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SCREENING OF MICROORGANISMS DISPLAYING ACETYL XYLAN ESTERASE ACTIVITY

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Abstract

Hemicellulose is a major component of the lignocellulosic biomass, located between lignin and cellulose fibers and is the second most abundant natural polymer on earth. The main constituent of hemicellulose is xylan, a polysaccharide comprised of β-1.4-linked xylopyranosyl residues. Due to its complex structure and heterogeneous nature, hydrolysis of xylan requires a synergic action of several enzymes generally named xylanases. Amongst them, acetyl xylan esterase (AXE) is an accessory enzyme that liberates acetyl groups from the side chains of the xylan backbone. These acetyl side-groups protect the backbone of xylan from the action of others enzymes, therefore their release facilitates the action of endoxylanases. Several microorganisms display acetyl xylan esterase activity, the main producers being fungi and bacteria. The aim of this study was to test different strains in regard to their ability to display acetyl xylan esterase activity. The microorganisms subjected to screening belonged to the genres: Aspergillus, Penicillium, Bacillus, Trichoderma and Fusarium. The strains subjected for screening were also studied for their xylanase activity, in order to compare their ability to produce both xylanase and acetyl xylan esterase. In addition, the microorganisms selected after the screening process were subjected to protein assay, in order to determine the specific enzymatic activities. The best acetyl xylan esterase activities were detected with Aspergillus brasiliensis ATCC 16404 UV 7, Aspergillus brasiliensis ATCC 16404 UV 5, Aspergillus niger UV 10 and Penicillium digitatum UV 11. These experimental results are significant for further studies related to hydrolysis of hemicellulose, regarding lignocellulosic biomass valorisation.

Key words*: acetyl, xylan, esterase, xylanase.*

INTRODUCTION

Lignocellulosic materials, such as forestry and agricultural wastes, are the major source for renewable organic matter (Pothiraj et al., 2006; Yoo et al., 2014).

Lignocellulose has three main components: cellulose, hemicellulose and lignin, with hemicellulose as the second most abundant natural heteropolymer on earth (Hendriks and Zeeman, 2009).

Xylan, as the main component of hemicellulose, consists of β-1.4-linked xylopyranosyl residues (Blum et al., 1999).

It can be degraded by the synergic action of several enzymes such as: β-1.4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase and phenolic acid (ferulic and p-coumaric acid) esterase (Dhiman et al., 2008; Motta et al., 2013).

Several reports suggest that enzymatic removal of xylan enhances cellulose hydrolysis, therefore it is important to improve xylan enzymatic degradation in order to obtain an efficient enzymatic hydrolysis of lingocellulosic materials (Zhang et al., 2011).

Acetyl xylan esterases (AXE, E.C. 3.1.1.72) are key accessory enzymes involved in deacetylation of xylans and xylo-oligosachharides. They are able to hydrolyse acetyl groups from D-xylopyranosyl residues in xylan chains (Hou, 2005).

After the hydrolysis of the acetyl ester groups, xylan main chain has new ester-free regions targeted by β-1.4-endoxylanase. Acetylxylan hydrolysis by endoxylanases proceeds with a higher and faster rate when AXEs are involved. (Hou, 2005; Zhang et al., 2011)

Due to the lack of low-cost and well characterized acetylated xylo-oligosaccharides and xylans, screening of microorganisms displaying AXE activity is usually conducted by using chromogenic or fluorogenic acetylesterase substrates such as α-naphtyl acetate, β-naphtyl acetate, 4-nitrophenyl acetate or 4-methylumbelliferyl acetate (Biely et al., 2014; Johnson et al., 1988; Shao and Wiegel, 1995). In addition, some methods use as a substrate

chemically acetylated xylan, obtained usually by Johnson's method (1988) (Hou, 2005).
Some studies have concluded that supplementation of culture medium with Tween 80 could improve significantly AXE and xylanase activities, due to the fact that Tween 80 has a strong impact on the microorganism ability to synthesize the enzymes (Atta et al., 2011).
There are several AXE producing microorganisms studied over the years, including fungi and bacteria: *Penicillium purpurogenum, P. notatum, P. chrysogenum, Phanerochaete chrysosporium, Schizophylum commune, Aspergillus awamori, A. niger, Bacillus pumilus, B. subtilis, Thermoanarobacterium* sp.*, Streptomyces lividans, S. flavogriseus, Fusarium oxysporum, Trichoderma reesei, T. longibrachiatum, Aureobasidium pullulans* etc. (Atta et al., 2011; Bajpai, 1997; Biely et al., 1988; Degrassi et al., 1998; Halgasová et al., 1994; Hou, 2005; Yang et al., 2017).
The aim of this study was to test different microbial strains regarding their ability to display acetyl xylan esterase activity, under different cultivation conditions.

MATERIALS AND METHODS

Microorganisms

The bacterial and fungal isolates studied were provided by the Department of Genetics and genetic engineering of the Faculty of Biotechnologies from USAMV Bucharest.
For this study, several microbial isolates were subjected to screening: wild strains (*Bacillus amyloliquefaciens B4, B. amyloliquefaciens BN7, Trichoderma viride TV2, Fusarium oxysporum, Aspergillus niger, A. brasiliensis ATCC 16404, Penicillium digitatum)* and mutant strains *(A. niger UV 5, A. niger UV 10, A. niger UV 20, A. brasiliensis UV 5, A. brasiliensis UV 7, A. brasiliensis UV 14, Penicillium digitatum UV 6, Penicillium digitatum UV 11, Penicillium digitatum UV 12*).
The mutant strains were obtained after random mutagenesis through UV irradiation according to Ho and Ho method (2015). Briefly, the spore suspensions were kept at a distance of 10 cm at 254 nm in a vertical laminar flow cabinet for different time exposure: 30 minutes (*A. niger UV 5* and *A. brasiliensis UV 14)*, 40 minutes (A. *brasiliensis UV 7* and *P. digitatum UV 6)*, 50 minutes (*A. niger UV 10* and *Penicillium digitatum UV 11*) and 60 minutes (*A. niger UV 20, A. brasiliensis UV 5* and *Penicillium digitatum UV 12).*

Primary screening of microbial strains for AXE activity

The selected isolates were cultivated on two different liquid mediums containing 0.8% corncob xylan as the only carbon source; only one medium supplemented with 0.5% Tween 80. Other components of the cultivation medium were for fungi (g/L): 0.05 g $MgSO_4 \cdot 7H_2O$, 0.005 g $CaCl_2$, 0.005 g $NaNO_3$, 0.009 g $FeSO_4 \cdot 7H_2O$, 0.002 g $ZnSO_4$, 0.012 g $MnSO_4$, 0.23 g KCl, 0.23 g KH_2PO_4, 2 g peptone (Adesina and Onilude, 2013) and for bacteria (g/L): 0.05 g $MgSO_4 \cdot 7H_2O$, 0.05 g NaCl, 0.01 g $CaCl_2$, 0.2 g yeast extract, 0.5 g peptone (Mahilrajan et al., 2012).
The Erlenmeyer flasks containing the cultivation medium were inoculated with the selected microorganism and incubated at 30 ± 2°C in an incubator with shaker at 140 rpm for 5-9 days depending on the strain. Samples were taken at every 24 hours and centrifuged at 4500 rpm for 30 minutes. The supernatants were subjected to acetyl xylan esterase assay.

Secondary screening

The isolates that displayed high acetyl xylan esterase activity were subjected to a second screening being cultivated on the same liquid medium, but with different carbon sources: wheat bran (WB) and de-starched wheat bran (DSWB), supplemented with 0.5% Tween 80.
De-starched wheat bran was prepared according to Mukherjee (2007) and Huang (2013). Samples were taken at every 24 hours and were analysed for their acetyl xylan esterase activity and xylanase activity.

Acetyl xylan esterase assay

The enzymatic activity of AXE was measured by hydrolysis of p-nitrophenyl acetate (pNPA) to p-nitrophenol (pNP), according to a modified method by Johnson et al. (1988) (Atta et al, 2011). The assay mixture consisted of 1mL 0.1M sodium phosphate buffer (pH 7), 0.9 mL 10 mM pNPA and 0.1 mL enzyme sample and

was incubated at 37ºC for 10 minutes. The release of pNP was measured by reading the absorbance at 410 nm using a spectrophotometer. One unit of acetyl xylan esterase activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol per minute under the specified assay conditions.

Xylanase assay

Xylanase activity was determined according to the DNS (3.5-dinitrosalicylic acid) assay for reducing sugars (Bailey et al., 1992). Assay mixture consisted of 0.5 mL sample and 0.5 mL of 0.6% oat spelt xylan and was incubated at 40ºC for 10 min. The reaction was terminated by adding 1 mL of DNS and heating for 5 min 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.

Protein assay

The samples from the secondary screening were analysed for their soluble protein quantification using Lowry method (1951). This analysis was necessary to determine the specific enzymatic activity.

RESULTS AND DISCUSSIONS

Primary screening

The wild and mutant strains obtained with random mutagenesis through UV exposure were evaluated for their ability to produce acetyl xylan esterases on xylan medium with or without Tween 80.

From the bacterial strains, *B. amyoliquefaciens B4* was detected with the highest AXE activity of 1.20 µmol/ml/min. on xylan medium and 1.29 on xylan medium supplemented with Tween 80 (Table 1). The results were higher than other data regarding AXE activity of *Bacillus* sp. (Christov et al., 1993).

As shown in Table 1, for the bacterial strains, the addition of 0.5% Tween 80 had a low improvement on AXE production (7.5% and 13.8%) under this study experimental conditions, lower than the results of other studies (Atta et al., 2011).

The best AXE activity were recorded with fungal strains (Table 1), such as: *A. niger UV 10, A. brasiliensis UV 5, A. brasiliensis UV 7* and *P. digitatum UV 11*. Amongst them, *A. brasiliensis UV 7* had the best AXE activity on corncob xylan medium (2.68 µmol/ml/min.), while *A. brasiliensis UV 5* had the highest activity of 3.24 µmol/ml/min. on corncob xylan medium supplemented with Tween 80, comparable with other studies (Christov et al., 1993; Khan et al., 1990). The results obtained with the mutant strains were comparable or slightly higher than the activities recorded with the wild type fungal strains (Table 1).

Although other studies (Christov et al., 1993; Christakopoulos et al., 1999) suggest that *Fusarium oxysporum* is an important producer for AXEs, under this experimental conditions, the strain displayed a low AXE activity on both cultivation mediums.

Table 1. Acetyl xylan esterase activity on xylan medium with or without Tween 80

Microorganism	AXE activity (µmol/ml/min)	
	Xylan medium	Xylan + Tween 80 medium
B. amyloliquefaciens B4	1.01	1.21
B. amyloliquefaciens BN7	0.29	0.33
T. viride TV2	1.40	1.67
Fusarium oxysporum	0.32	0.41
A. niger - wild type	1.78	2.19
A. niger UV 5	1.71	2.14
A. niger UV 10	**2.31**	**2.93**
A. niger UV 20	1.68	1.99
A. brasiliensis ATCC 16404 - wild type	1.81	2.15
A. brasiliensis UV 5	**2.50**	**3.24**
A. brasiliensis UV 7	**2.68**	**3.19**
A. brasiliensis UV 14	1.92	2.37
P. digitatum - wild type	2.05	2.66
P. digitatum UV 6	2.02	2.59
P. digitatum UV 11	**2.34**	**3.03**
P. digitatum UV 12	1.92	2.25

For *P. digitatum* mutant strains (Table 1), the AXE activities were comparable with the wild strain, except for *P. digitatum UV 11*, that displayed a relatively high AXE activity (3.03 µmol/ml/min) on xylan medium supplemented with Tween 80. This can suggest that UV mutagenesis could be an important method for

improvement of microbial strains, regarding their AXE activities.
According to these results (Table 1), the addition of Tween 80 improved the enzymatic activity of the isolates with 20-30%, comparable with other similar studies (Atta et al., 2011).

Secondary screening
After the first screening, several fungal isolates detected with the highest AXE activity were subjected to a second screening in order to examine the influence of the selected carbon source upon AXE and xylanase activities.

Table 2. AXE and xylanase activity on wheat bran (WB) and de-starched wheat bran (DSWB)

Microorganism	AXE activity (µmol/ml/min)		Xylanase activity (µmol/ml/min)	
	WB	DSWB	WB	DSWB
B. amyloliquefaciens B4	1.05	1.14	1.72	1.89
A. niger - wild type	2.10	2.31	2.44	2.67
A. niger UV 10	2.72	2.99	2.68	2.90
A. brasiliensis ATCC 16404 - wild type	2.08	2.27	2.51	2.73
A. brasiliensis UV 5	3.09	**3.41**	**3.24**	**3.52**
A. brasiliensis UV 7	**3.10**	3.35	2.87	3.1
P. digitatum - wild type	2.48	2.72	2.63	2.89
P. digitatum UV 11	2.89	3.17	2.80	3.05

The best AXE activity was detected with *A. brasiliensis UV 7* on wheat bran medium (3.10 µmol/ml/min.) and with *A. brasiliensis UV 5* on de-starched wheat bran medium (3.41 µmol/ml/min.). AXE activities of the mutant strains were higher than the activities of the wild type strains with almost 50% (Table 2).
Comparing the results of AXE activity from table 1 and table 2, it can be observed that when using wheat bran the microbial strains show a slightly lower activities compared with the activities on corncob xylan medium, both supplemented with Tween 80. However, with de-starched wheat bran, there's an increase in enzymatic activity of 8-10%, in comparison with the results obtained with wheat bran (Table 2).
According to the xylanase assay, the results show that the highest enzymatic activity was detected with *A. brasiliensis UV 5* on both wheat bran medium (3.24 µmol/ml/min) and de-starched wheat bran medium (3.52 µmol/ml/min), higher than the activities of the wild type strains with cca 29% (Table 2).
In addition, the use of de-starched wheat bran instead of wheat bran as the only carbon source was correlated with an increase of enzymatic activities of 7-10% (Table 2).
Regarding *P. digitatum,* AXE activities of the mutant isolates were higher with almost 16.5% than the wild strain, while xylanase activities were higher with cca 6% than the wild type strain (Table 2).

Protein assay
After the secondary screening, the samples from the strains cultivated on de-starched wheat bran with Tween 80 were also analysed for their protein content in order to determine their specific AXE activity.
The highest specific enzymatic activity of 1.34 µmol/mg protein was recorded with *A. brasiliensis UV 5* (Table 3), higher than the wild type strain with cca 12%.

Table 3. Specific AXE activity

Microorganism	Specific enzymatic activity (µmol/mg protein)
B. amyloliquefaciens B4	1.05
A. niger - wild type	1.22
A. niger UV 10	1.43
A. brasiliensis ATCC 16404 - wild strain	1.20
A. brasiliensis UV 5	**1.34**
A. brasiliensis UV 7	1.27
P. digitatum - wild type	0.78
P. digitatum UV 11	0.81

P. digitatum had a low specific enzymatic activity with both wild and selected mutant strain (Table 3).

CONCLUSIONS

Acetyl xylan esterases (AXE) are key accessory enzymes necessary for the complete hydrolysis of xylan, their action increasing the activity of β-1.4-endoxylanases.
In this study, several microbial strains were subjected to a screening regarding their ability

to produce acetyl xylan esterases on both corncob medium and corncob medium supplemented with Tween 80. Amongst them, 4 mutant strains displayed the highest AXE activities: *Aspergillus niger UV 10, Aspergillus brasiliensis ATCC 16404 UV 5, Aspergillus brasiliensis ATCC 16404 UV 7* and *Penicillium digitatum UV 11*.

The best AXE activity of 3.24 µmol/ml/min was detected with *Aspergillus brasiliensis ATCC 16404 UV 5* on corncob xylan medium supplemented with 0.5% Tween 80. The mutant strain was obtained after random mutagenesis through UV irradiation for 60 minutes.

The highest specific enzymatic activity of 1.34 µmol/mg protein was displayed by *Aspergillus brasiliensis ATCC 16404 UV 5*, higher than the wild type strain with almost 12%.

The addition of Tween 80 was correlated to an improvement in enzymatic activities with 20-30% for all the isolates.

The cultivation of the selected strains on both wheat bran and de-starched wheat bran, allowed the observation that by using de-starched wheat bran instead of wheat bran, the enzymatic activities were higher with 7-10% for the majority of the microbial strains, under this experimental conditions.

A less studied strain for its AXE activity, *Penicillium digitatum* displayed a relatively high enzymatic activity of 3.03 µmol/ml/min. (corncob xylan + Tween 80) and 3.17 µmol/ml/min (de-starched wheat bran + Tween 80).

Considering the results obtained, UV mutagenesis could be an important tool for obtaining improved mutant strains with higher AXE activities.

These results are significant for further experimental studies related to hydrolysis of hemicellulose, regarding lignocellulosic biomass valorisation.

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