

## Potential of Spent Substrate of *Pleurotus sajor-caju* for Methyl Violet 2B Decolorization

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This study presents the effect of different factors like initial concentration of dye, carbon and nitrogen sources, enzyme mediator, cofactor and heavy metal ions in culture medium for decolorization of Methyl Violet 2B, an azo dye widely used in textile and leather industries, through *Pleurotus sajor-caju* spent substrate. Spent substrate of *P. sajor-caju* was recorded for highest decolorization (100%) with lowest concentration (25 ppm) of Methyl violet 2B. Addition of carbon and nitrogen sources separately, except cellulose enhanced decolorization and highest was in presence of sucrose (87.75%), followed by starch (87.48%). Lower concentration (25 ppm) of glucose, starch and ammonium carbonate, while higher concentration of urea (100 ppm) supported highest decolorization. After 4 to 5 days of incubation, highest decolorization was with 0.1% Veratryl alcohol and Manganese ions added separately in culture medium, while presence of salts of lead, cadmium, cobalt and nickel (0.025%) in culture medium supported higher decolorization of 100, 94.60, 71.0 and 68.54%, respectively against 62.35% in control. Among the ligninolytic enzymes the spent substrate exhibited appreciable activities of laccase, manganese peroxidase and lignin peroxidase.

**Key words:** Methyl Violet 2B, *Pleurotus sajor-caju*, Spent substrate, Culture conditions.

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Synthetic dyes are used in a wide range of industries (textile, pharmaceutical, cosmetic and food industries) and during industrial processing, up to 40% of used dyestuffs are released in to processed water<sup>1</sup>, producing a highly colored wastewater that affects transparency and gas solubility in water bodies<sup>2</sup>. Currently several physico-chemical methods are in use for decolorization of these wastewaters, but these have limitations of high cost, high salt content in the effluent and difficulties in treating concentrated waste<sup>3</sup>. Being eco-friendly and cost-

competitive, biological processes involving bacteria, fungi and enzymes<sup>4</sup> have been tried in the past and among these, white rot fungi have been recognized as most efficient, degrading dyes with the involvement of their extracellular lignin-modifying enzymes<sup>5</sup>.

Spent Mushroom Substrate (SMS) released after cultivation of different mushrooms, contains residual mushroom fungal biomass along with a rich population of heterotrophic fungi and bacteria, which can act as an inexpensive source of phenoloxidases. SMS also has ability to chemically adsorb organic and inorganic pollutants, while diverse category of microbes it harbors have capability of biologically breaking down the organic xenobiotic compounds present in soil and water<sup>6</sup>. In several previous studies, role of extracellular ligninolytic enzymes and microbes from SMS of different mushrooms<sup>7</sup>, and mushroom mycelia,

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especially of *Pleurotus florida*, *P. ostreatus*, *P. flabellatus* and *P. sajor-caju* have been evaluated for their use in dye decolorization activities<sup>2,8</sup>. This study presents role of culture factors like initial concentration of dye, enzyme mediator/cofactor (veratryl alcohol,  $\text{MnSO}_4$ ), carbon and nitrogen sources, heavy metals, in decolorization of Methyl Violet 2B using fresh spent substrate of *P. sajor-caju*.

## MATERIALS AND METHODS

### Substrate and Chemicals

Methyl Violet 2B ( $\text{C}_{25}\text{H}_{30}\text{ClN}_3$ ) was procured from Sigma-Aldrich, while Potato Dextrose Broth (PDB) used as culture medium was procured from Hi-Media Lab. Heavy metals (Fig. 5) were procured from Merck, while manganese sulphate and veratryl alcohol were procured from s d Fine-Chem Limited and Hi-media Lab., respectively. Samples of spent substrate of oyster mushroom (*Pleurotus sajor-caju* strain, PL-1140) were collected from Directorate of Mushroom Research (DMR), Solan, (HP), India.

### Extracellular enzyme activity

*Pleurotus sajor-caju* was grown under conditions of solid state fermentation at DMR, Solan (HP) India. Crude enzymes were prepared by extraction with 0.1M sodium acetate buffer, pH 4.5 under magnetic stirring for 30 min at room temperature (1g solid substrate/ 5mL buffer) and filtration through sheet cloth. The filtrate was then centrifuged at 3,000 rpm for 10 min at 4°C. All enzyme activities were determined spectrophotometrically at 30°C. LiP activity was measured by the oxidation of veratryl alcohol to veratrylaldehyde and corrected for veratryl alcohol oxidase activity. One unit of activity represents 1  $\mu\text{mol}$  of veratryl alcohol oxidized to veratrylaldehyde per minutes. Activities of extracellular laccase was determined by monitoring the absorbance increase at 425 nm in the rxn. mixture. Laccase was assayed by monitoring the oxidation of ABTS. One unit was defined as the amount of enzyme that oxidized 1  $\mu\text{mol}$  of substrate per minute and the activities were expressed in  $\text{UL}^{-1}$ . Manganese peroxidase (MnP) activity was measured by using  $\text{Mn}^{2+}$  as the substrate. One unit defined as the amount of enzyme that oxidized 1  $\mu\text{mol}$  of substrate per minute<sup>9</sup>.

### Dye Concentration

Five concentrations (25, 50, 100, 150 and 200 ppm) of Methyl Violet 2B were used for evaluating the effect of initial dye concentration on its decolorization. PDB medium (potato dextrose, 24 g; distilled water, 1000 mL; pH, 7.2) was distributed in 250 mL Erlenmeyer flasks (100 mL/ flask) and sterilized at 15 p.s.i. pressure for 20 min. Different concentrations of dye were prepared in sterilized PDB by adding 0.025, 0.050, 0.10, 0.15 and 0.20 ml of dye stock solution (1 g dye/10 mL sterilized distilled water). Fresh spent substrate of *P. sajor-caju* was added aseptically @ 1%, w/v to dye mixed sterilized PDB and mixed thoroughly. Flasks devoid of spent substrate but with dye were kept as control. Three replications were kept for each treatment and all flasks including control were incubated at  $25 \pm 1^\circ\text{C}$  for next 5 days in BOD incubator. For recording optical density, 3 mL sample from each flask was withdrawn and centrifuged at 10,000 rpm for 10 min and clear supernatant obtained was used for recording decolorization, if any, by recording the decrease in optical density at  $\lambda_{\text{max}}$  (584 nm) using UV-Visible double beam Spectrophotometer (Unico-3802) starting from 0 d to 5 days of incubation.

### Carbon and Nitrogen Source

Six sources of carbon viz., fructose, glucose, lactose, sucrose, starch and cellulose were used to study the effect of carbon sources on decolorization of Methyl Violet 2B. All were used at a concentration of 0.5% in PDB medium and spent substrate of *P. sajor-caju* @ 1.0%, w/v was used as potential dye decolorizing agent. For further confirmation of the role of carbon and nitrogen sources in dye decolorization, glucose and starch (carbon source), and Urea and ammonium carbonate (nitrogen source) were studied at 3 different concentrations (25, 50 and 100 ppm). Protocol for medium preparation, dye/spent substrate addition, number of replications, incubation temperature/duration, sample withdrawal/centrifugation, data recording and statistical analysis was same as for role of initial dye concentration on its decolorization, excepting instead of 5 initial concentrations of dye only one (100 ppm) was used. Flasks devoid of any additional carbon and nitrogen source but with dye @ 100 ppm and *P. sajor-caju* spent substrate @ 1.0%, w/v were used as one control and second

control was dye with wheat straw, which used as main substrate for mushroom cultivation.

#### **Veratryl alcohol**

Veratryl alcohol, a natural secondary metabolite of *P. chrysosporium* as well as a substrate for Lignin peroxidase (LiP), is considered as an inducer of the ligninolytic enzymes system under *in vitro* conditions and has been reported to increase the rate of oxidative degradation of lignin and several other aromatic compounds<sup>10</sup>. For studying effect of veratryl alcohol on dye decolorization four concentrations (0.025, 0.05, 0.075 and 0.1%) were used in PDB medium. Flasks devoid of veratryl alcohol but with dye and spent substrate were kept as control. Rest of the protocol was similar to earlier steps of the study.

#### **Manganese ions**

Manganese peroxidase (MnP) oxidizes Mn(II) into Mn(III) as a nonspecific oxidant and then oxidizes a variety of other organic substances<sup>10</sup>. Effect of 5 concentrations of Manganese ions (0.025, 0.05, 0.075, 0.1 and 0.125%) was studied by adding 25, 50, 75 and 100 mg of MnSO<sub>4</sub> in 100 mL of sterilized PDB medium in 250 mL flasks, each containing dye and spent substrate @ 100 ppm and 1.0%, w/v, respectively. Flasks devoid of manganese sulphate but with 100 ppm concentration of dye in PDB and 1.0% *P. sajor-caju* spent substrate were used as control. Rest of the protocol was similar to earlier steps.

#### **Heavy Metal ions**

Effect of 6 heavy metal ions (Cd<sup>2+</sup>, Pb<sup>2+</sup>, Hg<sup>2+</sup>, Co<sup>2+</sup>, Zn<sup>2+</sup> and Ni<sup>3+</sup>) on decolorization of Methyl Violet 2B was studied using 0.025% concentration of heavy metal ions, 100 ppm concentration of dye and 1.0%, w/v of spent substrate of *P. sajor-caju* in PDB. Rest of the experimental protocol was similar to earlier steps. Flasks devoid of any heavy metal but with 100 ppm concentration of dye and 1.0%, w/v of spent substrate of *P. sajor-caju* were used as control.

#### **Measurement of Decolorization Extent**

Three mL sample was collected each time from each replication and centrifuged at 10000 rpm for 10 min, and clear supernatant thus achieved was used for measuring decolorization extent. Decolorization extent was determined by measuring absorbance of the supernatant at  $\lambda_{\max}$  (584 nm) of Methyl Violet 2B by using UV-Visible double beam Spectrophotometer (Unico-3802).

Decolorization extent was calculated as<sup>9</sup>.

Decolorization extent (%) =  $(OD_1 - OD_t / OD_1) \times 100$

Where OD<sub>1</sub> is initial absorbance at 0 d, OD<sub>t</sub> is absorbance after incubation for different periods under different experimental conditions and t is incubation time.

## **RESULTS AND DISCUSSION**

### **Extracellular ligninolytic enzymes activity**

Spent substrate of *P. sajor-caju* was recorded highest activity of MnP (55 U/mL<sup>-1</sup>), followed by laccase (47 U/mL<sup>-1</sup>). Lower amount of LiP were recorded as compared to two enzymes in both the spent substrate (Fig. 1). Fungal ligninolytic enzymes are commercially in use in textile industry and have potential for more industrial applications. The present study envisages that out of three enzymes, activity of MnP was higher and role of similar enzyme have specially highlighted in decolorization of Cibacron Red FN-2BL with *Schizophyllum commune*, while minor role of LiP and laccase<sup>11</sup>.

### **Different Concentrations of Dye**

Experiment conducted with 5 concentrations of dye revealed that after 1 and 3 day of spent substrate mixing, highest decolorization was in 100 ppm dye concentration. However, after 5 day of SMS mixing, the relationship between initial dye concentration and its decolorization was inversely proportional, as it was near 100% in lowest concentration (25 ppm), while only 90% in highest dye concentration (150 ppm) (Fig. 2). In several earlier studies, the response of initial concentrations of dyes towards their decolorization has been reported to vary from dye to dye<sup>10</sup>. However, in a good number of cases, where fungi have been used as decolorizing agents, decolorization has been recorded higher at lower initial dye concentration<sup>12</sup> and like present study this was more evident in studies with *Pleurotus florida*<sup>13</sup> and *Pleurotus ostreatus*<sup>8</sup>.

### **Carbon and Nitrogen Sources**

Data depicted (Fig. 3) reveal that decolorization of Methyl Violet 2B at different stages was higher in presence of different carbon sources, excepting Cellulose, where it was lesser than control. Decolorization stimulatory effect was more pronounced at early stage (after 1 and 2 day) of SMS mixing, which became lesser significant at

later stages (after 3 and 4 day). At the end of experiment, sucrose and starch added media exhibited highest decolorization of Methyl Violet 2B. Highest decolorization was recorded with sucrose (87.75%), followed by starch (87.48%), while only 80.29% in control and 5 to 7% in second control inoculated with wheat straw alone. Amongst different concentrations of carbon and nitrogen sources, 25 ppm concentration each of glucose, starch and ammonium carbonate, while 100 ppm of urea supported highest decolorization of dye (Table 1). Nearly 100% decolorization of dye was achieved on addition of 25 ppm either of glucose or starch. Amongst nitrogen and carbon sources, enhancement in decolorization was more pronounced on addition of carbon sources than nitrogen sources (urea and ammonium carbonate). In several earlier studies, different concentrations of carbon sources have been evaluated for their role in dye decolorization and a concentration ranging from 0.2 to 2.0% of glucose<sup>14</sup> has been reported to support higher level of dye decolorization. In a few studies urea and ammonium nitrate have also been used as nitrogen sources but only ammonium nitrate in combination with

organic nitrogen sources has been recorded to have decolorization stimulatory effect, while urea has been reported to decrease both decolorization as well as activity of LiP and MnP enzymes, considered responsible for dye decolorization in white rot fungi<sup>15</sup>.

#### Veratryl alcohol and Manganese ions

At an early stage, lower concentration of veratryl alcohol (0.025%) supported highest dye decolorization, while at later stages, highest concentration (0.1%) supported highest decolorization (Fig. 4a). Manganese ions also exhibited similar trend, as at initial stage, slightly enhanced decolorization was with lower concentration of Mn<sup>2+</sup> compared with other concentrations including control. However, at a later stage (5 day), all Mn<sup>2+</sup> treatments supported almost equal level of decolorization but slightly higher than control (Fig. 4b). Compared with control, both veratryl alcohol and Mn<sup>2+</sup> supported higher decolorization, though more with lower concentration (0.025%) at initial stage and with higher concentrations at later stages. Role of these chemicals on decolorization of molasses wastewater and ligninolytic enzymes activity of

**Table 1.** Effect of different concentrations of carbon and nitrogen sources on decolorization of Methyl Violet 2B through *Pleurotus sajor-caju* spent substrate

Carbon/ NitrogenSource	Concentration (ppm)	Dye decolorization (%) at different time interval (days)			
		1	2	3	4
Glucose	25	23.08	63.74	89.01	100.00
	50	66.34	70.30	71.29	89.11
	100	16.03	79.39	93.1	94.66
	Control	12.50	23.78	27.62	37.85
Starch	25	22.00	70.00	100.00	100.00
	50	33.99	41.83	99.35	100.00
	100	39.52	69.46	94.01	99.40
	Control	13.33	22.22	35.55	42.89
Urea	25	0.00	0.00	0.00	0.00
	50	0.00	0.00	0.00	0.00
	100	25.25	27.02	44.19	49.75
	Control	13.94	28.33	29.04	40.59
Ammoniumcarbonate	25	18.34	55.62	75.15	76.41
	50	0.00	7.00	10.50	10.61
	100	0.00	0.00	0.00	0.00
	Control	14.50	26.85	31.00	41.56

“a” ppm - part per million

“b” % - percentage

CD 0.05 = 4.557

*P. chrysosporium* have also been studied earlier and either absence or lower concentration of Mn(II) and absence of veratryl alcohol have been reported to support higher color reduction than their presence at higher concentration<sup>14</sup>. However, presence of Mn (II) in medium was recorded to induce highest activity of MnP<sup>15,16</sup>, while activity trend of LiP was reverse to it<sup>14</sup>. Contrary to this a few researchers have also reported decolorization stimulatory role of some laccase mediators (phloroglucinol, thymol, violuric acid and acetosyringone) and nearly complete decolorization (90-100%) have been reported for Remazol brilliant blue R, Coomassie brilliant blue and Acid red<sup>15,16</sup>. In present study also, the presence of veratryl alcohol and manganese sulphate at lower concentrations has shown higher decolorization at initial stage than other concentrations and control.

### Heavy metal ions

Data depicted (Fig. 5) reveal that out of 6 heavy metals, presence of only Hg<sup>2+</sup> and Zn<sup>2+</sup> decreased the decolorization of Methyl Violet 2B compared to control. Contrary to this, presence of Pb<sup>2+</sup> and Cd<sup>2+</sup> @ 0.5% in growing medium separately has enhanced dye decolorization and 94.6 to 100% decolorization of Methyl Violet 2B was recorded after 4 day of mixing of spent substrate along with aforesaid heavy metals (Fig. 5). Normally presence of heavy metal ions in growing medium is considered as detrimental for the growth of microorganisms including fungi, however in present study, presence of Lead, Cadmium, Cobalt and Nickel ions has supported higher decolorization compared to control. It is attributed to their ability to provide more stability to laccase, which is considered as the potential ligninolytic enzyme having role in dye

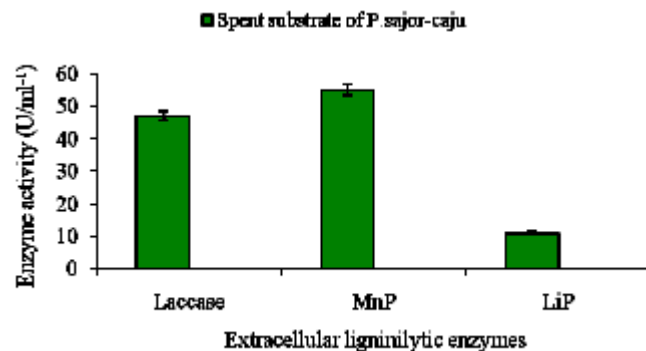


Fig. 1. Extracellular ligninolytic enzyme activity of spent substrate of *P. sajor-caju*

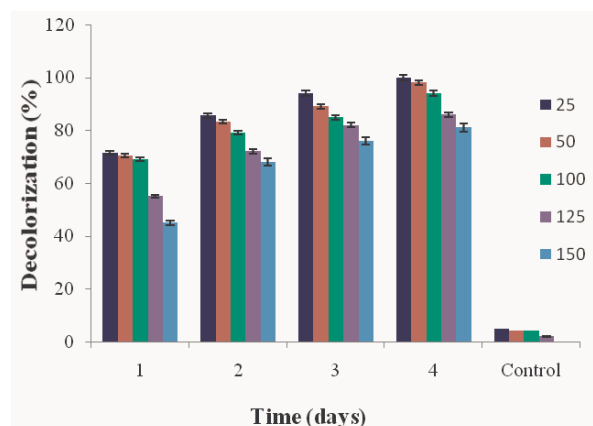


Fig. 2. Effect of initial concentrations of dye on its decolorization through *P. sajor-caju* spent substrate

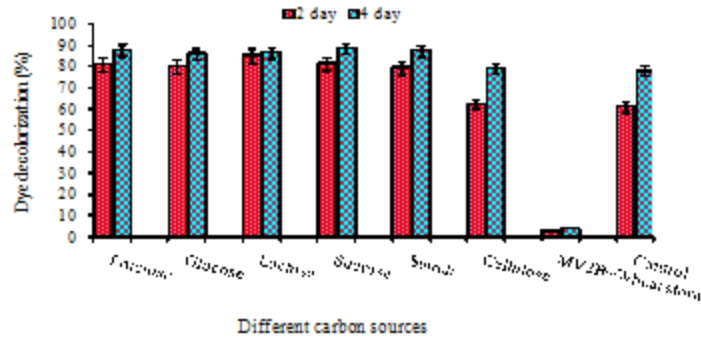
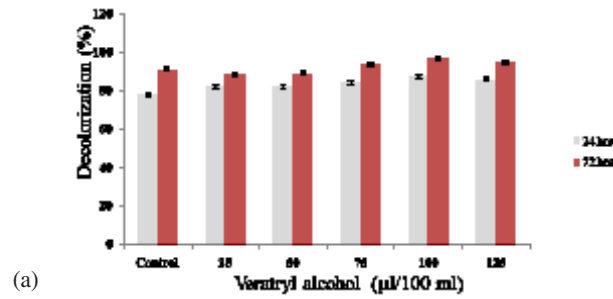
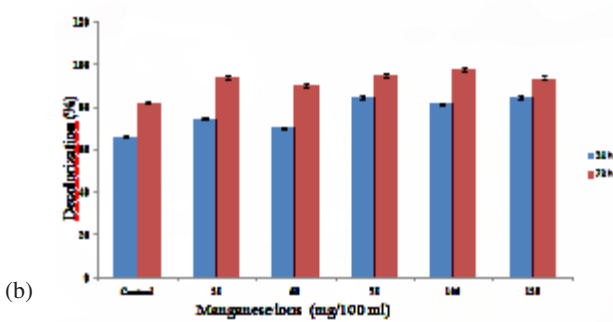


Fig. 3. Effect of different carbon sources in growing medium on decolorization of Methyl Violet 2B using spent substrate of *P. sajor-caju*



(a)



(b)

Fig. 4. Effect of different concentrations of mediator and cofactor on decolorization of Methyl Violet 2B through *P. sajor-caju* spent substrate: a, Veratryl alcohol; and b, Manganese ions

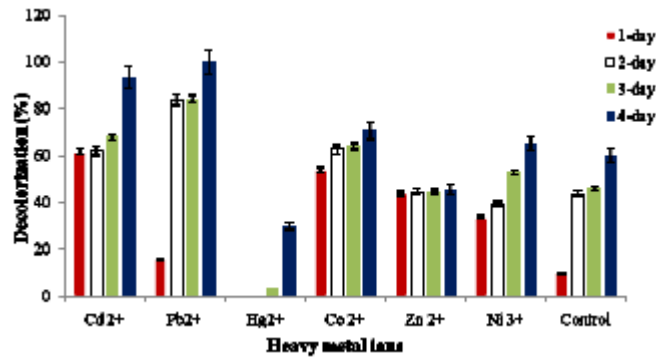


Fig. 5. Effect of different heavy metal ions on decolorization of Methyl Violet 2B through *Pleurotus sajor-caju* spent substrate

decolorization by the White rot fungi<sup>17</sup>. Presence of heavy metals in colored industrial wastewater has also been reported to affect succession of microbial community<sup>18</sup> and hence decolorization.

## DISCUSSION

Spent mushroom substrate (SMS) with appreciable level of nutrients and habitat of potential bioremediative microorganisms is presently finding limited utilities. The constituents of SMS viz., decomposed wheat straw, mushroom mycelia biomass and rich population of microorganisms with their ability to degrade synthetic dyes<sup>19</sup>. SMS from *P. sajor-caju* harbored highest fungal population, dominated by *Aspergillus fumigates*, followed by *Schizophyllum commune* and *Pezizomycotina* sp. SMS of *P. sajor-caju* did harbor highest population as well as diversity of bacteria comprised of four different species i.e. *Bacillus licheniformis*, *B. subtilis*, *Rummeliibacillus stabekisii* and *Pseudomonas fluorescens*. The microbial variability has the relevance in the light of SMS as source of potential microorganisms with their role in textile effluent decolorization<sup>9</sup>.

In present study, different culture parameters (initial concentration of dye, presence of additional amount of carbon and nitrogen sources in medium, presence of enzyme cofactor/mediator and heavy metals in growing medium) have been studied for their effect on decolorization of Methyl Violet 2B through *P. sajor-caju* spent substrate under laboratory conditions. As reported by many earlier workers that initial concentration of dye has direct bearing on its decolorization, as lesser concentration of dye supports more microbial growth and consequently affects dye decolorization and same is true with present study. In present study, presence of an additional amount of carbon source in growing medium has promoted dye decolorization, as additional amount of carbon source acts as an easily available substrate for growth of different types of microorganisms contributed by *P. sajor-caju* spent substrate, which ultimately decolorize the dye. Presence of nitrogen source acts dual way, as in earlier studies higher amount of nitrogen has been reported to decrease activities of several ligninolytic enzymes (LiP, MnP, laccase etc.), which have been reported to have role in dye decolorization and that

is why lower concentrations of these sources had supported more decolorization than their higher concentrations. Studies carried out on role of enzymes mediator, cofactor and stabilizers on dye decolorization including veratryl alcohol, manganese ions and some heavy metal ions have clearly elucidated role of these in dye decolorization and role of some metal ions including heavy metal ions in enhancing dye decolorization is attributed to their enzyme stabilizing ability. Similar are the findings of present study in which presence of Lead, Cadmium, Cobalt and Nickel ions is recorded to support higher decolorization than control. However, presence of veratryl alcohol and manganese ions has supported higher decolorization only at lower concentrations during initial stage of experiment.

## CONCLUSIONS

The results concluded that the lower conc. of dye (25 mg/L), manganese ions, veratryl alcohol and additional carbon and nitrogen sources enhances the decolorization of MV2B. In case of metal ions (lead, cadmium, cobalt and nickel ions) higher decolorization of MV2B was observed than the control and pellet form of mycelia with agitated conditions enhances the rate of decolorization up to significant level. Spent substrate of *P. sajor-caju* is available in plenty and found in the immobilized form with mushroom mycelia & microbes for synthetic dyes decolorization. Because of potential applications in the textile industry effluents treatment, the use of this spent substrate of *P. sajor-caju* having ligninolytic enzymes open up new possibilities for the development of green technology alternatives to existing chemical treatment. For further research work, there is need to optimize the potential ligninolytic enzymes of SMS of different strains of different edible mushrooms. Therefore, spent substrate of *P. sajor-caju* can be used as an economical and eco-friendly tool to minimize the pollution by textile and leather industries to a significant extent.

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