Bioremediation of anaerobically digested post-methanation distillery spent wash

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Anaerobically treated post-methanation spent wash is highly coloured with exceptionally high chemical oxygen demand (COD). An efficient isolate of Pseudomonas sp., viz., RG-2 for decolourisation was isolated from soil collected from distillery premises by enrichment culture method. RG-2 isolate was having considerable decolourisation (34.6 per cent) of 12.5 per cent diluted post-methanation spent wash after 8 days incubation. Addition of glucose to medium was essential to have decolourisation by this isolate. The effect of pH, carbon source, carbon source dose, nitrogen source, incubation period and post-methanation spent wash concentration have also been studied.

The distillery is one such industry that consume huge quantities of water and consequently generate large volumes of spent wash. Currently, there are 295 distilleries in India with an installed capacity of over 2.79 billion liters of ethanol production per annum. For every liter of alcohol, 10 - 15 L of spent wash is produced. Hence about 30 - 40 billion liters of spent wash is generated annually. It is highly coloured and its treatment is difficult by normal biological processes, viz., activated sludge process and anaerobic lagooning. It also has an extremely high COD load (=90,000 mg/L) and its disposal in natural water bodies results in lowering of pH of the water body, depletion of dissolved oxygen and decrease in primary productivity, etc. The distillery effluent were beneficial to Oryza sativa, Cajanus cajan, Helianthus annuus and Vigna mungo only up to 5 per cent dilutions and lethal at higher concentration (75-100 per cent). Disposal on land is also equally detrimental causing a reduction in soil alkalinity and manganese availability. Its recalcitrance is due to the presence of melanoid brown polymers that are formed by maillard amino-carbonyl reaction. These compounds have antioxidant properties which render them toxic to microorganisms used in various treatment processes. To reduce the organic pollution load, anaerobic digestion is used in 155 distilleries in India. The post-methanation spent wash (PSW) also have considerable COD (=25,000 mg/L) which is much higher than the recommended levels for disposal into inland surface water (250 mg/L).

Several investigators have suggested the potential use of microbial systems in the bioremediation of spent wash. Aspergillus oryzae Y-2-32 strain showed high decolourisation which was caused by the adsorption of melanoid in mycelia especially lower molecular weight fractions, which was influenced by the kind of sugars used for growth. Coriolus versicolor P44a strain had very high PSW decolourisation due to intra-cellular enzymes induced by molasses pigment. The induced enzyme consisted of two types, viz., a sugar dependent enzyme and a sugar independent enzyme. The efficiency of basidiomycetes isolated from distillery effluents for decolourisation varied from 2.5-64.5 per cent. Coriolus sp. no. 20 decolourised a melanoid solution to 80 per cent in darkness. The decolourisation occurred with an intracellular enzyme which required aeration and some kind of sugar, particularly glucose and sorbose. Mycelia sterilia was found most effective decolourising microbial system for molasses pigments among 228 strains of class deuteromycetes. The decolorization efficiency has been reported to be more than 50 per cent. In the present study, an attempt has been made to isolate and characterize a bacterial isolate and investigate its capability to further bioremediate the anaerobically treated post-methanation spent wash.

Experimental Procedure

Materials

Post-methanation spent wash (PSW) was obtained from the bio-methanation plant of a distillery in Haryana and stored at 4°C to avoid further oxidation.

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Physico-chemical analysis of the PSW was carried out as reported elsewhere. The various analysed parameters were pH, electrical conductivity, total carbon, total nitrogen, total sugar, COD, potassium, phosphorus and sulphate.

**Methods**

**Enrichment and isolation**

Several soil samples were collected from the vicinity of the anaerobic digester of the distillery in random manner. All the collected soil samples were mixed to have a composite soil sample that was used for bacterial isolation.

For enrichment of bacteria, a 2.5g soil sample was added to 50 mL of 12.5 per cent diluted PSW medium (0.5% peptone, 0.05% MgSO₄⋅7H₂O, 0.01% KH₂PO₄, 2% agar agar at pH 6.0). Three different enrichment media were used. In the first, PSW was the sole source of carbon and nitrogen; in the second PSW was considered as source of carbon and nitrogen source (ammonium sulphate; 0.2 per cent) was added and in the third, PSW was considered as the sole source of nitrogen while additional carbon source (glucose; 2 per cent) was added. The flasks were incubated at 37°C for 7 days. The flasks showing some decolourisation were subcultured successively several times at weekly intervals. The isolation of microbial cultures was carried out using the spread plate and streak plate method on agar medium (0.5% NaCl, 0.5% beef extract, 0.2% peptone, 0.05% MgSO₄⋅7H₂O and 2% agar agar at pH 7.5).

**Inoculum preparation and sampling**

The inoculum were prepared by inoculating a loopful of bacterial culture into nutrient broth from 24 h old slant cultures and incubated for 72h at 30°C on a shaker. The culture broth so obtained were used as an inoculum for further studies.

**Decolourisation assay**

Screening of bacterial isolate for decolourisation of PSW was carried out in 12.5 per cent PSW medium. Five mL of inoculum were added to 50 mL medium and incubated for pre determined time intervals at 30°C. A 5 mL sample was withdrawn from the incubated flask and analysed for decolourisation. Decolourisation of PSW was measured as a decrease in optical density at 475 nm spectrophotometrically of the cultured media supernatant against un-inoculated PSW medium and expressed the results as percentage decrease in absorbance.

**Results and Discussion**

The post-methanation spent wash used in the present study was having pungent odour and dark brown colour. A high organic load (20400 mg/L, COD) has been recorded in this PSW. It also contained 12000 mg/L total sugars, 1600 mg/L nitrogen, 35 mg/L phosphorus, 8000 mg/L potassium, 1100 mg/L sulphate with 11.2 dS m⁻¹ electrical conductivity and 6.8 pH.

Several bacterial isolates capable of decolorizing the PSW were isolated. All were isolated from those enrichment flasks in which additional source of carbon (glucose) was added. This indicates that the sugars present in the PSW are not readily available to the microorganisms. The per cent decolourisation [incubation period = 8 days] efficiency of the isolated bacterial isolates varied between 6 to 34.6 per cent. Among these isolates RG-2 showed the highest decolorizing efficiency (34.6 per cent). Therefore, all further decolourisation studies were carried out on RG-2 isolate. Based on cultural and biochemical tests such as Methyl red reaction, citrate utilization, catalase test, growth on Mac Conkey agar, gelatin liquefaction, starch hydrolysis, H₂S production and nitrate reduction, etc., RG-2, an isolate was identified as *Pseudomonas Sp.*

**Effect of pH on decolourisation**

To explore the effect of medium's pH on the decolourisation efficiency of RG-2 isolate, initial pH of PSW medium was varied from 3 to 8 keeping all other conditions constant [PSW concentration = 12.5 per cent; incubation period = 8 days; glucose dose = 2 per cent] respectively. The results showed that initial pH of the medium had significant effect on decolourisation. At pH 3.0 decolourisation was 12 per cent, which increased to 34.6 per cent at pH 7.0. There was slight decrease in decolourisation at pH 8.0 than neutral pH (Fig. 1). This indicates that the optimum pH for decolourisation of PSW is in the vicinity of 7.0.

**Effect of carbon source**

To explore the effect of carbon source on decolourisation, glucose was replaced by fructose, sucrose and ribose in PSW medium keeping the PSW concentration, carbon source dose and incubation period constant at 12.5 per cent, 2 per cent and 8 days respectively. A control was maintained with PSW as the sole source of carbon. The results showed that in absence of any additional carbon source, decolourisation was negligible (3 per cent) which
increased to 18, 24, 32 and 34.6 per cent in presence of ribose, fructose, sucrose and glucose respectively. This indicates that addition of sugar is essential for decolourisation of PSW as the sugars present in the PSW are not readily available to the RG-2 isolate. Further, glucose proved to be the best carbon source for RG-2 isolate (Fig. 2).

While studying the effect of glucose dose in the medium, it has been observed that decolourisation was increased with increase in glucose dose. Fig. 3 shows that there was a sharp increase in decolourisation up to one per cent dose. Afterwards increase in decolourisation was slow up to 3 per cent dose, whereas higher than 3 per cent glucose dose had no further effect on decolourisation.

**Effect of nitrogen**

To study the effect of different nitrogen sources, peptone was replaced by beef extract, yeast extract and sodium nitrate from PSW medium while maintaining the PSW concentration, nitrogen source dose and incubation period constant at 12.5 per cent, 0.2 per cent and 8 days respectively. A control was maintained with PSW as the sole source of nitrogen. In absence of any nitrogen source decolourisation was 20.4 per cent which increased to 26.5, 28.8, 32.4 and 34.6 per cent in the presence of sodium nitrate, beef extract, yeast extract and peptone respectively. These results showed that with the addition of nitrogen source there was moderate increase in decolourisation. Where as nature of different nitrogen sources too had considerable effect on decolourisation as compared to peptone. Similar observations have been reported in literature for different microbial systems to decolourise the PSW.

**Effect of incubation time**

To study the effect of incubation period on decolourisation by RG-2 isolate PSW concentration in the medium was maintained at 12.5 per cent. The results so obtained have been depicted in Fig. 4. The
Effect of PSW concentration

The effect of PSW concentrations in medium has been studied at 6.25, 12.5 and 25 per cent dilutions keeping all other factors constant as a function of different incubation periods. The results showed an increase in decolourisation with decrease in PSW concentration (Fig. 5) at all the incubation periods. Maximum decolourisation observed was 56.6 per cent at 6.25 per cent dilution after eight days. This may be due to dilution of the inhibitory compounds present in PSW. Presence of vanillic acid and gallic acid in PSW has been reported. These and related compounds are known to function as microbial inhibitors. The inhibitory effect of increasing PSW concentration may also be caused by a lowering of the water activity in the system leading to inhibition of growth due to osmotic effects.

The results of present study indicate that isolated bacterial isolate has considerable PSW decolourisation efficiency at low PSW concentrations. The major limitation to make the use of RG-2 isolate for bioremediation be realistic is the addition of sugar, which requires future efforts to overcome this shortcoming.

References
12. Central Pollution Control Board, Maximum permeable limits (mg/l) for effluent discharge, New Delhi.

Results showed that maximum decolourisation was attained by 6th day of incubation. The decolourisation was negligible between 6th and 8th day. It has been reported that the decolourisation by *Coriolus versicolor* Ps-4a strain is almost completely coincident with the growth of mycelia and the maximum decolourisation (70 per cent) was reached in 4 to 5 days. After that decolourisation was stopped as the mycelia growth was stopped. This has also been correlated with the availability of sugar in the medium that as long as readily available carbon is present in the medium, growth is fast. Once the available forms of sugar are exhausted, decolourisation slows down.
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