International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2020; 8(4): 2464-2469

Received: 28-05-2020
Accepted: 30-06-2020

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Optimization of culture conditions for enhanced decolorization of Amido black by *Calocybe indica* (CBE 1515) spent mushroom substrate

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DOI: https://doi.org/10.22271/chemi.2020.v8.i4ab.10005

Abstract
The objective of this study was to exploit the decolorization potential of Spent Mushroom Substrate (SMS) of *Calocybe indica* (Strain-CBE 1515) for the biodegradation of reactive textile dye Amido black. Initial studies have been made on screening of mycelia and crude enzyme extract of spent compost of Milky mushroom (*Calocybe indica*) strains for their dyes (Amido black, Congo red and RBBR) decolorization potential. It was observed that strains of *C. indica* were found to be hyper specific to laccase production and significant decolorization potential was showed by CBE 1515 for Amido black. Various process parameters like composition of basal nutrient medium, pH, temperature, additional carbon and nitrogen sources, and initial dyestuff concentration were optimized to develop an economic decolorization process. The optimum dye decolorization was achieved in LME medium containing dextrose and yeast extract as carbon and nitrogen sources respectively and adjusting the pH to7.5 and incubated at 30 °C.

Keywords: Dye decolorization, spent mushroom substrate, amido black, milky mushroom

1. Introduction
The textile finishing generates a large amount of dyes and pigments containing wastewater from dyeing and subsequent steps that forms one of the largest contributions to water pollution (Santhy and Selvapathy, 2006) [19]. Color present in dye-containing effluents gives a straightforward indication of water being polluted, and discharge of this highly colored effluent can damage directly the aquatic life in receiving water (Senthilkumar *et al.*, 2005) [20]. Due to their chemical structures, dyes are resistant to fading on exposure to light, water, and many chemicals (Robinson *et al.*, 2001) [18].

Conventional treatment methods of textile effluents are either ineffective, costly, complicated, or have sludge problems (Stolz, 2001; Robinson *et al.*, 2001) [24, 18]. The economic and safe removal of the polluting dyes is still an important issue. Among the most economically viable methods available for decolorization, the most practical in terms of manpower requirement and expenses appears to be biological system (Murugesan and Kalaichelvan, 2003; Boer, 2002) [14, 4]. Although decolorization is a challenging process to the textile industry, the great potential of microbial decolorizing can be adopted as an effective tool. In the recent past, there has been an intensive research on bioremediation of dyes, and the use of ligninolytic fungi is turning into a promising alternative to replace or supplement present treatment processes (Boer, 2002; Dos-Santos *et al.*, 2004; Asgher *et al.*, 2006) [4, 6, 2].

Lignolytic fungi can mineralize xenobiotics to CO_2 and H_2O through their highly oxidative and non-specific ligninolytic system, which is also responsible for the decolorization and degradation of a wide range of dyes (Boer *et al.*, 2004; Mazmanci *et al.*, 2005) [5, 13]. These ligninases including laccase, lignin peroxidase (LiP), and manganese peroxides (MnP) are able to decolorize dyes of different chemical structures (Levin *et al.*, 2004; Asgher *et al.*, 2006) [12, 1]. In continuation of our previous studies (Sowjanya *et al.* 2018) [23], decolorization of reactive textile dye Amido Black by *Calocybe indica* CBE 1515 is a part of our efforts for developing indigenous technology for decolorization of textile dyes and thus dye-containing effluents.

2. Material and Methods

2.1. Textile dye: Amido Black

2.2. Culture and maintenance: *Calocybe indica* (P and C) strain CBE 1515 was procured from Department of Plant pathology, Tamil Nadu Agricultural University, Coimbatore. The culture of *C. indica* strains were maintained on Potato Dextrose Agar (PDA) slants at 30± 2 °C by sub-culturing them fortnightly.

2.3. Basal nutrient media: Five different growing media Potato Dextrose Broth (PDB), LME broth, Complete Yeast Extract broth (CYM), Richard's Medium (RM), Malt Extract Broth (MEB) were used for studying effect of media composition on decolorization of Amido black by using spent substrate of CBE-1515 which was considered as potential dye decolourizer among the tested *C. indica* strains.

2.4. Optimization protocol

The decolorization process was optimized by studying the effect of different cultural parameters (medium, temperature, pH, initial concentration of dye, carbon and nitrogen source) on per cent decolorization by SMS of CBE-1515 strain in 250 mL flasks supplemented with different working concentrations of Amido black which had showed maximum decolorization in previous experiments. The classical method for medium optimization was followed, varying one parameter at a time and maintaining the pre optimized at constant level. The 0.5 mL of 0.5% (w/v) stock solution of dye was added in pre sterilized media flask to make up 50 ppm concentration of dye followed by addition of 5 g spent compost of *C. indica* in each flask using sterilized forcep. The dye supplemented flasks devoid of SMS were kept as control. Decolorization if any was recorded by recording decrease in optical density at λ_{max} from 0 day up to 3 days of incubation.

2.4.1. Step1: The modified LME basal medium (LME) was used in order to eliminate the possible dye absorbing effect (Pointing, 1999) [16] and the composition of LME can be easily altered to get optimized medium as it is not a synthetic medium.

2.4.2. Step2: LME was prepared by using 5 different carbon sources *viz.*, tartaric acid, sucrose, lactose, xylose and dextrose in combination with other ingredients of the medium and inoculated with dye and SMS.

2.4.3. Step3: Taking one carbon source (dextrose) which had shown maximum decolorization in previous trial as constant, modified LME was prepared with five different nitrogen sources viz., peptone, ammonium oxalate, yeast extract, tryptone and $NaNO_3$ in combination with other ingredients and inoculated with dye and SMS.

2.4.4. Step4: Three different pH (4.0, 6.0 and 8.0) were used for studying effect of pH on decolorization of Amido black using SMS of *C. indica*. The pH which showed maximum decolorization was further optimized with narrow range of pH value (8±0.5). Modified LME was prepared with optimized carbon & nitrogen source and the obtained broth's pH was adjusted with HCl/NaOH.

2.4.5. Step5: Modified LME was prepared by using optimized carbon (dextrose) and nitrogen (yeast extract) sources in combination with other ingredients, adjusted to optimized pH and inoculated with dye and SMS and incubated at four different temperatures (25 °C, 30 °C, 35 °C and 40 °C).

2.4.6. Step6: Modified LME was prepared with optimized carbon & nitrogen source and pH was adjusted to an optimized value (7.5) obtained from previous step using HCl/NaOH. Four different concentrations *viz.*, 25, 50, 100 and 200 ppm were made by adding 0.25 mL, 0.50 mL, 1 mL and 2 mL of 0.5% (w/v) stock solution of dye respectively in pre sterilized modified LME media flasks followed by addition of 5 g spent compost of *C. indica* in each flask using sterilized forcep.

2.5. Measurement of decolorization extent

Sample (4 mL) was collected at each step from each replication and centrifuged at 5000 rpm for 20 min. The decolorization extent was determined by measuring absorbance of supernatant at specific wavelength λ_{max} (610 nm) for Amido black by using UV-Visible spectrophotometer. Decolorization extent was calculated as

$$\text{Decolorization extent (\%)} = [100\times(OD_i-OD_t)]/OD_i$$

Where OD_i is initial absorbance at 0 day, OD_t is absorbance after incubation for different periods under different experimental conditions, t is incubation time.

3. Results and Discussion

3.1. Cultural medium

Statistically there was no significant difference among the five culture media used in this study. However, on 3rd day of incubation the % decolorization was recorded as follows: LME (75.5%) > RH (72.0%) > ME (71.5%) > CYM (70.0%) > PDB (64.5%) (Fig 1)

Maximum decolorization obtained may be related to the type of medium used in the mycelial growth of *C. indica* impregnated in SMS. Apart from being rich in salts, the medium also contained lignocellulose, which is essential for the enzyme induction. Elisashvili and co workers (2006) [7] found that the presence of a lignocellulosic substrate is obligatory for manganese peroxidase production by *P. dryinus* IBB 903 since there the enzyme production was observed to be ceased when the fungus was grown in a synthetic medium with various carbon sources.

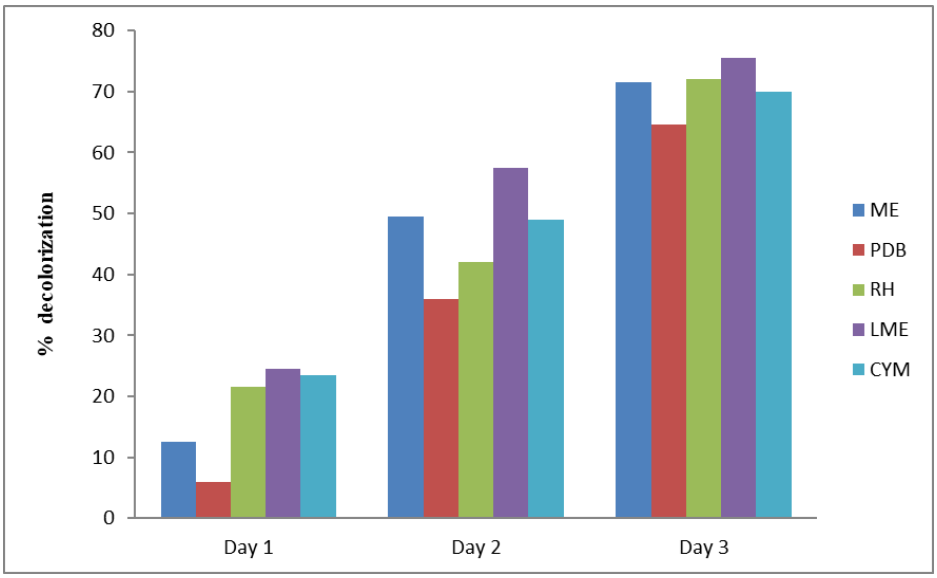

Fig 1: Per cent decolorization of Amido black by SMS of *C. indica* strain CBE 1515 in different cultural medium

3.2. Carbon source

Decolorization experiments were conducted using sucrose, lactose, xylose, dextrose and tartaric acid as additional carbon sources. A dramatic increase in decolorization of Amido black has been observed with dextrose (63.4%) addition after 24 hours incubation only. The decolorization efficiency showed a steady increase with the increase in incubation period. All the carbon sources enhanced decolorization of Amido black. However, addition of dextrose caused maximum decolorization (79.1%) followed by xylose (76.5%) and lactose (73.2%) whereas tartaric acid and sucrose could cause only 65.7% and 64.9% of decolorization respectively after 3 days of incubation (Fig 2).

Decolorization of Poly R 478 dye by ten white-rot fungi was reported to vary in response to different carbon regimes and fastest decolorization rates were achieved with monomers (glucose, xylose) as carbon source (Leung and Pointing, 2002) [11]. The findings of Leung and Pointing (2002) [11] are in similarity with present study in which dextrose and xylose supplementations enhanced the decolorization potential of SMS. As the glucose is a monomer, it can be easily consumable by fungi thereby causing a significant shortening of lag phase and increasing its productivity. Supplementation of monosaccarides (glucose, dextrose) to the medium containing dye provides easily metabolizable energy source to the fungus and creates an environment to enhance decolorization rate of dyes.

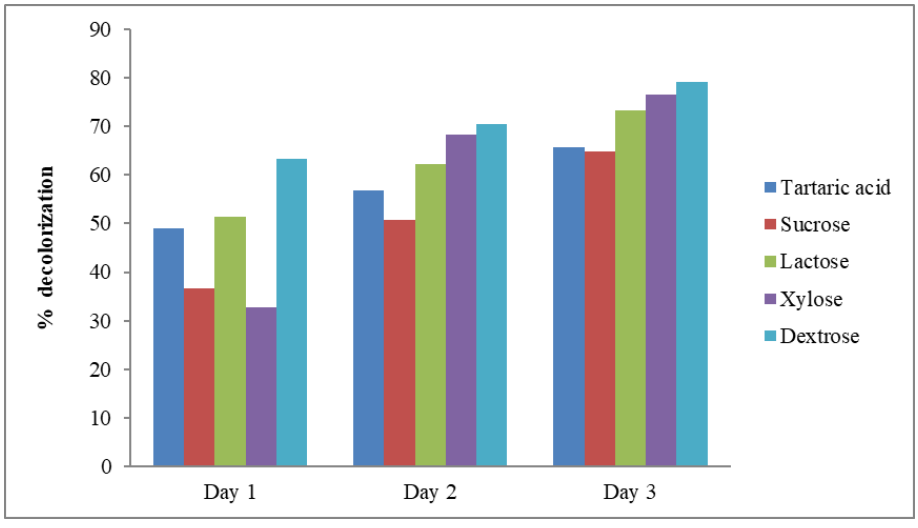

Fig 2: Per cent decolorization of Amido black by SMS of *C. indica* strain CBE 1515 in LME supplemented with different carbon sources

3.3. Nitrogen source

Effect of different additional nitrogen sources on percent decolorization was investigated under optimum conditions of dextrose as carbon source. After three days of incubation, 83.3% decolorization was observed in the medium supplemented with yeast extract which was recorded greater than other nitrogen sources. At initial days of incubation there was no significant difference among different nitrogen sources in enhancing dye decolorization potential but, statistically significant difference was observed on 2nd and 3rd day after inoculation with SMS. Dye decolorization potential by different nitrogen sources was observed to be as follows: yeast extract (83.3%) > tryptone (82.6%) ≥ Ammonium oxalate (79.2%) > $NaNO_3$ (75.4%) > peptone (66.8%). (Fig 3) The nitrogen levels in the medium influence the rate of lignolytic enzyme production and dye decolorization by white-rot fungi. In general the higher concentration of nitrogen source declines the enzyme production (Leung and Pointing, 2002) [11]. In contrast, Lee and co workers (2004) [10] has reported that the addition of nitrogen source has resulted

in significant enhancement of color removal efficiency by *S. commune*. The mycelial growth of fungi has increased with supplementation of nitrogen source. The results indicated that the rate of decolorization efficiency was increased up to a concentration of 0.5% due to the requirement of nitrogen as a nutrient whereas, the further raise in nitrogen levels declined the rate of color removal since the breakage of azo bonds decreased due to the presence of easily accessible excess nitrogen in the form of ammonium ions (Vahabzadeh *et al.*, 2004) [26].

Different combinations of carbon and nitrogen sources were studied by Iqbal *et al.*, (2011) [9] to record a variation in lignin degrading enzyme profile of *Trametes versicolor*. For enhancing production of peroxidases (LiP and MnP) maltose and urea were found to be best, while for laccase production glucose and yeast extract found to be the best combination.

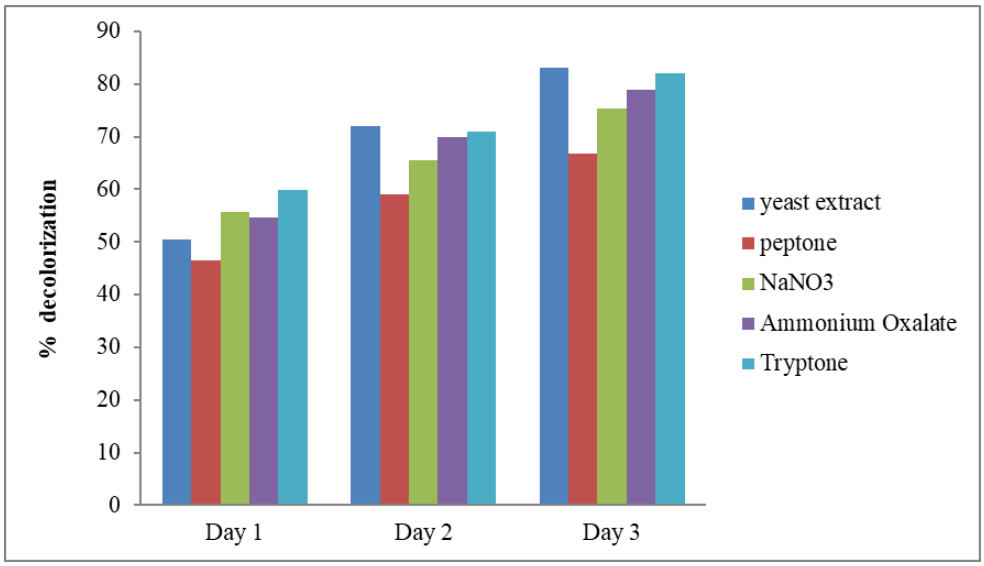

Fig 3: Per cent decolorization of Amido black by SMS of *C. indica* strain CBE 1515 in LME supplemented with different nitrogen sources

3.4. pH

Results for the effect of pH on% decolorization of Amido black showed that maximum decolorization efficiency (80.9%) was observed in medium adjusted with pH 7.5 after 3 days of incubation. The effect of pH on dye decolorization by SMS was obtained as follows: 7.5 (80.9%) ≥ 8 (79.5%) ≥ 8.5 (75.6%) > 6 (70.5%) > 4 (55.3%) on third day of incubation (Fig 4). The results show that basic pH is favouring growth of fungi and maximum decolorization of Amido black.

The chemistry of dye molecule and fungal biomass were greatly affected by the initial pH of growing medium. The effect of pH on the decolorization process is important, since it was noted that dyes are soluble over a certain basic pH ranges and insoluble at acid pH (Fu and Viraraghavan, 2001) [8]. In present study, maximum decolorization efficiency was observed at neutral to basic pH. The results are in accordance with previous studies of Senthilkumar and co workers (2011) [21], where 95 per cent of dye degradation efficiency was obtained by white-rot fungus *Phanerochaete chrysosporium* on synthetic dye bath effluent containing Amido black 10B at the pH of 7. The optimum pH for decolorization of RB 5 (150 mg/l) by *F. trogii* was pH 4.5–7.5 (Park *et al.*, 2004) [15].

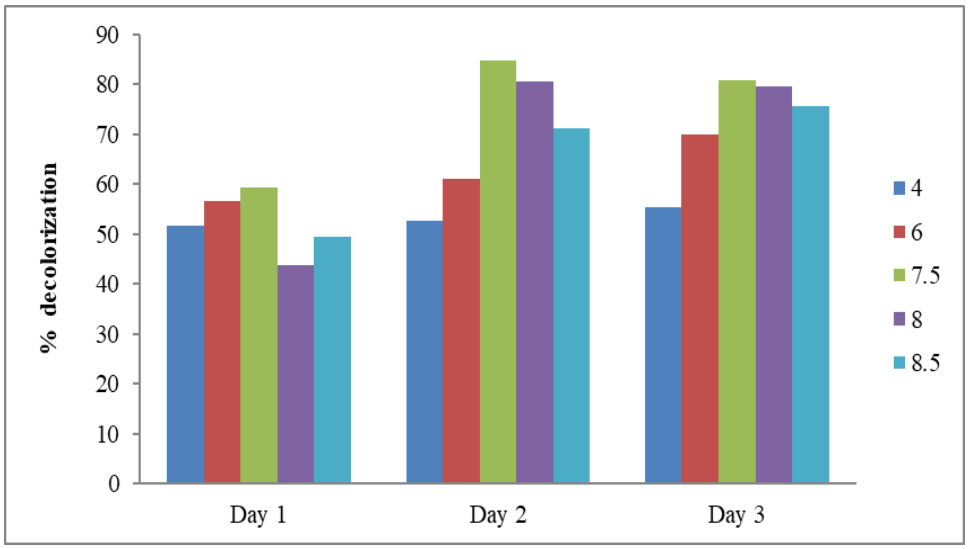

Fig 4: Effect of pH on per cent decolorization of Amido black by SMS of *C. indica* strain CBE 1515

3.5. Temperature

Results for the effect of temperature on% decolorization of Amido black showed that maximum percent decolorization (85.5%) was observed in flasks incubated at 30 °C followed by flasks incubated at 25 °C (80.6%) and 35 °C (73.2%) after 3 days of incubation. Minimum decolorization was observed in flasks incubated at 40 °C (Fig 5).

The results obtained in present study were in accordance with the reports of Poornima *et al.* (2016) [17], the optimum temperature for decolourizing Amido black was 30 °C, Bhatti *et al.* (2008) [3], at the low- medium temperature range (25–35 °C) fungus shown increased efficiency of dye decolorization and Singh *et al.* (2010) [22], the optimum temperature for decolorizing all the dyes (tested by author) except for crystal violet by SMS of *P. sajor caju* was between 30 °C – 35 °C and there was a decrease in the rate of decolourisation at temperatures above 35 °C. The maximum decolorization (71%) was observed in the shake flasks incubated at 30 °C for 7 days under optimum conditions. The decline in dye removal potential was observed at higher temperatures (40–45 °C). White-rot fungi show adverse growth under optimum temperature conditions as compared to at higher temperatures (Toh *et al.*, 2003) [25]. Temperature optima of 30–37 °C have also previously been reported (Ashger *et al.*, 2006 and Boer *et al.*, 2004) [1, 5] for different white-rot fungi for decolorization of chemically diverse dyestuffs.

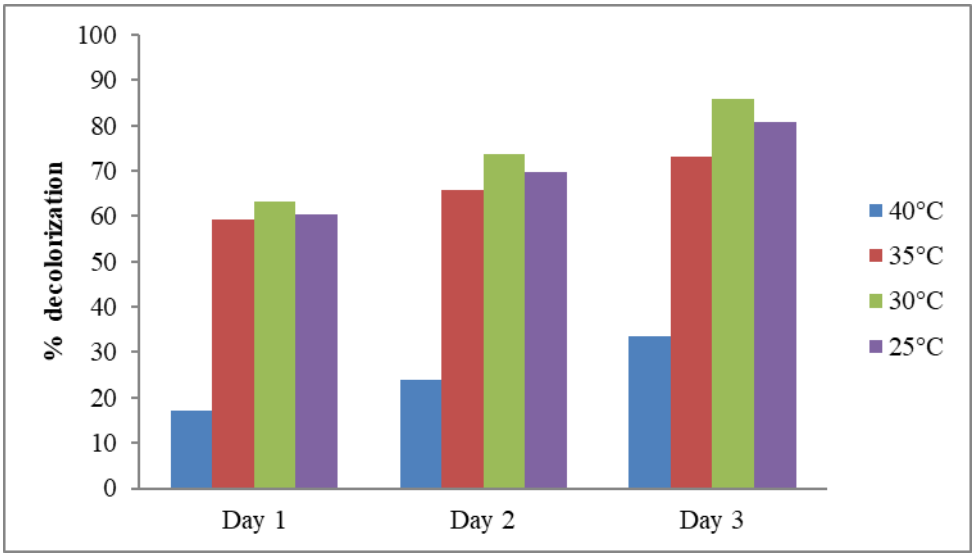

Fig 5: Effect of temperature on percent decolorization of Amido black by SMS of *C. indica* strain CBE 1515

3.6. Initial concentration of dye

Initial concentration of dye had diverse influence on per cent decolorization of Amido black using SMS of *C. indica.* At initial stages (1st and 2nd day after inoculation) the decolorization at different dye concentrations did not vary significantly. After 3 days of incubation the trend line for effect of dye concentration on% decolorization was obtained (Fig 6) as follows: 25ppm (85.9%) ≥ 100ppm (84.7%) ≥ 50ppm (80.2%) > 200ppm (78.8%)

The rate of decolourisation was increased with the increase in dye concentration exhibiting apparent first order reaction. In general, high dye concentration will cause a slower decolourisation rate (Young and Yu 1997) [27]. However, in the present study, the rate of decolourisation increased to a certain optimum concentration of dye after which the rate of decolourisation decreased. These results were in accordance with Singh *et al.* (2010) [22].

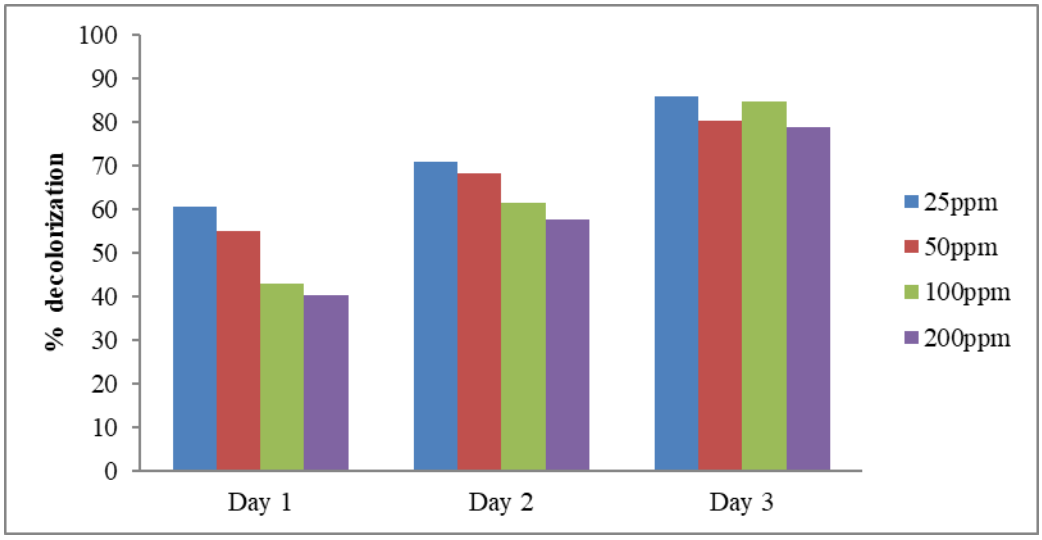

Fig 6: Effect of initial concentration of dye on decolorization of Amido black by SMS of *C. indica* strain CBE 1515

4. Conclusion

Among the variable concentrations of synthetic dyes used, 0.5 mL in of 0.5% of Amido black for 50 mL medium was best suited concentration for optimizing different cultural conditions to enhance the dye decolorization potential of *C. indica* spent mushroom substrate. The degradation potential can be enhanced in modified LME medium containing dextrose and yeast extract as carbon and nitrogen sources respectively and adjusting the pH to 7.5 and incubated at 30 °C.

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