BIOREMEDIATION OF DISTILLERY SPENT WASH USING *Pseudomonas aeruginosa, Aspergillus niger AND MIXED CONSORTIA*

Nikam S.B.*, Saler R.S. and Bholay A.D.

1. Department of Biotechnology, K. T. H. M. College, Nasik, Maharashtra (INDIA)
2. Department of Botany, K. T. H. M. College, Nasik, Maharashtra (INDIA)
3. Department of Microbiology, K.T.H.M. College, Nasik, Maharashtra (INDIA)

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ABSTRACT

Distillery spent wash contain melanoidins which are natural condensation products of sugar and amino acids produced by non-enzymatic Millard amino-carbonyl reaction taking place between the amino and carbonyl groups in organic substances. Melanoidins are very important from environmental aspects and due to their structural complexity, dark colour and offensive odor, these pose serious threat to soil and aquatic ecosystem. This causes the problem, like reduction of sunlight penetration, decreased photosynthetic activity and dissolved oxygen concentration in the water bodies whereas on land, it causes reduction in soil alkalinity and inhibition of seed germination. Bioremediation is an ecofriendly technology for treating chemical spills and hazardous waste. It is considered highly desirable to exploit the biodegradation potential of soil microorganisms from polluted sites. Use of microorganisms like *Pseudomonas aeruginosa, Aspergillus niger, Streptoccous sp., Bacillus sp.* and *Staphyloccous aurius* will be the cost effective biotechnology for treatment of water polluted by spent wash containing melanoidin. The individual organisms and their mixed consortia degraded the 75% to 80% concentrated spent wash. After the optimization of various physicochemical parameters the mixed consortia exhibited enhanced activity as compared to the individual cultures alone.

Key Words: Bioremediation, Melanoidin, *Pseudomonas aeruginosa, Aspergillus niger*, Consortia, Amino-carbonyl reaction

INTRODUCTION

Distilleries the alcohol producing industries are one of the major polluting industries. Distillery spent wash is the residual liquid generated during alcohol production. It has been observed that a typical cane molasses based distillery generates 15 L of spent wash effluent per liter of ethanol produced. Around 212 distillery units in India generate more than 30 billion liters of spent wash annually. The most important characteristic of spent wash is that it is strongly acidic, dark brown colored hydrophilic viscous liquid waste with strong objectionable odour. Dark brown color of spent wash is mainly because of the presence of polymeric melanoidin pigments formed by the non enzymatic amino carbonyl reaction means Millard reaction. Melanoidins are recalcitrant due to presence of caramel. Antioxidant nature of melanoidins make them toxic to many microorganisms, including those present in waste water treatment processes. Agricultural land loses their fertility due to disposal of the spent wash directly into river. It also harms the aquatic system as its colored pigments reduce photosynthetic activity and depletes the dissolved oxygen in the water bodies. Spent wash polluted water has high biological oxygen demands, chemical oxygen demands, low pH, obnoxious smell. To reduce the dark color, acidic pH, high BOD, high COD, it is considered highly desirable to exploit the biodegradation potential of soil microorganisms from polluted sites. As such polluted soils can facilitates selection of biodegradative capability in microorganisms and may act as reservoir of selective communities capable of degrading pollutants.
MATERIAL AND METHODS

Sample collections
Sample of biomethanated distillery effluent was collected from Niphad Sahkari Sakhar Karkhana - Distillery division, Niphad, Nasik, Maharashtra, India. The contaminated soil was collected from the site nearby the distillery unit. The soil was collected by scrapping the top layer of soil and subsurface soil and packed in an air tight sterile PP bag.

Isolation of organism by enrichment technique
Microorganisms screening was done by enrichment, the tubes showing decolourisation were subsequently sub cultured four times and isolation of microbial culture was carried out on minimal salt glucose medium by spread plate technique. The pure culture of different microbial isolates S1-S5 were maintained on minimal salt agar medium containing 5% spent wash.

Standard melanodin preparation
The dark brown colored standard melanodin was prepared in the laboratory by heating 1M glucose with 0.5M of glycine at 90°C and pH-5.5 for 6 hr. The absorption maxima for standard melanodin was measured at 450nm by double beam Spectrophotometer (Shimadzu) and a standard dose curve was prepared. For heating at 90°C hot air oven was used. The striking feature was that the absorption maxima for collected spent wash measured was also at 450nm. Hence, the further degradation studies were performed at 450nm.

Degradation studies
For mixed consortia loopful of pure culture of each isolate from minimal salt spent wash agar media plate was transferred to 100 ml minimal medium with 60% and spent wash in 500 ml erlenmeyer flask and incubated at room temperature 28±2°C to study the degradation ability. After 10 hr interval, 5ml aliquot was withdrawn for assaying degradation. Un inoculated minimal salt medium and minimal medium with 60% and spent wash were used as a blank and control respectively. Isolates showing excellent results were selected for further study. Same procedure was followed for Aspergillus niger and Pseudomonas aeruginosa.

COD and BOD measurement
Chemical Oxygen Demand and Biological Oxygen Demand of the samples before and after the treatment were determined using potassium dichromate and Winkler’s method respectively.

Biochemical and morphological studies of the isolates
Biochemical and microbiological characterization of the isolates was done according to standard protocols. Species identification was supported by VITEK 2 System at Bac-test laboratory Nasik, Maharashtra, India.

Optimization of physic-chemical parameters
Various parameters were optimized to achieve better degradation and COD removal activities by mixed consortia. In previous studies the same was performed for Aspergillus niger, Pseudomonas aeruginosa. To study the effect of externally added carbon source on degradation activity and COD reduction activity 1g% each of glucose, fructose, maltose, sucrose and starch was added separately in minimal salt medium containing 80% spent wash. Optimum glucose concentration required for color removal and COD reduction by the isolate was determined by using glucose concentrations in the range of 0.1-1.0%. To study the effect of various nitrogen sources, ammonium chloride, ammonium sulphate, peptone and yeast extract and casein hydrolysate were added in the minimal medium at 0.4 g% concentration.

Effect of pH on the degradation activity was determined by using the spent wash medium adjusted to pH values within the range of 4 to 9. Optimum temperature required for decolourisation was determined by incubating the isolates in the culture medium at different temperatures within the range of 25 to 45°C. Different concentrations of spent wash such as 20, 40, 60 and 80% supplemented in the mineral medium as optimized above, were inoculated with the isolates and mixed consortia the maximum spent wash concentration utilized by mixed consortia for decolorisation was determined.

HPLC analysis
Whether or not melanoidin is the main component responsible for color in the spent wash was confirmed by HPLC analysis.
analysis was carried out in Instrumen -tations laboratory K.T.H.M. College, Nasik, Maharashtra (India). 25 µl of sample was injected to the HPLC unit, mobile phase was acetonitrile: water in the proportion of 44:55 (v/v) +1.0 ml glacial acetic acid + 0.5g of sodium acetate 3H₂O, pH of the system was 5.2. Flow rate of the system was 0.8ml/min, changes in the peak height were the clue for the degradation of melanoidin in spent wash (Table 1).

### RESULTS AND DISCUSSION

As the melanoidin content molasses spent wash is highly recalcitrant waste product. Treatment studies were performed using various sugars as externally added carbon source in the spent wash medium on decolourisation and COD reduction activities were studied. Decolourisation was found to be more in presence of all carbon sources used with respect to control and was found to be maximum in the presence of glucose. Degradation of melanoidin was also confirmed by spectrophotometric analysis as decrease in optical density of melanoidin at its λmax, appearance of new peak in the spectra with respect to control was the clue for suggesting effective degradation.

#### Isolation of organism by enrichment technique

The soil samples were purposefully collected from the disposal site of the spent wash for screening efficient microorganisms. Several sets of enrichment culture were initiated before isolating some microbial strains capable of various degrees of decolourisation of spent wash. Twenty five S₁-S₂₅ microbial isolates were screened through enrichment technique, which had the ability for decolourisation of spent wash. From these 25 organisms maximum decolourisation effect was given by *Aspergillus niger* and *Pseudomonas aeruginosa*. Further de colourisation studies were performed by using these two organisms. Now results of mixed consortia were also effective. Routine biochemical and microbial tests were performed for identification of the isolates up to genus level and species was confirmed by VITEK 2 system. From overall microbial and biochemical tests the organisms identified as *Staphylococcus* sps., *streptococcus* sp., *Bacilli* sps., *Pseudomonas aeruginosa* and *Aspergillus* sps.

#### Biodegradation studies

Decolourisation was followed by spectrophotometric measurements at 450nm, which is λmax of melanoidin and spent wash. The effect of various sugars as externally added carbon source in the spent wash medium on decolourisation and COD reduction activities were studied. Decolourisation was found to be more in presence of all carbon sources used with respect to control and was found to be maximum in the presence of glucose. Degradation of melanoidin was also confirmed by spectrophotometric analysis as decrease in optical density of melanoidin at its λmax, appearance of new peak in the spectra with respect to control was the clue for suggesting effective degradation.

#### Optimisation of physico-chemical parameters

Glucose in 0.4% concentration as carbon source was found to be optimum for decolorizing activity and above 0.58% glucose there was decrease in decolorizing activity for mixed consortia (Fig. 1 and Fig. 2). This effect might be due to the acidic conditions produced in the medium after incubation by all organisms, inhibiting to the microbial growth. There was no effect of externally added organic and inorganic nitrogen on decolourisa-tion efficiency. Nitrogen from the spent wash might be sufficient for the growth of the mixed consortia. From the data, it is clear that the mixed consortia were utilizing nitrogen from spent wash contents. Maximum decolourisa-tion and COD reduction was found within the pH range of 6-6.5 (Fig. 3), the preferred range for the growth of the mixed consortia .Temperature range 30 of 37°C was found to be suitable for activity of all the isolates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention time</th>
<th>Area</th>
<th>Height</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(Before treatment)</td>
<td>2.423</td>
<td>1180608</td>
<td>50575</td>
<td>100.0000</td>
</tr>
<tr>
<td>B(48hr treatment)</td>
<td>2.435</td>
<td>1084859</td>
<td>46092</td>
<td>100.0000</td>
</tr>
<tr>
<td>C(72 hr)</td>
<td>2.699</td>
<td>895421</td>
<td>45650</td>
<td>87.3203</td>
</tr>
</tbody>
</table>
The medium composition was optimized as (g/l, glucose-4, KH$_2$PO$_4$-0.2, MgSO$_4$ - 0.009, pH-6.5 and temperature of 37°C. Under the optimum conditions mixed consortia were able to decolorize the spent wash by 59% and COD reduction by 57% after 72hr of incubation for 80% spent wash. Percent reduction in color and COD of treated spent wash under optimum performance conditions are shown in Fig. 1 to Fig. 4.

Fig. 1: Effect of different concentration of glucose % degradation and COD removal by mixed consortia

Fig. 2: Effect of different types of sugars on % degradation and COD removal by mixed consortia

Fig. 3: Effect of different pH on % decolorisation and COD removal mixed consortia
Fig. 4: Effect of different temp. on % decolorisation and COD removal by mixed consortia

Physicochemical analysis of the spent wash effluent before and after treatment with the mixed consortia is presented in the Table 2.1,2 Appreciable reduction in case of most of the parameters were observed, especially color, BOD, COD, etc. This is very significant from the toxicological implications for discharging spent wash effluent in the water bodies (Table 2).1,2,28

Table 2: Physico-chemical analysis of the spent wash effluent before and after microbial treatment by mixed consortia

<table>
<thead>
<tr>
<th>S/N</th>
<th>Parameters</th>
<th>Before treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Color</td>
<td>Dark brown</td>
<td>Light brown</td>
</tr>
<tr>
<td>2</td>
<td>Odor</td>
<td>Strong pungent</td>
<td>Mild</td>
</tr>
<tr>
<td>3</td>
<td>pH</td>
<td>4-4.3</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>BOD(mg/L)</td>
<td>60,540</td>
<td>34,350</td>
</tr>
<tr>
<td>5</td>
<td>COD(mg/L)</td>
<td>95680</td>
<td>43500</td>
</tr>
<tr>
<td>6</td>
<td>Total sugar(mg/L)</td>
<td>12,3000-90,000</td>
<td>700-1300</td>
</tr>
<tr>
<td>7</td>
<td>Total Dissolved Solids</td>
<td>7800</td>
<td>1600</td>
</tr>
<tr>
<td>8</td>
<td>Iron(mg/L)</td>
<td>124</td>
<td>80</td>
</tr>
<tr>
<td>9</td>
<td>Magnesium(mg/L)</td>
<td>2550</td>
<td>240</td>
</tr>
<tr>
<td>10</td>
<td>Sulphates (mg/L)</td>
<td>980</td>
<td>770</td>
</tr>
<tr>
<td>11</td>
<td>Free chlorides(mg/L)</td>
<td>7000</td>
<td>650</td>
</tr>
<tr>
<td>12</td>
<td>Phosphorus(mg/L)</td>
<td>4850</td>
<td>400</td>
</tr>
<tr>
<td>13</td>
<td>Oil and Grease(mg/L)</td>
<td>174</td>
<td>170</td>
</tr>
</tbody>
</table>

Several researchers have investigated the role of microbial community in the degradation of melanoidins in the spent wash. Bacillus and Xanthomonas in immobilized form are reported to degrade the color causing material in the spent wash.22,23 Most of the bacterial strains like Pseudomonas, Acetobacter, Aeromonas sp. are reported to be capable of degrading some of the recalcitrant compounds in the aerobically digested distillery spent wash. In a two stage bioreactor using Pseudomonas putida and Aeromonas sp. has achieved color and COD reduction by 60 and 44.4% respectively.22 The fungus Coriolus hirustus, exhibited ability to decolorize melanoidin by 74% in GPY medium.29,30 White rot fungi Phanerochaete chrysosporium, decolourised MSW (6.25% v/v) supplemented with glucose 9.25g/L) by 85% after 10 days of incubation.30 Lactobacillus hilgardi is reported to decolourise melanoidin solution 28 %. In all of these cases 0.4-3% sugar either glucose or sucrose with essential nutrients were added and decolourisation required 7-10 days also the spent wash concentration was less. Pseudomonas aeruginosa used in this work
was capable of giving 55% and 57% reduction in color and COD respectively with 80% spent wash within 72hr with externally added glucose 0.5% (Fig. 5). Also the indigenous isolate Aspergillus niger was capable of giving 58% and 57% reduction in color and COD, respectively with 80% spent wash (Fig. 6). Within 72hr with externally added dextrose 0.4%. While mixed consortia was capable to give 59% decolourisation and 57% COD removal for 80% spent wash with externally added glucose 0.4% (Fig. 7). Use of mixed consortia for decolourisation of 90% and 100% spent wash is in progress. Better decolourisation was observed with 20%, 40% and spent wash using the above strains like Pseudomonas aeruginosa, Aspergillus niger, Streptococcus sp and Bacilli sp and mixed consortia. 

**Fig. 5**: Optimization of spent wash concentration by *Pseudomonas aeruginosa*

**Fig. 6**: Optimisation of spent wash concentration by *Aspergillus niger*

**Fig. 7**: Optimization of spent wash concentration for degradation by mixed consortia
Addition of readily available external carbon source was found to be necessary for metabolism of microbes in the spent wash medium. Although spent wash contains huge amounts of sugar but its easily metabolisable carbon content is almost negligible. Growth pattern of the isolate with respect to color removal indicated that within first 24 hr growth was initiated but without any decolourisation, but after 24 hr gradual increase in growth with decolourisation was observed up to 72 hr. This effect can be explained as, during initial phase organism utilizes easily metabolisable carbon source added to the medium and later on begins to degrade spent wash components for carbon source. When both decolourisation and COD reduction were monitored as a function of time, the results showed that with the increasing decolourisation activity there was notable COD reduction and it was profound between 24-72 hr (Table 3).

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>% Decolorisation</th>
<th>% COD Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td>48</td>
<td>38</td>
<td>41</td>
</tr>
<tr>
<td>72</td>
<td>58</td>
<td>59</td>
</tr>
</tbody>
</table>

The probable mechanism of decolourisation and COD reduction might be through enzymatic degradation as laccase, sugar oxidase and manganese dependant peroxidase have been reported for microbial degradation of melanoidin.

CONCLUSION

It is clearly noted that the local isolates *Pseudomonas aeruginosa*, *Aspergillus niger* and their mixed consortia were found to be more efficient in decolourisation of 80% spent wash along with melanoidin degradation in comparison to earlier reports. As the highly concentrated spent wash is decolourised by the above strains and mixed consortia this approach can be further exploited to develop a cost effective, eco-friendly biotechnology package for the treatment of concentrated distillery spent wash.

ACKNOWLEDGEMENT

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