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Enzymatic clarification and fading of synthetic and real melanoidins by Laccase and Peroxidases in submerged fermentation by Phanerochaete chrysosporium BW808 (MTCC 787)

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ABSTRACT

P. chrysosporium BW808 (MTCC 787) was used for enzyme production to treat different synthetic melanoidins solutions namely sucrose-glutamic acid (SGA), sucrose-aspartic acid (SAA), glucose-aspartic acid (GAA) and glucose-glutamic acid (GGA) Maillard products which are mixed in equal proportions as well as real melanoidins. Treatment efficiency was monitored in terms of clarification, color removal, COD reduction, pH change for an incubation period of 10 days. Results showed that Laccase, Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP) activities reached a peak on the eighth day incubation and started to decline on the tenth day with both SMM (Synthetic melanoidin mixture) and RM (real melanoidin). The peroxidase enzymes were developed to a similar extent in both the SMM and real melanoidins whereas the Laccase activity was higher at 120 U/l with SMM than with real melanoidins which was 100 U/l. The optimum pH range of the three enzymes under investigation laccase, lignin peroxidase and manganese peroxidase is 4.5-6; 4.5 -5.5 and 4.5-5.5 respectively. Laccase showed higher activity with SMM than with real melanoidins throughout the entire pH range from 3-8. There is maximum decolorization and appreciable reduction of COD from 10% to 60 % in SMM and 10% to 56% in RM at ph 5.5. The clarification and decolorization were measured by % transmittance and absorbance values respectively. The synthetic melanoidins were clarified and decolorized a little better than the melanoidins collected from the distillery. The development of enzymes in the broth culture is crucial to the clarification, decolorization and reduction of COD of in both real melanoidins (RM) and mixture of synthetic melanoidins (SMM).

Keywords: Melanoidins, Molasses, clarification, Phanerochaete chrysosporium (PC).

INTRODUCTION

Melanoidins are dark brown to black colored natural condensation products of sugars and amino acids produced by non-enzymatic browning reactions called Maillard reactions (1). These compounds are highly resistant to microbial attack, conventional biological processes such as activated sludge treatment are inefficient to decolorize melanoidin-containing wastewaters, such as molasses wastewaters from distilleries and fermentation industries [2]. Normally Melanoidins are found at concentrations of 2.0 % in effluents discharged from molasses processing industry. Untreated distillery effluents or spent wash are well known to cause enormous pollution in the natural resources by increase in organic load, depletion of oxygen content, discoloration and destruction of aquatic life [3].

They exist extensively not only in foods but also in wastewaters released from various agro-based industries as sugarcane molasses based distillery and fermentation industries. The distillery wastewater colorants are mainly polyphenols, melanoidins, alkaline degradation products of hexoses, and caramels. Due to hazardous nature of melanoidins, its chemical and microbial degradation has been attempted to reduce its toxicity and also to characterize its chemical structure so that better strategies could be achieved for its degradation and decolorization. A lot of efforts have been made by the research community to remove the colorants including biological methods employing different fungi, bacteria ,algae, enzymatic treatment, chemical oxidation, coagulation/precipitation, oxidation and membrane filtration [4]. These technologies could also be employed in the industries to remove the colorants as a final treatment step after the anaerobic digestion [5].

Microorganisms (bacteria/fungi/actinomycetes) due to their inherent capacity to metabolize a variety of complex compounds have been utilized since long back for biodegradation of complex, toxic and recalcitrant compounds present in various industrial wastes for environmental safety. Microbial degradation and decolourisation of industrial wastes is an environment-friendly and cost competitive alternative to chemical decomposition process of wastes minimization (6, 7).

Moreover, the utility of microbes in industrial wastes treatment process is largely depends on the enzymatic setup, nutrient requirement of microbes as well as nature chemical structure of recalcitrant compounds and environmental conditions (8). Microbial decolorization of anaerobically treated effluent reduced the hazard from toxic effect which indicated that there is necessity for microbial degradation at secondary or tertiary stage prior to its disposal for environmental safety [9].

The decolorization of four types of synthetic melanoidins i.e., glucose–glutamic-acid (GGA), glucose–aspartic-acid (GAA), sucrose–glutamic acid (SGA), and sucrose–aspartic-acid (SAA), were investigated using three different isolates, viz. *Bacillus thuringiensis*, *Bacillus brevis* and *Bacillus* sp. [10].The degree of decolorization of the melanoidins separately by each isolate was in the 1–31% range. The results also indicated that the GAA polymer was the most recalcitrant among the melanoidins tested. The clarification and decolorization of melanoidin by bacteria are mediated due to prevalence of manganese peroxidase (MnP) as decolourising enzyme [11] and play an important role in degradation of several dyes [12].Lactic acid bacteria also prove to be useful in the degradation of melanoidins from sugarcane molasses processing industries and phenolics from palm oil mills (13) Thus, the microbial decolorization can be exploited to develop a cost effective, eco-friendly biotechnology package for the treatment of distillery effluent.

Phanerochaete chrysosporium (*P. chrysosporium*) is known to secrete a large number of hydrolytic and oxidative enzymes for degradation of natural lignocellulosic material, which includes cellulases, hemicellulases, lignin peroxidases, manganese peroxidases (14, 15, 16, 17 ,18,19) and pectinases (20). It also produces acid proteinase, glutami-nase and five kinds of peptidases which are related to aspartyl protease family (21). The protease activity investigated in the batch cultures of *P. chrysosporium* (22) had showed both primary (81.5, 71.5, 52, 28, 24 and 22 KDa) and secondary activity (75.5 and 25 KDa). Rahul etal demonstrated that *Phanerochaete chrysosporium* BW808 (MTCC 787) is capable of producing a protease enzyme system with agro-substrates (23).

Phanerochaete chrysosporium PC has been shown to produce a relatively nonspecific ligninolytic enzyme system which appears irrespective of the presence of lignin (13), and this fungus can oxidize a broad spectrum of chemical compounds (5). So the decolorization of four types of synthetic melanoidins i.e., glucose–glutamic-acid (GGA), glucose–aspartic-acid (GAA), sucrose–glutamic acid (SGA), and sucrose–aspartic-acid (SAA), were investigated using Phanerochaete chrysosporium, the wood rot fungus. Our study is a preliminary attempt to decolorize the synthetic and real melanoidins with the crude enzyme preparations from PC BW808 (MTCC 787) cultured with 10% melanoidins.

The possibility and efficiency of laccase and peroxidase producing wood rot fungus Phanerochaete chrysosporium for the clarification and degradation of synthetic melanoidin mixture (SMM) and real melanoidins (RM) solutions were evaluated in this study. Samples of waste water collected from a distillery in Maharashtra (90% v/v) were studied under submerged culture conditions. Phanerochaete chrysosporium was selected because it is known to produce Laccase and peroxidases which might prove to be useful in the biodegradation of melanoidins from distillery effluents.

MATERIALS AND METHODS

Media and strain used inoculum

Organism

P. chrysosporium BW808 (MTCC 787) was obtained from IMTECH, Chandigarh India. It was maintained at 37°C on 1% malt extract agar slants and subcultured every week.

Inoculum and crude enzyme preparation

All the culture media used in this study were of biological grade. The reagents and chemicals were procured from Hi Media, Merck and SD fine chemicals all from Mumbai, India.

The inoculum was taken from stationary cultures containing fresh conidial suspension with a conidium concentration of 5×10^5 spores per ml. The inoculation ratio of the experimental cultures was 2% (v/v), and the incubation temperature was maintained at 37°C in all cases. The growth and maintenance medium used was YMD broth medium [1% (w/v) malt extract, 1% (w/v) yeast extract, 0.1% (w/v) KH2PO4, 2% (w/v) dextrose and YMD agar contain 1.5% agar] at pH 6.5. Basal medium used for enzyme production contained 3% (w/v) starch (carbon source), 0.3% (w/v) NaNO3 (nitrogen source), 0.05% (w/v) KCl, 0.1% (w/v) KH2PO4 and 0.01% (w/v) FeSO4.7H20 and maintained at 37°C in an orbital shaker (120 rpm) for 24 to 96 h. At the end of incubation, the whole fermentation medium was filtered, centrifuged at 5000 rpm for 15 min and the clear supernatant was used as crude enzyme preparation.

Enzyme assays

Briefly, the laccase activity was determined using 2 2' azinobis (3 ethyl benthiazoline-6-sulfonic acid) [ABTS] (as per sigma Aldrich Method) as substrate. The absorbance will be monitored by using spectrophotometer at 420 nm. Lignin peroxidase activity was determined by measuring the production of veratryl aldehyde from veratryl alcohol at 310 nm in glycine HCl buffer at pH 3 at 30°C by addition of H2O2.Manganese peroxidase activity was determined by monitoring the oxidation of guaiacol (2-methoxyphenol) at substrate at 465 nm.

Preparation of synthetic melanoidins

The synthetic melanoidins solution used in this study was prepared by refluxing the equimolar (1M) solution of sucrose ,aspartic acid and 0.5M sodium carbonate at 100 ◦C for 7 h whereas; natural melanoidins solution was prepared from distillery effluent. Four types of melanoidins i.e., sucrose-aspartic-acid (SAA), sucrose-glutamic acid (SGA), glucose-aspartic-acid (GAA) and glucose-glutamic-acid (GGA) were synthesized SAA was synthesized using sucrose, aspartic acid and sodium carbonate, SGA from sucrose, glutamic acid and sodium carbonate, GAA from glucose, aspartic acid and sodium carbonate and GGA was prepared utilizing glucose, glutamic acid and sodium carbonate under above conditions and then after adjustment of the reaction mixture to pH 7.4 with 1N NaOH. These solutions contained mg/l COD from 267000 to 418000 mg/l. All of them were mixed in equal proportions and used to amend the medium.

The enzyme assays were carried out in the presence of both real melanoidin pigment collected from the distillery and the synthetic melanoidin mixture .To determine the optimum pH of the enzymes the assays were set up in triplicate with the crude enzyme preparation at different ph values from 3-8**.**

Enzyme Production

Enzyme production was done in 500 ml flask containing 100ml of culture media (pH-5.5-6) containing glucose 1%, peptone 0.3%, KH2PO4 0.06%, ZnSO4 0.0001%, KH2PO4 0.04%, FeSO4 0.0005%, MnSO4 0.05% and MgSO4 0.05% as previously reported. The media were inoculated with seven days old sporulated culture, suspension prepared from the culture grown in Petri-plates. For growing Phanerochaete chrysosporium in suspension, inoculum (250 mg) was prepared by homogenizing the six day old mycelium obtained from Petri-plates. Enzyme activity was determined at three day intervals, after which the culture will be centrifuged at 10,000 g of 30 min at 40°C.

Inoculum preparation and decolorization studies

The inoculum was prepared for decolorization studies in two steps. In the first stage, a pure culture was transferred to a 50 ml modified YMD broth consisting of (w/v): 1.0% glucose, 0.05% peptone, 0.1% K2HPO4 and 0.05% MgSO4 · 7H2O, incubated for 24 h in shaking flasks (140 rpm) under aerobic condition at 30 ±2 °C. Subsequently, it was transferred into flasks containing synthetic melanoidin mixture (5%) mended YMD broth.

In the first experiment, the decolorization experiments were carried out by the addition of fungal inoculums (OD590 0.05- 0.08) in 250 ml flasks containing modified YMD broth containing 5% synthetic melanoidin mixture at pH 5.5 and 30 ±2 °C for 10 days. A separate set of uninoculated flasks was maintained in parallel as control.

In the second experiment *the fungal* isolates consisting of PC were assessed for their decolorization potential in natural melanoidins pigment (10% (v/ v)) amended with yeast extract (0.1% w/v) under similar conditions as the first experiment conditions (140 rpm) at pH 5.5 and 30 ±2 °C for 5 days. A loopful of PC inoculums to 250 ml flasks containing 50 ml real melanoidins (5% v/v) and 90% sterilized water containing yeast extract (0.1% w/v) at pH 5.5 and 30 ±2 °C. A separate set of uninoculated flasks was maintained in parallel as control.

Samples were withdrawn from culture media at regular 24 h intervals for decolorization measurements. The supernatant was taken after centrifugation (10000g for 10 min) for determination of color and chemical oxygen demand (COD) [16]. Melanoidins removal or adsorption activity was determined as a decrease of optical density in the absorbance at 475 nm against the control. The pH was determined in supernatant. Experiments were repeated three times to reduce experimental error. Percent transmittance (%T) was determined from absorbance (A) in all the flasks by using the formula $A = 2 - \log_{10} \%T$.

RESULTS AND DISCUSSION

The fungal isolates that were maintained with effluent and without effluent for a period of 10 days served as tests and controls respectively. They were assessed for the growth and activity of enzymes on modified YMD agar plates amended with synthetic melanoidin mixture and 0.1% phenol red (w/v).

Fig 1&2:
Fig 1 showing the streaked plate of *Phanerochaete chrysosporium* on YMD agar
Fig 2: showing luxuriant growth of *Phanerochaete chrysosporium* on YMD agar

Fig 3&4:
Fig 3 showing the growth of *Phanerochaete chrysosporium* on YMD broth with RM
Fig 4: showing clarified and decolorized broth shown in fig 3 in 10 days

The culture isolates of PC showed optimum growth and exhibited peroxidase activity by changing the deep red color of dye to light yellow. PC was found to exhibit laccase, LiP and MnP peroxidase activities which mean the capability to degrade melanoidin compounds. The development of enzyme activities with SMM and the real melanoidin pigment is investigated at throughout a study period of ten days and the results are recorded at each 24 hr interval. The results of the enzyme assays are given below.

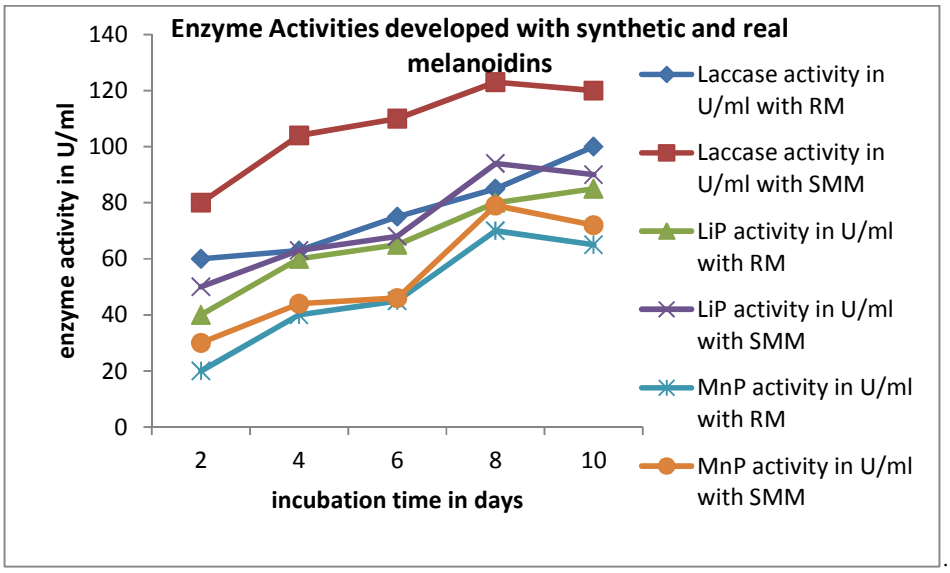

Fig 5: The results of enzyme assays with SMM and RM in graphical representation

The results showed that the enzyme activities reached a peak on the eighth day incubation (Fig 5) and start to decline on the tenth day. This result was observed with both SMM and real melanoidins. One of the important trends that were observed is that the peroxidase enzymes were developed to a similar extent in both the SMM and real melanoidins whereas the Laccase activity was higher at 120 U/l with SMM than with real melanoidins which was 120 U/l/.

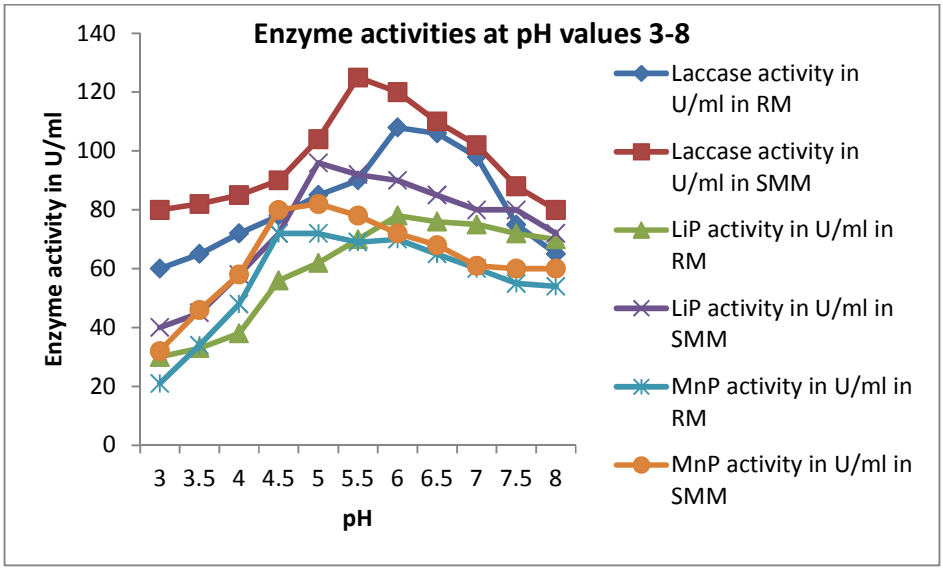

Fig 6: The graph shows the optimum pH range of Laccase and peroxidases

The optimum ph range of the three enzymes under investigation laccase, lignin peroxidase and manganese peroxidase is 4.5-6; 4.5 -5.5 and 4.5-5.5 respectively. It is interesting to note that both the peroxidases show the same range and the laccase is rising till ph 6. The results showed that the peroxidases developed to a similar level in both real melanoidins and SMM throughout the ph range of 3-8.Laccase showed higher activity with SMM than with real melanoidins throughout the entire ph range from 3-8.The results are shown in the graph in Fig 6.

CLARIFICATION STUDY AND COD REDUCTION

Melanoidin pigments are complex products formed as a result of non enzymatic reactions between sugars and amino acids and the first and foremost aspect to be addressed is the dark brown color along with the thick and viscous nature which pollutes and damages the place or the thing where they are dumped. So clarification and decolorization studies are taken up with respect to incubation time and ph to test the potential of PC to degrade melanoidins. The fungus was used to study the visual clarification and decolorization of the real melanoidins and the synthetic melanoidin mixture (SMM). Decolorization study along with monitoring of reduction in COD levels at each 24 hr interval was carried out by growing PC in the optimized conditions namely at room temperature **30**±2C,ph range 4.5-5.5 for 10 days in the presence of SMM and RM separately as described in materials and methods.The synthetic and real melanoidins were visually clear and transparent after the 10 day treatment with PC.

40% decolorization was observed in both SMM and RM at pH 3 and it increased as the ph is raised from 3-6.5.At ph 6.5 the maximum clarification and decolorization was observed with both SMM and RM.As the ph is raised beyond 6.5 the % decolorization decreased and only 50% was observed at ph 8 with RM and 60% was shown with SMM. On the whole, SMM were decolorized more than the RM throughout the ph range from 3-8.The scenario of the results is shown in the graph in fig 7.

During the initial stage of the experiment on the 2nd day the COD reduction that was observed was 10% and COD was reduced to 75% and 70% with SMM and RM respectively as the experiment went on till 10th day. A 10% reduction of COD was observed at ph 3 and the COD was reduced further as the ph is increased to 5.5.This trend was observed in the case of both SMM and RM. At ph 5.5 and 6, 60% and 50% reduction in COD was observed with SMM and RM respectively. The results are shown in the graph below in Fig 8.

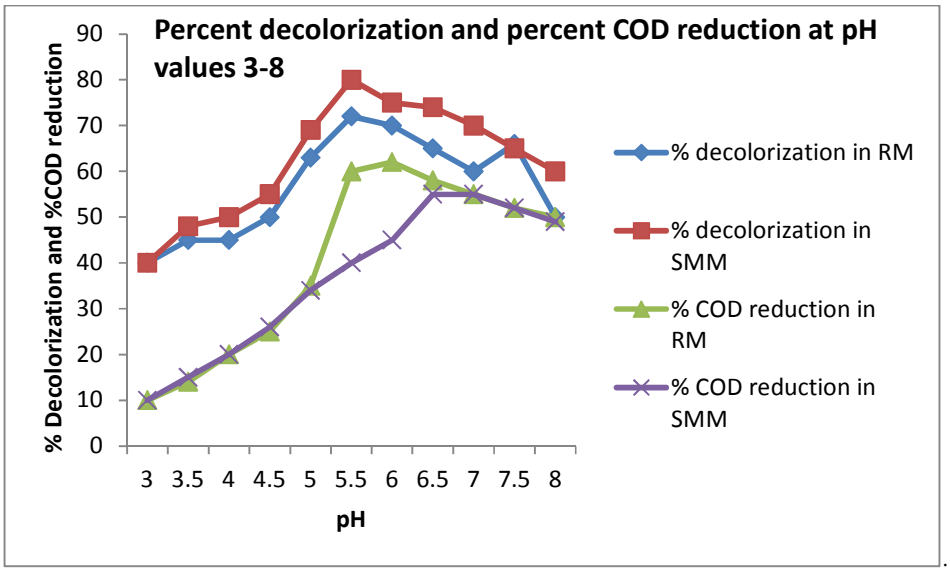

Fig 7: Decolorization and COD reduction at pH range 3-8

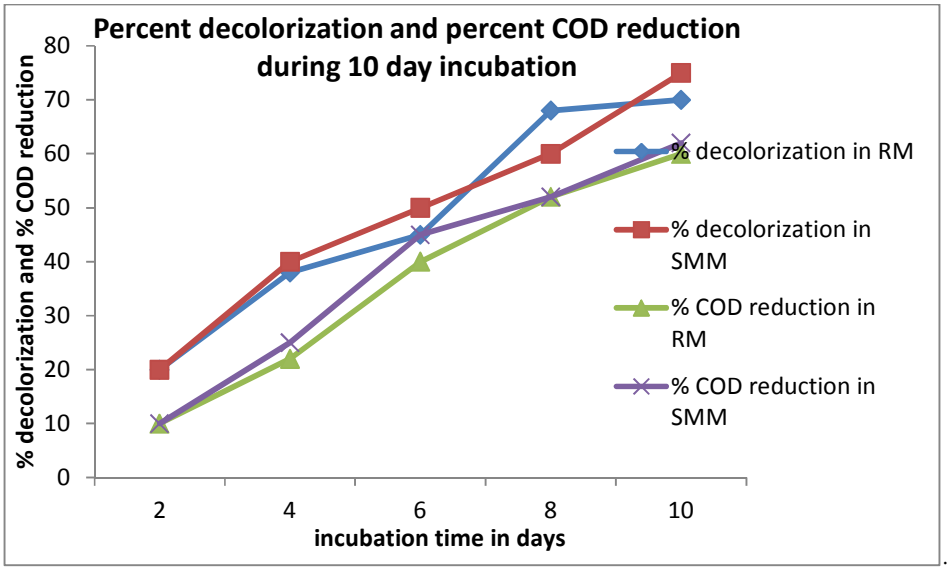

Fig 8: Decolorization and COD reduction at different days of incubation

To understand whether the fungal fermented broths with the effluent and SMM are clarified by the enzymes developed the percent transmittance (%T) was monitored every day of the study period and the results are given below in fig 9.The %T was increased from 27.54% on the first day to 77.62% on the tenth day of the study period in SMM whereas it rose from 25.11% to 63% in RM showing that about 50% increase in transmittance demonstrates the proof for clarification of SMM and RM by the enzyme activity. One of the important points is that increase in %T is more in SMM than in RM as the results showed that the difference between first day and tenth day was 50.1% in SMM whereas it was 38% in RM (Fig 9).

The results showed that the decolorization and reduction of COD were maximum at a ph range 4.5 -6.5 when the enzyme activity was maximum. It means that the enzymes play a key role in the decolorization of melanoidins whether synthetic or real. 80% decolorization of the SMM and 70% decolorization of RM was observed in the broth cultures. This might be because of the fact that in SMM exclusively melanoidins are present whereas in RM

collected from the distillery other substances like the unreacted sugars and proteins which do not react with the enzyme but can interfere with the reactions are also mixed up with the RM. It seems that due to this reason there is a little more decolorization in SMM than RM.

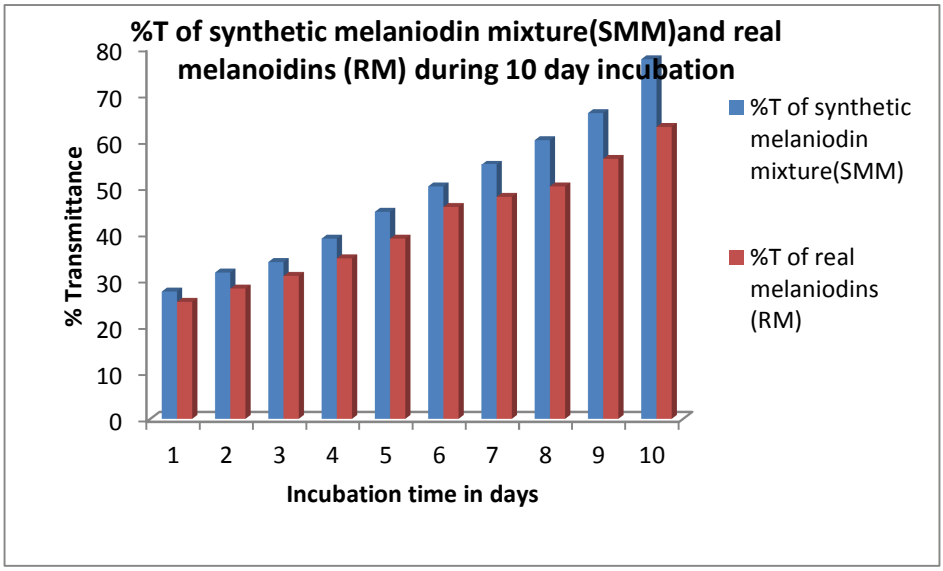

Fig 9: %T of synthetic melaniodin mixture(SMM) and real melanoidins (RM) during 10 day incubation

The decolorization of both synthetic and real melanoidins was successfully accomplished by the wood rot fungus *P. chrysosporium* BW808 (MTCC 787).It is evident from the study that the optimum pH range for the laccase and peroxidases, 4.5-6.5, is the pH range where the enzyme activity and decolorization were maximum. It also follows from the study that there is a remarkable reduction in COD up to 55% at pH 6.

CONCLUSION

The decolorization of melanoidin by PC was shown to be closely related to the composition and pH of the medium and the nature of melanoidins.

Thus, it can be concluded that the environmental factors like pH, temperature, and nutrients play a vital role in microbial degradation process of industrial wastes because the activity of enzymes is greatly influenced by them various environmental factors. It can be inferred that the development of enzymes in the broth culture is crucial to the clarification and decolorization and reduction of COD of SMM and RM. Further experimental and analytical work is required to understand the exact function of each enzyme in degradation of melanoidin pigments.

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