Biodegradation and decolourization of anaerobically treated distillery spent wash by a novel bacterial consortium

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Abstract

The aim of this study was to isolate microorganisms capable of decolourizing and degrading anaerobically treated distillery spent wash. A bacterial consortium DMC comprising of three bacterial cultures was selected on the basis of rapid effluent decolourization and degradation, which exhibited $67 \pm 2\%$ decolourization within 24 h and $51 \pm 2\%$ chemical oxygen demand reduction within 72 h when incubated at 37 °C under static condition in effluent supplemented with 0.5% glucose, 0.1% KH_2PO_4, 0.05% KCl and 0.05% $MgSO_4 \cdot 7H_2O$. Addition of organic or inorganic nitrogen sources did not support decolourization. The cultures were identified as *Pseudomonas aeruginosa* PAO1, *Stenotrophomonas maltophila* and *Proteus mirabilis* by the 16S rDNA analysis.

Keywords: Bacterial consortium; Distillery spent wash; Decolourization; Melanoidins; 16S rDNA

1. Introduction

Alcohol distilleries are one of the most polluting industries generating an average of 8–15 l of effluent ("spent wash") per liter of alcohol produced (Saha et al., 2005). There are 285 distilleries in India alone producing 2.7 billion liters of alcohol and generating 40 billion liters of wastewaters annually (Raghukumar et al., 2004). Due to high biochemical oxygen demand of raw spent wash, application of anaerobic treatment technology with biogas recovery has been reported to be highly effective (Nandy et al., 2002). Anaerobic treatment is an accepted practice and various high rate anaerobic reactor designs have been tried at pilot and full-scale operation (Lata et al., 2002). However, anaerobically treated effluent still contains high concentrations of organic pollutants and as such cannot be discharged directly (Nandy et al., 2002). Its recalcitrance is due to presence of brown polymers melanoidins, which are formed by Maillard amino carbonyl reaction (Kumar et al., 1997). Melanoidins are analogous to humic acid substances of soil or melanins in regard to their chemical properties (Hayase et al., 1984). Since conventional treatment processes can accomplish only low degradation of melanoidins, it is necessary to explore additional treatments to remove the colour from molasses effluent and prevent serious environmental problems that coloured waste waters can promote in river courses such as reduction of photosynthetic activity and dissolved oxygen concentration. Melanoidins can be removed by physiochemical treatments. However, these methods require high reagent dosages and generate large amount of sludge (Pena et al., 2003). Microbial decolourization and degradation is an environment friendly and cost competitive alternative to chemical decomposition processes (Moosvi et al., 2005). So far, there has been limited success in search for bacteria and fungi, which can efficiently degrade melanoidins, in order to reduce the colour and COD (Kumar et al., 1998). It is considered highly desirable to exploit the biodegradation potential of soil microorganisms from polluted sites exposed to recalcitrant compounds of distillery spent wash for prolonged periods. As such polluted soils can facilitate selection of biodegradative capability in microorganisms and may act as reservoir

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of bacterial communities capable of degrading pollutants (Gosh et al., 2004).

The present study was focused on the screening and isolation of distillery spent wash decolourizing organisms from contaminated soil, optimizing various operational parameters for maximal decolourization and biodegradation of anaerobically treated distillery effluent and identification of bacterial cultures by the 16S rDNA based molecular approach.

2. Methods

2.1. Collection and physiochemical analysis of anaerobically treated distillery spent wash

Distillery spent wash was collected from the anaerobic effluent treatment plant of Kanoria Chemicals Ltd., Ankleshwar, Gujarat, India which treated its effluent with an Upflow anaerobic sludge blanket (UASB) reactor. The effluent samples were stored at 4 °C. The effluent was characterized and analyzed for pH, chemical oxygen demand (COD), biological oxygen demand (BOD), total solids (TS), total suspended solids (TSS), total organic carbon (TOC), total nitrogen (TN), phosphates and sulphates based on the Standard Methods for Examination of Water and Wastewater (APHA, 1995).

A colourimetric method with closed reflux method was used for measurement of COD using Hach DR 2010 spectrophotometer at 620 nm to measure absorbance of COD samples. The dissolved oxygen concentration in wastewater was monitored with a BOD probe and dissolved oxygen meter (YSI 5010 BOD probe & YSI 5100 Dissolve oxygen meter, Ohio, USA). Total solids was determined by drying known volume of whole effluent at 103 °C till constant weight and total suspended solids was determined by drying filtrate at 103 °C. TOC and TN were measured on a TOC-V_{CSN} analyzer (Shimadzu, Japan). Phosphate was determined by Vanadomolybdophosphoric acid colourimetric method and sulphate was determined by turbidimetric method (APHA, 1995). Total phenol content was determined according to the Folin Ciocalteau method (Jimenez et al., 2003). Total and reducing sugars were determined by the 3,5-dinitrosalycylic acid method (Kumar et al., 1997).

2.2. Enrichment and isolation of distillery spent wash decolourizing consortium

Soil samples collected from distillery spent wash contaminated sites of Ankleshwar were used for screening of effluent decolourizing cultures by enrichment culture technique. Ten grams soil was suspended in 100 ml sterile distilled water. Diluted anaerobically treated distillery spent wash was inoculated with the soil suspension and incubated at 37 °C under static condition. Cultures that showed decolourization were tested further by transferring in fresh effluent as the only source of carbon and nitrogen for consistent decolourization in every successive transfer. The effluent was diluted to obtain an absorbance of 2.8–3 at 475 nm and was used in treatment studies. Bacterial inoculum was prepared by growing the consortium DMC overnight in nutrient broth under shaking at 150 rpm at 37 °C.

2.3. Decolourization assay and growth

Decolourization and growth were monitored spectrophotometrically using a Spectonic 20D+ spectrophotometer (Milton Roy, USA). Decolourization of the effluent was measured as decrease in optical density at 475 nm of the supernatant obtained upon centrifugation (6000*g* for 20 min) of 5 ml culture against uninoculated effluent and expressed as the percentage decrease in the absorbance. The cell pellet obtained on centrifugation was resuspended in 5 ml distilled water and its absorbance was measured at 660 nm and reported as growth of the consortium.

2.4. Optimization of culture conditions for decolourization and COD reduction

The medium composition for obtaining maximum decolourization and COD reduction was examined by supplementing the anaerobically treated distillery spent wash with basal salts (%) 0.1 KH_2PO_4; 0.05 KCl; 0.05 $MgSO_4 \cdot 7H_2O$ and various carbon sources and organic and inorganic nitrogen sources. Decolourization was also studied at different inoculum size, temperature range (20–40 °C) and pH values (4–9). The pH of the medium was adjusted with 1 N HCl or NaOH.

2.5. Identification of effluent decolourizing consortium

2.5.1. Cultivation and DNA extraction

The effluent decolourizing consortium DMC comprised four morphologically different types of bacteria. Each of the bacterial cultures was grown on effluent agar plates and purified by streaking on the same culture medium. A pure colony of each bacterium was grown in nutrient broth till log phase growth was obtained. Genomic DNA from each isolate was extracted as described by Ausubel et al. (1997).

2.5.2. 16S rDNA amplification

Amplification of 16S rDNA fragment of each isolate was performed with 20–30 ng of bacterial DNA as template using 8f (AGAGTTTGATCCTGGCTCAG) and 1492r (GGTTACCTTGTTACGACTT) eubacterial universal primers (Eden et al., 1991; Weisburg et al., 1991) in a reaction mixture which consisted of 2.5 mM $MgCl_2$, 10 mM each of dNTPs, 1.5 U of Taq polymerase (BIORON, GmbH, Ludmigshafen, Germany), in an Ependorff Mastercycler® gradient. The thermocycler program included an initial denaturation of 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min annealing at 57 °C for 1 min, extension at 72 °C for 1 min and a final extension at

72 °C for 15 min. The amplified products were resolved on 1.2% (w/v) agarose gel.

2.5.3. Amplified rDNA restriction analysis (ARDRA) of 16S rRNA genes retrieved from spent wash decolourizing bacterial consortium

16S rDNA fragments amplified from the isolates were further screened by ARDRA. Ten microliters (200 ng) of these PCR products containing (1.48 kb) 16S rDNA from each sample were digested with 5 U of *Msp*I, *Hae*III and *Hha*I in 20 µl reaction mixtures and incubated at 37 °C for 12 h. Separation of the digested PCR fragments was performed by urea denaturing polyacrylamide gel electrophoresis (PAGE) with 6% (w/v) polyacrylamide [1:19], 6 M urea, and 1X Tris borate EDTA buffer in a gel of 1 mm thickness. Electrophoresis was carried out for 90 min at 250 V, followed by ethidium bromide staining. Gels were analyzed using UVP-GDS-8000 Gel Documentation Systems and scanned by Lab Works image acquisition and analysis software (UVP, Inc., Upland, California, USA).

2.5.4. 16S rDNA sequence analysis

16S rDNA (1.4 kb) amplified products were purified using polyethylene glycol (PEG) 6000 in presence of 2.5 M NaCl (Dunn and Blattner, 1987) and were sequenced using 8f and 1492r eubacterial universal primers in an automated DNA sequencer using Big Dye terminator Kit v.2 (Applied Biosystems, Rotkreuz, Switzerland).

3. Results and discussion

Decolourization and degradation of distillery spent wash has been a serious environmental concern, which is evident from the magnitude of research done in this field in the last decade (Pena et al., 1996; Dahiya et al., 2001a,b; Sirianuntapiboon et al., 2004a,b). The recalcitrance of melanoidins to biodegradation is apparent from the fact that these compounds escape various stages of waste water treatment plant and finally, enter the environment. Stringent regulations on the discharge of coloured effluent help to check the direct discharge of such effluent into the environment.

3.1. Characteristics of the anaerobically treated distillery spent wash

The chemical properties of the distillery spent wash obtained from the biomethanation treatment plant of Kanoria Chemicals Ltd., Ankleshwar Gujarat, India are shown in Table 1.

Table 1
Characteristics of anaerobically treated distillery spent wash

Parameters	Values of anaerobically treated effluent
pH	7.5–8
BOD (mg/l)	8000–10,000
COD (mg/l)	45,000–52,000
TN (mg/l)	4284
TOC (mg/l)	31,090
TS (mg/l)	72,500
TSS (mg/l)	40,700
Phosphates (mg/l)	1625
Sulphates (mg/l)	3875
Chlorides (mg/l)	7997
Phenols (mg/l)	7202
Total sugar (g %)	0.36
Reducing sugar (g %)	0.17

3.2. Enrichment and isolation of distillery spent wash decolourizing consortium

Decolourization of distilleryspent wash has been mainly observed in fungi (Ohmomo et al., 1987; FitzGibbon et al., 1998; Kahraman and Yesilada, 2003; Jimenez et al., 2003). There are a good number of reports showing role of fungi in decolourization of melanoidins by adsorption of melanoidins to mycelia (Pena et al., 1996) as well as role of ligninolytic enzymes (Watanabe et al., 1982; Raghukumar and Rivonkar, 2001; Vahabzadeh et al., 2004). However, there are very few reports indicating the role of bacteria in bioremediation of distillery spent wash. The bacterial consortium DMC was found to decolourize the effluent up to 27% and reduce COD up to 25% when incubated in static condition at 37 °C. Four different bacterial cultures were obtained upon isolation on effluent agar plates from the mixed consortium DMC, of which three were Gram negative cocco bacilli and one was Gram negative bacillus. When each pure culture was tested individually for its decolourization and COD reducing ability, none of the cultures showed appreciable level of decolourization or COD reduction.

3.3. Optimization of culture conditions for decolourization and COD reduction

3.3.1. Effect of media composition

The decolourizing and COD reducing ability of consortium DMC was investigated in different conditions such as distillery spent wash (DSW) only, with basal salts (DSW S), with glucose only (DSW G), with glucose and basal salts (DSW SG) and with nitrogen source basal salts and glucose (DSW SGN). The consortium DMC decolourized the anaerobically treated distillery spent wash most effectively in presence of basal salts and glucose and in absence of nitrogen source (67% reduction). In absence of basal salts or glucose the decolourization and COD reduction was very low (27%) and (25–36%), respectively (Fig. 1). Supplementation of a readily available carbon source such as glucose appeared to be necessary for decolourization. Although effective decolourization in absence of glucose has not been reported, COD reduction of anaerobically treated distillery effluent in absence of additional nutrients has been reported in bacteria such as *Aeromonas hydrophila*, *Enterobacter disslovens*, *Pseudomonas putida*, *Stenotrophomonas maltophila*, etc. (Gosh et al., 2004).

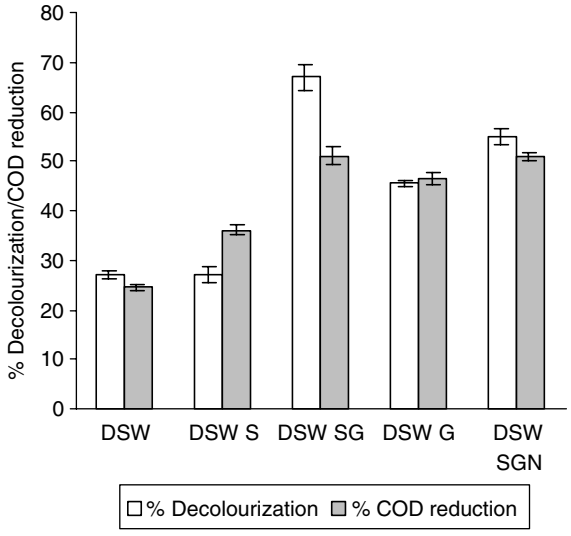

Fig. 1. Effect of media composition on decolourization and degradation of anaerobically treated distillery spent wash by consortium DMC at 37 °C within 72 h.

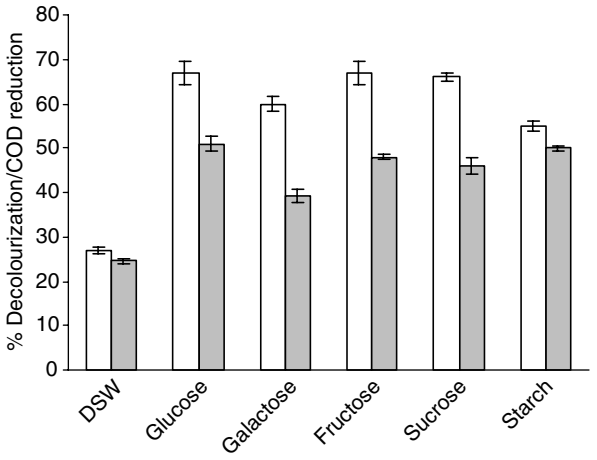

Fig. 2. Effect of carbon source on decolourization and degradation of anaerobically treated distillery spent wash by consortium DMC at 37 °C within 72 h.

3.3.2. *Effect of carbon source*

Various carbon sources have been used in attempting decolourization of distillery effluent (Pena et al., 1996; Kumar et al., 1997; Sirianuntapiboon et al., 2004a,b). Decolourization and COD reduction were monitored by using different carbon sources such as glucose, galactose, fructose, sucrose and starch at a concentration of 0.5% (w/v). Glucose was the best carbon source, allowing maximum decolourization (67%) and COD reduction (51%) at 0.5% concentration. The decolourizing ability of the consortium DMC was tested at different glucose concentrations ranging from 0.1% to 1% (w/v). The decolourizing efficiency of the consortium increased with increase in glucose concentration from 0.1% to 0.5% reaching maximum (67%) at 0.5%. Further increase in glucose concentration did not improve decolourization rather the COD reduction was less. Fructose, sucrose and galactose were found to be fairly good substrates allowing 60–67% decolourization and 40–48% COD reduction. Starch was relatively poor co-substrate, aiding only 55% decolourization (Fig. 2).

Gosh et al. (2002) reported the use of 1% glucose obligatory for growth and decolourization activity of *Pseudomonas putida*. A facultative anaerobic culture L-2 reported by Kumar et al. (1997) showed 31% decolourization and 57% COD reduction of effluent in presence of 1% glucose in 7 days. Sirianuntapiboon et al. (2004a,b) reported an acetogenic bacterium and yeast belonging to *Citeromyces* sp. decolourizing distillery waste in presence of 2–3% glucose in 8 days.

3.3.3. *Effect of nitrogen source*

The results of the effect of various organic and inorganic nitrogen sources at a concentration of 0.05% (w/v) on effluent decolourization and degradation showed that supplementation of nitrogen sources harmfully affected bacterial decolourization of the effluent. Addition of organic nitrogen sources such as yeast extract, peptone, beef extract and tryptone brought down the level of decolourization to 55% whereas inorganic nitrogen sources such as sodium nitrate and ammonium nitrate reduced the decolourization values to 36% and 18%, respectively (Fig. 3).

Fahy et al. (1997) reported similar findings of colour intensification of effluent in presence of nitrogen source. A similar phenomenon was reported for a number of bacterial strains (Fahy et al., 1997). Miyata et al. (2000) found inhibitory effects of organic nitrogen on melanoidin decolourization by *Coriolus hirsutus*. However, Sirianuntapiboon et al. (2004a,b) reported sodium nitrate for inducing decolourizing activity in yeast *Citeromyces* sp. and yeast extract and peptone in acetogenic bacterium strain no. BP103.

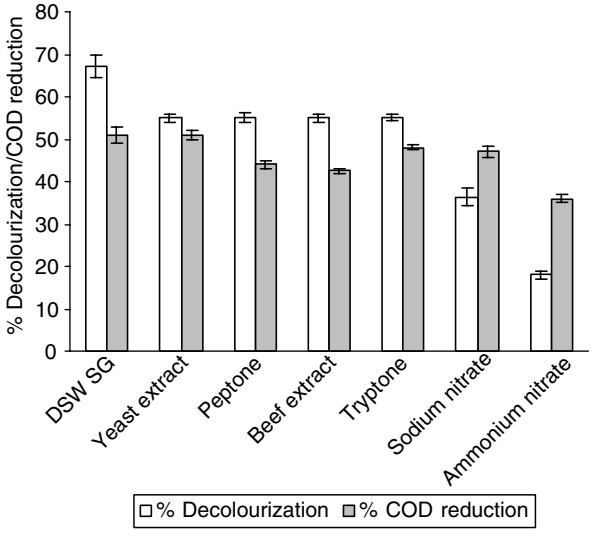

Fig. 3. Effect of nitrogen source on decolourization and degradation of anaerobically treated distillery spent wash by consortium DMC at 37 °C within 72 h.

3.3.4. Effect of initial pH, temperature, inoculum size and agitation

Results showed that the highest decolourization (67%) and COD reduction (51%) was at pH 7 (data not shown). Melanoidin solubility depends on pH; it is less soluble in acidic pH than in alkaline pH (Pena et al., 1996). Higher and lower than pH 7 led to decrease in decolourization activity as well as the growth of the consortium. Sirianuntapiboon et al. (2004a) have reported highest decolourizing activity by an acetogenic strain in the pH range of 5–7.

Effluent decolourization is affected by incubation temperature (Kumar et al., 1997). It was observed that increase in temperature from 20 to 37 °C was accompanied with increase in decolourization and COD reduction from 35% to 67% and 27% to 51%, respectively (data not shown). Further increase in temperature to 40 °C adversely affected the decolourization and COD reduction ability of the consortium.

It was observed that decolourization increased with increase in inoculum size. Maximum decolourization and COD reduction was observed at 15% (v/v) inoculum concentration (approximately 11×10^6 CFU/ml in the medium). Further increase in the inoculum size did not improve the decolourization (data not shown). Sirianuntapiboon et al. (2004b) reported similar results on studying the relationship between inoculum size and decolourization. Dahiya et al. (2001a) also carried out studies on effect of mycelial inoculum size on effluent decolourization and found that 5% (w/v) inoculum was the optimum and further increase in inoculum concentration produced more fungal biomass where as the decolourization of the medium was not improved.

Bacterial consortium DMC exhibited maximum decolourizing activity only when incubated under static condition (data not shown), whereas agitated cultures although grew well and also brought about higher COD reduction (66%) but showed low decolourization (38%).

3.3.5. Time course of growth, decolourization and COD reduction

Time course of effluent decolourization and COD reduction was studied along with the growth of the consortium. With increase in time there was increase in cell mass, increased decolourization and reduction in COD. Maximum decolourization ($67 \pm 2\%$) was attained in 24 h (Fig. 4) and there was no further colour removal; however, increase in cell mass continued. Maximum COD reduction ($51 \pm 2\%$) was obtained in 72 h and after this the consortium entered stationary growth phase showing no significant increase in COD reduction and cell mass (data not shown).

3.4. Differentiation of bacterial isolates in the consortium and analysis of the sequenced data

Restriction fragment analysis was carried out of all isolates and when these patterns were observed on the gel

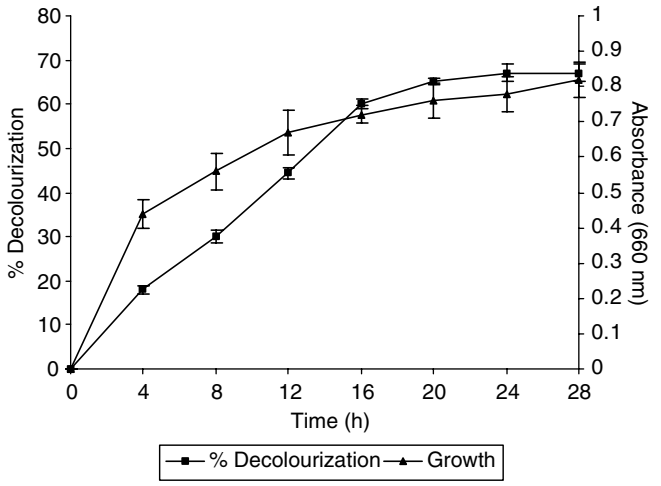

Fig. 4. Time course of decolourization of anaerobically treated distillery spent wash by consortium DMC at 37 °C.

(ARDRA), two patterns were noticed i.e. cultures 1 and 3 were identical, whereas different patterns were observed for cultures 2 and 4, differentiating them from the rest. This was further confirmed by subjecting the 16S rDNA amplified products to DNA sequencing (Figs. 5 and 6).

The 16S rRNA gene, a 1484 bp fragment, obtained from the isolates was sequenced using 8f and 1492r along with internal primers. These complete sequences were then probed using NCBI BLASTn program (Altschul et al., 1997). Pair wise alignments giving a closest match of 99% with sequences analyzed were chosen. The sequences

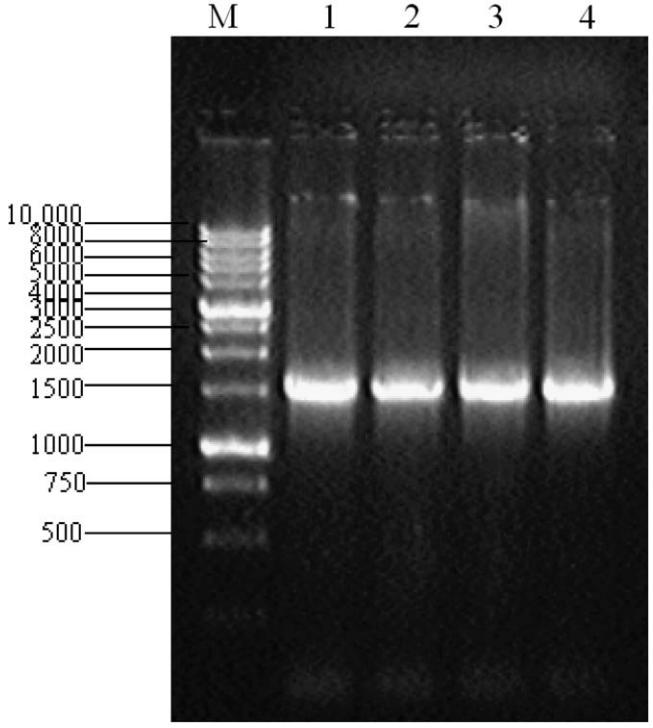

Fig. 5. PCR amplicons of 16S rDNA fragment.

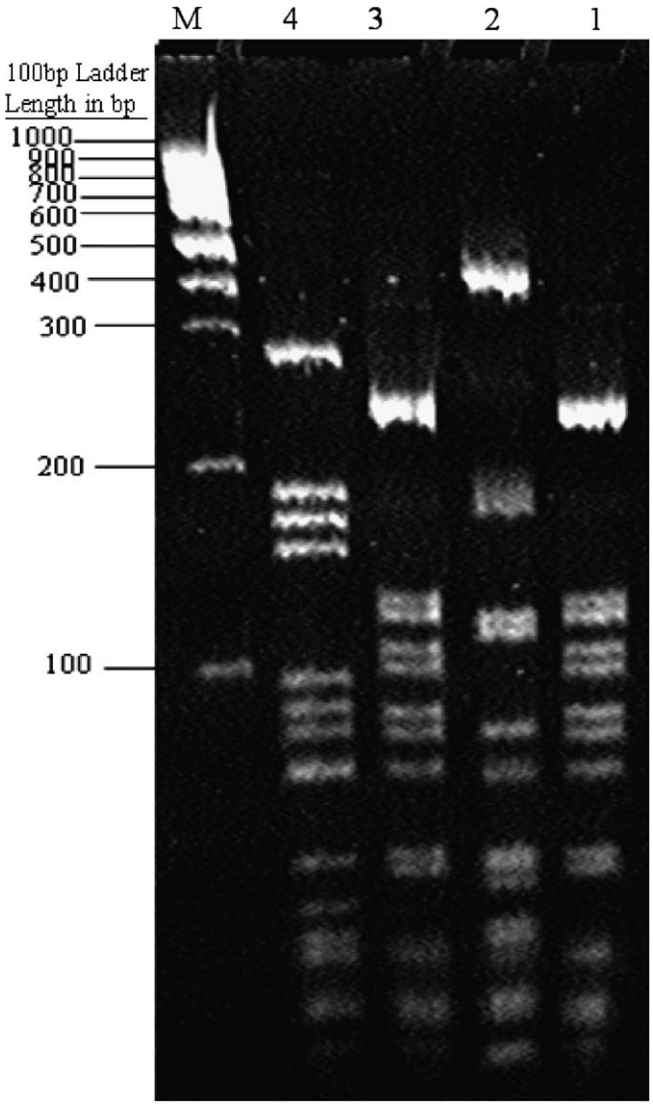

Fig. 6. ARDRA profiles of isolates from distillery spent wash decolourizing consortium DMC.

retrieved from the NCBI database giving the closest match in pair wise BLAST were identified as *Pseudomonas aeruginosa* PAO1 (cultures 1and 3), *Stenotrophomonas maltophila* (culture 2) and *Proteus mirabilis* (culture 4).

4. Conclusions

Application of bacterial cultures for biodegradation and decolourization of post biomethanated distillery effluent could be to be a pragmatic approach. Bacterial consortium DMC, comprising *Pseudomonas aeruginosa* PAO1, *Stenotrophomonas maltophila* and *Proteus mirabilis* appeared a good choice one than earlier reported cultures in terms of low additional nutrient requirements i.e. basal salts and glucose only and without any nitrogen source for its activity. Ability of the culture to decolourize the effluent in static condition within 72 h and to reduce COD signified the novelty of the consortium DMC.

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