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Decolorization of molasses effluent using isolated strain

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ABSTRACT

After use of molasses as a raw material for fermentation such as in alcohol and amino acid production, a large amount of colored substances remain in the fermentation as effluent after recovery of the product. Molasses waste water is one of the most difficult waste products to dispose off because of low p H and dark brown color. Laboratory experiments were conducted to decolorize molasses effluent using the fungal strain isolated from the effluent. The physicochemical characteristics of molasses effluent were evaluated. The optimum pH and temperature for the decolorization activity of the isolated fungal strain was found to be 6.0 and 30^{0}C respectively. About 30% color removal was achieved on the second day in all effluent concentrations (0.5-5%) studied, without any carbon and nitrogen source. However, maximum decolorization of 60% with added glucose (carbon source) and maximum reduction in COD of 50% was obtained on the sixth day. In all the studies, it was found that decolorization increased upto 6 days and then decreased. This was due to the adsorption and desorption of pigments to the fungal mycelial pellets. Further characterization studies for the identification of the fungus revealed it to be *Aspergillus*. It was concluded that this fungal culture might have high potential application in reducing the pollution of molasses effluent prior to its disposal.

KEYWORDS

Decolorization;
Effluent;
Biodegradation;
Melanoidin.

INTRODUCTION

Recalcitrant and pollutants are becoming a major concern to global ecology and environment. An awareness of environmental problems and potential hazards caused by industrial wastewaters has prompted many countries to limit the discharge of untreated effluents[1]. Widespread contamination of soil as well as ground water and surface water near the industrial area has brought this problem to the forefront. Cleaning up of environmental pollution also presents a serious economic burden to the society. Considering the magnitude of this financial burden, it becomes apparent that cost effective yet efficient methods of decontamination are vital to our success in solving the hazardous waste problem[2].

Molasses is an important by product of sugar industry and is widely used as a carbon source in microbial fermentation industries because of its availability[3]. After use of molasses as a raw material for fermentations such as in alcohol and amino acid productions, a large amount

of colored substances remain in the fermentation as effluent after recovery of the product. Molasses wastewater or spent wash is one of the most difficult waste products to dispose off because of low pH and dark brown colour[4]. The recalcitrant nature of molasses is due to the presence of melanoidins, which are formed in the Maillard amino-carbonyl reaction[5]. These compounds have antioxidant properties, which render them toxic to aquatic micro and macro organisms[6]. The highly colored components disposed into natural water bodies may result in their eutrophication. Melanoidins also lead to a reduction of sunlight penetration in rivers, lakes or lagoons which in turn decreases both photosynthetic activity and dissolved oxygen concentrations which is detrimental to aquatic life. Disposal of effluent in the land is equally hazardous, causing a reduction in soil alkalinity and manganese availability, inhibition of seed germination and ultimately ruin the vegetation[7].

In order to minimize pollution along with toxicity in wastewaters, these effluents are treated by biological oxidation in aerated lagoons and/or activated sludge systems[8]. Such treatments have the ability to reduce BOD and COD of effluents, but they fail to reduce colour significantly. Efforts have also been made by the industries to develop methods for colour removal at least to some extent, using metal coagulants such as compounds of alumina, calcium, magnesium and iron. Physical and chemical processes such as precipitation and sorption remove high molecular weight materials, color, toxicity, suspended solids, and COD, but BOD and low molecular weight compounds are not removed efficiently[9]. Presently available biological and physical methods do not appear to be the ultimate solution to waste treatment problems but rather methods to transform waste to another form.

One method that has become increasingly popular is bioremediation. The underlying basis of bioremediation is the natural process of biodegradation which can reduce the concentration of pollutants and completely oxidize some organic pollutants to carbon dioxide, water, nitrate and other inorganic components that can be accommodated in the environment[10]. Micro organisms are especially useful for bioremediation because of their great metabolic diversity. It is the harnessing of these microbiological activities and their implementation in advanced engineering applications that form the basis for the increasing use of bio treatment systems for biodegradation of even the most difficult hazardous chemical[11].

In this study, an effort was made to decolorize molasses effluent with the aid of microbes isolated from effluent and soil collected from the area of disposal of wastewater.

MATERIALS AND METHODS

Collection of molasses effluent

Cane molasses effluent was collected from Sakthi sugars limited, Tamilnadu where about 12 lakh litres of effluent is drained out from the plant per day. The effluent samples were stored at 4°C

Analysis of physicochemical characteristics of molasses effluent

The effluent sample was analysed for various physicochemical parameters. The colour of molasses effluent was found to be dark brown, and pH was 3.84. The amount of dissolved oxygen, BOD and COD were found to be 3.5 mg/L,200mg/L and 600mg/L respectively. The amount of TDS and TSS are 91g/L and 25.5g/L respectively. The values of reducing sugar and non reducing sugars were found to be 9.878mg/L and 11.215mg/L respectively. Lignin peroxidise activity was estimated by the method of Tien and Kirk[16] and was found in trace amounts.

Isolation and screening of melanoidin degrading microbes

Sample sources used for isolation of melanoidin degrading microbes were soil from the area of disposal of the effluent and molasses effluent. The samples were serial diluted and spread plated on isolation medium containing 0.5-1% molasses effluent and agar. These plates were incubated at 30°C for 48hrs and were checked for microbial growth as well as for clear zones.

Primary screening was carried out by plate assay method[13] for selection of melanoidin degrading microbes. Molasses effluent was used as carbon source. The strains screened through plate assay method were subjected to secondary screening on giant colony medium[14] with molasses effluent of 5% and 10% as carbon source. The screened strains were inoculated onto

the centre of agar plate and cultivated at 30°C for 10 days to obtain a giant colony. The decolorization ability of the cultures was compared. Colonies producing decolorization zones were selected and the isolates were maintained in molasses effluent agar slants.

Decolorization experiments

The absorbance at 475 nm is commonly used as a standard index to determine molasses pigment decolorization[1]. Melanoidin decolorizing activity was measured as decrease in optical density or absorbance at 475 nm. Decolorization assay was carried out to select the organisms producing maximum decolorization.

Microbes producing decolorization zones in giant colony method were inoculated in Vogel's mineral media and incubated at 30°C for 48 hours. 1%(v/v) of microbial inoculum from the preinoculum was inoculated into molasses effluent medium. The medium was incubated at 30°C for 10 days at 200rpm. Samples were withdrawn at 24 hour intervals for chemical analysis and decolorization measurement.

For decolorization measurement, 1ml of culture medium was centrifuged at 8000 rpm for 15 minutes, and the supernatant was assayed for decolourization activity.

Optimization/standardization of conditions for decolorization

Various parameters were optimised to achieve better decolourization. To study the effect of pH, isolated microbial culture was inoculated in the molasses effluent medium and adjusted to different pH levels[3-7] and incubated at room temperature. Optimum temperature needed for decolorization was determined by incubating the culture medium at varying temperatures (4 °C, 11°C, 30°C and 37°C). Decolourization assay was carried out to study the effect of pH and temperature on the decolorization activity of the isolated strain.

Molasses effluent medium of different concentrations (0.5%, 1.4%, 2.5% and 5%) were inoculated along with the isolated strain and assayed for decolorization to optimize the maximum concentration required for decolorization. To study the effect of different carbon sources on decolorization, glucose, fructose, sucrose, galactose and glycerine were added to molasses effluent medium at 0.5% concentration and assayed for decolorization.

To optimise the effect of glucose concentration on color removal 0.2%, 1%, 2.5% and 5% of glucose was added to the molasses medium along with the isolated strain Nitrogen sources at 0.2% (w/w) concentration were added to molasses effluent medium. Both organic (yeast extract, beef extract and peptone) and inorganic nitrogen sources (ammonium sulphate and sodium nitrate) were used to study the effect on the decolorization ability of the isolated strain.

Effect of pre-treatment

As molasses effluent contained nonreducing sugars, pretreatment was carried out to covert nonreducing sugar to reducing sugar. In this case, 2N hydrochloric acid was added to the culture sample and kept in a boiling water bath for 25 min. Contents were cooled immediately and neutralised with 2N sodium hydroxide solution. The pretreated sample was used for DNS assay.

Changes in selected physicochemical parameters after treatment

After treatment of the effluent with the isolated strain, effluent samples were tested for pH, COD and lignin peroxidise activity.

RESULTS AND DISCUSSION

Molasses wastewater is a dark brown colored recalcitrant effluent which has a high chemical oxygen demand and high pollution potential. Accordingly, increasing attention has been directed towards utilising microbial activity for the decolourisation of molasses effluent. TABLE 1 give the result of physicochemical parameters of molasses effluent.

TABLE 1 : Physicochemical characteristics of molasses effluent

S.No.	Parameters	Results
1.	Color	Dark brown
2.	pH	3.84
3.	Total dissolved solids (g/L)	91.0
4.	Total suspended solids (g/L)	25.5
5.	Dissolved oxygen (mg/L)	3.5
6.	Biochemical oxygen demand (mg/L)	200
7.	Chemical oxygen demand (mg/L)	600
8.	Reducing sugar (mg/L)	9.878
9.	Non- reducing sugar (mg/L)	11.215
10.	Lignin peroxidise activity (mole/mL)	0.05

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The amount of total dissolved solids (TDS) and total suspended solids (TSS) are 91g/L and 25.5 g/L respectively. The estimated values were much higher than the reported values given by Trivedy[18]. The higher amounts of TDS and TSS may be due to the presence of melanoidins and other colloidal substances.

The amount of dissolved oxygen, biochemical oxygen demand and chemical oxygen demand were found to be 3.5 mg/L, 200 mg/L and 600 mg/L respectively. The high BOD may be due to the presence of organic load and also due to the presence of sugar and oil from the machineries. The COD/BOD ration of the mill effluent was found to be 1:3. A similar result was reported by Rao and Dutta[12] indicating that the waste is amenable to biological treatment.

The values of reducing sugar and non-reducing sugars were found to be 9.878 mg/L and 11.215 mg/L respectively. It was reported by Satttler and Zerban[13] that about 10% of the reducing power of the fermentable substances in cane molasses was due to volatile ingredents, such as hydroxyI methyI furfural, acetoin, formic acid and levulinic acid, which were the decomposition products of he sugars. Unfermentable substances in cane juice are accelerated by heat and the fact that the high temperature used in processing cane juice in sugar mills and the subsequent storage of molasses under hot tropical sun increase the amount of non-fermentable substances.

Isolation and screeing of melanoidin degrading microbes

Low concentration of molasses effluent (0.5% and 1%) was used for isolation. A total of 36 organism including bacteria and fungi were isolated. Among them 10 organisms were selected trough primary screeing by plate assay method. 3 microbial strains- one fungal species, BS-I and two bacterial strains PC-I and CR-I were selected by secondary screening using giant colony method. The cultural characteristics of the three strains following secondary screeing are depicted in TABLE 2.

TABLE 2 : Cultural characteristics of isolated strains from molasses effluent

Strains	Molasses effluent Agar	Vogel's mineral medium	Decolorization zones
BS-I (Fungus)	White mycelial growth with black spores	Turbid, homogenous with pellicle formation. Mycelial mat was formed	+++
PC-I (Bacteria)	Pink, concentric, ring shaped, circular, entire and moist colonies	Turbid with pink colored growth	++
CR-I (Bacteria)	Cream, circular and smooth colonies	Uniform and fine turbidity	+

From TABLE 2 it is clear that the fungal strain BS-I produced highest decolorization zone compared to PC-I and CR-I.

Decolorization assay was carried out to select the organism producing maximum decolorization. Different concentration of molasses effluent (0.5%,1.5%,2.5% and 5%) were used for comparing the decolorization activity of 3 strains. Though the two bacterial strains produced decolorization zones, the percentage of decolorization was found to be low and hence the two strains were not used for further studies.

Among the different molasses effluent concentrations (0.5%, 1.5% and 5%) tested 1.5% exhibited maximum decolourisation activity of 48.62% on the fourth day (Figure 1).

Increased concentrations generally reduced both percentage decolorization and COD removal. Photographs 1 and 2 exhibits the decolorizing activity of BS-I and different growth patterns of BS-I at different effluent concentrations.

The inhibitory effect of increasing molasses effluent concentrations is likely due to the presence of inhibitory compounds such as phenolics, gallic and vanillic acid. These results show that substantial dilution is required for molasses effluent before biological treatment. For further optimisation, 1.5% dilution was used.

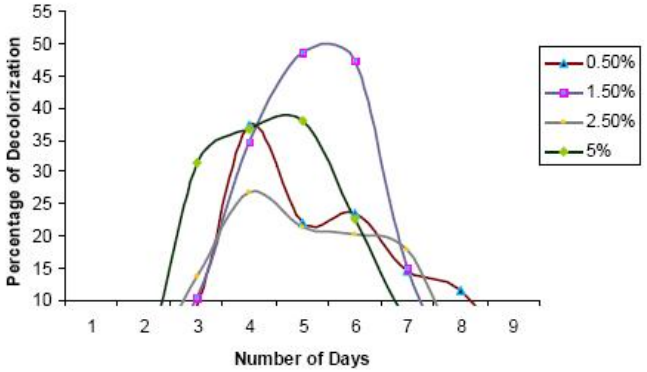
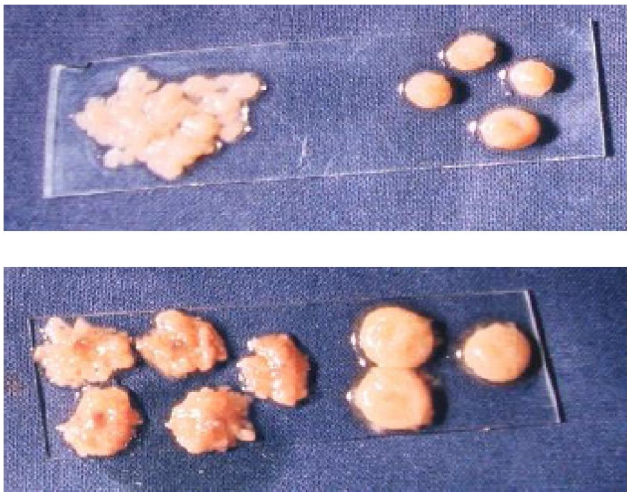


Figure 1 : Effect of different effluent concentrations on decolorization activity of *BS-I* strain

Photograph 1 : Decolorization activity of BS-I strain at different effluent concentrations (0.5%, 1.5%, 2.5% and 5%)



Photograph 2 : Growth characteristics of *BS-I* strain at different effluent concencentrations (0.5%, 1.5%, 2.5% and 5%)

Optimisation of conditions for decolorization of effluent using BS-I strain

The influence of pH in the decolorisation process was very important, as it was noted that some compounds responsible for waste water colour were soluble over a certain pH range. The melanoidin solubility depends on the pH, it is less soluble at acidic pH than at alkaline pH[1]. Therefore the pH of the wastewater was modified by the addition of hydrochloric acid and sodium hydroxide and tested for decolorization (Figure 2).

The maximum percentage decolorisation was around 49% with an initial pH of 6.0 on the sixth day of the experiment. The degradation was better than other pH even on the second and fourth day. But there was no steady increase in the decolorisation due to the adsorption and desorption of the melanoidin pigments on the mycelia.

The effect of culture temperature on melanoidin decolorising activity was examined in the range of 4-37°C (Figure 3).

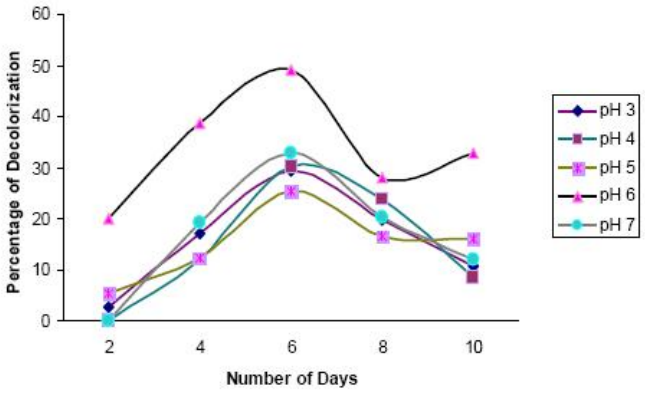


Figure 2 : Optimum pH range for decolorization activity of the isolate.

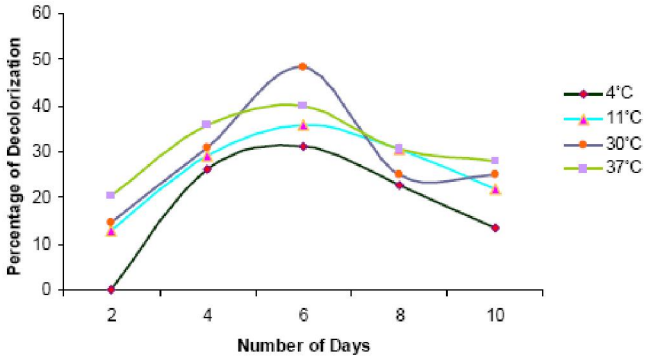


Figure 3 : Effect of temperature on decolorization activity of isolate

The optimal temperature for maximum decolorisation was found to be 30°C on the sixth day. The achieved decolorization might be due to the degradation of smaller molecular weight fractions of melanoidin[14]. Preliminary experiments proved that colour removal was higher when the culture medium was sterilised. As a result sterilised culture medium was used for all the experiments. Thus better colour elimination could have been due to structural changes in compounds when the wastewater was treated at high temperature (Pena et al., 1996).

The presence of a readily available carbon source was necessary for the growth of the culture and also for melanoidin degradation. Different carbon sources exhibiting decolorization activity is shown in figure 4.

Glucose gave the highest growth and decolorization of 48.26% on the 10th day. This indicates that the effluent contained little amount of easily metabolisable carbon content. Therefore, addition of readily available carbon source was necessary for microbial metabolism in the effluent medium.

Since glucose gave the highest decolorization, dif-

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ferent concentrations of glucose were used (Figure 5).

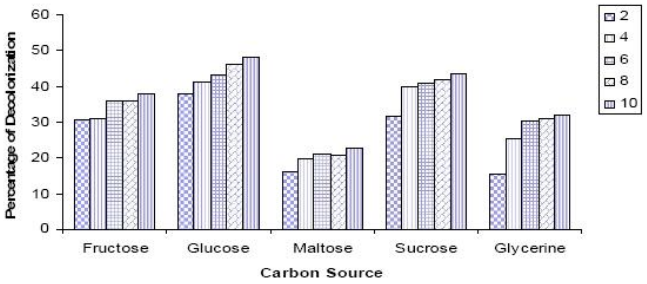


Figure 4 : Effect of supplementary carbon sources on decolorization activity of isolate

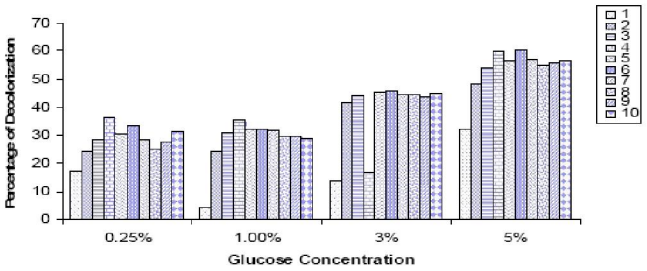


Figure 5 : Effect of different concentration of glucose on decolorization activity

5% glucose gave the highest decolorization activity of 60% on the 6th day under optimum conditions. Higher concentration of glucose results in greater adsorption and pellet formation of the culture in shaking conditions of 200rpm. This might be the reason for higher decolorization.

Among the different nitrogen sources tested, peptone was the most advantageous. For a high activity, an organic nitrogen source like yeast extract, peptone and beef extract was found effective whereas inorganic nitrogen sources like ammonium sulphate and sodium nitrate was unsuitable (Figure 6).

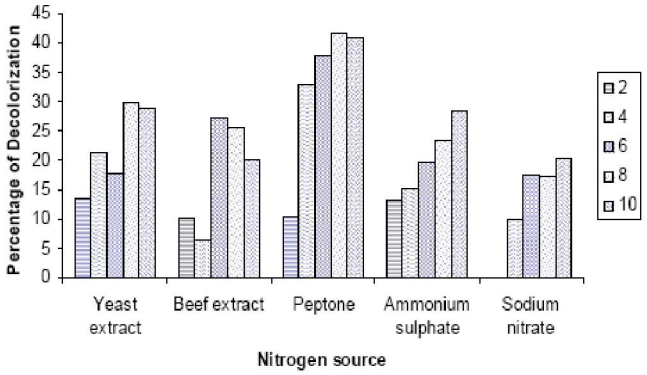
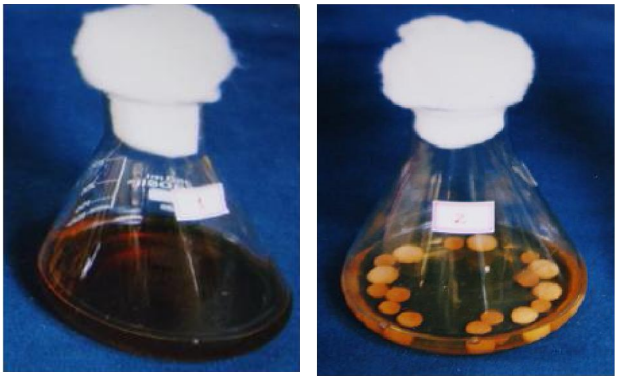


Figure 6 : Effect of nitrogen sources on decolorization activity of isolate

However, lack of additional nitrogen did not inhibit either growth or decolorization, as did the lack of additional carbon source.

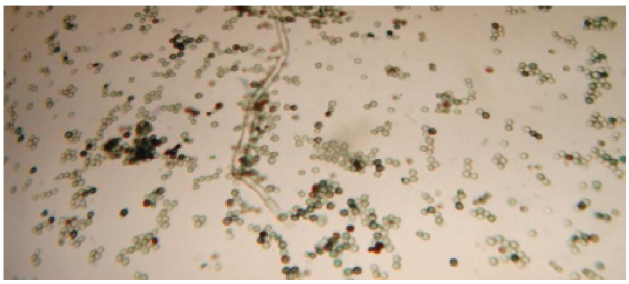
Since molasses effluent contained non-reducing sugars, pretreatment was carried out to convert non reducing sugar to reducing sugar. This pretreatment helped in greater degree of decolorization initially. Non reducing sugar was converted to reducing sugar such as glucose and fructose following acid hydrolysis and this would have been the reason for more degradation in the beginning. Photograpgh 3 shows the effluent sample used and the decolorized sample following inoculation with the fungal strain.



Photograph 3 : Molasses effluent and the decolorized sample following inoculation with the fungal strain *BS-I*

Decolorized effluent sample was checked for pH, COD and lignin peroxidise activity. There was a decrease in pH indicating the presence of acidic metabolites in the decolorized effluent sample. Maximum percentage of COD reduction was 50% on the 6th day. Lignin peroxidise activity was found in trace amounts.

Further characterization studies carried out for the identification of the isolated BS-I strain revealed it to be *Aspergillus sp* (Photograph 4). This isolated strain was found to be efficient in decolorizing the effluent in the presence of externally added carbon source as well as dilution upto 1.5%. Genetic improvement of the



Photograph 4 : *Aspergillus* strain following lactophenol staining

strain could be explored in future for improvement of its efficiency.

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