SCREENING OF XYLANASE PRODUCING MICROORGANISMS

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Abstract: *Hemicellulose, the second most abundant natural polymer on earth, is a mixture of polysaccharides and vegetable gums, found, together with cellulose and lignin, in plant cell walls. Xylanases are enzymes involved in the hydrolysis of xylan, the major component of hemicellulose. These enzymes are able to hydrolyze xylan, by breaking the β-1,4-glycoside linkages, in order to produce xylose and other degradation compounds. Many degrading microorganisms such as fungi, bacteria, yeast etc. have been found to produce xylanases. In this study, our aim was to test different strains regarding their ability to produce xylanases. A screening of the bacterial and fungal strains was performed in order to select the microorganisms that could produce higher amount of xylanases. The strains were cultivated on minimal agar medium with 0.5% oat spelt xylan as the carbon source. The plates were incubated at 28ºC, for three to ten days (depending on microorganism) and analyzed at every 24 hours for the occurrence and evaluation of the halo diameter, using Congo red staining. Based on the xylan hydrolysis area, several microbial strains were selected for further analysis: Bacillus amyloliquefaciens B4, B.amyloliquefaciens BN7, Aspergillus flavus T11, A. flavus AFR A. niger prot., A.niger An4, A.brasiliensis, Trichoderma atroviride TK20, T.viride UV, T. viride Tv2 T.harzianum TK25, T. harzianum P8, Fusarium sp., Penicillium digitatum, Rhizoctonia solani, P. verruculosum KUCC 47345. For enzymatic analysis and the pH variation, the microorganisms were cultivated in liquid medium containing 0.5% oat spelt xylan, at 28ºC for 5-9 days. In addition, the protein assay was carried out in order to calculate the specific enzymatic activity. The best activities were detected in B.amyloliquefaciens B4, A. brasiliensis ATCC 16404, Penicillium digitatum and A.niger An4. These results are significant for further studies regarding lignocellulosic biomass biodegradation by an enzymatic complex.*

Keywords: *Xylanase, Bacillus, Aspergillus, Trichoderma, Fusarium, Penicillium*

INTRODUCTION

Hemicellulose, first introduced in 1891 by Schulze, is the second most abundant natural polymer on earth (20-50% of lignocellulose biomass) (HENDRIKS AND ZEEMAN, 2009; AGBOR, 2011) that consists of xylans, mannans, galactans and arabinans as the main heteropolymers (BEG, 2001). The fractions proportion depends upon the source, therefore, in hardwood hemicelluloses are dominantly found as xylan and in softwood as glucomannan (BEG, 2001; HENDRIKS AND ZEEMAN, 2009; AGBOR ET AL., 2011, BUGG, 2011).

Xylan is the most abundant renewable non-cellulosic polysaccharide present on earth (DHIMAN et al., 2008). It's located between the lignin and the cellulose fibers, being covalent linked to lignin and non-covalent to cellulose, thus helping protect the fibers against degradation to cellulases (BEG, 2001). Xylan is composed of xylopyranosyl residues linked by β-1,4-glycosidic bonds. Due to heterogeneity and chemical complexity of xylan, the complete breakdown requires the action of several hydrolytic enzymes with diverse specificity and modes of action (MOTTA ET AL., 2013; UDAY et al., 2016).

Xylanases, a group of enzymes that hydrolyse xylan backbone into small oligomers, are ubiquitous and diverse by nature (COLLINS ET AL., 2005). The xylanolytic enzyme system includes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase and phenolic acid esterase (DHIMAN ET AL., 2008). β-1,4-endoxylanase (EC 3.2.1.8) is responsible for the hydrolysis of the main chain xylosidic linkages and β-xylosidase

(EC 3.2.1.37) releases xylosyl residues after attacking the xylooligosaccharides (SUBRAMANIYAN AND PREMA, 2002). If the main two enzymes are used for the breakdown of the backbone of xylan, the rest of them are necessary for the cleavage of the side-chain of the molecule (UDAY ET AL., 2016). The combined action of all these enzymes converts the xylan molecule into constituent sugars.

Studies have shown that xylanases are produced by a variety of sources, including bacteria, fungi, yeast, algae (MANDAL, 2015), seeds, snails, crustaceans (POLIZELI ET AL., 2005). However, the major producers of these enzymes are fungi and bacteria. Bacterial xylanases have different characteristics from fungal xylanases. Therefore, xylanases produced by bacteria and actinomycetes (*Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp.) are efficient in a broader pH range of 5 to 9 and temperature of 35-60ºC (BEG ET AL., 2001; MANDAL, 2015; MOTTA ET AL., 2013) being useful in different industries due to their alkali tolerance and thermostability, like the pulp and paper industry (MANDAL, 2015). Fungal xylanases (*Aspergillus* sp., *Fusarium* sp., *Penicillium* sp.) are effective at a pH range of 4 to 6 and temperature below 50ºC (MANDAL, 2015), thus being used in limited industrial applications. However, fungi are important producers due to their higher xylanase activity (compared with bacteria, yeast) (MANDAL, 2015; MOTTA ET AL., 2013), their high yields and extracellular release of the enzymes (Nair and Shashidhar, 2008). Another difference between bacterial and fungal xylanases is linked to the presence of cellulase, few studies reporting fungal xylanase without cellulase activity (SUBRAMANIYAN AND PREMA, 2002).

The purpose of the study was to test different isolated strains regarding their ability to produce xylanases. Over the years, the use of xylanase in different industries (bio-processing of fabrics, biobleaching of pulp, waste paper recycling, bioconversion into higher value products, food and feed) has increased significantly (DHIMAN ET AL., 2008; TECHAPUN ET AL., 2003; HAKI AND RAKSHIT, 2003;). Thus, the interest in this field has increased, scientists isolating newer microbial strains for xylanase production (DHIMAN ET AL., 2008).

MATERIALS AND METHODS

Microorganism

Bacteria (*Bacillus amyloliquefaciens B4, B.amyloliquefaciens BN7, B.licheniformis B40, B.subtilis ICPC, B.subtilis 832 S, B.subtilis USA 2, B.subtilis ATCC 11774, Bacillus spp. B5, Bacillus spp. B6, B.amyloliquefaciens BIR, Streptomyces spp. S6, Str S1* and *Str. S9),* fungi (*Trametes versicolor, Alternaria sp., Rhizoctonia solani, Aspergillus flavus T11*, A.*flavus AFR, A.niger prot.*, *A.niger An4, A.brasiliensis ATCC 16404, Trichoderma atroviride TK20, T.viride UV, T. viride Tv 2, T.harzianum TK25, T. harzianum P8, Fusarium graminearum G82*, *F. oxysporum, F.culmorum FC28, Penicillium digitatum, P. verruculosum KUCC 47345*) were provided by the Department of Genetics and genetic engineering of the Faculty in USAMV Bucharest.

Screening of microbial isolates for xylanase activity

Primary screening of isolates – qualitative evaluations

The bacterial and fungal isolates were subjected to screening for their xylanase activity using the plate screening method on minimal agar medium with 0.5% oat spelt xylan as

the only carbon source (Sridevi and Charya, 2013). For bacteria the medium consisted of (g/L): 0.05g $MgSO_4 \cdot 7H_2O$, 0.05g NaCl, 0.01g $CaCl_2$, 0.2g yeast extract, 0.5g peptone, 2g agar (MAHILRAJAN, 2012). The medium for the fungal strains contained the following constituents (g/L): 0.05g $MgSO_4 \cdot 7H_2O$, 0.005g $CaCl_2$, 0.005g $NaNO_3$, 0.009g $FeSO_4 \cdot 7H_2O$, 0.002g $ZnSO_4$, 0.012g $MnSO_4$, 0.23g KCl, 0.23g KH_2PO_4, 2g peptone, 19g agar (ADESINA AND ONILUDE, 2013). The Petri dishes containing the medium were inoculated with the microorganism in the center of the dish. The plates were incubated at 28±2ºC for 3 to 10 days, depending on the strain and analyzed at every 24 hours for the occurrence and evaluation of the halo diameter. The Petri dishes were flooded with 0.4% Congo red dye and after 10 minutes washed with 1M NaCl (ADESINA AND ONILUDE, 2013).

Secondary screening of isolates – quantitative determinations

The selected isolates after the first screening were cultivated on liquid medium with 0.5% oat spelt xylan as the carbon source. Other components of the medium were for bacteria (g/L): 0.05g $MgSO_4 \cdot 7H_2O$, 0.05g NaCl, 0.01g $CaCl_2$, 0.2g yeast extract, 0.5g peptone (Mahilrajan, 2012) and for fungi (g/L): 0.05g $MgSO_4 \cdot 7H_2O$, 0.005g $CaCl_2$, 0.005g $NaNO_3$, 0.009g $FeSO_4 \cdot 7H_2O$, 0.002g $ZnSO_4$, 0.012g $MnSO_4$, 0.23g KCl, 0.23g KH_2PO_4, 2g peptone (ADESINA AND ONILUDE, 2013). The Erlenmeyer flasks (250 mL) containing the medium were inoculated with the selected microorganism and incubated at 28±2ºC in an incubator with shaker at 120 rpm for 5-9 days.

Tertiary screening of isolates

The isolates that showed high xylanase activity were subjected to a third screening, being cultivated on liquid medium with 0.5% wheat bran as the carbon source. Other constituents of the medium were (g/L): 0.05g $MgSO_4 \cdot 7H_2O$, 0.005g $CaCl_2$, 0.005g $NaNO_3$, 0.009g $FeSO_4 \cdot 7H_2O$, 0.002g $ZnSO_4$, 0.012g $MnSO_4$, 0.23g KCl, 0.23g KH_2PO_4, 2g peptone. The inoculated media flasks were incubated at 28±2ºC in an incubator with shaker at 120 rpm for 7 days.

Xylanase assay

Samples were taken at every 24h, xylanase activity being determined according to the DNS assay for reducing sugars (MILLER, 1959; BAILEY ET AL., 1992). Assay mixture consisted of 0.5 mL sample and 0.5 mL of 0.6% oat spelt xylan (0.6g in 100 ml of 0.05M Na-acetate buffer at pH 5.3) and was incubated in water bath at 40ºC for 10 min. The reaction was terminated by adding 1 mL of 3,5-dinitrosalicylic acid (DNS) reagent and heating for 5min at 80ºC. 3 mL of distilled water was added to the mixture and after 30 min the absorbance was read at 540 nm using a spectrophotometer to determine de amount of sugar released by the enzyme. One unit of xylanase was defined as the amount of enzyme that released 1 μmol reducing sugar as xylose equivalent per minute in the reaction mixture under the specified assay conditions. In addition, the protein assay by Lowry method (1951) was carried out in order to calculate the specific enzymatic activity.

RESULTS AND DISCUSSION

Primary screening of isolates

The xylanolytic activity was detected based on the clear zones of hydrolysis of xylan around the microbial colonies. After the evaluation of the halo diameter (Figure 1.), several microbial isolates were selected for the quantitative determination of xylanase activity: bacteria (*Bacillus amyloliquefaciens B4, B.amyloliquefaciens BN7*) and fungi (*Aspergillus flavus AFR, A. flavus T11, A.niger An4, A. niger prot., A.brasiliensis ATCC 16404, Trichoderma atroviride TK20, T.harzianum TK25, T. harzianum P8, T. viride Tv2, T.viride UV, F.culmorum FC 28, Rhizoctonia solani, Penicillium digitatum, Fusarium graminearum G82, F. oxysporum, P. verruculosum KUCC 47345*).

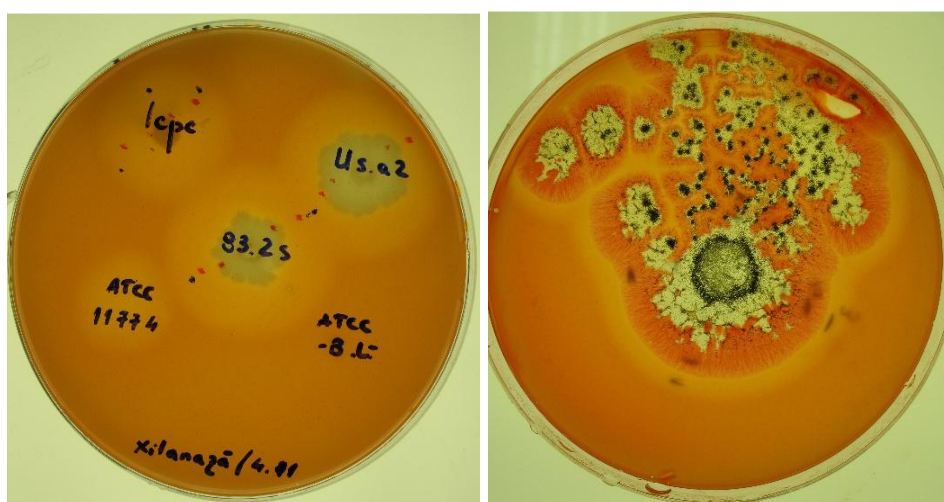

Figure 1. Evaluation of the xylan hydrolysis area in bacteria (left) and fungi (right) on selective media containing xylan 0.5%, after staining with Congo red 0.4% solution

Secondary screening of isolates

The abilities of microbial strains used in the first step of screening for xylan degradation were quantitative evaluated after cultivation in selective medium with 0.5% xylan. Differences among bacterial strains and fungal strains were detected.

Bacteria

Bacillus amyloliquefaciens BN7 had a xylanase activity of 0.03 - 0.22 µmol/mL/min during the incubation period, lower than in other studies (Amore et al., 2014), the pH range being between 7.8-8.68. Therefore, the maximum xylanase activity of 0.22 µmol/mL/min was on the 4th day and was correlated with a pH of 8.63 (Table 1). The specific xylanase activity was only of 0.11 µmol/mg protein, being much lower than the one of *B. amyloliquefaciens B4.*

Table 1.

Xylanase activity by different isolated strains

Microorganism	Xylanase activity (µmol/mL/min)		Specific enzymatic activity (µmol/mg protein)
	Xylan medium	Wheat bran medium	
Bacteria			
Bacillus amyloliquefaciens B4	1.71	1.65	1.35
Bacillus amyloliquefaciens BN7	0.22	-	0.11
Fungi			
Aspergillus flavus AFR	2.71	1.2	0.48
A. flavus T11	2.42	2.43	0.57
A.niger An4	2.03	1.74	1.87
A.brasiliensis ATCC 16404	3.05	2.39	1.14
Trichoderma atroviride TK20	1.89	-	-
T.harzianum TK25	2.01	-	-
T. viride Tv2	2.03	1.95	1.06
T.viride UV	0.81	-	-
F.culmorum FC 28	0.72	-	-
Rhizoctonia solani	0.09	-	-
Penicillium digitatum	*2.42*	*2.52*	*0.49*
Fusarium graminearum G82	0.32	-	-
F. oxysporum	0.41	-	-
T. harzianum P8.	2.9	2.1	0.74
A.niger prot.	2.63	2.05	1.03
P. verruculosum KUCC 47345	-	1.26	0.43

The best xylanase activity was recorded with *B. amyloliquefaciens B4* strain: 0.04 - 1.71 µmol/mL/min during the incubation period, the values being similar to other studies (Ten et al., 2004). The pH range varied between 8.06-8.89 during the incubation period. The maximum xylanase activity was 1.71 µmol/mL/min for B4 strain, and corresponded to a pH of 8.69 recorded in the 5th day of the incubation period (Figure 2.). This strain also showed a high specific xylanase activity of 1.35 µmol/mg protein.

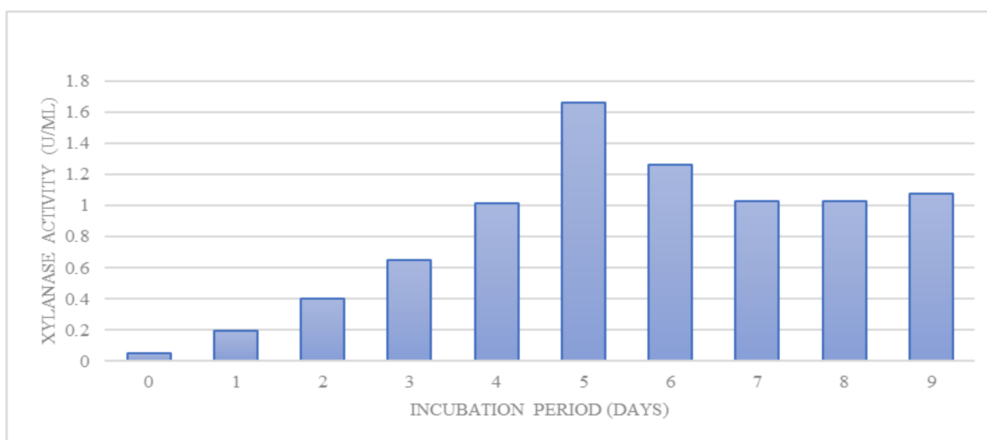

Figure 2. Xylanase activity of *B. amyloliquefaciens*

Fungi

The *Aspergillus* isolates used in these experiments exhibited a xylanase activity similar to other studied strains (Huitron et al., 2008), but the specific enzymatic activity was higher compared with the literature data (Ja'afaru, 2013). The highest xylanase activity was showed by *A.brasiliensis ATCC 16404* with 3.05 µmol/mL/min activity determined on the 4th day of the incubation period (Table 1).

Other microorganisms that showed high xylanase activity were: *T. harzianum P8* (4th day), *Aspergillus AFR* (4th day), *A. flavus T11* (4th day), *Penicillium digitatum* (4th day), *A.niger An4* (5th day), *A. niger prot.* (5th day), *T.harzianum TK25* (5th day), *T. viride Tv2* (5th day). When the protein assay was conducted, the results determined that the highest specific xylanase activity had *Aspergillus niger An4* with a value of 1.87 µmol/mg protein.

Tertiary screening of isolates

The purpose of the third screening was to examine the influence of culture media composition on the xylanase activity of the microbial isolates. For this aim, the microorganisms were cultivated in liquid xylan medium and in a medium where the carbon source was represented by wheat bran. The results showed that no significant differences between the values of the xylanase activity were detected (Table 1).

Of all the bacterial and fungal isolates analyzed, literature provides little information on xylanase production by *Penicillium digitatum*, in this research the strain having a high xylanase activity of 2.42 µmol/mL/min on xylan medium.

The highest xylanase activity on xylan medium was determined by *A.brasiliensis ATCC 16404*, while on wheat bran medium the highest value was obtained with *P.digitatum* strain: 2.52 µmol/mL/min.

Regarding the specific activity, the best results were obtained with the strains *A.niger An4, A.brasiliensis ATCC 16404* and *Bacillus amyloliquefaciens B4.* For this reason, in the further studies the combination of the best xylanolytic strains will be evaluated.

The data obtained with the strains tested in these experiments are comparable with those communicated by other authors for *Aspergillus niger, A. flavus, A brasiliensis, Trichoderma viride, T. harzianum* (HUITRON ET AL., 2008; JA'AFARU, 2013). For *P.digitatum* the synthesis of xylanases is less documented in literature (Cole and Wood, 1970) and the results obtained suggest that the strain used in this experiment is promising for further studies related the xylan degradation.

CONCLUSION

In this work, 31 microbial strains (collection or new isolates from various sources) were subjected to a screening for their ability of xylan degradation. Among them, 18 were examined for xylanase activity from both qualitative and quantitative point of view. The highest xylanase activity was obtained with *Bacillus amyloliquefaciens B4* and *Aspergillus brasiliensis ATCC 16404*. However, the best specific xylanase activities were detected in *B.amyloliquefaciens* B4 and in *Aspergillus niger* An4.

The cultivation of selected microorganisms in xylan medium and in a medium where the carbon source was represented by wheat bran allows the observation that no significant differences in enzymatic activity are related to the medium composition.

A less studied microorganism for xylan degradation, *Penicillium digitatum*, showed a high xylanase activity in both xylan medium and wheat bran medium.

It was determined that wheat bran could be useful as a cheap alternative substrate for screening of xylanase producing microorganisms.

These results are significant for further studies regarding lignocellulosic biomass biodegradation by a microbial enzymatic complex.

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