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BIODEGRADATION OF LIGNOCELLULOSIC SUBSTRATES WITH IMPROVED FUNGAL STRAINS

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Abstract

Lignocellulosic biomass is considered to be the most abundant renewable biomass in the world. However, since only 5% of it is valorised, research is still focused on a better degradability of this waste due to its potential in various applications. Given the structural complexity, recalcitrance and variety depending on the source, several enzymes are involved in a better degradation of lignocellulose. This research study is focused on testing two fungal isolates on their ability to depolymerise two lignocellulosic substrates with a different chemical composition: beech sawdust and wheat bran. The fungal isolates were obtained through random UV mutagenesis from Aspergillus brasiliensis ATCC 16404. The fungal strains were compared based on their enzymatic activities regarding the production of endoxylanase, acetyl xylan esterase, feruloyl esterase and laccase. The highest specific enzymatic activities for all the enzymes analysed were registered for Aspergillus brasiliensis UV_{14}, a strain obtained after exposure to UV irradiation for 50 minutes.

Key words*: acetyl xylan esterase, Aspergillus brasiliensis, endoxylanase, feruloyl esterase, laccase.*

INTRODUCTION

Lignocellulosic biomass is one of the most abundant renewable resources, with an amount of approximately 181.5 billion tons every year. Unfortunately, only 5% of this biomass is currently utilised (Singh et al., 2022), the rest being wasted or burned in land fields which has a major impact on the environment (Liang et al., 2020).

Lignocellulosic waste has a tremendous potential as a substrate for producing various products used in a multitude of applications such as: food, beverages and feed industries, bleaching of pulp for the paper industry, obtaining antioxidants, enzymes and low-cost chemicals etc. However, the valorisation of this biomass is not fully achievable due to its problematics with biodegradability. Therefore, several studies are still focused on complete degradation of lignocellulose by targeting the main components: cellulose, hemicellulose and lignin and their structural units (Monica et al., 2024; Rusu et al., 2022). The composition of these three polymers varies with the source of the waste, usually being 25-65% cellulose (higher contents found in sugarcane bagasse, softwood or hardwood stalks, grains straw etc.), 11-50% hemicellulose (higher contents evaluated in *Gramineae*, wheat or barley straw, softwood or hardwood stalks etc.) and 5-40% lignin (higher contents detected in nut shells or wood stalks etc.) (Harmsen et al., 2010; Palonen, 2004; Pandey, 2015).

Due to the structural variability of lignocellulosic waste, several enzymes are required for best yield regarding lignocellulose degradation (Dincă et al., 2024; Giurescu et al., 2023), such as: cellulases (β-glucosidase, β-1,4-endoglucanase, β-1,4-exoglucanase), hemicellulases (β-1,4-endoxylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetyl xylan esterase, feruloyl esterase) and ligninases (laccase, heme peroxidase, versatile peroxidase) (Li et al., 2022).

In view of the fact that cellulases are widely studied since their isolation from *Trichoderma reesei* in 1940 (Liu et al., 2021), this research was focused more on analysing the biodegradation of lignocellulosic substrates (wheat bran and beech sawdust) with hemicellulases (endoxylanase, acetyl xylan esterase, feruloyl esterase) and ligninases (laccase).

MATERIALS AND METHODS

Microorganisms

Two fungal strains were tested in this study: *Aspergillus brasiliensis* UV_7 and *Aspergillus brasiliensis* UV_{14}, both obtained through random UV mutagenesis from a parental strain *Aspergillus brasiliensis* ATCC 16404, after exposure to a UV lamp for 20 min (UV_7) and 50 min (UV_{14}), as described in previous research (Burlacu et al., 2017).

Growth medium

Two growth mediums were used for testing the ability of the fungal strains to degrade lignocellulosic substrates:

Medium 1 (g/L): 8 g beech sawdust, 5 g Tween-80, 0.005 g $NaNO_3$, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.005 g $CaCl_2$, 0.012 g $MnSO_4$, 0.009 g $FeSO_4 \cdot 7H_2O$, 0.002 g $ZnSO_4$, 0.23 g KCl, 0.23 g KH_2PO_4, 2 g peptone;

Medium 2 (g/L): 8 g pre-treated wheat bran, 5 g Tween-80, 0.005 g $NaNO_3$, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.005 g $CaCl_2$, 0,012 g $MnSO_4$, 0.009 g $FeSO_4 \cdot 7H_2O$, 0.23 g KCl, 0.002 g $ZnSO_4$, 0.23 g KH_2PO_4, 2 g peptone.

The wheat bran was pre-treated according to the protocol described by Sarangi et al. (2009). Thus, they were repeatedly washed with distilled water, then filtered and washed again with an aqueous solution of 1.5% Tween-80. The wheat bran was then dried and incubated for 20 minutes at 121ºC and stored at -20ºC.

The Erlenmeyer flasks with the inoculated liquid mediums were incubated at 30±2ºC, 140 rpm agitation for 7 days. At every 24 h samples were collected, centrifuged, filtrated and subjected to analysis for different enzymatic activities: xylanase, acetyl xylan esterase, feruloyl esterase and laccase.

Endoxylanase assay

The method used for evaluating endoxylanases activity was based on the quantification of reducing sugars using DNS reagent, endoxylanases catalysing the hydrolysis of xylan, and the reducing carbohydrates formed (xylose) are determined spectrophotometrically at 540 nm. A modified assay described by Chidi et al. (2008) was followed. Briefly, 0.5 ml diluted filtrate (collected at every 24 h) was mixed with 0.5 ml xylan (0.6% in sodium acetate buffer 0.05M, pH 5.3) and the mixtures were incubated at 40ºC for 10 minutes. After adding 1 ml DNS reagent, the samples were boiled for 5 minutes and left to cool down before adding 3 ml distilled water. After 30 minutes, the absorbance was read at 540 nm. A standard calibration curve was created with known concentration of D-xylose. One unit of endoxylanase activity was defined as the amount of the enzyme that released one µmol of xylose per minute under these experimental conditions.

Acetyl xylan esterase assay

The activity of acetyl xylan esterase was evaluated based on the quantification of p-nitrophenol (pNP) released following the hydrolysis of p-nitrophenyl acetate (pNPA), according to the method proposed by Atta et al. (2011). The assay mixture contained 0.1 ml diluted filtrate, 0.9 ml pNPA (10 mM in DMSO) and 1 ml sodium phosphate buffer (0.1M, pH 7.00). After incubation at 37ºC for 10 minutes, the absorbance was read at 410 nm. A standard calibration curve was created with known concentration of p-nitrophenol (pNP). One unit of acetyl xylan esterase activity was defined as the amount of enzyme able to release one µmol of pNP per minute under these experimental conditions.

Feruloyl esterase assay

The method for measuring the enzymatic activity of feruloyl esterase is based on the release of ferulic acid following the hydrolysis of the substrate (Mastihuba et al., 2002). Therefore, 0.2 ml diluted filtrate was mixed with 15 µL ethyl ferulate (10 mg/mL in dimethylformamide) and 0.8 ml sodium phosphate buffer (100 mM, pH = 6.00). After incubation at 37°C for 2 h, the samples were boiled at 100°C for 3 minutes and the absorbance was read at 338 nm. A standard calibration curve was created with known concentration of ferulic acid. One unit of feruloyl esterase activity was defined as the amount of enzyme able to release one µmol of ferulic acid per minute under these experimental conditions.

Laccase assay

Laccase activity is measured based upon the degradation of the substrate (guaiacol) in one minute based on a continuous spectrophotometric method, according to the protocol described by Desai et al. (2011). Briefly, 1 ml diluted filtrate was mixed with 1 ml guaiacol (2 mM) and 3 ml sodium acetate buffer (10 mM pH = 5.00). After incubation at 30ºC for 15 minutes the absorbance was read at 450 nm.

The enzyme activity is calculated based on the formula:

$$\text{A.E.} = \frac{A*V}{e*t*v}$$

where:

A = absorbance value at λ = 450nm;

V = Total volume of the reaction mixture;

e = extinction coefficient of guaiacol at 450 nm (12,100 $M^{-1}cm^{-1}$);

t = incubation time (min);

v = volume of the filtrate (that contains the enzyme).

One enzyme unit was defined as the amount of enzyme required to oxidize one µmol of guaiacol per minute, under the described experimental conditions.

Total soluble protein assay

Lowry's method (Lowry et al., 1951) was used for the evaluation of protein concentration in the collected filtrates at every 24 h. For this, 1 mL of properly diluted sample was mixed with 1 mL reagent A+B (A: NaOH, Na_2CO_3, $KNaC_4H_4O_6 \cdot 4H_2O$ and B: $CuSO_4$ 0.5%). After 10 minutes, 10 ml of dilluted Folin-Ciocâlteu reagent was added and the mixtures were incubated for 10 minutes at 50°C. The extinctions were read at 660 nm. A standard calibration curve was created with known concentration of bovine seric albumin (BSA).

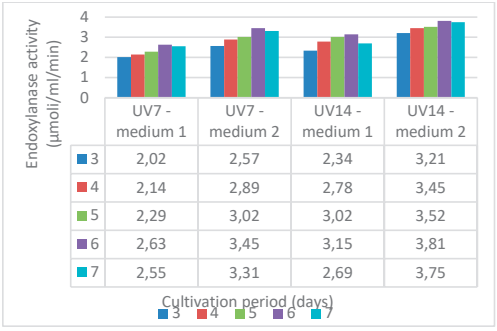
RESULTS AND DISCUSSIONS

The importance of fungal hemicellulases resides from their range of optimal parameters such as efficiency at temperatures below 50ºC and especially acidic pH range of 4-6, since bacterial hemicellulases are active mainly in alkaline conditions (Beg et al., 2001).

The substrates were selected for this study based upon their different composition in major lignocellulosic components. Therefore, beech sawdust contains: 38-50% cellulose, 23-32% hemicellulose and 15-26% lignin (Liu et al., 2023; Świątek et al., 2020). Wheat bran has a relatively different composition: 11-19% cellulose, 11-35% hemicellulose, 5-6% lignin and other components such as 14-25% starch, 13-18% protein etc. (Glaser et al., 2023; Merali et al., 2015). The pretreatment applied to wheat bran was carried out for the removal of starchy residues, which tend to affect the activity of lignocellulases.

Following the cultivation of the two fungal strains on the medium containing beech sawdust, it was found that better results were obtained with the mutant strain *Aspergillus brasiliensis* UV_{14}, compared to *Aspergillus brasiliensis* UV_7 (Figure 1). *A. brasiliensis* UV_{14} had the maximum endoxylanase activity of 3.81 µmoles/mL/min, when it was cultivated on wheat bran medium.

Analysing the results with ANOVA with a single variable, significant differences in the endoxylanase activity are determined ($p<0.05$) between the two fungal strains and also between the same fungal strain cultivated on different substrate.

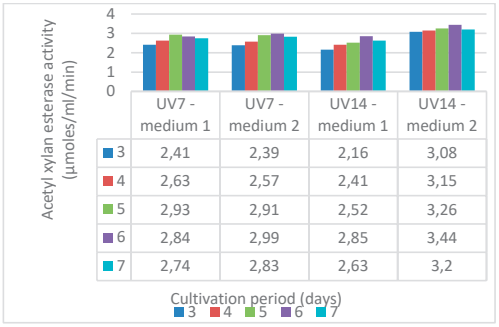


	UV7 - medium 1	UV7 - medium 2	UV14 - medium 1	UV14 - medium 2
3	2,02	2,57	2,34	3,21
4	2,14	2,89	2,78	3,45
5	2,29	3,02	3,02	3,52
6	2,63	3,45	3,15	3,81
7	2,55	3,31	2,69	3,75

Figure 1. Endoxylanase activity of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

Comparing the endoxylanase activity of each microbial strain cultivated on both mediums, it is noticeable that the medium containing wheat bran determined a higher enzymatic activity, mainly due to the higher hemicellulose content of this substrate.

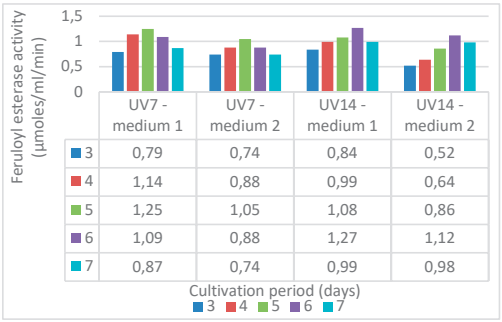
In addition, it is noticeable that the highest endoxylanase activities were registered on the 6th day of cultivation for all the strains cultivated on both mediums (Figure 1).
Regarding acetyl xylan esterase activity, better results were obtained with the mutant strain *A. brasiliensis* UV_{14} compared to *A. brasiliensis* UV_7, on both cultivation media, but more pronounced on the medium containing wheat bran (Figure 2).



	UV7 - medium 1	UV7 - medium 2	UV14 - medium 1	UV14 - medium 2
3	2,41	2,39	2,16	3,08
4	2,63	2,57	2,41	3,15
5	2,93	2,91	2,52	3,26
6	2,84	2,99	2,85	3,44
7	2,74	2,83	2,63	3,2

Figure 2. Acetyl xylan esterase activity of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

The maximum acetyl xylan esterase activity was 3.44 µmoles/ml/min for the mutant strain grown on the medium containing 0.8% wheat bran as the sole carbon source. In addition, by using medium 1, which had beech sawdust as a carbon source, it was observed that the differences in acetyl xylan esterase activity were insignificant ($p>0.05$), following statistical processing with a single variable ANOVA. Moreover, by using medium 2 which had pre-treated wheat bran as a carbon source, it was observed that the differences in acetyl xylan esterase activity were significant ($p<0.05$), following statistical processing with one-variable ANOVA.
In addition, it is noticeable that the highest acetyl xylan esterase activities were registered on the 6th day of cultivation for all the strains cultivated on both mediums (Figure 2), except for *A. brasiliensis* UV_7 when cultivated on beech sawdust (5th day).
Analysing the results regarding feruloyl esterase activity, it was found that by cultivating the fungal strains on medium with beech sawdust, better results were obtained compared to their cultivation on medium with pre-treated wheat bran (Figure 3). This is explained by the high lignin content of beech sawdust, compared to that of wheat bran, thus there are more ester bonds between lignin and hemicellulose.



	UV7 - medium 1	UV7 - medium 2	UV14 - medium 1	UV14 - medium 2
3	0,79	0,74	0,84	0,52
4	1,14	0,88	0,99	0,64
5	1,25	1,05	1,08	0,86
6	1,09	0,88	1,27	1,12
7	0,87	0,74	0,99	0,98

Figure 3. Feruloyl esterase activity of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

The differences in feruloyl esterase activity of the fungal strains compared between cultivation on the medium with beech sawdust and that with pre-treated wheat bran were insignificant by statistical processing with one-variable ANOVA ($p>0.05$). The highest feruloyl esterase activity (1.27 µmoles/ml/min) was observed in the case of the mutant strain of *A. brasiliensis* UV_{14}, cultivated on medium containing beech sawdust as the only carbon source (Figure 3). Regardless of the cultivation mediums used, the highest feruloyl esterase activities were registered on the 6th day of cultivation for *A. brasiliensis* UV_{14} and 5th day for *A. brasiliensis* UV_7 (Figure 3).
When measuring laccase activities, it was determined that *A. brasiliensis* UV_{14} clearly has a higher enzymatic activity, unrelatedly to the medium in which it was cultivated (Figure 4). Moreover, the differences between the laccase activities of the strains regarding their activity compared between the two-cultivation media are ensured by statistical processing with one-variable ANOVA ($p>0.05$).

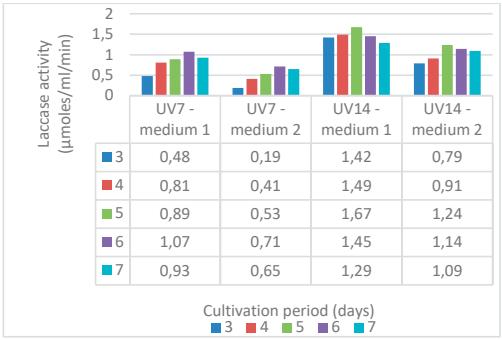


Figure 4. Laccase activity of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

The maximum laccase activity for both strains was determined when the fungal strains were grown on medium 1 containing beech sawdust as a carbon source (Figure 4), which could be correlated with the higher lignin content of this substrate, the phenolic compounds being more easily hydrolysable by laccases.

Measuring the amount of soluble protein synthesized by the tested strains allowed the calculation of specific enzymatic activities for endoxylanases, acetyl xylan esterases, feruloyl esterases and laccases (Table 1). The specific enzymatic activity allows a relevant comparison of the action of the enzymes synthesized by the tested strains.

Table 1. Specific enzymatic activities of mutant *A. brasiliensis* strains cultivated on beech sawdust (medium 1) and wheat bran (medium 2)

Fungal strain	Specific endoxylanase activity (µmol/mg protein)	Specific acetyl xylan esterase activity (µmol/mg protein)	Specific feruloyl esterase activity (µmol/mg protein)	Specific laccase activity (µmol/mg protein)
UV_7 medium 1	1.15	1.26	0.49	0.47
UV_7 medium 2	1.35	1.34	0.44	0.33
UV_{14} medium 1	1.21	1.15	0.52	0.68
UV_{14} medium 2	2.05	1.61	0.61	0.59

In the case of the specific activity of endoxylanases, the maximum value was obtained with the strain *A. brasiliensis* UV_{14} cultivated on medium with pre-treated wheat bran (Table 1).

Regarding the specific activity of acetyl xylan esterases, the maximum value was recorded with the strain *A. brasiliensis* UV_{14} cultivated on medium with pre-treated wheat bran. When cultivated on medium with beech sawdust, the *A. brasiliensis* UV_7 strain registered a higher acetyl xylan esterase activity than the activity of the mutant strain *A. brasiliensis* UV_{14} (Table 1).

The isolate *A. brasiliensis* UV_7 had a lower specific feruloyl esterase activity than the enzymatic activity of the mutant strain *A. brasiliensis* UV_{14}. However, *A. brasiliensis* UV_7 had a higher specific enzymatic activity when cultivated on beech sawdust, compared with *A. brasiliensis* UV_{14} who had a higher activity when cultivated on wheat bran (Table 1).

In regards to specific laccase activity, the highest values were registered for *A. brasiliensis* UV_{14} regardless of the cultivation media. Also, both isolates had a higher specific enzymatic activity when cultivated on beech sawdust than on wheat bran (Table 1).

CONCLUSIONS

In this experimental study, two fungal strains were tested: *Aspergillus brasiliensis* UV_7 and *Aspergillus brasiliensis* UV_{14} - mutant strains obtained by random mutagenesis with UV radiation, after exposure for 20 and 50 minutes. These strains were cultivated on two lignocellulosic substrates that had different compositions: medium with 0.8% wheat bran and medium with 0.8% beech sawdust.

The results of this study led to the conclusion that the synthesis of the components of the xylanolytic system and laccase is regulated by the substrate used, obtaining maximum enzyme activities of endoxylanase and acetyl xylan esterase following cultivation on medium with pre-treated wheat bran, and higher feruloyl esterase and laccase activities when they were grown on medium with beech sawdust. The different proportions of the majority components (cellulose, hemicellulose and lignin) of the substrates used explain these differences.

The results obtained can be important contributions to the development of biodegradation processes of lignocellulosic waste and, in addition, to the valorization of the

compounds obtained in biotechnological applications, in areas of acidic pH, given the fact that most applications are based on bacterial enzymes that act mainly in the alkaline range.

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