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Decolorization and COD Reduction of Anaerobic Digested Molasses Spent Wash by Native Microbial Consortium

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The aim of this study was to isolate native microbial consortium capable of decolorizing and degrading anaerobically treated molasses spent wash (MSW). We reported decolorization and COD reduction of MSW by native microbial consortium isolated from MSW disposal site and using *Bacillus megaterium,* a standard culture for comparison. Microbial decolorization and COD reduction was found to be dependent on specific carbon (glucose) concentration. Results indicate that under optimal condition of carbon for each treatment, the maximum COD reduction (62%) and decolorization (56%) was achieved at 2.5% glucose concentration with native microbial consortium, followed by *Bacillus megaterium*. (49.72% and 52.92%). The concentration of MSW up to 6.25% was good for higher percentage of color removal and COD reduction, but, with increasing concentration of MSW i.e. 12.5%, 18.75% and 25%, resulted in decreased decolorization and COD reduction. Experiment on fresh or used microbial cells on fresh or used medium was also done to understand the reason of decolorization.

Key words: Native microbial consortium, COD, Decolorization, Molasses spent wash.

Molasses is one of the important raw materials for various fermentation industries. In an ethanol distillery in India, 10-15 liters of MSW are produced for every liter of alcohol with approximately 20,000 liter of spent wash produced per day in each distillery AIDA (1993). Molasses spent wash (MSW) is a highly colored effluent with a potential to cause eutrophication of waterways due to it's high pollution load, with a COD in the order of 90,000 mg/l Fahy *et al.* 1997. The colored nature of MSW is due to the presence of natural polymers called melanoidins formed by the Maillard Amino Carbonyl reaction.

MSW leads to an environmental problem due to high organic load, intense coloration, foul smell, phenolic compounds, melanoidins and the presence of thermal and alkaline degradation

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product of sugar (caramels) Jain *et al.* (2001). Melanoidin has shown to have antioxidant property and are toxic to many microorganisms, typically used in waste water treatment. Due to its recalcitrant nature, conventional waste water treatment is unable to remove color. However, many methods such as blazoning, dilution with fresh water and anaerobic digestion are used to treat spent wash from distilleries. MSW also causes manganese deficiency when disposed in soil leading loss to soil fertility Aggarwal and Pandey (1994). MSW is highly acidic and have antioxidant property and generally microorganisms do not survive in the solution, but bacterial strains like *Xanthomonas fragariae, Bacillus cereus* have been used for the distillery waste water treatment Jain *et al.* (2001). Some heterogeneous bacteria decolorize the spent wash effluent and have the ability to adapt to all the unfavorable environmental condition and also reduce the BOD and COD of the effluent Kumar *et al.* (1997). Biological treatment method are considered to be more efficient and ecofriendly than the chemical method of effluent treatment. The bacterial species such as *Pseudomonas, Serratia, Bacillus,* and *Cornybacterium* are involved in the bacterial decolorization Jothimani *et al.* (2003).

Pure bacterial or fungal cultures have been studied in order to decolorization of MSW, however, the performance of fungal decolorization was limited by long growth cycle and moderate decolorization rate. But decolorization by bacteria is comparatively faster but it may require a mixed community of *Bcillus* to decolorize melanoidins through combined metabolic mode of individual culture Kumar and Chandra (2006). Hence, the bacterial consortium seems to be more competent for MSW treatment due to co-metabolism to enhance the efficiency of decolorization and COD reduction.

It is highly desirable to isolate and utilize the potential of MSW prolonged period polluted site soil microbes, since these soil acts as selection agent of principle microbes bearing biodegradative ability. Therefore, it is necessary of explore the possible efficacy of native consortium for decolorization and COD reduction. In the present study, we compared and standardized decolorization and COD reduction of MSW using native microbial consortium isolated from MSW polluted site and a standard strain of *Bacillus megaterium* (MTCC 2949).

MATERIAL AND METHODS

Collection of sample

The samples were collected from Doon Valley Pvt. Ltd. Distillery situated in Kuanwala, Dehradun (Uttarakhand) using washed and sterilized container and stored at 4°C until used. Samples were centrifuged at 8000 rpm for 10 min to remove suspended solids before using as substrate for bioremediation studies

Chemical analysis of MSW

The MSW was analyzed for COD, BOD, pH, total suspended solids (TSS), total solids (TS), total organic carbon (TOC), total nitrogen (TN), sulphates and phosphates as described by APHA, (1999). Total and reducing sugar was determined as in Kumar *et al.* (1997) and total phenol was determined as by Jimenez *et al.* (2003).

Enrichment and Isolation of native consortium from MSW

For the isolation of native micro flora from MSW, the medium $(NH4)_2SO_4$ 0.26%; K_2HPO_4 0.01%; $MgSO_4$ 0.02%: $CaCl_2$ 0.001%; $FeSO_4$ 0.001%; Yeast extract 0.01% and glucose (0.02%) of pH 7.2-7.4 was used. Broth was used for enrichment of native microbial flora in the medium and agar was added for isolation of bacterial strains after incubation at 35°C for 48 h. Four different types of bacterial colonies were observed on plates. These colonies were purified by repeated streaking and mixed cultures of different isolated strains have been used for consortium preparation. On the basis of morphological and biological tests the bacterial strains were identified as *Pseudomonas aeroginosa, P. fluoresces, P. putida* and *Aeromonas hydophila*.

Basal medium

The common basal medium was used for performing the experiment i.e. glucose 20 g, Peptone 5 g, K_2HPO_4 1 g, $MgSO_4.7H_2O$ 0.5 g; pH 6.0; per 1000 ml of distilled water and 10% MSW was inoculated into each containing basal medium. The freeze dried lyophilized strain of *Bacillus megaterium* MTCC 2949, was collected from culture collection of IMTECH (Institute of

Microbial Technology), Chandigarh, India.

Bioremediation studies

The influence of various concentrations of carbon and MSW using above mentioned basal medium in presence of native microbial consortium and *Bacillus megaterium* was studied in triplicate. Different concentration of glucose (1.5%, 2.5%. 3.5% and 4.5%) and MSW (2.5%, 6.25%, 12.5%, 18.75% and 25%) were used along with a set of control having no additional carbon. In each experiment, 5 ml of broth culture of native microbial consortium and *Bacillus megaterium* was inoculated to 50 ml of basal medium containing 10 % MSW and incubated for 2, 4 and 6 days at 35°C at stationary conditions to determine the percent decolorization and COD reduction. Samples were centrifuged at 8000 rpm for 5 min and the supernatant diluted to 5 fold with distilled water. The decolorization activity was measured by determining absorbance of sample at 475 nm wavelength and expressed as percent of the initial absorbance of the sample prior to the treatment.

Decolorization by living and autoclaved cells

To understand, whether the decolorization of MSW was due to biological or non biological activity. The living and autoclaved cells of bacterial consortium with different cell concentrations (5-30% v/v) were added into 250 ml Erlenmeyer flask containing 50 ml each of MSW. The flasks were kept at 30°C for 48 h at 200 rpm shaking conditions. At respective time points samples were withdrawn, centrifuged at 8000 rpm for 15 min. The OD of the supernatant was read using spectrophotometer at 475 nm.

Study on decolorization limitation

The native microbial consortium was inoculated into MSW and cultured with shaking (200 rpm) for 48 h at 30°C. Cells were centrifuged at 10,000 rpm, 10 min and 4°C and harvested. Harvested cells were washed with sterile solution to remove the culture medium residue. Washed bacterial cells were resuspended in the fresh medium of the same volume and kept at 48h, 30°C and 200 rpm. The used culture medium was centrifuged at 10,000 rpm at 4°C to completely remove bacterial cells, afterward inoculated with fresh native microbial consortium (5%v/v) and grown as above.

RESULTS AND DISCUSSION

The chemical analysis of MSW obtained from Doon Valley Pvt. Ltd. Distillery, Kuanwala, Dehradun are given in Table 1. There are several reports about fungal bioremediation and decolorization of distillery spent wash (Fahy *et al.* (1997); Dahiya *et al.* (2001) and Raghukumar and Rivonkar (2001). However, a few reports are there regarding the role of bacteria for MSW decolorization and COD reduction. Four different stains were isolated and coinoculum of all these four strains constituted native microbial consortium which have been used in the present study along with a standard strain of *B. megaterium* (MTCC 2949) in the present study for comparison.

Table 2 shows the highest COD reduction (62%) and decolorization (56%) activity was achieved at 2.5% glucose concentration with native consortium, followed by *B. megaterium* (49.7% and 52.9%). Further, increase in glucose concentration did not improve decolorization; rather the COD reduction was also less. Therefore, decolorization and COD reduction in all the treatments were dependent upon concentration of carbon source. The observation draws support from the findings of Kumar *et al.* (1997); Singh *et al.* (2007) and Sivakumar *et al.* (2006).

The concentration of MSW up to 6.25% resulted in higher percentage of color removal and COD reduction by native microbial consortium and *B. megaterium*. But, using higher concentration of MSW i.e. 12.5%, 18.7% and 25%, decreased decolorization and COD reduction was observed (Fig. 1-4). The inhibitory

Table 1. Chemical analysis of raw molasses spent wash

COD (mg/l)	42000-53000
BOD (mg/l)	7800-9700
pH	7.1-8.4
TSS (mg/l)	38870
TS (mg/l)	71650
TOC (mg/l)	29814
TN (mg/l)	3978
Sulphates (mg/l)	3698
Phosphates	1607
Total sugar (g %)	0.16
Reducing sugar (g %)	0.32
Phenols (mg/l)	7183

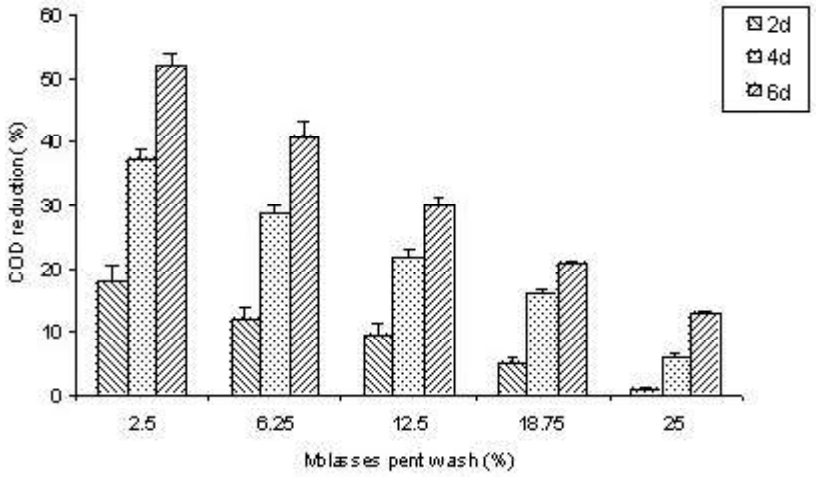

Fig. 1. Effect of different MSW concentrations on COD reduction by native microbial consortium

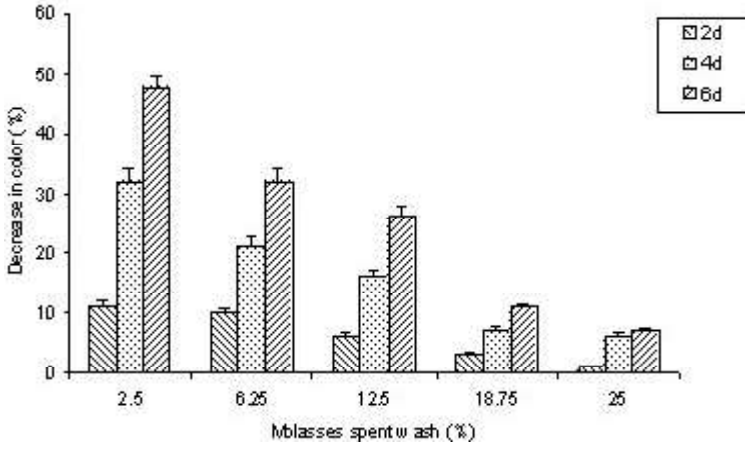

Fig. 2. Effect of different MSW concentrations on decrease in color by native microbial consortium

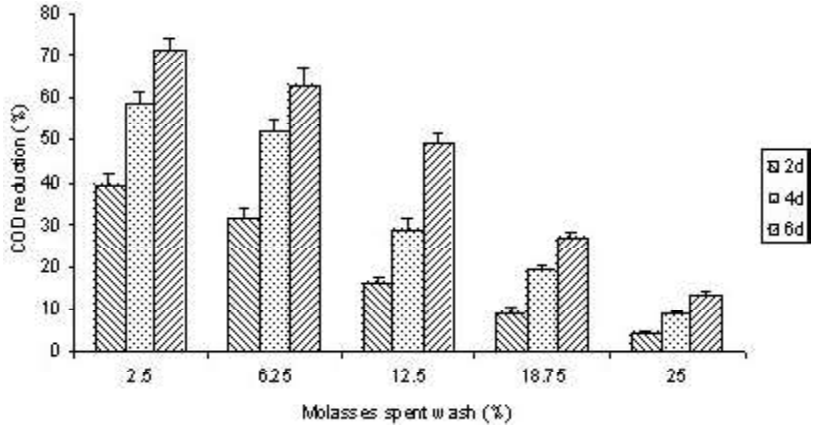

Fig. 3. Effect of different MSW concentrations on COD reduction by *Bacillus megaterium*

effect of MSW concentration is likely to be due to the presence of inhibitory compound such as phenolic, gallic and vanillic acid (Kumar *et al.*, 1997).

The maximum decolorization and COD reduction was achieved in medium supplemented with MSW 2.5% after 6 days. The highest COD reduction and decolorization was achieved by the native consortium i.e. (63.1%, 52.1%) respectively which was quite higher than the value achieved by the *B. megaterium* (52.2 %, 42.12%) after 6 days using 6.25% MSW (Fig 1-4). Further increase in MSW concentration rather decreased the process of decolorization and COD reduction. Decolorization of living and autoclaved cells was studied to verify weather it is due to biological or non biological activity. Inoculation with (5-30% v/v) of autoclaved native microbial consortium at 48h showed no MSW decolorization showed no MSW decolorization, rather the decolorization was exhibited by living cells of microbial consortium (Fig. 5).

Again, to confirm that decolorization is by biological activity and not by adsorption on to microbial cells. The cell pellets of living and autoclaved were suspended with equal volume of NaOH (0.1M) to extract color substances adsorbed to cell surface. The extracts were centrifuged and at 475 nm OD was measured. The final fraction of color substance was negligible extracted by

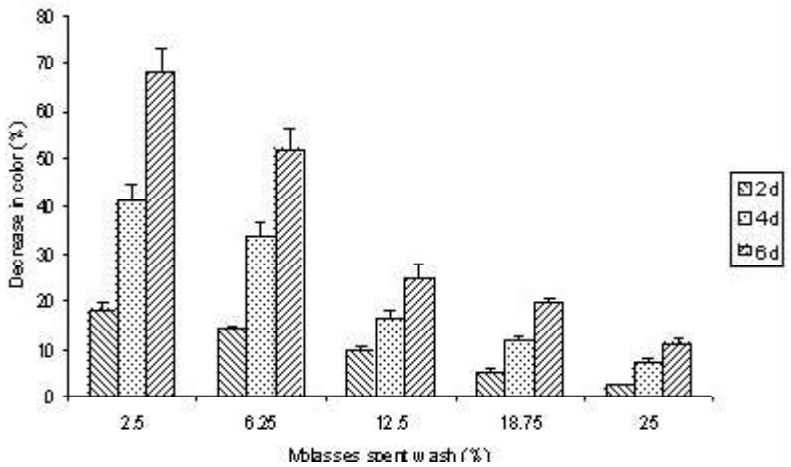

Fig. 4. Effect of different MSW concentrations on decrease in color by *Bacillus megaterium*

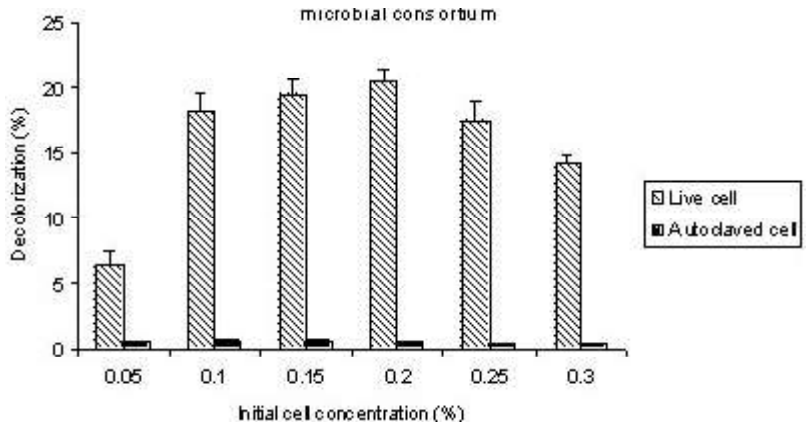

Fig. 5. Decolorization by living cells and autoclaved cells of native microbial consortium

NaOH, therefore, the decolorization was due to biological activities.

To understand the limitation of decolorization by microbial consortium, the used microbial cells were inoculated into fresh medium and used medium was inoculated with fresh microbial consortium cells (Fig 6-7). The fresh microbial cells decolorized the MSW medium during first 24 h of incubation, afterward decolorization was decreased. Using fresh bacterial cells in used medium, the decolorization was hardly achieved (Fig 7). This could be due to absence of nutrients or accumulation of toxic metabolites therefore, no decolorization by fresh cells in used media.

The decolorization of MSW was increased with increase in inoculum size. Maximum decolorization and COD reduction was observed with 5 ml of inoculum (1×10^9 cfu/ml). Further increase in the inoculum size did not result in any improvement of decolorization and COD reduction (data not given). Dahiya *et al.* (2001) also studied the relation between decolorization and inoculum size and concluded the 5% (w/v) inoculum was sufficient and further increase resulted only in fungal biomass, while there was no improvement in decolorization. The native microbial consortium exhibited increased decolorization and COD reduction compared to that shown by single isolate of *B. megaterium*.

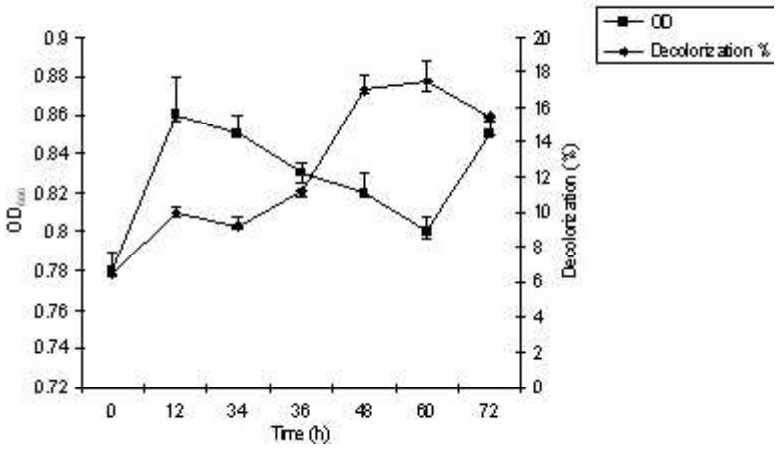

Fig. 6. Study of MSW decolorization by used native microbial consortium in fresh medium

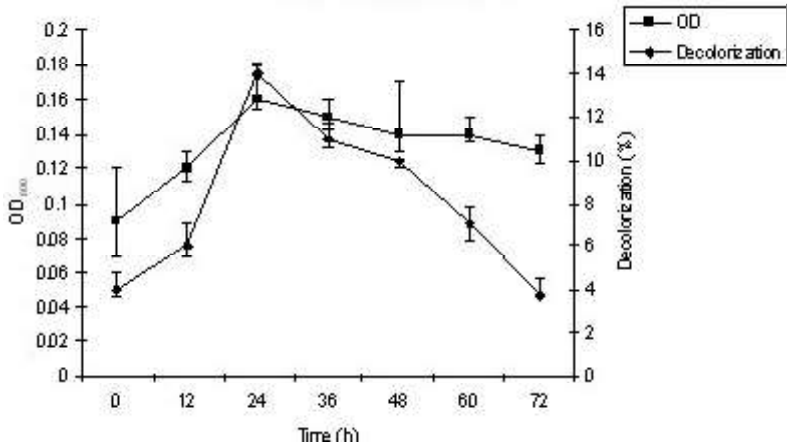

Fig. 7. Study of MSW declolorization by fresh native microbial consortium in used medium

Table 2. Influence of different glucose concentrations as additive in basal medium + 10% MSW on the decolorization and COD reduction by native microbial consortium and *B. megaterium*

Treatment	Native microbial consortium				*Bacillus megaterium*			
Parameter	% COD reduction (Days)		% Decolorization (Days)		% COD reduction (Days)		% Decolorization (Days)	
Glucose%	4.1±1.52	10.2±2.13	21.0±3.76	3.2±0.55	10.5±3.63	23.0±4.78	3.2±0.85	7.2±2.32	15.6±5.20	3.9±1.02	14.4±3.65	18.2±4.20
Control	7.3±0.92	23.8±4.05	46.5±6.27	6.2±1.63	18.2±6.14	42.2±7.89	7.2±2.36	17.2±4.21	30.2±9.36	15.0±3.62	26.5±4.23	45.8±8.71
1.5	10.0±1.01	30.2±4.85	62.0±7.25	15.1±3.69	32.9±8.02	56.0±9.25	14.8±5.20	28.9±9.21	49.7±11.21	11.4±5.01	27.9±8.71	52.9±12.3
2.5	15.1±1.85	29.2±6.70	44.5±6.85	19.2±4.74	39.7±6.35	47.9±5.54	12.0±2.65	22.3±6.58	35.6±7.27	7.9±2.57	18.0±6.20	30.0±6.59
3.5	8.1±2.0	23.6±4.20	36.2±6.14	8.0±2.10	15.7±3.25	42.1±7.23	4.0±1.14	16.2±4.08	21.8±5.88	2.7±0.9	10.9±2.18	24.1±4.29
4.5	4.1±1.53	10.2±2.10	21.0±3.76	3.2±0.55	10.5±3.63	23.0±4.78	3.2±0.85	7.2±2.32	15.6±5.20	3.9±1.02	14.4±3.65	18.2±4.20

This may be due to the enhanced effect of coordinated metabolic interactions on decolorization and COD reduction.

CONCLUSIONS

The use of native microflora isolated from the site of distillery for COD reduction and decolorization could be a realistic approach for treating MSW. Bacterial native flora *Pseudomonas aeroginosa, P. fluoresces, P. putida* and *Aeromonas hydophila* appeared to be suitable choice rather than using a single organism.

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