Biocatalysis and Agricultural Biotechnology 17 (2019) 160–167

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Isolation and characterization of non-cellulolytic *Aspergillus flavus* EGYPTA5 exhibiting selective ligninolytic potential

Mohamed S. Hasanin[a,*], Osama M. Darwesh[b], Ibrahim A. Matter[b], Houssni El-Saied[a]

[a] Cellulose and Paper Department, National Research Centre, 33 EL-Buhouth St., Dokki, Cairo, Egypt
[b] Agricultural Microbiology Department, National Research Centre, 33 EL-Buhouth St., Dokki, Cairo, Egypt

ARTICLE INFO

Keywords:
Agriculture wastes
Lignocelluloses
Non-cellulolytic lignolytic enzymes
Molecular identification
Biotechnology

ABSTRACT

Environmental applications of selective lignin-degrading fungi and enzymes are of great interest as alternate technologies for paper industry (biopulping), biofuels, organic fertilizers, animal feeds. For this reason, the current study aimed to isolate selective delignifying fungi to convert the abundant lignocellulosic agricultural wastes into value-added products. From five lignocellulolytic fungi isolated from agricultural soil rich with partial decayed wooden trimmings, one isolate was selected due to its selectivity towards lignin degradation. The selected non-cellulolytic fungus isolate was identified according to morphological and molecular techniques as *Aspergillus flavus* EGYPTA5 with accession number MH425453. This fungal strain has been proven to cause an efficient and selective degradation of lignin in agricultural lignocellulosic wastes without affecting cellulose content. Lignin peroxidases, laccase, polyphenol oxidase, nitrate reductase and cellulase enzymes from investigating strain were assayed in the current research. All the tested enzymes except cellulase were produced with various activity degrees. Lignin peroxidases were the most active enzyme produced under experimental conditions (reached to 2.45 U/ml). Physical and chemical analysis of lignocellulosic agricultural wastes as well as FTIR analysis confirmed that *A. flavus* EGYPTA5 could be used as an alternative technology for biotreatment of lignin-rich cellulosic wastes to degrade lignin without affecting cellulose fibers. The isolated fungus and/or its specific-lignolytic enzymes could be applied in various environmental and industrial applications.

1. Introduction

Covering the demands of tremendous increased human populations requires a continuous searching for sustainable resources. The abundant lignocellulosic agricultural wastes are of great interest in this regard as a feedstock for many applications such as paper industry, biofuels, organic fertilizers and animal feeds (Paramjeet et al., 2018; Ruqayyah et al., 2013). Lignin, the most abundant terrestrial aromatic compound (Hammel, 1997), is a heterogeneous polymer of 4- hydroxyl phenyl propanoid units that support rigidity and strength to plants (Hasanin et al., 2018). It represents around 25% in woody plants and around 10% in case of non-woody plants (Tandon, 2015). Presence of lignin with cellulose fibers considered as a disadvantage in some applications such as paper industry. The recalcitrant of lignin towards both biological and chemical degradation is a mandatory reason to look after active degradation methods (Sette and Santos, 2013). Non-biological treatments of lignocellulosic wastes could be effective step for delignification of biomass but it not an ecofriendly strategy (Kong et al., 2017). Thermochemical treatments produced many toxic and corrosive by-product substances (black liquor) which liberated due to lignin degradation. Also, the hydrolysis of cellulose and hemicellulose could be a main drawback in some industries (Fang et al., 2018).

Many microorganisms (bacteria or fungi) known to degrade xenobiotic compounds (includes organic pollutants, pesticides etc.) as well as decaying of agricultural organic wastes such as lignocelluloses with the superiority of fungus in this regard (Abdel-Hamid et al., 2013; Abou-Shanab et al., 2012; Fernández-Fueyo et al., 2016; Hoballah et al., 2014). Selective lignin-degrading microorganisms or their lignin-degrading enzymes are attractive choice to pretreatment lignocellulosic agricultural wastes for bioethanol production and paper industry in (bio-pulping process) (Fang et al., 2018; Singh and Singh, 2014; Wang et al., 2013). Lignolytic microbes produce extracellular enzymes that convert lignin into smaller intermediates (through different metabolic pathways) enabling it to involve in “the central carbon metabolism” (Beckham et al., 2016). White-rot fungi were the most studied lignin-degrading fungi due its high degradation ability. Brown-rot fungi also are well-known for production of oxidative enzymes (Wang et al., 2013). *Aspergillus* sp. reported to has the ability to produce lignin

* Corresponding author.
E-mail address: sido_sci@yahoo.com (M.S. Hasanin).

https://doi.org/10.1016/j.bcab.2018.11.012
Received 19 September 2018; Received in revised form 14 November 2018; Accepted 15 November 2018
Available online 17 November 2018
1878-8181/

degradation enzymes (Su et al., 2016). Lignolytic activity reported for different lignin-degrading microorganisms usually attributed to peroxidases (lignin peroxidases and manganese peroxidases), laccases and polyphenol oxidases (Abdel-Hamid et al., 2013; Remédios et al., 2016; Upadhyay et al., 2016).

The potential environmental and industrial applications of lignolytic enzymes could be summarized as following: Food industry (enhance color appearance due to degradation of phenolic compound using laccases); bio-pulping and paper industry (delignification of wood pulps); textile industry (Enzymatic dye bleaching); bioremediation (degradation of xenobiotics industries wastewater) as well as different applications in the fields of pharmaceuticals, medicals and nanotechnology (Darwesh et al., 2014, 2015, 2018; Maciel and Ribeiro, 2010).

In this context, screening and isolation of selective lignin degrading (non-cellulolytic) fungi with high efficiency extracellular lignolytic enzymes is an important issue to investigate. Hence, this article aimed to isolate such fungi adapted with Egyptian conditions to be used in fibers-delignification related industries as well as other environmental applications.

2. Materials and methods

2.1. Samples and isolation sources

Lignocellulosic agricultural wastes samples *i.e.* citrus trees trimmings were collected from some local farms located in Giza governorate at Egypt to be used as a substrate in this study. Collected raw materials were subjected to drying in air oven at 70 °C followed by mechanical grinding and sieving. Samples for isolation of lignin-degrading fungi were obtained from soil containing partial decayed wooden trimmings under trees in the same citrus farms.

2.2. Isolation of non-cellulolytic lignin decomposing fungi

Samples suspected to contain lignin-degrading fungi were enriched in liquid mineral salts medium (MSM) (Darwesh et al., 2014) contained 10% (w/v) of grinded oven-dried citrus trees waste material as a sole carbon source. Enriched cultures were inoculated with 10 ml fungal spore suspension (106 spore/ml) and incubated at ambient temperature on orbital shaker (120 rpm) for 2 weeks followed by sub culturing under the same conditions for another 2 weeks. A liquate of 0.1 ml from each enriched flask was spread on the surface of MSM agar plates amended with 10% (w/v) wood trimmings (very fine grinded) as a sole carbon source. Emerged fungal colonies (after 7 days of incubation) were re-cultured (by fungal discs) on fresh MSM agar plates with the same conditions to insure its ability for utilization and degradation of lignin-rich wastes. The most active lignin-degrading fungal isolate was selected on the base of good growing and production of lignolytic enzymes. Purification of obtained isolate was performed by streaking on PDA plates supplemented with 200 mg/l amoxicillin antibiotic followed by maintenance on PDA slants for further studies.

From one day to ten days treated fibers harvest were washed with distilled water several times. The treated fibers were centrifuged for 5 min at 3000 rpm several times to eliminated the fungal mycelia and spores. The fibers dried in oven at 70 °C overnight and subjected to further investigations.

2.3. Identification and characterization of the selected lignolytic fungus

The selected lignolytic fungus was identified according to its morphological characteristics and molecular biology techniques. The morphological characteristics were examined using light microscope (Olympus cx41) after 4 days of growth on PDA agar plates. A loop of aerial mycelia of the tested fungi was conducted on glass slide and examined by light microscope at magnification of 40x. For molecular identification, fungal mycelium from a 3 days old culture in potatoes dextrose broth (PDB) was harvested using Whatman No. 1 filter paper. The total genomic DNA was extracted using CTAB protocol (Benito et al., 1993). In brief, the fungal mycelium was dried and grinded using liquid nitrogen and incubated at 60 °C for 1 h with CTAB buffer. Chloroform/isoamyl buffer was added and incubated in ice water bath for 10 min. Genomic DNA was purified by isopropanol after centrifugation of the previous mixtures as previously described by (Barakat et al., 2017). DNA of the fungal isolate was amplified by polymerase chain reaction (PCR) using ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC - 3′) designed for sequencing. The PCR amplification was carried out in a 50 µl reaction mixture containing 1 × PCR buffer (NEB, England), 1 nmol of dNTPs, 1 pmol of 2 mM $MgSO_4$, 0.25 pmol of forward and reverse primers, 1 unit Taq DNA polymerase (NEB, England) and 10 µl template DNA. The PCR started with the following conditions: initial denaturation at 95 °C for 10 min and 35 cycles of denaturation at 95 °C for 30 s, annealing at 56°C for 30 s and elongation at 72 °C for 60 s with final extension at 72 °C for 10 min. Agarose gel (1% in 1X TBE buffer) was used to electrophorese PCR products (Kheiralla et al., 2016). The gel was visualized under UV light (254 nm). The PCR product is purified by QIAquick Gel Extraction Kit (QIAGEN, USA) according to the manufactures. The identification was achieved by comparing the contiguous DNA sequence with data from the reference and type strains available in public databases GenBank using the BLAST program (National Centre for Biotechnology Information) (http://www.ncbi.nlm.nih.Gov/BLAST). The obtained sequences were aligned using Jukes Cantor Model and the isolate was registered in Gen Bank (Eida et al., 2018).

2.4. Lignocellulolytic enzymes assays

Liquid filtrate from various incubation times (0–10 days) old fungal cultures grown on PDB or liquid MSM amended with waste as sole carbon source were collected and used directly as the crude enzyme in enzymes assay experiments. All experiments were carried out in triplicate and the numbers appeared in average value. On the other hand, to treat the fibers, the fungal isolate was used directly with the raw material.

2.4.1. Cellulase enzyme assay

In order to detect ability of fungal isolate to produce cellulases enzymes, one disc of fungal growth was inoculated on MSM agar plates containing 1% microcrystalline cellulose (MCC) as sole carbon source. After incubation at 28 °C for 3 days, the formation of clear zone around fungal disc was noted as a positive result for cellulases. On the other hand, for quantification of cellulase enzymes production, one disc of fungal strain was inoculated in cellulose-containing MSM broth medium and incubated in an incubator shaker at 28 °C with speed of 120 rpm for 3 days. Cellulase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method (Ghose, 1987).

2.4.2. Lignin-peroxidases assay

Lignin peroxidases activity of culture filtrate was determined spectrophotometrically at 420 nm in a reaction mixture containing 2.4 ml of 100 mM potassium phosphate buffer pH 6.0, 0.3 ml of 5.33% pyrogallol, 0.2 ml of 10 mM H_2O_2 according to the method described by Darwesh et al. (2014).

2.4.3. Laccases enzymes assay

Spectrophotometric assay for Laccases activity was performed at 460 nm by the oxidation of 10 mM 2.6-dimethoxyphenol (DMP) in 100 mM sodium acetate buffer (pH 5) by Laccase enzyme (culture supernatant) according to the method of Darwesh et al. (2014).

2.4.4. Polyphenol oxidase enzyme assay

Polyphenol oxidase activity was determined for culture filtrate in a

reaction mixture of 2 ml, containing 0.01% catechol in 0.1 M phosphate buffer (pH 6). Absorbance (495 nm) measured after 3 min of incubation. The enzyme activity was expressed as changes in absorbance min^{-1} mg^{-1} of protein (Darwesh et al., 2014).

2.5. Analysis of fibers composition

2.5.1. Lignin contents

Lignin was determined as Klason lignin method. In brief, about one g of the air-dried sample was treated with 15 ml of 72% sulfuric acid for 2 h at room temperature (25–30 °C), then 3% sulfuric acid, followed by refluxing for 4 h. The mixture was filtered on a pre-weighed ash-less filter paper and washed with hot distilled water till neutrality, and then the lignin was gravimetrically estimated (El-Saied et al., 2012).

2.5.2. Holocellulose and cellulose contents

Holocellulose was quantified with sodium chlorite according to the procedure of (Wise et al., 1946) as adapted by (Timell, 1961) with slight modifications. In brief, 150 ml of 0.2 M acetic acid buffer was poured on 2.5 g of extractives-free sample. Then, 1 g of sodium chlorite was added, followed by 0.2 ml of glacial acetic acid and the sample was put in a water bath for 1 h at 70–80 °C. Sodium chlorite with acetic acid was further repeated for successive cycles of chlorination. After the necessary number of chlorination, the solution was filtered, washed with 500 ml of cold water followed by 50 ml of acetone and the holocellulose content as the remaining residue was determined gravimetrically. Holocellulose was treated with 17.5%(w/w) NaOH to obtain alpha-cellulose and calculated gravimetric according to El-Saied et al. (2012).

2.5.3. Resin and wax contents

Resins and waxes content of the used oven-dried grinded plant-origin wastes were gravimetrically calculated. Soxhlet extraction using methanol: benzene mixture (1:1) was accomplished for 6 h. The extracted substances were air-dried followed by oven heating at 100–105 ºC for 8 h (El-Saied et al., 2012).

2.5.4. Ash estimation

Ash content was gravimetrically estimated by igniting of the waste material in muffle furnace in a porcelain crucible first at 400 °C for 30 min, then raised the muffle temperature up to 850 °C for 45 min (El-Saied et al., 2012).

2.5.5. Fourier transform infrared (FTIR)

The structure change between treated and untreated fibers was studied by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Spectrum Two IR Spectrometer - Perkin Elmer, Inc., Shelton, USA). All spectra were obtained by 32 scans and 4 cm^{-1} resolution in wave numbers ranging from 4000 to 450 cm^{-1}.

2.5.6. Differential scanning calorimetry (DSC)

The DSC instrument (SETRAM DSC evo-131, France) with scan rate 5 °C/min is used to study the thermal behavior of treated and untreated fibers. The crystallinity was calculated via CALISTO software for thermal analysis data acquisition and processing by SETRAM Company, France.

3. Results and discussion

3.1. Isolation of selective lignin-degrading fungus

Partially decayed wooden trimmings samples of citrus trees were collected from some agricultural soils for isolation of highly selective lignin degrading fungi. Hardness and color of woody particles in soil samples supported the supposition of containing lignin-degrading fungi. Enrichment of isolation sources in liquid mineral salts medium (MSM) containing 10% (w/v) of grinded oven-dried citrus wood as a sole carbon source results in the growth of lignin degrading microorganisms. Sub culturing after two incubation weeks under the same conditions was conducted to obtain more selectivity towards lignin degradation. Similar isolation procedures were reported in several studies and found to be useful for isolation of lignin-degrading microorganism (Sasikumar et al., 2014). Representative fungal colonies were selected according to morphological features and re-inoculated on solidified (MSM) with same conditions. Five fungal isolates were selected based on growth and secretion of lignolytic enzymes in agar plates amended with lignin-rich wastes as sole carbon source as tabulated in Table (1). All selected isolates were examined against their ability for cellulase production. Only one fungal isolate gives negative results against cellulase and considered as non-cellulolytic lignin degrading fungus. The selected isolate showed good growth on the medium containing agriculture waste as the sole carbon source while it can't grow on cellulose as the sole carbon source under the same conditions. This isolate was purified, slanted and subjected for identification. This is not the first report isolation has non-cellulolytic activity in ascomycetes fungi (Juhász et al., 2003) isolated *A. niger* that lake the ability to degrade cellulose which has potential industrial applications.

Table 1
Enzymes activity of fungal isolates grown on MSM agar medium.

Isolate No.	Lig. peroxidase	laccase	NO_3 reductase	Polyphenole oxidase	Cellulase
EGYPTA1	+	+	+	++	+
EGYPTA2	+	+	+	+	+
EGYPTA3	+	+++	+	+	+
EGYPTA4	+++	+	+	+	+
EGYPTA5	+++	+++	++	+++	Un

*(+) weak productivity, (++) moderate productivity, (+++) strong productivity and (Un) undetected productivity.

3.1.1. Morphological and molecular identification of the most potent isolated fungus

The selected non-cellulolytic lignolytic fungal isolate (encoded EGYPTA5) was subjected to morphological characterization and molecular techniques identification. The colonies of this isolate on the surface of PDA medium were contained yellow-green spores on the upper surface and reddish-gold below as shown in Fig. 1. Microscopic examination showed that, hyphae are hyaline and septate, and the conidia produce thick mycelial mats while the conidiophores are rough and colorless, phialides are arranged in one and two rows. All previous morphological features are related to *Aspergillus flavus* (Rodrigues et al., 2007).

The identification was completed and confirmed using molecular techniques. Total fungus genomic DNA of isolate EGYPTA5 was extracted and applied for their rRNA amplifications. The obtained sequences of this amplification were compared with related sequences on the National Centre for Biotechnology Information (NCBI) data base and found closely with *Aspergillus flavus* confirming the morphological identifications. This sequence was recorded in GenBank under accession number of MH425453 with the name of *Aspergillus flavus* isolate EGYPTA5. The closely phylogenetic tree illustrated in Fig. 2.

Most studies referred to white rot fungi as the most potent selective-lignin degrading microorganisms. Nevertheless, few researchers reported different fungi as non-cellulolytic lignin degrading fungus other than white rot fungi such as *Aspergillus* sp. (Milstein et al., 1984; Otjen et al., 1987; van Kuijk et al., 2016). *Aspergillus flavus* proven to produce some lignin degrading enzymes such as laccases (Ghosh and Ghosh, 2017).

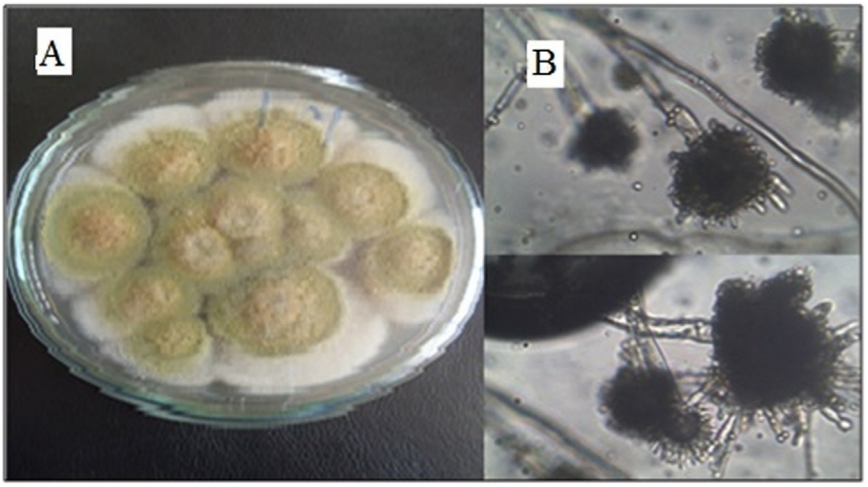

Fig. 1. Colonies of non-cellulolytic lignolytic fungal isolate EGYPTA5 on PDA plate (a) and its image using light microscopic (b).

3.2. Assay of enzymes related for lignolytic agriculture wastes

Microbial enzymes as biological catalysts play a crucial role in many biochemical reactions and involve in many industries. Many enzymes are useful in agriculture wastes decomposition as lignin peroxidases, laccase, polyphenol oxidase, nitrate reductase and cellulase enzymes (Darwesh et al., 2015). Lignolytic enzymes were assayed during study to determine the ability of isolated strain *i.e. Aspergillus flavus* EGYPTA5 to degrade lignin of wooden agricultural wastes. Enzymes assay helps to recognize the optimum period for production of the highly amount of lignolytic enzymes (Brito et al., 2008; Chan Cupul et al., 2014). The cellulase enzyme activity was absent over all tested days. This result indicates that this fungal strain was not cellulolytic organism, and that was confirmed by its negative ability to grow on cellulose as a sole carbon source in liquid MS medium and this is in agreement with other previous study (Juhász et al., 2003).

In case of lignolytic enzymes of *A. flavus* EGYPTA5 cultivated in lignin-free PDA medium for 10 days, various activities were recorded as illustrated in Fig. 3. The results indicate that the activities of tested enzymes increased up to 6 or 7 days of incubation then started in decreasing. Despite lignin peroxidases recorded, the highest activity in PDA medium, all activities of the examined enzymes generally were very low (doesn't exceeded 0.15 U/ml). This finding indicates that lignolytic enzymes could be secreted but in low concentrations even in the absence of lignin in cultivation medium. Asemoloye et al. (2018) reported the production of lignin peroxidases and laccase by different rhizospheric fungal strains including *A. flavus* in lignin-free cultivation media.

For induced lignolytic enzymes assay, *Aspergillus flavus* isolate EGYPTA5 was cultivated in MSM medium containing lignin-rich agriculture wastes as a sole carbon source. The results illustrated in Fig. 4 clearly show that, the activity of tested enzymes was increased when using the induction medium comparing with PDB medium (Fig. 3). Lignin peroxidase enzyme was the superior one in this experiment and their activity reach to 2.45 U/ml after 9 days of incubation followed by the other enzymes. These results clearly indicate that, the presence of suitable substrate *i.e.* lignin induce the production of related enzymes (lignolytic enzymes). Results obtained from other researcher support our data regarding secretion of lignolytic enzymes in presence and absence of lignin (Han et al., 2014). Additionally the other researchers reported the highest value of lignin peroxidases enzymes was 0.51 U/ml (Pant and Adholeya, 2007). This indicated that the reported isolate has the potential and recommend as Lignin peroxidase enzymes producer.

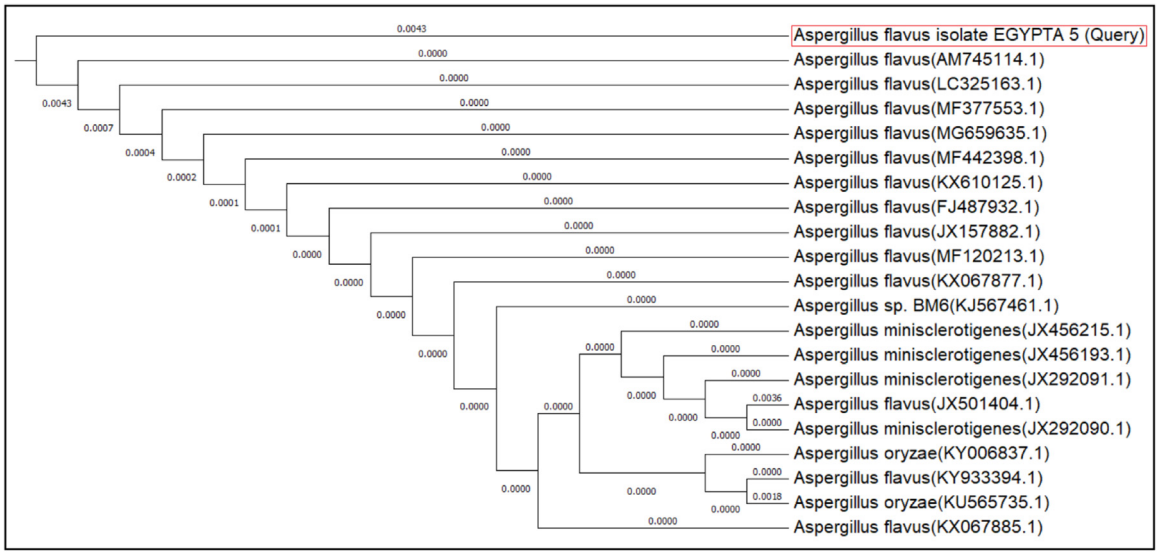

Fig. 2. Phylogenetic tree of *Aspergillus flavus* isolate EGYPTA5 and closely related strains.

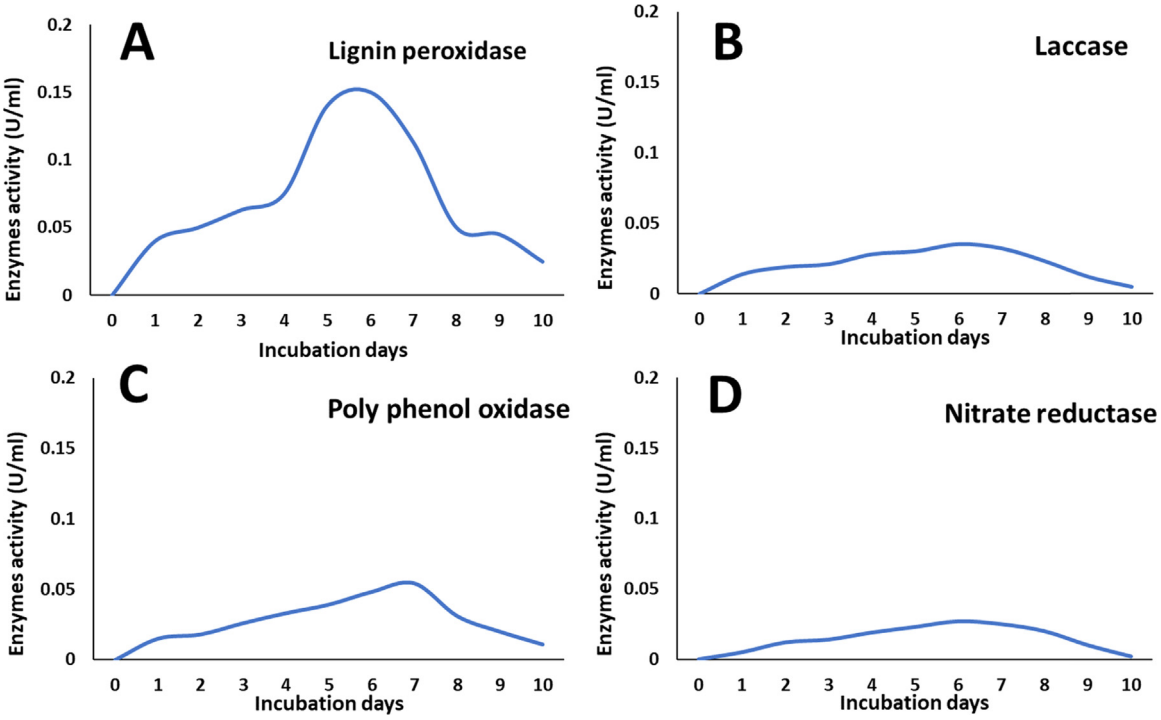

Fig. 3. Non-inducible lignolytic enzymes activities of *Aspergillus flavus* isolate EGYPTA5 grown in lignin-free medium (PDB medium).

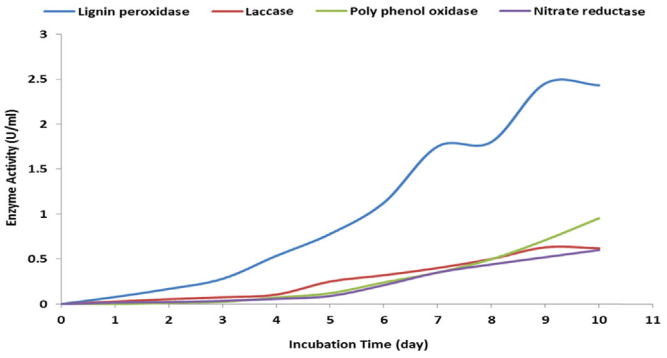

Fig. 4. Inducible changes in lignolytic enzymes activities of *Aspergillus flavus* isolate EGYPTA5 grown in MSM medium containing lignin-rich agriculture waste as carbon source.

Table 2
Changes in agriculture waste content (%) before (control) and after fungal treatment during 10 days of incubation.

Incubation days	Lignin, %	Holocellulose, %		Ash, %	Wax/ Resin, %
		Alpha-cellulose	Hemicellulose		
0 (Control)	17	68	6.5	7.21	1.03
1	17	68	6.7	7.21	1.03
2	17	67	6.7	8.2	1.03
3	17	67	6.7	8.2	1.03
4	17	69	4.6	8.2	1.03
5	17	71	2.7	8.2	1.03
6	17	72	1.6	8.2	1.03
7	16.5	71	1.6	9.4	1.5
8	15.5	70	1.6	11	1.8
9	11	73	1.6	12	2.24
10	9	75	1.6	12	2.24

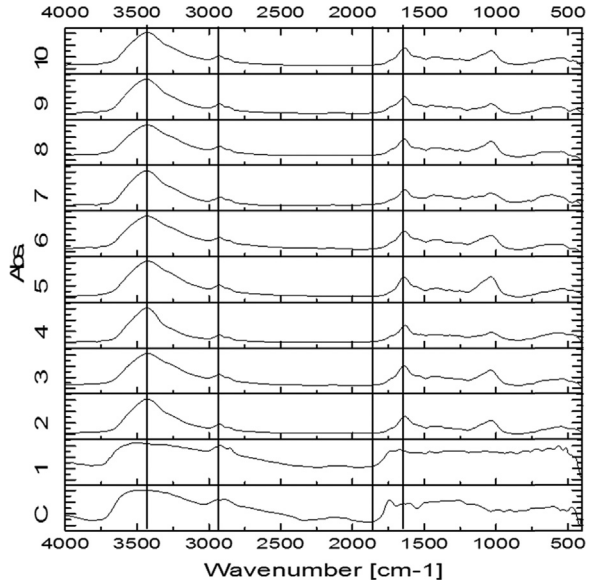

Fig. 5. FTIR of original (untreated) lignin-rich agricultural wastes (C) and biologically treated with *Aspergillus flavus* EGYPTA5 during the 10 days of incubation (1 – 10).

3.3. Fibers analysis before and after enzymatic decomposition of agriculture waste

The untreated agricultural wastes were analyzed as a control and the 1–10 days treated wastes were analyzed as well to understand the effect of the producing enzymes on the fibers constituent. Lignin, holocellulose (alpha, beta, and gama celluloses as well as hemicellulose), ash, wax and resin are estimated. The analyses were tabulated in Table (2). The cellulose and ash content were increased by the incubation time until day eight in which these components contents were not changed. Fibers analysis cleared that the lignin content was affected by incubation period; this emphasized that the enzymes of fungal isolate

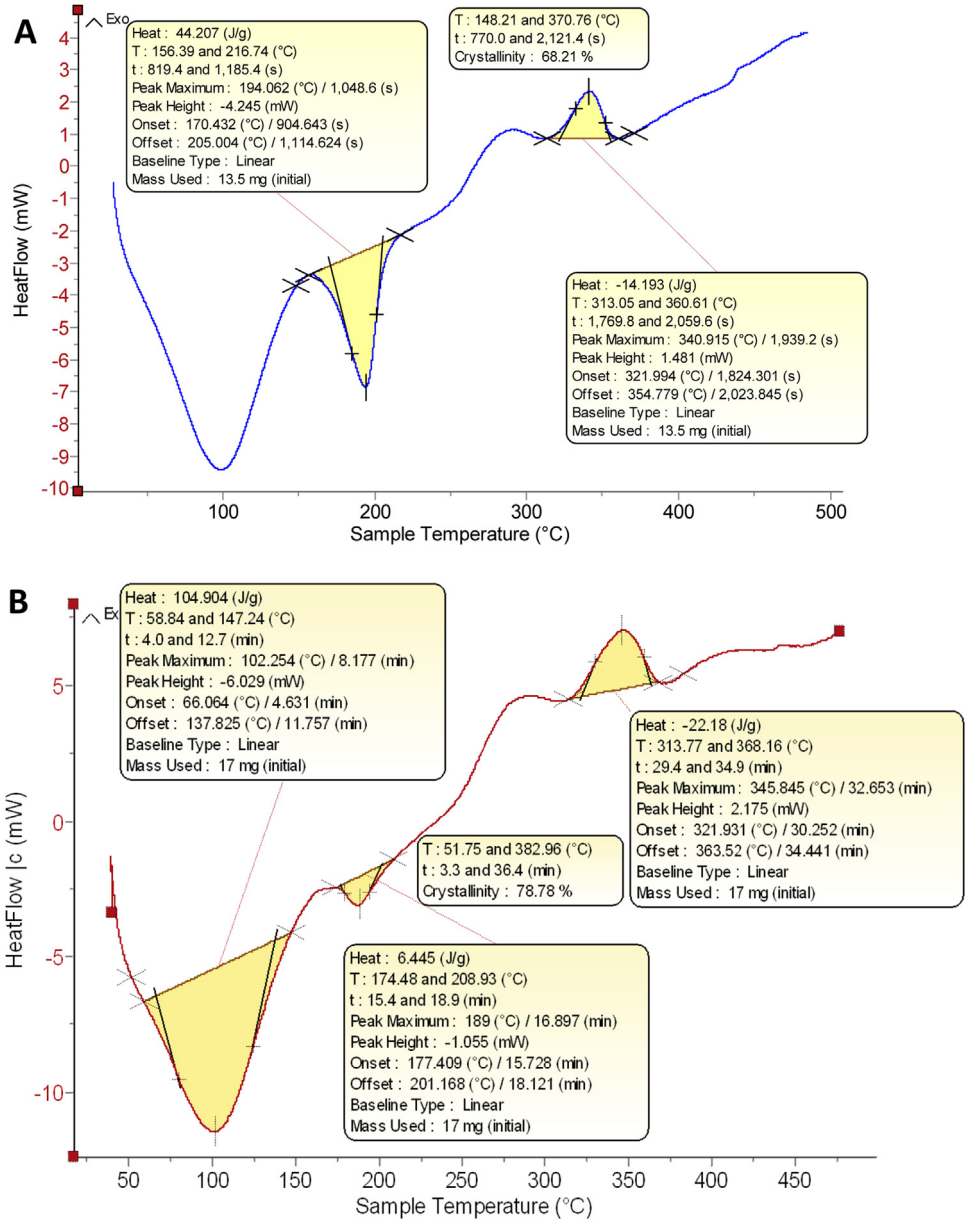

Fig. 6. DCS of original (untreated) lignin-rich agricultural wastes (C) and biologically treated with *Aspergillus flavus* EGYPTA5 during the 10 days of incubation (1 − 10).

have the ability for selective lignin degradation without any cellulytic activity on fibers. These results are in parallel with lignin degradation enzymes assay. The obtained results clear that *Aspergillus flavus* isolate EGYPTA5 could be used for selective fibers-delignification pretreatments to reduce lignin content without negative effects on cellulose content.

3.4. Fiber instrumental analysis

FTIR spectroscopy tool was used to give conformation on the lignocellulosic constituent changing during treatment with fungal enzymes (Khalil et al., 2017). Fig. 5 illustrate FTIR spectra of original (untreated) lignin-rich agricultural wastes as well as changes occurred through ten days (from day 1 to day 10) cultivation with *Aspergillus flavus* EGYPTA5 isolate. Each lignocelluloses constituent has been characteristic peaks identified in the literature, and this characteristic peak must be found only in this constituent not in others. The most characteristic peaks of cellulose were b-glycosidic linkage at around 900 cm^{-1} and O-H stretching vibration around 3417 cm^{-1} (broad band) (Brienzo et al., 2009). Aromatic skeleton vibrations at around 1600 cm^{-1} are standard for lignin indicator bands, so presence of these bands confirms the presence of lignin and acetyl or uronic ester groups which characterized the lignin structure and not found in any other lignocelluloses material constituents also appeared from 1730 to 1750 cm^{-1}. Untreated fibers showed bands at 3431, 2951, 1749, 1651 cm^{-1} which refer to free starch O-H of cellulose, C–H stretching vibration which characterized all lignocellulosic fibers, C-O stretch of lignin and C-C in plane symmetrical starch of lignin respectively. On the other hand, the biologically treated fibers (with *Aspergillus flavus* EGYPTA5) after one day and up to 7 days showed the same peaks approximately with non-significant changes. Where, the peak of free starching of O-H was shifted to higher frequency from day 1 to day 7 by about 3 cm^{-1} as well as the peaks which characterize lignin were shifted to low frequency with little value 5 cm^{-1}. In contrast the treated fibers from day 8 to day 10 showed the highly significant difference. At day 8, spectra recorded remarked break down in lignin from 1749 cm^{-1}

at day 7–1696 cm^{-1}. Additionally, the peak of O-H stretching shift to higher frequency at 3467 cm^{-1} this refer to liberation the OH group as results to eliminate the lignin and hemicellulose. Day 9 spectra showed little pit changes in the two main peaks (cellulose and lignin). Day 10 spectra showed the highly efficacy of enzymes effect where the characteristic peak of ß-glyscidic linked which characterize cellulose is appear at 903 cm^{-1} and increased in O-H frequency to3524 cm^{-1}. This is related to removal of impurities from fibers and liberated the O-H as free starching frequency (El-Saied et al., 2018). Also peak of lignin was shifted to 1677 cm^{-1}. This is indicating to elimination of adequate amount of lignin, which makes cellulose characteristic bands appeared. Obtained FTIR results of lignin-rich agricultural wastes in this study are compatible with fibers chemical analysis (Mohamed et al., 2015).

Fig. 6 showed the two DSC curves for started untreated fibers (a) and 10 days treated fibers (b). Untreated fibers curve shows after adsorbed water region two peaks. First one has onset 156 °C and offset 216 °C with as well as peak temperature (T_P) 194 °C. The second peak with onset 313.0 °C and offset 360.61 °C as well as peak temperature (T_P) 340.915 °C. Similarly, in 10 day treated fibers the DSC curve has two humps peaks after adsorbed water peak with T_P189 °C for first and 345 °C for second. The increase in lignocellulosic material crystallinity is referring to elimination of lignin, hemicellulose, and/or the amorphous region. In our work the fungal isolate showed no cellulolytic activity, so the increase of crystallinity emphasized to the partial elimination of lignin and hemicellulose (Basta et al., 2016; Basta, El-Saied et al., 2018). Hence, crystallinity percentage showed the significant difference in both fibers' types where the crystallinity percentage in treated fibers increase by about 10% in comparison with untreated ones (Ibrahim et al., 2018). This result is confirming that the undesirable materials, which found in amorphous form (lignin and hemicellulose) are eliminated and that in agreement with all further investigations.

4. Conclusion

In this paper, the main aim was to isolate and characterize of selective lignin degrading fungi to be used in various industrial and environmental applications. *Aspergillus flavus* EGYPTA5 considered as efficient non-cellulolytic lignin degrading fungus. Production of lignolytic related enzymes (lignin peroxidases, laccase, polyphenol oxidase, nitrate reductase) were proven to be produced in low amounts in lignin-free medium and in reliable amounts when lignin-rich substrate was used as carbon source in cultivation medium. Thus, it could be concluded that lignolytic enzymes are inducible in certain fungal isolate. *A. flavus* (EGYPTA5) would be used as a biological treatment for lignin-rich cellulosic wastes to degrade lignin without affecting cellulose content of fibers. The isolated fungus and/or its specific-lignolytic enzymes could be applied in various environmental and industrial applications.

Acknowledgements

The authors express their sincere thanks to National Research Centre of Egypt for providing the necessary research facilities. The authors would like to acknowledge the facilities available at Central Laboratory Network (CLN), National Research Centre, Dokki, Egypt.

References

Abdel-Hamid, A.M., Solbiati, J.O., Cann, I.K., 2013. Insights into lignin degradation and its potential industrial applications. Adv. Appl. Microbiol. 1–28.
Abou-Shanab, R., Khalafallah, M., Emam, N., Aly, M., Abou-Sdera, S., Matter, I., 2012. Characterisation and identification of carbofuran-utilising bacteria isolated from agricultural soil. Chem. Ecol. 28 (2), 193–203.
Asemoloye, M.D., Ahmad, R., Jonathan, S.G., 2018. Transcriptomic responses of catalase, peroxidase and laccase encoding genes and enzymatic activities of oil spill inhabiting rhizospheric fungal strains. Environm. Poll. 235, 55–64.
Barakat, K.M., Hassan, S.W., Darwesh, O.M., 2017. Biosurfactant production by haloalkaliphilic Bacillus strains isolated from Red Sea, Egypt. Egypt. J. Aq. Res. 43 (3), 205–211.
Basta, A.H., El-Saied, H., El-Deftar, M.M., El-Henawy, A.A., El-Sheikh, H.H., Abdel-Shakour, E.H., Hasanin, M.S., 2016. Properties of modified carboxymethyl cellulose and its use as bioactive compound. Carb. Pol. 153, 641–651.
Basta, A.H., El-Saied, H., Hasanin, M.S., El-Deftar, M.M., 2018. Green carboxymethyl cellulose-silver complex versus cellulose origins in biological activity applications. Int. J. Biol. Macromol. 107, 1364–1372.
Beckham, G.T., Johnson, C.W., Karp, E.M., Salvachúa, D., Vardon, D.R., 2016. Opportunities and challenges in biological lignin valorization. Curr. Opin. Biotechnol. 42, 40–53.
Benito, C., Figueiras, A., Zaragoza, C., Gallego, F., De La Pena, A., 1993. Rapid identification of Triticeae genotypes from single seeds using the polymerase chain reaction. Plant Mol. Biol. 21 (1), 181–183.
Brienzo, M., Siqueira, A., Milagres, A.M.F., 2009. Search for optimum conditions of sugarcane bagasse hemicellulose extraction. Biochem. Engin. J. 46 (2), 199–204.
Brito, J., Silva, F., Leão, M., Almeida, G., 2008. Chemical composition changes in eucalyptus and pinus woods submitted to heat treatment. Biores. Technol. 99 (18), 8545–8548.
Chan Cupul, W., Heredia Abarca, G., Martínez Carrera, D., Rodríguez Vázquez, R., 2014. Enhancement of ligninolytic enzyme activities in a Trametes maxima-Paecilomyces carneus co-culture: key factors revealed after screening using a Plackett-Burman experimental design. Electr. J. Biotechnol. 17 (3), 114–121.
Darwesh, O.M., Moawad, H., Barakat, O.S., Abd El-Rahim, W.M., 2015. Bioremediation of textile reactive blue azo dye residues using nanobiotechnology approaches. . Res. J. Pharm. Biol. Chem. Sci. 6 (1), 1202–1211.
Darwesh, O.M., Sultan, Y.Y., Seif, M.M., Marrez, D.A., 2018. Bio-evaluation of crustacean and fungal nano-chitosan for applying as food ingredient. Tox. Rep. 5, 348–356.
Darwesh, O., Moawad, H., Wafaa, M., Olfat, S., Sedik, M., 2014. Bioremediation of textile reactive blue, RB) azo dye residues in wastewater using experimental prototype bioreactor. Res. J. Pharm., Biol. Chem. Sci. 5 (4), 1203–1219.
Eida, M.F., Darwesh, O.M., Matter, I.A., 2018. Cultivation of oleaginous microalgae *Scenedesmus obliquus* on secondary treated municipal wastewater as growth medium for biodiesel production. J. Ecol. Engin. 19 (5), 38–51.
El-Saied, H., Basta, A.H., Hassanen, M.E., Korte, H., Helal, A., 2012. Behaviour of rice-byproducts and optimizing the conditions for production of high performance natural fiber polymer composites. J. Pol. Env. 20 (3), 838–847.
El-Saied, H., Mostafa, A.M., Hasanin, M.S., Mwafy, E.A., Mohammed, A.A., 2018. Synthesis of Antimicrobial Cellulosic Derivative and its Catalytic Activity. J. King Saud. Univ. -Sci (in press).
Fang, X., Li, Q., Lin, Y., Lin, X., Dai, Y., Guo, Z., Pan, D., 2018. Screening of a microbial consortium for selective degradation of lignin from tree trimmings. Biores. Technol. 254, 247–255.
Fernández-Fueyo, E., Ruiz-Dueñas, F.J., López-Lucendo, M.F., Pérez-Boada, M., Rencoret, J., Gutiérrez, A., Pisabarro, A.G., Ramírez, L., Martínez, A.T., 2016. A secretomic view of woody and nonwoody lignocellulose degradation by Pleurotus ostreatus. Biotechnol. Biof. 9 (1), 49.
Ghose, T., 1987. Measurement of cellulase activities. Pure. Appl. Chem. 59 (2), 257–268.
Ghosh, P., Ghosh, U., 2017. Statistical optimization of laccase production by Aspergillus flavus PUF5 through submerged fermentation using agro-waste as cheap substrate. Acta Biol. Szeged. 61 (1), 25–33.
Hammel, K., 1997. Fungal degradation of lignin. Driven Nat.: Plant litter Qual. Decompos. 33, 45.
Han, Y., Shi, L., Meng, J., Yu, H., Zhang, X., 2014. Azo dye biodecolorization enhanced by Echinodontium taxodii cultured with lignin. PloS One 9 (10), e109786.
Hasanin, M.S., Mostafa, A.M., Mwafy, E.A., Darwesh, O.M., 2018. Eco-friendly cellulose nano fibers via first reported Egyptian *Humicola fuscoatra* Egyptia X4: isolation and characterization. Envir. Nanotechnol. Monit. Manag. 10, 409–418.
Hoballah, E., Saber, M., Matter, I., Zaghloul, A., 2014. Bioremediation of polychlorinated biphenyl, PCBs) in a sewaged soil by certain remediative amendments followed by phytoremediation. RJPBCS 5, 91–103.
Ibrahim, S., El Saied, H., Hasanin, M., 2018. Active Paper Packaging Material Based on Antimicrobial Conjugated Nano-Polymer/Amino Acid as Edible Coating. J. King Saud. Univ.-Sci (in press).
Juhász, T., Kozma, K., Réczey, K., 2003. Production of β-glucosidase in mixed culture of Aspergillus niger BKMF 1305 and Trichoderma reesei RUT C30. Food Technol. Biotechnol. 41 (1), 49–53.
Khalil, A.M., Abdel-Monem, R.A., Darwesh, O.M., Hashim, A.I., Nada, A.A., Rabie, S.T., 2017. Synthesis, characterization, and evaluation of antimicrobial activities of chitosan and carboxymethyl chitosan schiff-base/Silver nanoparticles. J. Chem. 2017.
Kheiralla, Z.H., Hewedy, M.A., Mohammed, H.R., Darwesh, O.M., 2016. Isolation of pigment producing actinomycetes from rhizosphere soil and application it in textiles dyeing. . Res. J. Pharm. Biol. Chem. Sci. 7 (5), 2128–2136.
Kong, W., Fu, X., Wang, L., Alhujaily, A., Zhang, J., Ma, F., Zhang, X., Yu, H., 2017. A novel and efficient fungal delignification strategy based on versatile peroxidase for lignocellulose bioconversion. Biotechnol. Biof. 10 (1), 218.
Maciel, M.J.M., Ribeiro, H.C.T., 2010. Industrial and biotechnological applications of ligninolytic enzymes of the basidiomycota: a review. Electr. J. Biotechnol. 13 (6), 14–15.
Milstein, O.A., Haars, A., Sharma, A., Vered, Y., Shragina, L., Trojanowski, J., Flowers, H.M., Gressel, J., Hüttermann, A., 1984. Lignin degrading ability of selected aspergillus Spp. Appl. Biochem. Biotechnol. 9 (4), 393–394.
Mohamed, A.A., Ali, S.I., Darwesh, O.M., El-Hallouty, S.M., Sameeh, M.Y., 2015. Chemical compositions, potential cytotoxic and antimicrobial activities of Nitraria retusa methanolic extract sub-fractions. Int J Toxicol. Pharmacol. Res 7, 204–212.

Otjen, L., Blanchette, R., Effland, M., Leatham, G., 1987. Assessment of 30 white rot basidiomycetes for selective lignin degradation. Walter de Gruyter, Berlin/New York.
Pant, D., Adholeya, A., 2007. Enhanced production of ligninolytic enzymes and decolorization of molasses distillery wastewater by fungi under solid state fermentation. Biodegr 18 (5), 647–659.
Paramjeet, S., Manasa, P., Korrapati, N., 2018. Biofuels: production of fungal-mediated ligninolytic enzymes and the modes of bioprocesses utilizing agro-based residues. Biocat. Agric. Biotechnol (In press).
Remédios, M., Carvalho, F.A., Enguita, F.J., Cardoso, C., Martins, I.C., Santos, N.C., Leitao, A.L., 2016. Effects of Penicillium chrysogenum var. halophenolicum on kraft lignin: color stabilization and cytotoxicity evaluation. 3 Biotech. 6 (1), 102.
Rodrigues, P., Soares, C., Kozakiewicz, Z., Paterson, R., Lima, N., Venâncio, A., 2007. Identification and characterization of *Aspergillus flavus* and aflatoxins. Commun. Curr. Res. Educ. Top. Tr. Appl. Microbiol. 527–534.
Ruqayyah, T.I., Jamal, P., Alam, M.Z., Mirghani, M.E.S., 2013. Biodegradation potential and ligninolytic enzyme activity of two locally isolated Panus tigrinus strains on selected agro-industrial wastes. J. Envir. Manag. 118, 115–121.
Sasikumar, V., Priya, V., Shankar, C.S., Sekar, S., 2014. Isolation and preliminary screening of lignin degrading microbes. J. Acad. Ind. Res. 3 (6), 291–294.
Sette, L.D., Santos, R.C.B., 2013. Ligninolytic enzymes from marine-derived fungi: production and applications. Marine Enzymes for Biocatalysis. Elsevier, pp. 403–427.
Singh, A.P., Singh, T., 2014. Biotechnological applications of wood-rotting fungi: a review. Biomass-. Bioeng. 62, 198–206.
Su, Y., Xian, H., Shi, S., Zhang, C., Manik, S.N., Mao, J., Zhang, G., Liao, W., Wang, Q., Liu, H., 2016. Biodegradation of lignin and nicotine with white rot fungi for the delignification and detoxification of tobacco stalk. BMC Biotechnol. 16 (1), 81.
Tandon, G., 2015. Bioproducts from residual lignocellulosic biomass. In: Nawani, N.N., Khetmalas, M., Razdan P.N., Pandey, A. (Eds), Advances in Biotechnology, pp, 52–75.
Timell, T., 1961. Isolation of galactoglucomannans from the wood of gymnosperms. Tappi 44, 88–96.
Upadhyay, P., Shrivastava, R., Agrawal, P.K., 2016. Bioprospecting and biotechnological applications of fungal laccase. 3 Biotech. 6 (1), 15.
van Kuijk, S.J., José, C., Rencoret, J., Gutiérrez, A., Sonnenberg, A.S., Baars, J.J., Hendriks, W.H., Cone, J.W., 2016. Selective ligninolysis of wheat straw and wood chips by the white-rot fungus Lentinula edodes and its influence on in vitro rumen degradability. J. Anim. Sci. Biotechnol. 7 (1), 55.
Wang, Y., Liu, Q., Yan, L., Gao, Y., Wang, Y., Wang, W., 2013. A novel lignin degradation bacterial consortium for efficient pulping. Biores. Technol. 139, 113–119.
Wise, L.E., Murphy, M., D Adieco, A., 1946. A chlorite holocellulose, its fractionation and bearing on summative wood analysis and studies on the hemicelluloses. Pap. Tr. J. 122 (2), 35–43.