purification and purification is a naturally expensive process. One of the main approaches in industries, the cheap material and easy processes is preferred. Corn husk is one of the abundant agricultural waste material which is not widely used. Corn husk including insoluble xylan could be a choice cost-effective purification material since it produced results similar to DEAE sepharose. The enzyme ASX42 xylanase produced by *Bacillus* sp. is an alkaline and cellulase free enzyme. According to the results, ASX42 has potential for pulp and paper, biofuels, detergent, food industries, and arboriculture.

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