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Research Paper

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Azo dye degradation by bacterial laccases produced from mushroom spent

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***Abstract*-** Laccases are multi-copper enzymes which catalyze the oxidation of a wide range of phenolic and non-phenolic aromatic compounds and participate in several applications such as bioremediation, biopulping, textile, and food industries. It is widely distributed in higher plants, fungi and also found in bacteria. Azo dyes containing effluents from different industries pose threats to the environment. Though there are physico-chemical methods to treat such effluents, bioremediation is considered to be the best eco-friendly method. The present study emphasises on isolation of efficient laccase producers from mushroom spent and their use for azo dye degradation. The primary isolates were isolated by enrichment culture technique using guaiacol as substrate. About 10 secondary isolates were studied for laccase production using raw substrate like saw dust. The isolates were identified culturally, microscopically and biochemically. The laccase production cycle for the isolates was studied and found that peak production was by 72 hours. The selected laccase producers were tested for their different azo dye degradation potential both in isolation and as a consortium. The different azo dyes studied were trypan blue, congo red, malachite green and methyl red dyes for degradation at regular intervals of 24 hours for a period of 3 days. The different concentrations studied were 10mg/L, 25mg/L, 50mg/L, 75mg/L, 100mg/L. The consortium of Azo dye degrading cultures (samples 1–5) could totally degrade all azo dyes with variation in degradation times, whereas isolates could only degrade methyl red with variation in time. The samples 2 and 5 showed complete degradation of methyl red in 48 hours and other applications can be further studied.

Key words: *Laccases, Guaiacol, Azo dyes, Consortium, Degradation.*

I. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are a group of polyphenol oxidases and are also called multicopper oxidases that oxidizes polyphenols, methoxy-substituted phenols, aromatic diamines and a range of other compounds [1]. Laccase is versatile oxidoreductase enzyme capable of oxidising a wide range of phenolic and non-phenolic compounds [2]. Laccases typically contain three types of copper, one of which contributes to its characteristic blue colour. Similar enzymes which lack the Cu atom responsible for the blue colour are called 'yellow' or 'white' laccases, but are not considered true laccases [1]. Laccases are majorly found in higher plants and fungi and have also been discovered in insects and bacteria, however, they are not common enzymes to be found in certain prokaryotic groups. Bacterial laccase are intracellular or periplasmic [1], [3].

The dyes are used to impart color to different substrates, such as fabric, paper, leather. The global consumption of dyes and pigments is approximately 700,000 tons/year, 2/3rd of it being consumed by the textile industry. Large amounts of dyes residues are generated due to the inefficiency in the colouring during the dyeing process. These residues are directly released into water bodies, consequently, contaminating the environment [4] causing pollution, eutrophication, including decrease in the photosynthetic activity and dissolved oxygen (DO), and change in the pH, increase in the biochemical oxygen demand (BOD) and chemical oxygen demand (COD), in aquatic life [5] hence the removal of these effluents becomes the most significant environmental problem [6]. Some azo dyes can exhibit more or less carcinogenic and/or mutagenic activity [4]. Therefore, treatment of these industrial effluents is important before their discharge into the environment.

The biological processes are advantageous over conventional processes which includes conversion of organic compounds to non-toxic products (water and carbon dioxide), economically reliable and sustainability [7]. In recent years, bacterial laccases have gained much attention because of their diverse applications in dye decolourization, waste detoxifications and bioremediation applications [8]. Bacterial laccases possess many valuable properties compared to fungal laccases as they are thermostable, alkaline tolerant, stable at high chloride concentrations, highly active and highly expressive and could be used for

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industrial and biotechnological applications [9], [10].

The present work focuses on isolation of laccase producing bacteria from mushroom spent, and the potential use of this laccase enzyme extracted from the bacteria in Azo dye degradation.

II. RELATED WORK

Laccase enzymes degrade the azo dye by a non-specific free radical mechanism to form phenolic compounds and thereby prevent the formation of toxic aromatic amines and have been extensively studied for their degradation of azo dyes [11], [12], [13], [14]. Laccase is generally found in higher plants, fungi and bacteria.

The catalysis of substrates by laccase involves reduction of oxygen to two molecules of water and simultaneous one-electron oxidation of the substrates [15].

Azo dyes are the largest class of water soluble synthetic aromatic dyes made up of one or more (N=N) groups and sulfonic (-SO3-) groups with high commercial value [16], [17]. Among the 80% of azo dyes that are presently used in the dyeing process in textile sector, approximately 10% of the dyes do not bind to the fiber and are released into the environment [18] possessing toxicity like lethal effect, genotoxicity, mutagenicity, and carcinogenicity to plants and animals [19]. It also interrupts the aquatic environment by reducing light penetration, gas solubility and interference of phytoplankton's photosynthesis [20].

Azo dyes are toxic and considered as xenobiotic compounds which are resistant to natural biological degradation [21]. Thus, treatment of dye is one of the challenging tasks till date. Physical and chemical treatment methods such as precipitation, coagulation, adsorption, flocculation, flotation, electrochemical destruction, and mineralization and decolorization process [22] have some disadvantages such as cost, time, and release of residues. Recently, researchers have been focusing their attention to enzymatic treatment.

The bacterial diversity in spent mushroom compost (SMC) is greatly influenced by the initial material, pasteurization treatment and the potential unintended colonization of the mushroom substrate by microorganisms during the cultivation process. Various agricultural wastes such as saw dust, banana peel and rice bran have commonly been used as a substrate for laccase production because of their low cost and accessibility.

Laccase activity by bacterial culture can be indicated by decolorization of the polymeric dye. [23], [24]. A very common method for identifying laccase activity is by supplementing the medium with a substrate, like DMP/guaiacol/ABTS/naphthol that will lead to the conversion of the colorless agar medium into a brown/reddish brown/green/blue color [25], [26].

Bacterial laccase comprises many unique properties over fungal laccases, as they work efficiently in extreme conditions and salt tolerance. Bacterial laccase was first isolated from Azospirillum lipoferum, in the year 1993 from rice rhizosphere. Various bacteria are known to produce laccase extracellularly but cannot secrete it outside the cell [27]. The bacteria are mainly Gram positive. e.g., Bacillus, Geobacillus, Streptomyces, Rhodococcus, Staphylococcus, Azospirillum, Lysinibacillus and Aquisalibacillus [28]. However, some Gram-negative bacteria like Pseudomonas, Enterobacter, Delfia, Proteobacterium and Alteromonas have also been found to secrete laccase [29].

III. MATERIALS AND METHODS

Isolation of bacteria

Mushroom spent samples were collected from 'S' Mushroom Agritech, Kukatpally, Hyderabad. The samples were inoculated in Nutrient broth (NB) incorporated with 0.02% guaiacol (HiMedia Labs, India) and incubated at 30° C for 24 hours. The broth samples were plated on Nutrient agar medium (NA) incorporated with 0.02% guaiacol (HiMedia Labs, India) and incubated at 30° C for 96 hours.

Screening of laccase producing bacteria

The broth tubes were checked for turbidity and agar plates were screened for bacterial growth. Laccase assay was performed at different intervals of time to determine the sample(s) with maximum amount of laccase enzyme produced (based on absorbance). The plates of the sample(s) with maximum laccase activity were identified. From the primary isolates that were obtained from 5 Mushroom spent samples 10 secondary isolates were selected based on their growth and laccase production in guaiacol substrate and raw substrate like saw dust by laccase assay method. The isolates were identified by cultural, microscopic and biochemical characteristics.

Laccase assay

The fermented samples of culture supernatant were assayed for laccase enzyme activity by guaiacol assay method using UV-Vis spectrophotometer at 450 nm.

Oxidation of guaiacol has been reported for laccase assay [30]. The oxidation of guaiacol by laccase resulted in reddish brown color which was measured at 450 nm. The reaction mixture can be prepared as follows:

(a) Guaiacol (2 mM) 1 ml.
(b) Sodium acetate buffer (10 mM) 3 ml.
(c) Enzyme source 1 ml (bacterial supernatant).

A blank was also prepared that contains 1 ml of distilled water instead of enzyme. The mixture was incubated at 30

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°C for 15 min and the absorbance was read at 450 nm using UV spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 µmol of guaiacol per min. The laccase activity in U/ml is calculated by this formula:

E.A = A*V/ t*e*v

Where,
E.A = Enzyme activity
A = Absorbance
V = Total mixture volume (ml)
v = enzyme volume (ml)
t = incubation time
e = extinction coefficient for guaiacol (0.6740 µM/ cm).

Evaluation of azo dye degradation

Different concentration of different azo dyes like Congo Red (CR), Methyl Red (MR), Trypan Blue (TB) and Malachite Green (MG) were added to fermented samples and incubated at room temperature for a period of 5 days. The degradation of the azo dyes was evaluated by visual change in color intensity of the azo dyes.

IV. RESULTS AND DISCUSSION

Screening and isolation of laccase producing bacteria from Mushroom spent:

Screening and isolation of laccase producing bacteria was performed using different sources. In the present work 5 different mushroom spent samples (Table 1) were screened for laccase producing bacteria and 57 primary bacterial samples were isolated by enrichment cultural technique using NA with 0.02% guaiacol as substrate shown in (Figure 1). Not much work has been done using mushroom spent but similar method was used where 96 different colonies were isolated from pulp and paper wastewater and were then screened on NA medium containing 0.5 mM guaiacol [31].

Out of 5 mushroom spent samples S 1 to S 5. From S 5 mushroom spent sample maximum no. of laccase producing bacteria were isolated (Figure 1 and 2).

The laccase enzyme production both for mushroom spent samples and bacterial isolates were tested using guaiacol as substrate. Based on laccase enzyme production, 10 efficient bacterial isolates with maximum enzyme production were selected. These isolates were identified by cultural, microscopic and biochemical characteristics. The colony morphology of these isolates was indicated in table 2 and figure 3. The microscopic examination indicates that majority of the isolates were found to be gram negative cocci (designated as CI 1 to CI 7 and CI 9 and CI 10) and few of them are gram positive bacilli (designated as CI 8) (Table 3 and Figure 4 to 9). Biochemical test like IMViC reactions were performed (Table 3). All these 10 secondary isolated were tested for azo degradation.

The enzyme activity was found to be maximum for 15 mins incubation for the S 2, S 4, CI 2 and CI 7, and 30 mins for S 2, S 5, CI 2 and CI 7 respectively (Figure 10 and 11).

Azo dyes degradation by bacterial laccase

Azo dyes are the largest class of water-soluble synthetic aromatic dyes made up of one or more (N=N) groups and sulfonic (-SO3-) groups with high commercial value [32] and are used in textile industry for coloring the fabrics. Laccases are able to degrade phenolic, aromatic amine, and non-phenolic compounds. Therefore, laccase enzyme can be used in the textile industry for the elimination of textile dyes and phenols and waste detoxification [33].

The 10 selected secondary isolates were subjected for laccase enzyme production and the fermented sample of each of these isolates were studied for azo dye degradation.

It was found that these samples didn’t show any degradation when used in isolation. However, the combination of these enzyme samples indicated azo dye degradation, therefore, the consortium of enzyme samples obtained from mushroom spent samples of (S 1 to S 5) were studied for azo dye degradation (Figure 12, 13, 14, 15). All five enzyme samples of consortium showed same catalytic activity with color change indicating azo dye degradation.

All enzyme samples S 1 to S 5 were studied for dye degradation with different azo dyes like congo red, methyl red, trypan blue and malachite green; similar to the work conducted by Narayanan *et al.* 2015 [25], where they reported the decolorization of synthetic dye T Blue, Yellow, GR, Orange 3R by immobilized laccase from *B. subtilis* MTCC.

The consortium S 1 to S 5 showed complete degradation of all the four azo dyes with variation in time (Figure 16, 17, 18, 19). The result was found to be different when guaiacol was replaced with 0.1% saw dust as laccase substrate, where methyl red was completely degraded by S 2 and S 5 but other dye did not get degraded as shown in figure 20.

Use of bacteria laccase for the degradation of textile dye has been extensively explored due to their fascinating properties like short production time, inexpensive media and stability.

V. CONCLUSION

Dye degrading bacteria were isolated from Mushroom spent using NA medium supplemented with 0.02% guaiacol yielding 10 isolates showing high laccase activity. The isolates CI 2 and CI 7 showed highest laccase enzyme activity with 0.1% saw dust as a laccase substrate. The

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enzyme samples of mushroom spent (S 1 to S 5) with guaiacol as substrate for enzyme production showed total degradation of all the four dye with variation in their degradation time. Whereas the enzyme samples S 2 and S 5 showed total degradation of only methyl red dye within 48 hours when saw dust was used as a substrate for enzyme production. Bacterial laccase extracted from mushroom spent can not only be used for dye degradation, but can also be effectively used in paper and pulp industries, xenobiotic degradation, and bioremediation.

Table 1: Mushroom spent sample

S.No	Mushroom spent sample	Sample
1	After 1st crop	Sample 1 (S 1)
2	Currently 3rd crop	Sample 2 (S 2)
3	After 3rd crop	Sample 3 (S 3)
4	One year old crop	Sample 4 (S 4)
5	Two year old crop	Sample 5 (S 5)

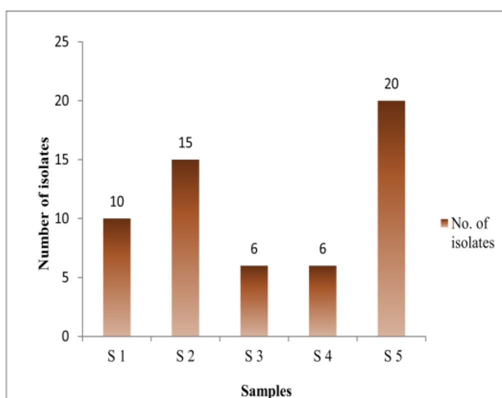

Figure 1. Bacterial isolates of Mushroom spent samples.

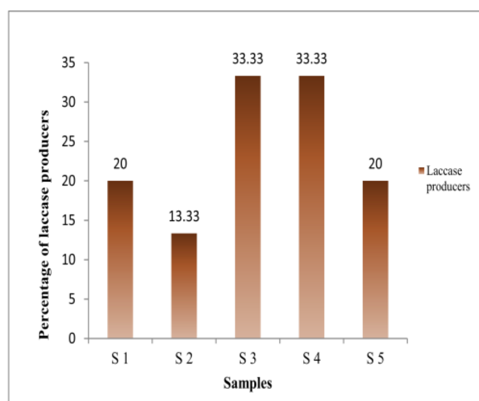

Figure 2. Percentage of laccase producers of mushroom spent samples.

Table 2: Colony morphology of the isolates.

Mushroom spent	Sample (Isolates)	Isolates	Colony morphology
Currently 3rd crop (S 2)	2nd plate Isolate 1	CI 1	White, shiny, smooth, slightly raised.
	2nd plate Isolate 2	CI 2	White, rough, raised centre with irregular margin.
After 3rd crop (S 3)	3rd plate Isolate 1	CI 3	Yellowish transparent, smooth, shiny.
	3rd plate Isolate 1	CI 4	Orange, smooth, slightly raised.
One year old crop (S 4)	4th plate Isolate 1	CI 5	White, flat, rough.
	4th plate Isolate 2	CI 6	White, large, flat, rough.
Two year old crop (S 5)	5th plate Isolate 1	CI 7	Yellow, round, raised, small, smooth.
	5th plate Isolate 2	CI 8	White, small, raised, smooth.
	5th plate Isolate 3	CI 9	White, large, flat, irregular margins.
	5th plate Isolate 4	CI 10	Brown, flat, entire margin

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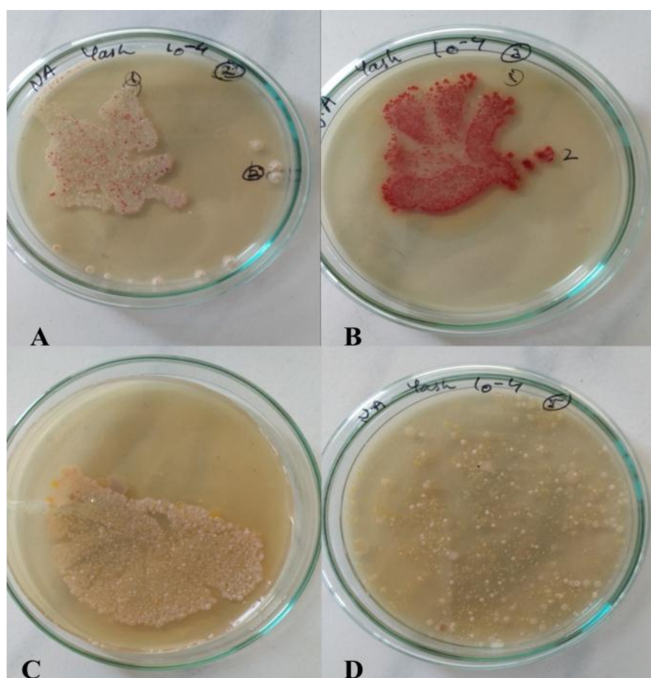

Figure 3. (A) Different isolates present in mushroom spent sample which is currently growing as 3rd crop,
(B) Different isolates present after 3rd crop in mushroom spent sample,
(C) Different isolates present in one year old mushroom spent sample,
(D) Different isolates present in two year old mushroom spent sample,

Table 3: IMViC and Gram's reaction results of the isolates of the mushroom spent samples.

S.No	Isolates	Indole test	Methyl Red test	Voges-Proskauer test	Citrate utilization test	Gram reaction
1	CI 1	-	+	-	-	Gram negative cocci
2	CI 2	-	-	-	-	Gram negative cocci
3	CI 3	+	+	-	-	Gram negative cocci
4	CI 4	+	+	-	-	Gram negative cocci
5	CI 5	+	+	-	-	Gram negative cocci
6	CI 6	+	+	-	-	Gram negative cocci
7	CI 7	+	+	-	-	Gram negative cocci
8	CI 8	-	-	-	-	Gram positive bacilli
9	CI 9	-	+	-	-	Gram negative diplococci
10	CI 10	-	-	-	-	Gram negative cocci

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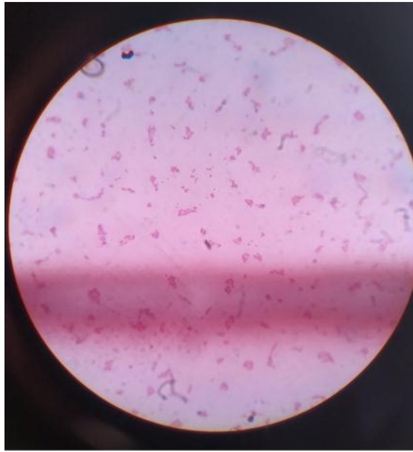
Figure 4. Gram negative cocci

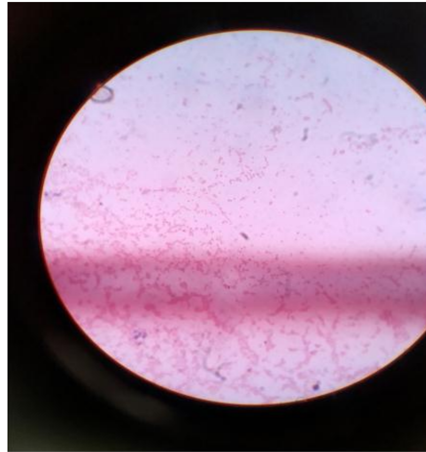
Figure 5. Gram negative cocci

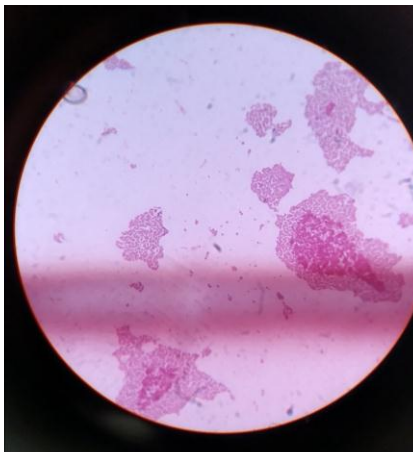
Figure 6. Gram negative cocci

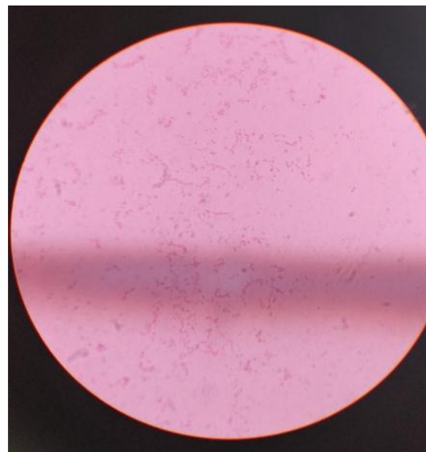
Figure 7. Gram negative cocci

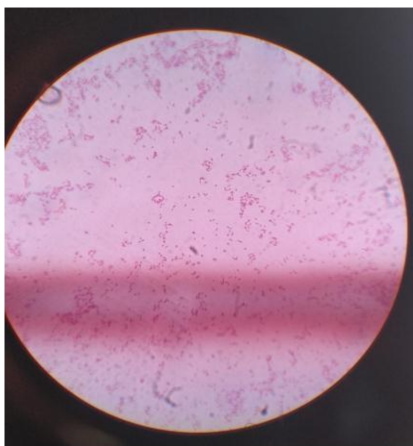
Figure 8. Gram negative diplococci

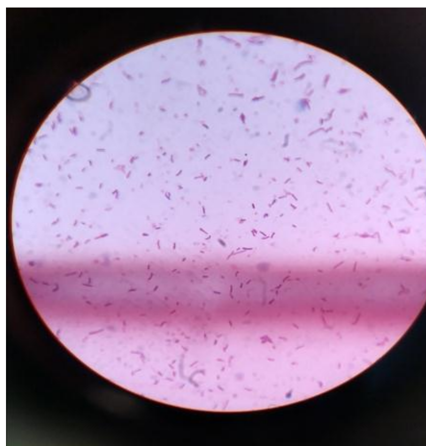
Figure 9. Gram positive bacilli

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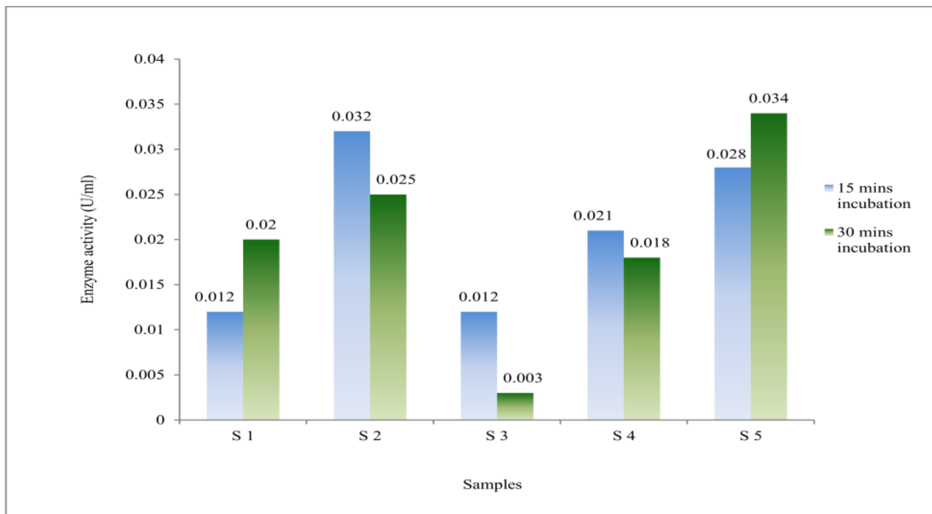

Figure 10. Laccase enzyme production of mushroom spent samples

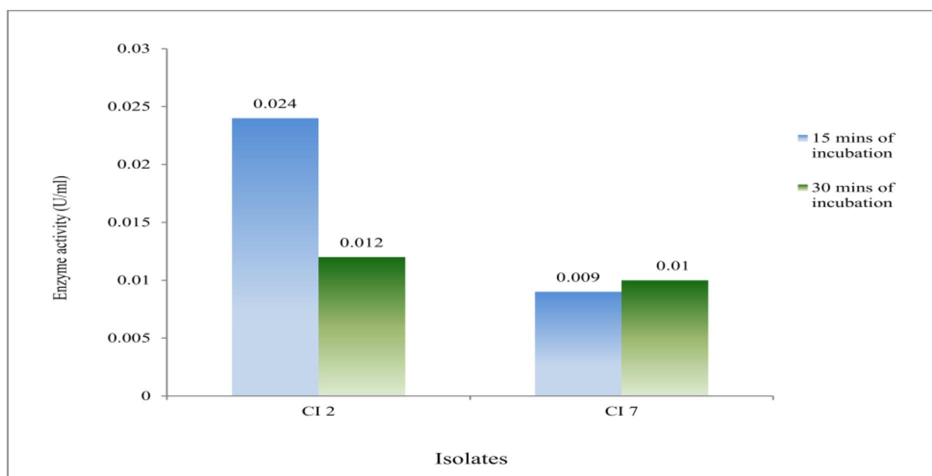

Figure 11. Laccase enzyme production of isolates of mushroom spent samples.

Figure 12.
(A) Different concentrations of Congo red dye inoculated with different consortium.
(B) Dye of all the concentrations were degraded within 90 hours.

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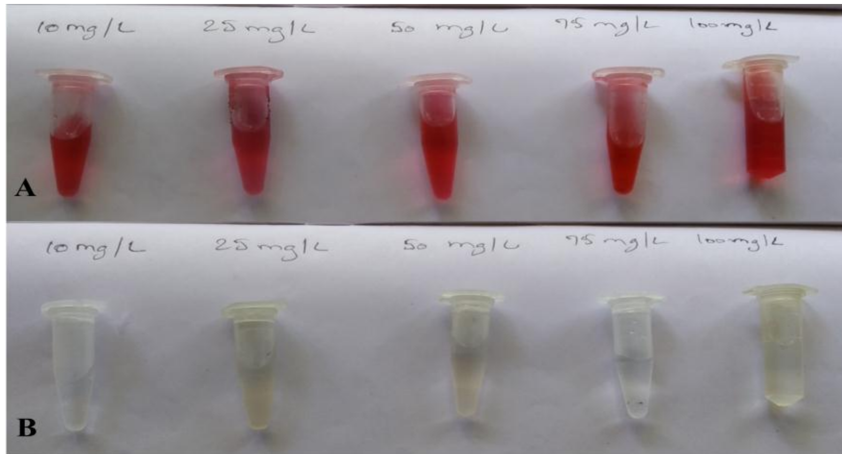

Figure 13.
(A) Different concentrations of Methyl red dye inoculated with different consortium.
(B) Dye of all the concentrations were degraded within 60 hours.

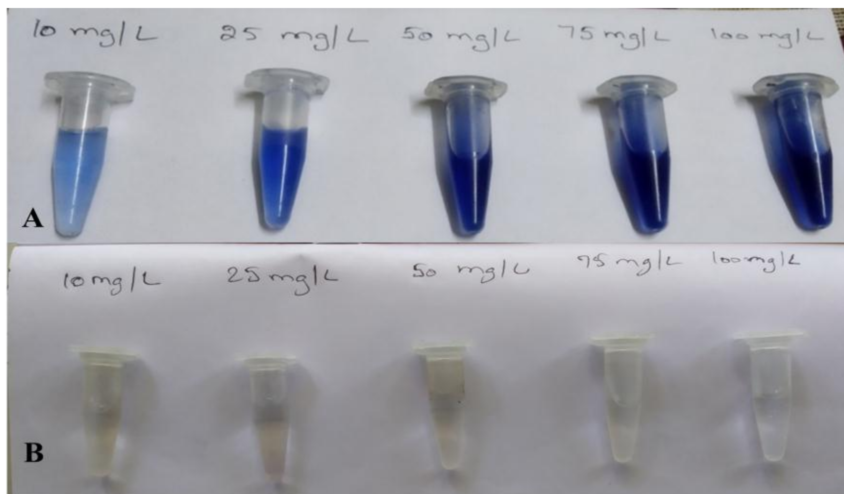

Figure 14.
(A) Different concentrations of Trypan blue dye inoculated with different consortium.
(B) Dye of all the concentrations were degraded within 90 hours.

Figure 15.
(A) Different concentrations of Malachite green dye inoculated with different consortium.
(B) Dye of all the concentrations were degraded within 112 hours.

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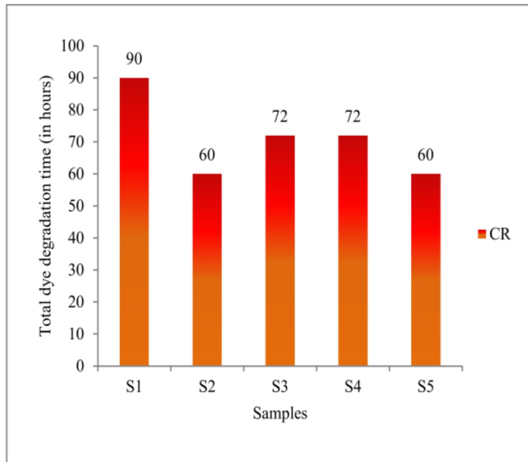

Figure 16. Time profile of degradation of congo red dye.

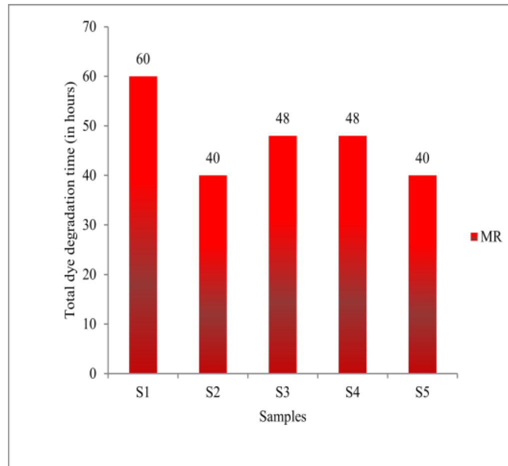

Figure 17. Time profile of degradation of methyl red dye.

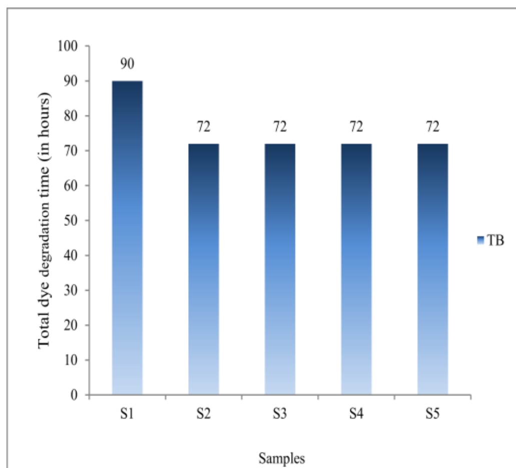

Figure 18. Time profile of degradation of trypan blue dye.

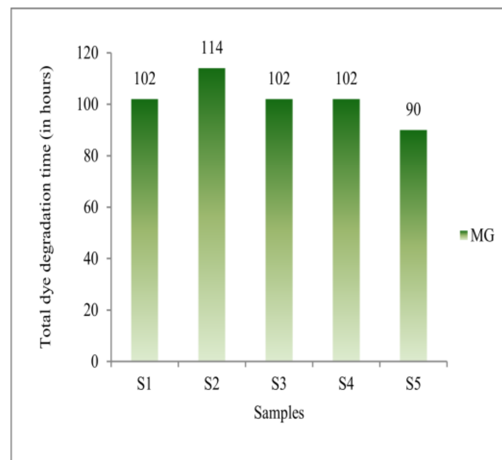

Figure 19. Time profile of degradation of malachite green dye.

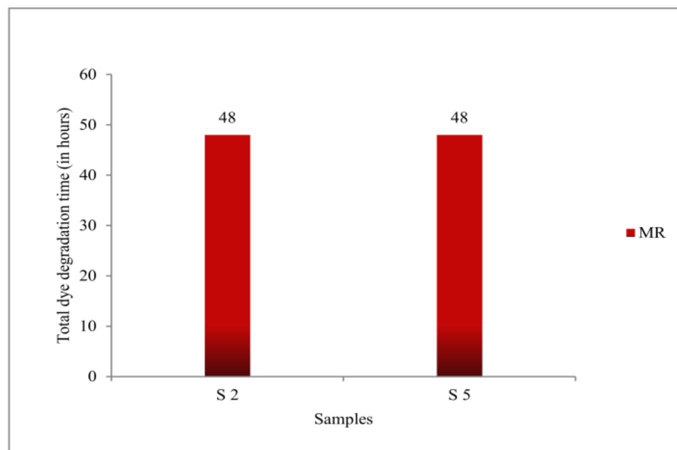

Figure 20. Time profile of degradation of Methyl red dye

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