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Decolorization of biomethanated distillery effluent by immobilized enzymes

Suneeta Panicker, Arveen Singh and Snehal Agnihotri*
Dr. D. Y. Patil Arts, Commerce and Science College, Sant Tukaram Nagar, Pimpri, Pune

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Abstract: The massive quantity of colored distillery wastewater called molasses spent wash (MSW), if disposed untreated can cause considerable stress on the water sources leading to widespread damage to aquatic life. Microbial decolourization is an environment-friendly technique for removing this color.Recent research has focused on the development of enzymatic processes for the treatment of wastewaters. Enzymes like peroxidases, oxido reductases, cellulolytic enzymes cyanidase, proteases, amylases, etc.from variety of different plants and microorganisms have been reported to play an important role in waste treatment. Practical problems, such as the high-cost isolation, purification, instability in organic media and high temperatures, restrict their use. In present work, the possibility of using immobilized enzyme to enhance the biodegradability of the distillery-spent wash has been investigated. Four bacterial strains showing decolorization of 30% Biomethanated wastewater were isolated from the sites where distillery effluent being discharged. The extracellular enzyme responsible for decolorization was purified, using a series of purification steps that included ammonium sulphate precipitation and gel permeation using sephadex G-100 column chromatography. The purified enzymes from each isolate were with a molecular mass of 68.7kDa and were capable of 61.8% decolorization of 60% effluent. Alginate immobilization of these enzymes led to 62.97% decolorization after 120 hours. Reusability of the immobilized enzyme showed that it could be used with similar efficiency for 3 cycles and thereby it decreased. This data can be referred to in developing a technology for the removal of a specific colorant from effluent in future.

Key words: Biomethanated Distillery Effluent, Decolorization, Enzyme purification, Immobilized Enzymes, Reusability.

INTRODUCTION

According to recent estimate, the alcohol production in India has reached the 3.29X 10^9 liters and generating 40.40 X10^{10} liters of wastewater called molasses spent wash (MSW), annually (1). This massive quantity, if disposed untreated can cause considerable stress on the water sources leading to widespread damage to aquatic life. A wide variety of aerobic microorganisms capable of decolorizing spent-wash include bacteria, fungi, cyanobacteria and yeasts. Recently, it was shown that the biomass of *Aspergillus oryzae JSA-1* could effectively reduce the total colour of undiluted BME samples up to 99.16 % (2). The enzymatic treatment falls between the physicochemical and biological treatment processes. It has some potential advantages over the conventional treatment. These includes: operation over a wide range of pH, temperature and salinity, absence of shock loading effects, absence of delays associated with the acclimatization of biomass, reduction in the sludge volumes and the ease and simplicity of controlling the process (3). Recent research has focused on the development of enzymatic processes for the treatment of wastewaters, solid wastes, hazardous wastes and soils in recognition of these potential advantages (4).

A large number of enzymes (e.g. peroxidases, oxidoreductases, cellulolytic enzymes cyanidase, proteases, amylases, etc.) from a variety of different sources have been reported to play an important role in an array of waste treatment applications (5). Laccases are common enzymes in nature, especially in plants and fungi. Practical problems, such as the high-cost, isolation & purification process and instability in organic media and high temperatures, restrict their use. To overcome these limitations, several methods have been suggested and the most important of which are immobilization techniques (6). Enzyme entrapment uses natural and synthetic polymers, such as agarose, agar and gelatin. In the present work, the possibility of using immobilized enzyme to enhance the biodegradability of the distillery-spent wash has been investigated. This integrated technique is being considered in light of a new possibility offered in the field of wastewater treatment. Thus the aim of the present study was to purify and immobilize the bacterial enzymes capable of decolourizing the distillery effluent.

***Corresponding Author:**
Dr. Snehal Agnihotri,
Dr. D.Y. Patil Arts, Commerce and Science College,
Pimpri, Pune,
Maharashtra, India.

MATERIALS AND METHODS

Collection of Biomethanated Distillery Effluent (BME) and soil samples

Biomethanated effluent sample was obtained from anaerobic treatment plant set up at molasses distillery Malegaon Sahakari Sakar Karkhana, Baramati, Maharashtra, India. The sample was centrifuged at 10,000 rpm for 30 minutes and centrifuged at 4°C to avoid further oxidation. In the present, soil sample was collected from sites where distillery effluent being discharged continuously for last few years in nearby vicinity of biomethanation plant from Malegaon Sahakari Sakar Karkhana, Baramati, Maharashtra, India.

Screening and isolation of organisms having the ability to decolorize the BME

Screening of the organisms having the ability to decolorize the biomethanated effluent was carried out by spreading the diluted soil sample on sterile nutrient agar plates, supplemented with 30% effluent for bacterial strains. Soil sample was serially diluted in sterile saline was spread on sterile agar plates. The plates were incubated at room temperature until visible microbial growth. The organisms showing zone of decolorization of the effluent in the agar plate after four days were selected for further studies. To check the decolorization potential of the strains, they were subjected for decolorization in liquid medium supplemented with 30% effluent. The isolated bacterial colonies were sub-cultured and maintained on sterile NA slants respectively. Identification of bacterial isolates showing potential effluent

decolorization was done by the standard methods of microbiology with respect to colony characteristics and biochemical characterization using Bregey's manual.

Enzyme production by microorganisms

Enzyme production by decolorizing bacterial isolates was performed by growing the isolates in yeast extract (0.1%) broth supplemented with 60% effluent and incubating for 4 days at 150rpm.Presence of effluent decolorizing enzymes was checked by agar well diffusion technique and by the reduction in the absorbance of effluent at 475nm.

Purification of enzymes

The extracellular enzyme responsible for decolorization was purified, using a series of purification steps that included ammonium sulphate precipitation (80% saturation) and gel permeation using sephadex G-100 column chromatography. These enzymes were further checked for their stability and activity by well diffusion technique on effluent supplemented basal agar media plates. The molecular weight of the isolated enzyme(s) was determined by 12.5% SDS PAGE and commassiee blue.

Immobilization of enzyme

Partially purified enzyme was mixed with 3% sodium alginate in 1:1 ratio (i.e. 250µl: 250µl) under continues stirring. The mixture was syringed into a stirring 0.2M $CaCl_2$ solution, and the resulting beads were left overnight at 4°C for hardening. The entrapped enzyme beads were washed three times by deionized water, before using them for the decolorization study.

Decolorization of effluent by immobilized enzyme and free enzyme

Decolorization of effluent by immobilized enzyme, free enzyme, was investigated by adding 250µl of each sample (i.e. immobilized enzyme, free enzyme) in 3ml of 60% effluent and kept for incubation at 37°C for different time periods. Relative percentage of decolorization was determined by measuring the absorbance at 475nm after different time periods for 5 days and comparing it with the absorbance of untreated effluent.

Reusability of immobilized enzymes for decolorization

Reusability of alginate immobilized enzymes and cells were investigated to check the economy of the immobilized enzyme. The used beads were filtered at the end of each cycle and washed three times with the 10mM phosphate buffer prior to their use for the treatment of the fresh 60% effluent.

RESULTS

Screening, isolation and identification of organisms having the ability to decolorize the BME

Screening of the bacterial strains, having the ability to decolorize the biomethanated effluent on sterile nutrient agar plate supplemented with 30% effluent, showed the presence of decolorizing zones around four bacterial isolates. Bacterial strains showing decolorization were isolated on nutrient agar plates and sub-cultured them on nutrient agar slants. Through colony morphology, microscopic observations and biochemical tests, isolate 1 was identified as *Desulfotomaculum sp.*, isolate 2 as *Methylococcus sp.*, isolate 3 as *Saccharococcus sp.* and isolate 4 as *Acidomonas sp.* Isolate 1 and isolate 2 were more promising showing significant decolorizationof60% effluent (Figure 1). These strains were selected for further studies.

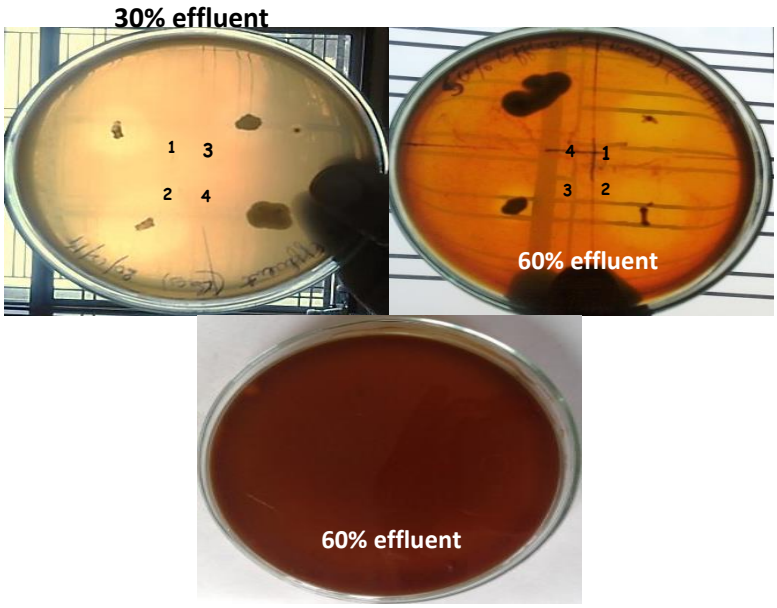


Figure 1: Isolates showing zone of decolorization of 30 % and 60% effluent after 48 hours

Enzyme production by microorganisms

Agar diffusion technique, performed to check the presence of decolorizing enzymes in the culture supernatant, on effluent supplemented basal agar plates, showed significant zones of decolorization within two hours and went on increasing for 4 days. On last day the complete decolorization of the plate was observed. The decolorization efficiency of the enzymes secreted by the isolates checked in the yeast extract broth supplemented with 30% effluent showed that the isolate 2 could decolorize the effluent maximally (58.05%) after 144hrs (Figure 2).

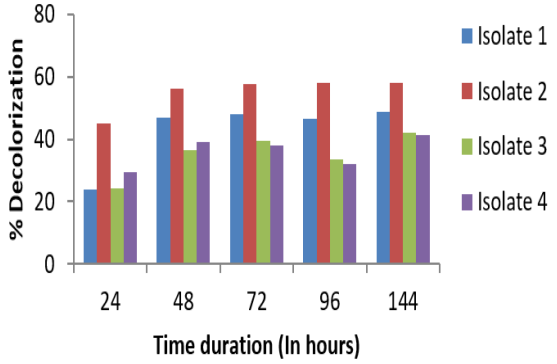


Figure 2: Percentage decolorization of BME by culture supernatant

Purification of enzymes

The culture filtrates of bacterial isolates were concentrated using ammonium sulphate (80% saturation) and the concentration of the protein was determined to be in a range of 0.5 mg/ml to 0.9 mg/ml. The concentrated enzyme from each isolate (2 ml each) was subjected to gel filtration using Sephadex G-100 column. The enzyme efficiency was monitored by agar diffusion and the decolorization was achieved within 2 hours and went on increasing for 8 hours (Figure3 and 4). The partially purified enzyme from isolate 2 showed a maximum of 61.80 % decolorization in the broth supplemented with 60% effluent after 24hrs and decreased thereafter (Figure 5).

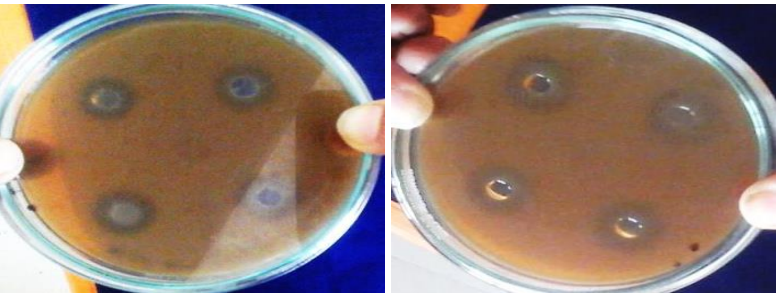


Figure 3: Decolorization by decolorizing
Figure 4: Decolorization by decolorizing enzyme after 2 hours enzyme after 8 hours

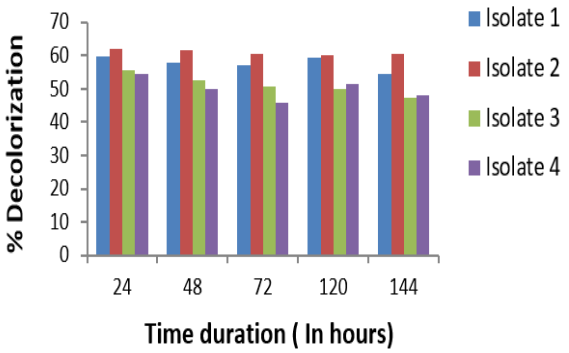


Figure 5: Percentage decolorization of 60% effluent by decolorizing enzyme

Gel electrophoresis

Purified proteins were loaded on a 12.5% SDS-PAGE and the gel was stained with commassie blue. The purified enzyme from each isolate was homogeneous showing a single protein band on SDS-PAGE with a molecular mass of 68.7 kDa when compared to standard molecular weight marker (Figure 6). Based on the size and the literature search, this enzyme probably could be the known melanoidin decolorizing laccase enzyme.

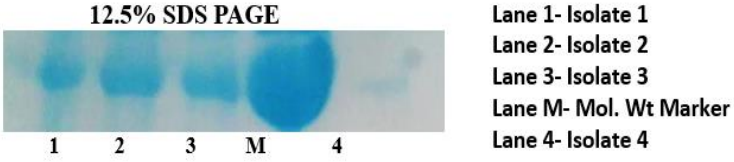
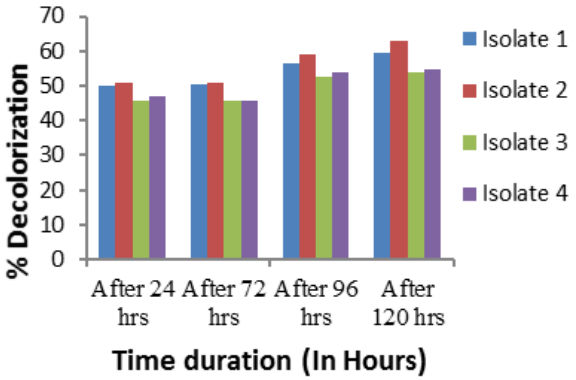


Figure 6: 12.5% SDS-PAGE with standard molecular weight marker

Decolorization of effluent by immobilized enzyme and free enzyme

Spherical and regular-shaped enzyme-alginate beads were obtained on gelation. Percent decolorization went on increasing from 50.89% after 24hrs to 62.97% after 120hrs, whereas in case of the free enzymes, the decolorization percent went on decreasing from 58.38 after 24 hrs to 55.94 after 120hrs. However the comparison of decolorization results indicated high stability by immobilized enzymes and confirmed the protective role by alginate immobilization. The results are depicted in Figures 7 and 8.



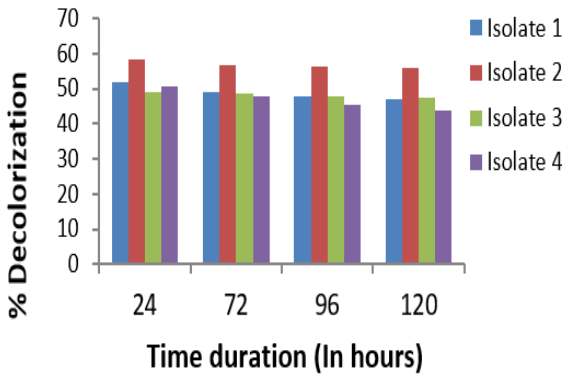


Figure 7: Percentage decolorization by
Figure 8: Percentage decolorization by Immobilized enzyme free enzyme

Reusability of immobilized enzymes and cells for decolorization

In this work, reusability of the immobilized enzyme showed that the immobilized enzyme could be used with similar efficiency for 3 cycles and thereby it decreased. The residual activities are presented in Figure 9.

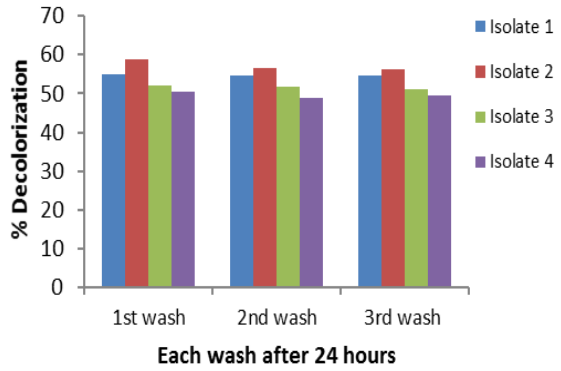


Figure 9: Percentage decolorization by immobilized enzyme during reusability

DISCUSSIONS

Distillery effluent is one of the most difficult waste products to dispose, because of low pH and dark brown color. The dark color remains as a problem, which requires pre-treatment before its safe disposal into the environment. Physical or chemical methods of waste treatment are invariably cost intensiverequire high reagent dosages and generate large amount of sludge (7) and cannot be employed in all industries. While some scientists have screened the standard basidiomycetous fungi for their decolorization potential (8), others have isolated bacteria from natural habitats and obtained good decolorization yields (9).

The present investigation was carried out with the objective of studying the decolorization efficiency of bacteria isolated from natural habitats adjacent to the effluent dumpsites and to make an effort to increase the stability and economy of the enzymes responsible for the degradation of colorants in distillery effluent by immobilization. Four Bacterial isolates from the molasses distillery of Malegaon Sahakari Sakar Karkhana, Baramati, Maharashtra, India, showed about 60% decolorization of 30% effluent on agar plates, indicating that they can be used as bio-remediating agents. Attempts were also made by different researches to isolate bacteria having different capacities to decolorize the effluent (10, 11). In this investigation, maximum decolorization was recorded at pH 6.0-7.0 by the bacteria. Similar results were reported when soil samples were used as inoculum instead of isolated organisms (12). Isolate 1 and isolate 2 were promising with more than 60% decolorization of 60% effluent.

It has been previously shown by several workers that the microbial degradation and decolorization of melanoidins is mediated by enzymes such as glucose oxidase, sorbose oxidase, manganese dependent peroxidases etc. (13). To ascertain the involvement of any enzyme in decolorization process by our isolates, enzyme purification by APS and Gel Filtration chromatography was performed. It was interesting to note that all organisms resulted in good decolorizing enzymes that yielded decolorization ranging from 54 to 61%, and showed the presence of 68.7 kDa protein, thus upholding the role of laccase, which convert glucose to gluconic acid, with H_2O_2 as a byproduct in the reaction. On SDS PAGE, most laccases show mobilities corresponding to molecular weight 60-100 kDa, of which 10-15% may be attributed to glycosylation (14).

The storage, stability and repeated usability of the immobilized microorganisms in wastewater treatment, is well known (15). Thus, sodium alginate beads were used for immobilization of the purified enzyme. Immobilized enzyme showed maximum of 62.97% decolorization after 120hrs as compared to the free enzymes which showed only 55.94% after same time period. The entrapped enzyme took time to reach its maximum activity whereas the free enzyme was active initially and showed decrease in its active as the time increased. This increase in time for the immobilized enzyme may be due to the time required by the substrate molecules to penetrate into the beads and reach the active sites of the enzyme. Calcium alginate entrapped dextran sucrose took 60 minutes to achieve the maximum enzyme activity, which was 4 time higher than the free enzyme (16)

The study showed that these immobilized enzymes could be effectively used for a minimum of three cycles repeatedly to decolorize effluent which will provide a good economy for the effluent treatment. It was slightly decreased with repeated loadings in case of bacterial cultures. Diffusion limitations may be one of the possible explanations for this result. This decrease in activity may be due to the leakage of enzyme from the beads, occurred due to the washing of beads at the end of each cycle. In another study it was reported that alpha amylase entrapped in Ca-alginate beads was reused for 6 cycles with ~ 30% loss in activity (17).

The bacterial strains employed during the present investigation might have brought about the cleavage of ethylinic C=C and azomethine C=N linkages. Therefore, conjugation is broken due to enzymatic oxidation; hence UV intensity (at 475 nm) is reduced (i.e., polymers being broken down to monomers or dimmers) (18).

CONCLUSION

Our results thus, indicate that the efficiency of microbiological color removal could be further augmented by use of the method of immobilizing enzymes. The investigation has come out with efficient enzymes isolated from microbial isolates capable of decolorization of 60% effluent by immobilizing these enzymes and created an understanding of the extent of degradation of the colorant by these efficient isolates. This data can be referred to in developing a technology for the removal of a specific colorant from effluent in future.

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