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A NOVEL METHOD OF USING INDIGENOUS WOOD ROTTING FUNGI IN LIGNIN DEGRADATION OF TOBACCO STALKS

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ABSTRACT

Tobacco stalks represent a type of abundant renewable resources whereas wood rotting fungi are of renowned industrial interest. This study was aimed at assessing the biotechnological potentiality of indigenous wood rotting fungi on lignin degradation of tobacco stalks. 21 isolates were isolated and screened for phenol red oxidation in Potato Dextrose Agar (PDA) containing 250 µg/mL phenol. 12 isolates oxidized phenol and were compared on decolorization of five dyes; acid fuchsin, indigo carmine, congo red, methyl orange, and malachite green supplemented independently at 250 µg/mL in PDA. Further, *In vitro* studies were carried out on lignin degradation. The isolates were incubated with ground tobacco stalks and final residual lignin content calculated. Isolate 9, 6 and 2 effectively reduced lignin content from 29.15% to 16.26%. 16.72% and 19.22% respectively, suggesting them as a suitable cost effective mechanism that can be considered for use on lignin degradation in Zimbabwe. The fungal isolates 9, 6 and 2 have great biotechnological potential in the paper industries in both dye discoloration and delignification and hence recommended for biotechnological reduction of pollutants from the paper industry.

Keywords: Decolorization; degradation; IWFRI; lignin cellulosic.

INTRODUCTION

Over the last century, cellulose pulp has been made from wood species which contributed 95% of the raw materials for pulp [1]. Paper pulp packaging has recently become attractive and world paper consumption has nowadays become high. The forestry resources have succumbed an imposed immense pressure due to the growing demand for paper [2]. An increased competition for raw materials in paper manufacturing will soon be undoubtedly triggered by the demand for various forest products as a result of rampant population growth and economic development [3].

With severe pressure emanating from the depleted forest resources in most countries, there is however the need to search for new solutions to meet the growing demands for paper [3] by utilizing non-woods to curb the fiber shortage that is anticipated to emerge in the near future. In

light of perceived high demand for forest products, non-wood and recycled fibers can be a replacement to wood fiber [3]. Non wood and recycled fibers offer a great substitute for wood in terms of fiber resource especially in regions with insufficient forest resources [3].

Three components of the non-wood materials are involved in the paper making process; cellulose, hemicellulose and lignin [1]. In comparison to wood species, tobacco stalks contain the major constituents: cellulose, hemicellulose and lignin in comparable amounts. Tobacco stalks on average usually constitute cellulose 30 to 40%, hemicellulose10 to 20%, and lignin 20 to 30% on a dry mass basis [4]. Pulp and paper manufacturing process at industrial level, utilizes large amounts of water and lignocellulosic materials. It is an energy demanding and highly polluting industry hence environmental impacts for this industrial sector have recently been closely examined [5]. With the thrust premised on reducing environmental pollution impacts, processes of pulping and bleaching in the paper industry coupled with biotechnological innovations have been applied. Biological methods for industrial application imply the use of fungal strains with ligninolytic enzyme activity [6]. In particular, the use of oxidative fungal enzymes laccase (Lac) manganese peroxidase (MnP), lignin peroxidase (LiP) may replace the use of chemical reagents used for the bleaching effect. The extracellular digestive enzymatic complex is capable of depolymerizing lignin, the aromatic polymer into lower molecular weight compounds [7].

The study was aimed at assessing the biotechnological potentiality of indigenous wood rotting fungi isolates on lignin degradation of tobacco stalks and it also proposed for the recommendation of tobacco stalks as a raw material for paper making in Zimbabwe. The study combined various approaches to support the assessment of biotechnological potential of studied isolates for use in the paper industry. These approaches included: phenol red oxidation, dye decolorization assay and lignin degradation assay. Results from these approaches were discussed and evaluated for their contribution in the paper industry.

MATERIALS AND METHODS

Pulp Processing

Tobacco stalks were obtained from Kutsaga land fields. They were cut into pieces of 15cm and dried until they reached moisture content of 15-20%. They were milled into pulp at Analytical Chemistry Services using a mill grinder and stored at room temperature until use for the bioassays. Upon use, they were autoclaved for sterilization twice at 120 °C for 15 minutes in glass jars.

Fungi Isolates

Twenty-one indigenous wood rotting fungi isolates (IWFRI) were randomly collected from decaying wood in Mushagashe North, Masvingo of altitude 1204 on longitude 30° 47’E and latitude 19° 50’S.

Culturing Media

Potato dextrose agar (PDA) (Merck & Co., Inc.) was used to culture the fungal isolates, maintain the strains at 4 °C and produce inoculum. Potato Dextrose Broth (PDB) (Merck & Co., Inc.) was used in

assays of enzyme activity in the presence of tobacco pulp. All media were prepared as indicated by the supplier and sterilized by autoclaving (Biobase Group® Model BKQ-B150/200II) at 120°C for 15 minutes.

Fungal Inoculum Preparation

Inoculum was generated using 6-mm cylindrical plugs removed with a cork borer from the margin of colony in the log growth phase and inoculated on the center of a 90-mm PDA petri dish. The dishes were incubated in darkness at 28°C until the mid-log phase and 6-mm plug of the inoculum was cut from the margin of the colony as described. These mycelia plugs were used for both solid and liquid media assays [8].

Phenol Oxidation Assay

The assay was done following an edited protocol by Wilberg et al. [9]. The oxidation assays for phenol were performed in PDA. The 21 isolates were first screened based on their growth on media containing 250ug/mL phenol red and their potentiality to oxidize phenol red, [10]. Phenol oxidation was visually observed by a color change from yellow to red around the culture growth. This indicated the ligninolytic nature of the fungi isolates.

Dye Decolorization Assay

The fungi isolates that oxidized phenol were further tested for their potentiality to decolorize five dyes supplemented independently in PDA. The decolorization assays of the dyes were performed in 90-mm Petri dishes. The assays were performed in PDA supplemented in independent experiments with acid fuchsin (triphenylmethane dye), malachite green (triphenylmethane dye), indigo carmine (indigoid dye), congo red (azo dye) and methyl orange (azo dye). The five compounds were used at a final concentration of 250 ug/mL and added to the media. The media with dyes was inoculated at the center of the plate with a 6-mm mycelium plug obtained as previously described and incubated in darkness at 28°C for 12 days. A control plate with media containing dye but without fungal inoculum was incubated to confirm that the color change was not induced by physicochemical factors during fungal growth. The decolorization of phenolic dyes was determined visually by the clearance and loss of dye color of the media [11]. All experiments were performed in pentaruplicate.

Lignin Degradation Assay

Lignin content was determined via acid detergent fibre using the Ankom method which is an alternative approach for lignin analysis [12]. Inoculum for the isolates was independently generated using 6-mm cylindrical plugs removed with a cork borer from the margins of mycelia colony in the log growth phase and inoculated in 200ml Potato Dextrose Broth (PDB). The PDB with the mycelia plugs were incubated in a shaker for 48 hours to produce inoculum. Autoclaved milled tobacco stalks were then added into the inoculum and incubated for 14 days. After 14 days of incubation the tobacco stalks were filtered from the broth and dried in an oven.

The dried tobacco stalk samples were then separately boiled in sulphuric acid solution of cetrimethyl bromide (CTAB) under controlled temperature (100°C). 0.6g (W1) of the tobacco stalk samples were weighed precisely into 250ml round-bottom flasks with glass joints to fix a reflux condenser. 100ml CTAB and a few drops of anti-foam agent Octyl alcohol were added to each sample. The condensers and the reflux

were connected for 1 hour from the time the boiling started. (ASTM 40-60) of known weight (W2) was filtered through a sintered glass crucible under gentle suction. The residues were washed with at least four portions of 100ml boiling water until they were acid free and this was tested using blue litmus paper. Acetone was used to wash in order to remove any other pigment for 1 hour and the residue fibre was air-dried by applying suction.

The crucible and the content were then placed in an oven at 105°C for 2 hours, cooled to slightly above room temperature and reweighed, both the crucible and content (W3). The glass crucible containing the dried acid detergent fibre was placed over a small beaker and 20ml concentrated sulphuric acid was added and stirred immediately with a glass rod for every 30 minutes. The procedure was repeated three times within 2 hours. The fibre was washed with hot water several times, filtered under suction and washed similarly as well with acetone. The crucible and the content were dried for 2 hours at 105°C in a desiccator and weighed (W4). The ash content was ashed at 500-550°C for 1 hour and allowed to cool in a desiccator and weighed (W5).

Data Collection

Experimental design

The research was carried out as a Completely Randomized Design with 13 treatments replicated 5 times. Data was analyzed using Analysis of variance (ANOVA) at 5 % significance level using the Genstart 18th Edition software.

Phenol oxidation

Phenol oxidation assessment was done at 5 days after inoculation (5 DAI) on PDA plates supplemented with phenol red 250ug/mL using the phenol oxidation assessment scale +/-:

+ = phenol oxidation
- = no phenol oxidation

Dye decolorization

Dye discoloration assessment was done at 12 days after inoculation (12 DAI) on PDA plates supplemented in independent experiments with acid fuchsin, congo red, malachite green, methyl orange and indigo carmine using the Dye discoloration assessment scale 0 – 5:

0= No discoloration
1= <25% of dye discolored
2= 25 – 50% of dye discolored
3= 50 – 75% of dye discolored
4= > 75% of dye discolored
5= Total dye discoloration

Lignin degradation (Grzegorz et al., 2017)

Lignin content (%) = $(\mathrm{W3} - \mathrm{W4}/\mathrm{W1}) \times 100$

RESULTS AND DISCUSSION

Ligninolytic enzymes are now in focus at different industrial levels throughout the world and the production of extracellular enzyme producing fungi has become essential to mitigate their increasing demand. IWRFI have since evolved complex enzymatic machinery meant to deal with environmental substances such as lignin that are difficult to degrade. Sources exhibiting hydrolytic enzyme activities were studied by some other researchers with a high percentage of these studies supporting hydrolytic enzyme producing fungi occurrence in decaying wood chips and other iomasses. Amongst diverse groups of enzyme producing microorganisms, major producers of lignocellulolytic enzymes are

largely terrestrial fungi isolates particularly *Trichoderma* and *Aspergillus species* [13].

Phenol red Oxidation Assay

The 12 IWRFI performed almost the same on phenol oxidation.

In the present study, a total of 21 IWRFI were isolated and 12 exhibited positive results for phenol oxidation assay, dye decolorization assay and lignin degradation assay. A color change from yellow to red was observed for phenol red oxidation in PDA media incorporated with phenol red [10]. (Fig. 1). There were significant differences amongst the IWRFI on phenol oxidation, ($p<0.001$, Table 1). Isolates with the same letter have statistical similarities on phenol oxidation means according to Tukey's α 0.05. This served as a screening basis for isolates with potential ligninolytic enzyme production [10]. The control had zero oxidation score and was significantly lower than the isolates. Isolate 12 had a mean oxidation score of 0.667 which was significantly higher than the control, and significantly lower than isolates 1-11.

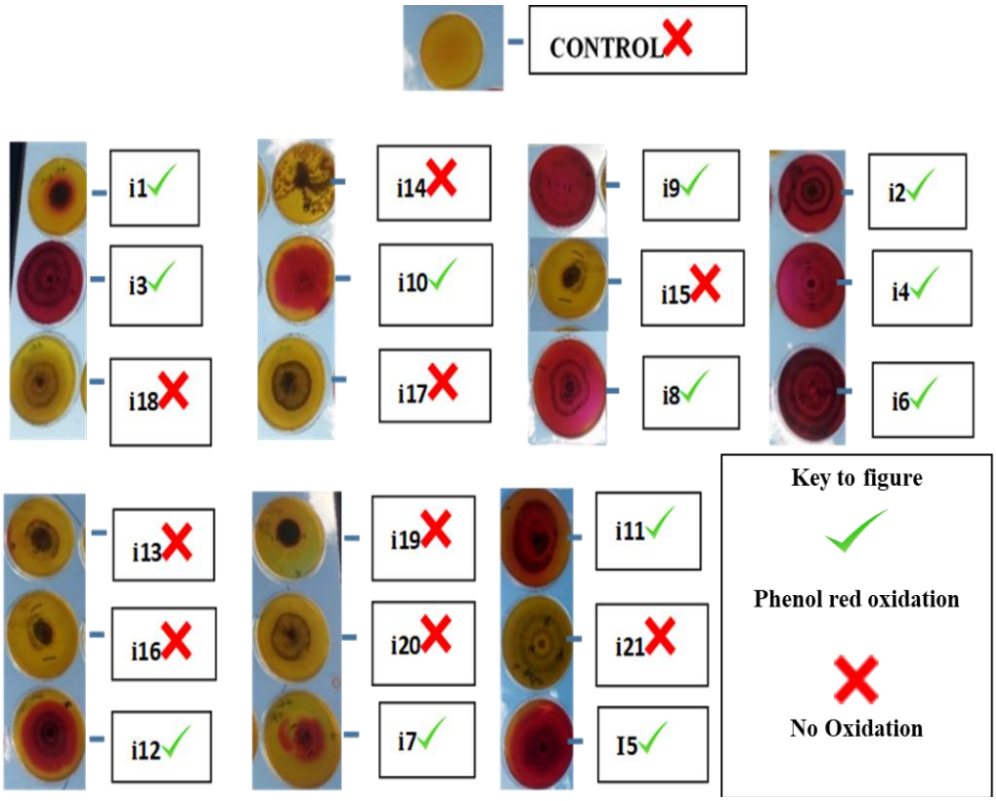

Fig. 1. Oxidation of phenol red from yellow to red

*IWRFI denoted by a green tick (✓) oxidized phenol red on PDA media as shown by the color change from yellow to red. Isolates that did not oxidize phenol red are denoted by the red **X** (✗). 12 out of 21 isolates oxidized phenol red. The top plate is the control without an isolate*

Table 1. Phenyl Red Oxidation statistical analysis report

Isolate	Oxidation
Control	0
1	1
2	1
3	1
4	1
5	1
6	1
7	1
8	1
9	1
10	1
11	1
12	0.666667
F-prob	<.001
s.e.d.	0.13
l.s.d.	0.27
Cv (%)	17.80

Table 2. Tukey's 95% Confidence Interval on phenol oxidation

Isolate	Mean
Control	0^{a}
12	0.6667^{b}
1	1^{c}
2	1^{c}
3	1^{c}
4	1^{c}
5	1^{c}
6	1^{c}
7	1^{c}
8	1^{c}
9	1^{c}
10	1^{c}
11	1^{c}

NB: Numbers with same letter supercsripts have same level of significance

Decolorization Assays

Preliminary tests conducted in solid media, PDA were used to determine whether the 12 IWRFI were able to decolorize five structurally different phenolic dyes; indigo carmine (indigoid dye), methyl orange (azo dye), congo red (azo dye), acid fuchsin (triphenylmethane dye) and malachite green (triphenylmethane dye). Recently, to select white-rot fungi that produce LiP, Azure B has been used in decolorization assays in solid medium and has shown the efficiency, [14]. In light of this, it has been found that to select fungi with the desired ligninolytic enzyme activities, using a set of appropriate dyes is of relevance.

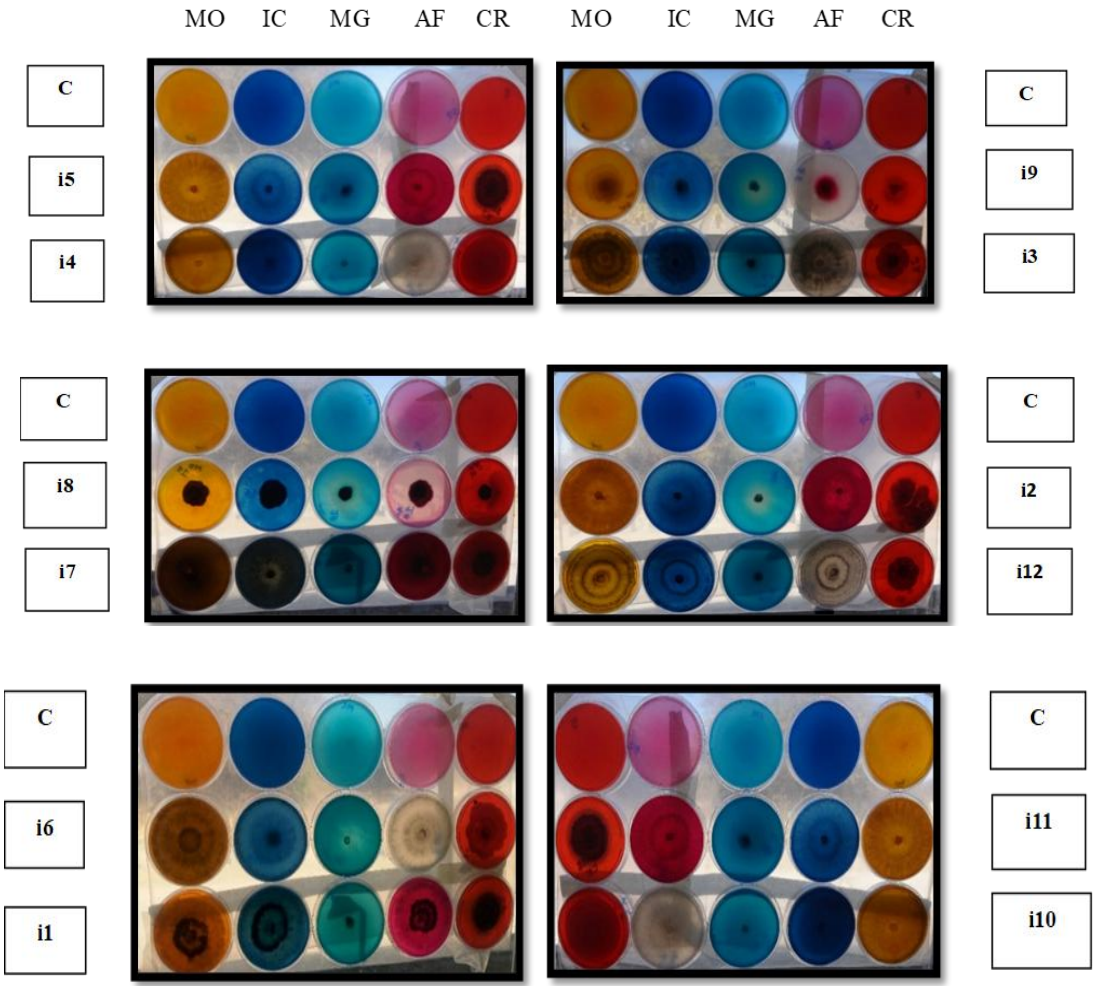

Fig. 2. Decolorization of MO, IC, MG, AF, CR dyes by 12 IWRFI

Key: MO = Methyl Orange dye, IC = Indigo Carmine dye, MG = Malachite Green dye, AF = Acid Fuchsin dye, CR = Congo Red, C = Control, I = isolate

Table 3. Dye decolorization scores of the 12 IWRFI

DYES	Fungal Isolates													
	Control	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**Mean**
Acid fuchsin	0.00	4.60	5.00	0.80	4.60	1.40	5.00	3.80	1.80	4.60	3.40	4.40	0.80	3.09
Malachite green	0.00	0.20	0.40	1.00	0.60	0.20	0.40	0.40	1.40	1.00	0.00	0.40	0.20	0.48
Indigo carmine	0.00	1.20	1.80	2.80	1.20	2.60	2.60	1.20	2.00	3.00	2.80	1.80	2.60	1.97
Methyl orange	0.00	0.00	0.80	1.80	0.60	1.80	1.40	0.80	1.80	1.20	1.00	1.20	1.00	1.03
Congo red	0.00	1.00	1.80	2.20	0.80	1.40	0.80	2.20	0.60	0.80	0.80	1.40	1.80	1.20
Fungal Isolate Average	0	1.4	1.96	1.72	1.56	1.48	2.04	1.68	1.52	2.12	1.6	1.84	1.28	
	F-prob	s.e.d.	l.s.d.											
Dyes	<.001	0.18	0.35											
Fungal Isolates	<.001	0.29	0.56											
Interaction	<.001	0.64	1.26											

Moreover, to select delignification fungi that are able to highly produce MnP, the use of azo/triphenylmethane dyes can be of adequate indication. Different types of azo dyes are used in pulp and paper, textile and leather industry from where the effluent containing these dyes comes out as waste stream [15,16]. These toxic and carcinogenic wastes need to be treated rather than degraded before release to the environment [17-20]. Previous experimental evidence has pointed out MnP as the main and instrumental enzyme in the decolorization of azo dyes [21,22] although laccase, cellulase, lignin peroxidase and xylanase enzymes are also instrumental in dye decolorization. Filamentous fungi secrete high levels of xylanase enzymes into culture medium which portray them as interesting sources of xylanases. All the 12 IWRFI decolorized the five dyes although with significant differences on the rate of dye decolorization [23-26]. The performances of the 12 IWRFI to decolorize the five dyes were significantly different as shown ($p<0.001$, Table 3). The key of the fungal isolates is shown at the left side of each column. Control dishes without fungal isolates are shown in the first column of each picture (Fig. 2).

The results show significant differences on dye decolorization ($p<0.001$). The performances of the 12 IWRFI to decolorize the five dyes were also significantly different as shown by the p value (Table 3; $p<0.001$).

Table 4. Tukey's 95% Confidence Interval of relativity on dye decolorization

Dye	Mean Score
Acid fuchsin	3.09^{d}
Malachite green	0.48^{a}
Indigo carmine	1.97^{c}
Methyl orange	1.03^{b}
Congo red	1.20^{b}

There were significant differences on dye Decolorization amongst the 5 dyes (Fig. 3). Acid fuchsin dye decolorization had the highest mean score, 3.09 thus it was decolorized rapidly than the other dyes. Malachite green dye was not effectively decolorized by the 12 IWRFI and it gave the lowest mean score of 0.48 which was significantly lower than the other dyes. The other three dyes had moderate mean scores. According to Tukey's α 0.05, dye decolorization of methyl orange and congo red dyes by the 12 IWRFI were statistically similar (LSD=0.35 Table 4) while for acid fuchsin dye, indigo carmine dye and malachite green dye, they were significantly different.

Table 5. Tukey's 95% Confidence Interval of relative performances of the 12 IWRFI

Fungi Isolate number	Fungi Isolate Average
Control	0^{a}
12	1.28^{bc}
1	1.40^{bc}
5	1.48^{bc}
8	1.52^{bc}
4	1.56^{bc}
10	1.60^{bc}
7	1.68^{bc}
3	1.72^{bc}
11	1.84^{bc}
2	1.96^{c}
6	2.04^{c}
9	2.12^{c}

There were significant differences amongst the 12 isolates on their dye decolorization efficiency ($p<0.001$, Table 3). Isolate means with the same letter are similar according to Tukey's α 0.05 (Table 5). Isolate 9 gave the highest mean score, 2.12 and it decolorized the dyes more rapidly than the other isolates. Isolate 9 performance was statistically similar to

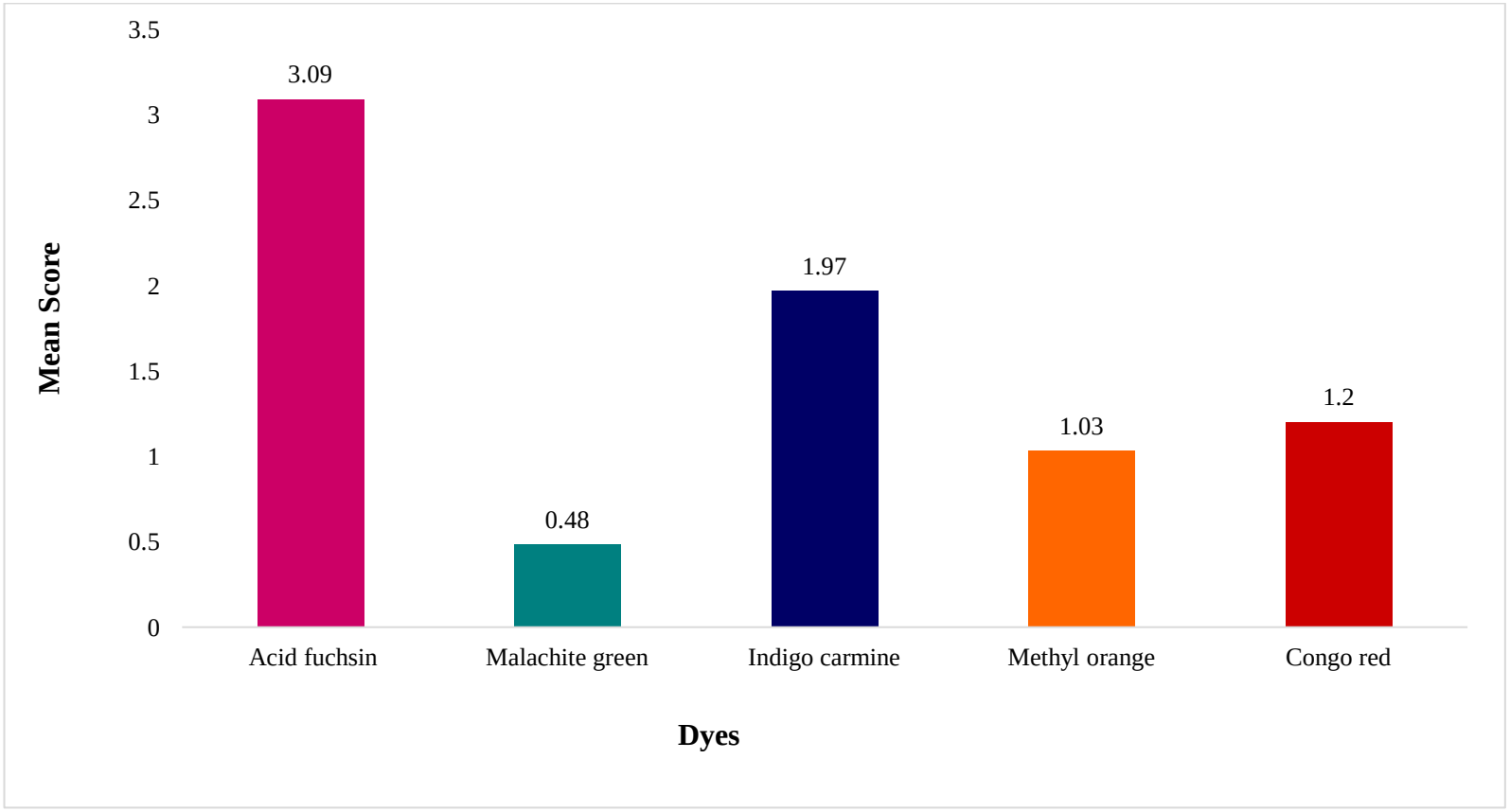

Fig. 3. Graphical illustration of dye discoloration mean scores

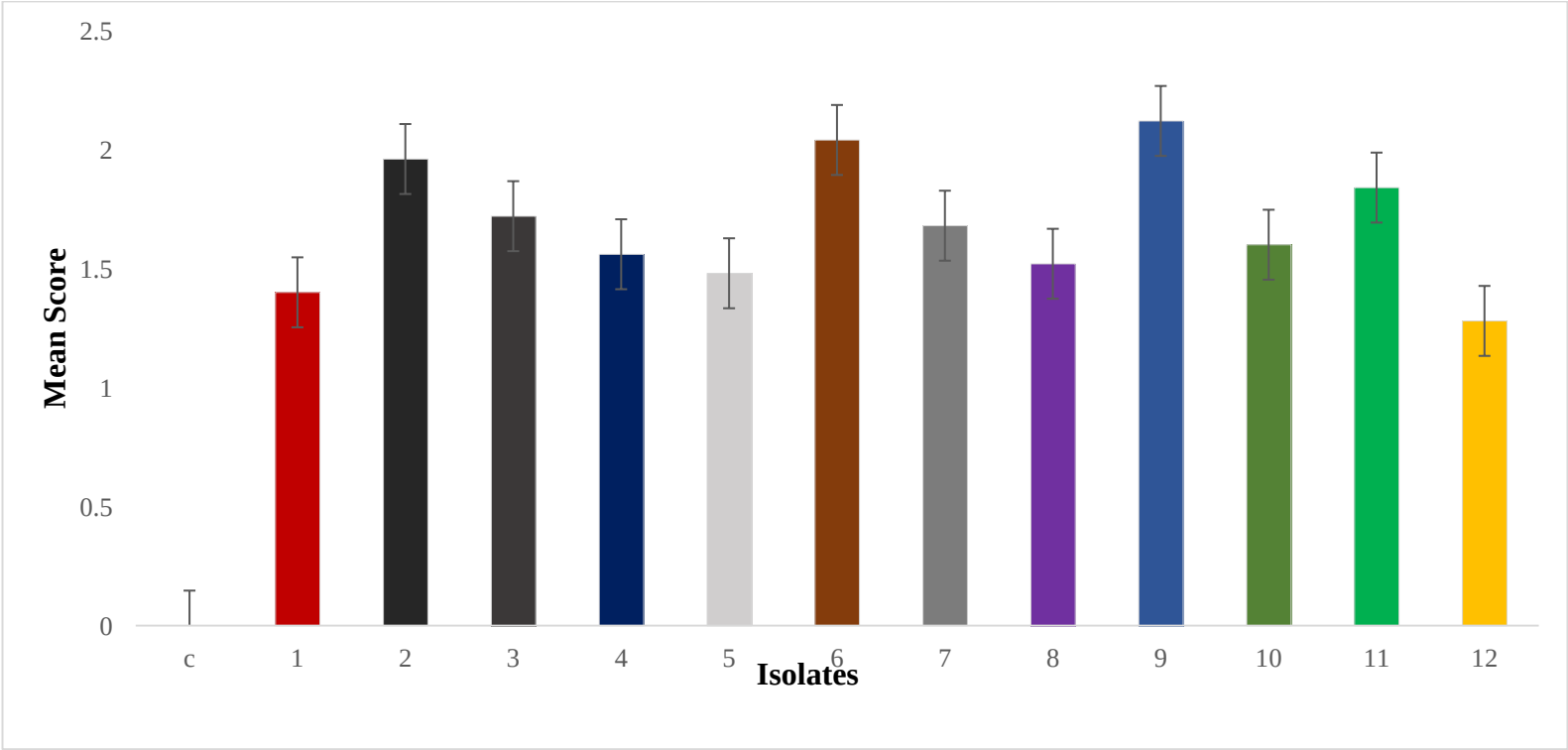

Fig. 4. Overall isolate performance on dye discoloration (Std dev. in the chart kept constant at ±1)

isolates 2 and 6 (LSD=0.56, Table 3). Isolates 12, 1,5,8,4,10,7,3,11 performed moderately and were statistically similar (LSD=0.56, Table 3). The control was significantly different from the isolates (LSD= 0.56 Table 3) and had the lowest score of 0.

There were overall significant differences on the performance of the 12 IWRFI across all the 5 dyes (Fig. 4). I9 had the highest mean score, 2.12 but its performance was statistically similar to i2 and i6. The performance of i11 was statistically similar to i10, i8, i7, i4 and i3. The performance of i5 was statistically similar to 1, 12, 8, 4, 7, 10, and 3. The control was significantly different from the 12 IWRFI. Further, investigation on dye decolorization by isolates 9, 6 and 2 reveals greater proportion of above said enzymes in them. It has been reported by many workers that a positive correlation exists between ligninolytic enzyme production and decolorizing efficiency of white rot fungi [27].

There were interactions ($p<0.001$) amongst all the 12 IWRFI on their performance as they decolorized the five dyes. The effect of dye decolorization depended on the chemical composition of the dyes. A trend was noted on the dyes with acid fuchsin dye being rapidly decolorized than the other dyes because of the highest isolate mean scores. Acid fuchsin dye perfectly binds to fungi cell walls staining the cells red [28]. This enables it to be decolorized more rapidly. The 12 IWRFI performed poorly on malachite green dye hence to the contrary, a trend of poor performance and low mean scores was noted. Malachite green dye could not be effectively decolorized because it is an organic chloride salt that has fungicidal properties [29]. It slowed the growth of the isolates and this resulted in the failure of other isolates to grow in PDA supplemented with it. For indigo carmine dye, the mean scores of the 12 IWRFI were also higher although they could not surpass highest mean scores for acid fuchsin dye. The isolates performed moderately on methyl orange dye and congo red dye and a moderate trend of mean scores was noted across the performance of all the 12 IWRFI. There was a trend of no performance for the control across all the dyes.

Lignin Degradation Assay

Ground tobacco stalks were incubated independently with IWRFI in an incubator shaker for 14 days, dried and tested for the residual lignin content after the acid detergent fibre test using the Ankom method. Tobacco stalks without an isolate were the control.

The mineral component of lignocellulosic material recovered after lignin degradation is generally ash content and also different metal salts including: silicates, carbonates, magnesium, oxalates and potassium phosphates, manganese, calcium and iron [30-33]. As an undesirable polymer, lignin removal during pulping and bleaching require enormous energy and chemical amounts. Before any pulping stage, hemicelluloses and lignin removal are fundamental stages in dissolving pulp [34]. Lignin has some alkali-resistant bonds resulting from formation of complexes with polysaccharides such as xylan which during Kraft process, fail to be completely hydrolyzed (Buchert et al., 1992).

Thus at the bleaching and post-treatment stages, remaining xylan and lignin is removed [34]. The interest in xylan-degrading enzymes and its applications at different industrial levels have increased significantly over the last two decades and

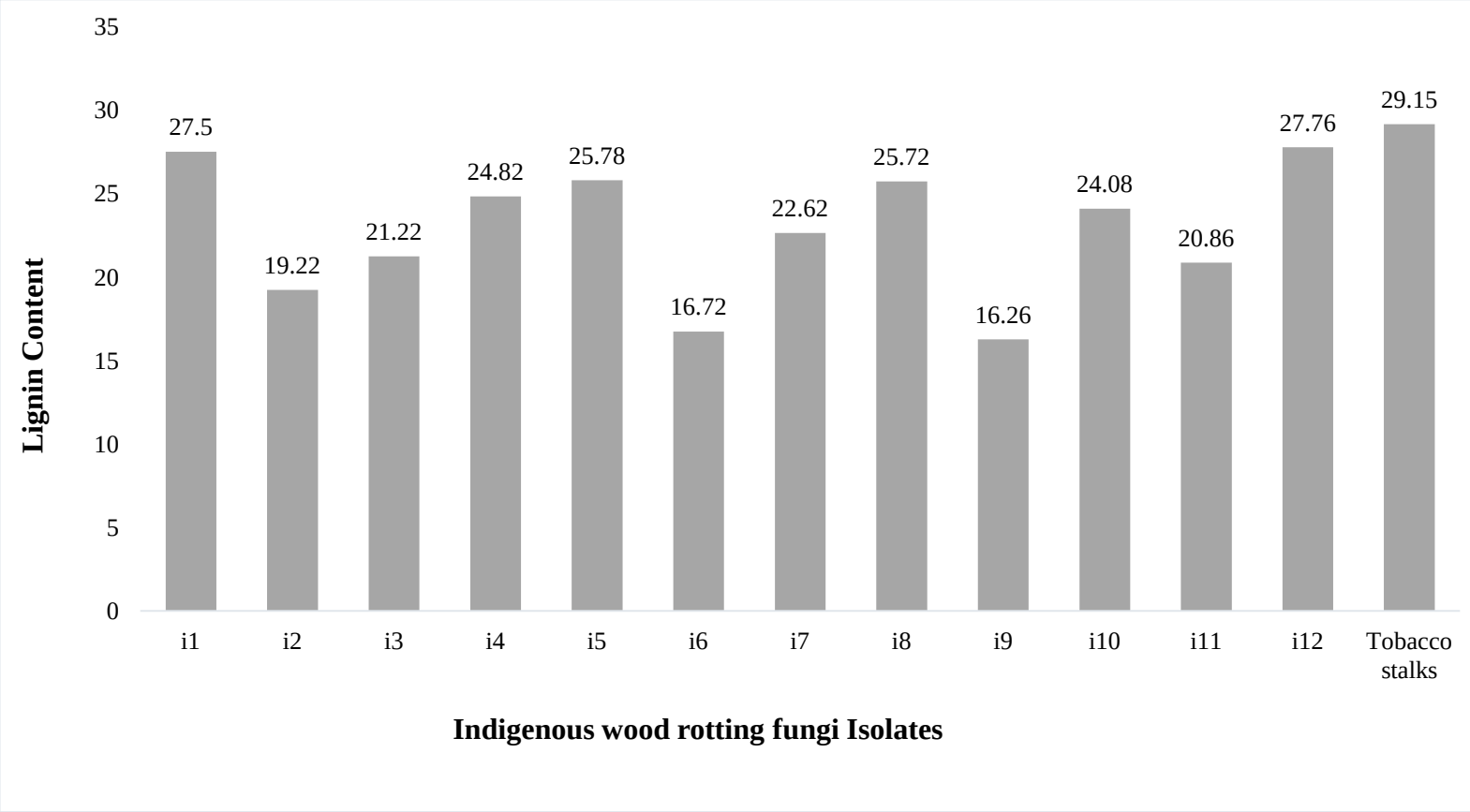

Fig. 5. Graphical illustration of residual lignin content of tobacco stalks

they have been widely detected in filamentous fungi and bacteria [35]. Major components of tobacco stalks' weight percentages are 32.9% glucan, 26.6% lignin, 13.0% xylan, and 7.4% ash. Like any other non-woody biomasses, tobacco stalk ash content is higher than wood [36], and the lignin content is lower than wood fiber (14 -37%) [37].

The varying ability to produce MnP, Lac, LiP, xylanase and cellulase enzymes by the 12 IWRFI brought about different residual lignin contents after the lignin degradation assay. Residual lignin contents after incubation of the ground tobacco stalks with the IWRFI was significantly different amongst all the isolates. The control, tobacco stalks without an isolate had lignin content of 29.15% (Table 6). All the 12 IWRFI degraded lignin but isolates 9, 6 and 2 effectively degraded lignin from 29.15% and had lower residual lignin contents of 16.26%, 16.72% and 19.22% respectively (Fig. 5) indicating their effectiveness in lignin degradation. This also indicated their potential to be greatly producing the ligninolytic enzymes. Conversely isolate 1 and 12 had significantly the highest residual lignin contents, 27.5% and 27.76% respectively, while isolates 2, 3, 4,5,7,8 had moderate residual lignin contents (Fig. 5).

CONCLUSION AND RECOMMENDA-TIONS

Paper industries discharge effluents containing high concentrations of polluting materials hence prior discharge, an effective treatment is necessary [38]. Since excess chlorination in wastewaters may pose health hazards, reduction of chlorine consumption via enzymatic pre-treatment is not only advantageous economically but also proves that enzyme treatment is an environmentally friendly and less hazardous alternative. Additionally the enzymes are capable of decolorizing and detoxifying the industrial effluents, degrading lignin on tobacco stalks which can be used to make paper while simultaneously managing environmental waste accumulation from tobacco stalks.

The present experiment also concludes that hydrolytic enzymes from fungi species can effectively degrade some industrial dyes and that they can also degrade lignin on tobacco stalks. Taken altogether, the data suggest that fungal isolates 9, 6 and 2 have great biotechnological potential in the paper industries in both dye discoloration and delignification. This strengthens the argument to continue searching for new strains and species to discover promising tools for this biotechnological application. The study will also help in commercializing the technology in reducing the pollution load of tobacco stalks and minimizing detrimental effects of chemical compounds in the environment by the paper industries [39-41].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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