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Decolourization of molasses based distillery wastewater using a bacterial consortium

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ABSTRACT: The effluent from sugarcane molasses based distilleries leads to environmental pollution due to its large volume and the presence of dark brown coloured compounds. In this study, a bacterial consortium isolated from waterfall sediments was selected as a molasses-decolourizing agent. The effect of culture conditions and medium composition on decolourization activity and growth of the bacterial consortium was investigated. The bacterial consortium was able to grow and decolourize molasses wastewater under facultative and anaerobic conditions in general. Aerobic culture conditions at pH 7 and 9 in molasses wastewater containing LB medium exhibited high growth but poor decolourization. The addition of a supplementary nutrient source in molasses wastewater medium significantly increased the decolourization activity of the bacterial consortium by up to 26.5% within 48 h under anaerobic conditions. Comparison of 16S rDNA sequences indicated that the bacterial consortium which showed decolourization activity under aerobic conditions consisted of *Acinetobacter* sp., *Pseudomonas* sp., *Comamonas* sp., *Klebsiella oxytoca*, *Serratia marcescens* and unidentified bacteria, whereas, the anaerobically enriched consortium consisted of *Pseudomonas* sp., *Klebsiella oxytoca*, *Bacillus cereus* and *Citrobacter farmeri*, a mercury-resistant bacterium, and an unidentified bacterium. Denaturing gradient gel electrophoresis profiles indicated that the community structures of aerobically and anaerobically enriched bacterial consortium were different.

KEYWORDS: 16S rDNA, DGGE, melanoidins, effluent, biological treatment

INTRODUCTION

Molasses is the one of the by-products of cane sugar production and is used in various fermentation processes, biofertilizer production, and feed for domestic animal[1]. In Thailand, molasses is commonly used as a raw material in alcohol distillery industries because of its low cost, availability, and suitability for fermentation processes. However, wastewater from molasses processing contains an extremely high chemical oxygen demand (COD) and biochemical oxygen demand (BOD), as well as a low pH and strong dark brown colour. After conventional biological treatment, most of the organic load is removed but nevertheless the dark brown colour still persists and it can even increase due to repolymerization of coloured compounds. The major coloured compounds are melanoidins which are the products of the Maillard reaction between sugar and amino acid produced upon heating[2]. This coloured polymer is a major water pollutant. Melanoidins also reduce the penetration of sunlight though rivers or lakes which as a result decreases both photosynthetic activity and dissolved oxygen concentration. This adversely affects many aquatic plants and animals. Its disposal into soil is equally detrimental, causing inhibition of seed germination and depletion of vegetation by acidified soil[3].

Several alcohol distillery industries in Thailand have attempted to treat molasses wastewater by anaerobic methods such as methane fermentation and using a waste stabilization pond system followed by aerobic treatment using an activated sludge system, aerated lagoon, or oxidation pond[4]. However, after these treatment processes, almost all of melanoidins in

molasses wastewater still remain and the COD of the treated wastewater is higher than the permitted value given by of Department of Industrial Works, Ministry of Industry, Thailand[5].

Decolourization of molasses wastewater has been attempted, but with limited success so far[1,6]. Treatment processes such as chemical precipitation, chemical adsorption[7], flocculation treatments[8], physiochemical treatments such as ozonization[9], and activated carbon adsorption[10] have been used for removal of melanoidins from treated wastewater. However, these processes still have disadvantages due to the high operation cost, high consumption of chemical agents, fluctuation of colour removal efficiency, high volume of solid waste produced, formation of hazardous byproducts, and intensive energy requirements. Biological treatments with microbial biodegradation methods are drawing attention because they are environmental friendly and cost competitive alternatives to chemical decomposition processes[11]. Biological treatments, using fungi such as *Coriolus* sp.[12], *Aspergillus* sp.[13], *Aspergillus niger*[14], *Trametes versicolor*[15], *Phanerochaete* sp.[16], *Phanerochaete chrysosporium*[17], *Coriolus versicolor*[17], *Coriolus hirsutus*[18] and *Penicillium decumbes*[19], yeasts such as *Citeromyces* sp.[20], and certain bacteria such as *Bacillus* sp.[21,22], *Pseudomonas fluorescence*[1], and acetogenic bacteria[6] have been reported.

There are a good number of reports showing the role of fungi in the decolourization of melanoidins by adsorption to mycelia as well as the role of ligninolytic enzymes[23,24]. However, the long growth cycle and spore formation limit the performance of the fungal system. In contrast, bacterial decolourization is usually faster, but it may require a mixed culture to decolourize molasses wastewater through combined metabolic modes of bacterial strains[25,26]. Adikane and his co-workers[25] reported that 69% decolourization of molasses spent wash was achieved by using soil samples as inoculum instead of isolated microorganisms[25]. Also, the mixed culture of *Bacillus* spp. exhibited a two- to four-fold increase in melanoidins decolourization over that shown by any individual *Bacillus* isolate[26]. Hence, mixed culture studies seem to be more promising for decolourization molasses wastewater. Until now, there have been very few reports describing the use of bacterial consortia in decolourization of distillery spent wash.

Here we explore the feasibility of using a mixed culture for molasses wastewater decolourization, with the ultimate aim of applying it to effluent treatment. In this work, the bacterial consortium capable of decolourizing molasses wastewater was isolated from sediments obtained from Moh-Peng waterfall at Maehongsorn Province in the North of Thailand. Various parameters for maximal molasses wastewater decolourization were obtained. Identification of bacteria in the consortium was performed using a 16S rDNA based molecular approach.

MATERIALS AND METHODS

Molasses wastewater

Molasses wastewater was obtained from Sangsom Co., Ltd., a sugarcane molasses alcoholic distillery in Nakornpathom Province, Thailand. The effluent samples were stored at 4 °C. The effluent was characterized and analysed for pH, COD, and BOD, based on the methods in Ref. 27. The wastewater showed the following characteristics: COD 100 000 mg/l, BOD 35 000 mg/l, TDS 7600 mg/l, SS 15 000 mg/l, dark brown colour, and pH 3.8–4.5.

Screening of molasses-decolourizing bacterial consortium

Various samples including soil, sediment, and wastewater were collected from various sources in Thailand for isolation of the bacterial consortium. In order to enrich molasses-decolourizing bacteria, 5 ml of each sample was transferred into 50 ml of LB medium supplemented with cyclohexamide (50 µg/ml) and cultivated at 30 °C on a rotary shaker at 200 rpm. For primary screening, enriched bacterial cultures were loaded into agar wells of 0.7 cm diameter on the modified molasses wastewater (MM) agar plates with sterile LB medium as control. Decolourization was observed as a clear zone around the holes after incubation at room temperature for 48 h under either aerobic or anaerobic conditions. As secondary screening, bacterial consortia which showed relatively high molasses decolourization in the primary screening were cultured in the modified molasses wastewater medium (MM) at 30 °C on a rotary shaker at 200 rpm. The determination of decolourization was carried out from the supernatants by measuring the optical density at 475 nm with a UV/VIS spectrophotometer and expressed as the percentage of decrease in the absorbance[17].

Preparation of molasses wastewater medium

The decolourization of molasses wastewater by the selected bacterial consortium was evaluated using three types of culture media. Molasses wastewater medium (WW) was prepared by diluting molasses wastewater with distilled water to 20% (v/v). Molasses wastewater containing LB medium (LBWW)

was prepared by adding 20% of molasses wastewater to LB medium. Modified molasses wastewater medium (MM) consisted of 20% of molasses wastewater, 0.01% $NaNO_3$, 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.01% $MgSO_4 \cdot 12\,H_2O$, 2% glucose, and 0.1% yeast extract.

Decolourization experiments

The molasses-decolourizing activity of the bacterial consortia were determined in various media and culture conditions. The selected bacterial consortium was cultivated in LB medium under either aerobic or anaerobic condition at 30 °C. After 48 h, the cultured medium was then centrifuged at 11 000*g*, 4 °C for 10 min. Bacterial cells were washed twice and resuspended in 0.85% NaCl to an OD_{600} of 1. The decolourization experiments were carried out by first transferring washed bacterial cells into test tubes containing a culture medium (WW, LBWW, or MM) and incubating at 30 °C for 48 h. Each tube was withdrawn for determination of cell dry weight and residual colour by OD_{475} as described above. Each experiment was carried out in triplicate. In addition, controls containing culture media without inoculum were also prepared to observe abiotic decolourization.

The effect of initial pH and aeration on the decolourization was also investigated. Culture media (WW, LBWW, and MM) were prepared at pH 4, 7, and 9 and the bacterial cells were then inoculated and cultivated under different aeration conditions: agitation at 200 rpm (aerobic), without agitation (facultative), and air replaced with non-oxygen gas in an anaerobic jar (anaerobic). Similarly, cell dry weight and absorbance were determined as mentioned above.

Identification of effluent decolourizing consortium by 16S rDNA sequence

Each bacterium in the consortium enriched in aerobic and anaerobic conditions was isolated into pure culture. Each isolate was transferred into liquid medium for 12 h. The cultures were centrifuged at 11 000*g* for 2–5 min and cells were resuspended in 100–500 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Bacteria were lysed by boiling for 10–15 min. Genomic DNAs were separated by centrifugation at 9200–13 000*g* for 5–10 min and used as a template for 16S rDNA amplification using primers 20F (5′-GAGTTTGATCCTGGCTCAG-3′) and 802R (5′-TACCAGGGTATCTAATCC-3′). The PCR products were used as template for PCR based DNA sequencing with UFUL primer (5′-GCCTAACACATGCAAGTCGA-3′) and Bigdye Termination v3.1 cycle sequencing kit (Master Mix)[28]. The sequences of 16S rDNA were compared with those available in the GenBank, EMBL, and DJB databases using the gapped BLASTN 2.0.5 program through the National Centre for Biotechnology Information.

Analysis of the microbial community using denaturing gradient gel electrophoresis (DGGE) of 16S rDNA

DNA was directly extracted from the bacterial culture with a bead-beating instrument and UltraClean Soil DNA Kit (MO BIO Laboratories). Then, each DNA sample was amplified by 16S rDNA PCR technique using PRBA 338F+CG clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3′) and PRUN518R (5′-ATACCGCGGCTGCTGG-3′) primers as described by the manufacturer. PCR product was then run on 8% polyacrylamide gel with a denaturing gradient of urea and formamide denaturant ranging from 25–60% for 5 h at 130 V with 1 × TAE. The DGGE gel was stained in 50 µg/ml ethidium bromide for 20 min. DNA band profiles were obtained under the UV transilluminator[29,30].

RESULTS AND DISCUSSION

Screening of molasses wastewater-decolourizing bacteria consortia

After aerobic enrichment of bacterial consortia from various sources in LB medium, the results of the primary screening showed that 9 different bacterial consortia were capable of decolourizing molasses wastewater under aerobic or anaerobic conditions or both as observed by a clear zone around agar wells on the MM agar plate. The molasses decolourization of those 9 consortia was re-tested in MM medium as a secondary screening. The bacterial consortium enriched from waterfall sediments in Maehongsorn province, Thailand was selected for further study due to its highest decolourization of 20% when cultivated in the MM medium under aerobic conditions for 48 h.

Optimization of culture conditions for decolourization

Fig. 1 shows the molasses-decolourizing activity of the bacterial consortium which had been enriched in LB medium under various media and culture conditions. The results showed that the decolourizing activity of bacterial consortium incubated in LBWW medium at pH 4 under anaerobic conditions had the highest decolourizing activity of 26.5% (Fig. 1a). In contrast, only 4.8% of decolourization was observed with WW medium with the same culture conditions.

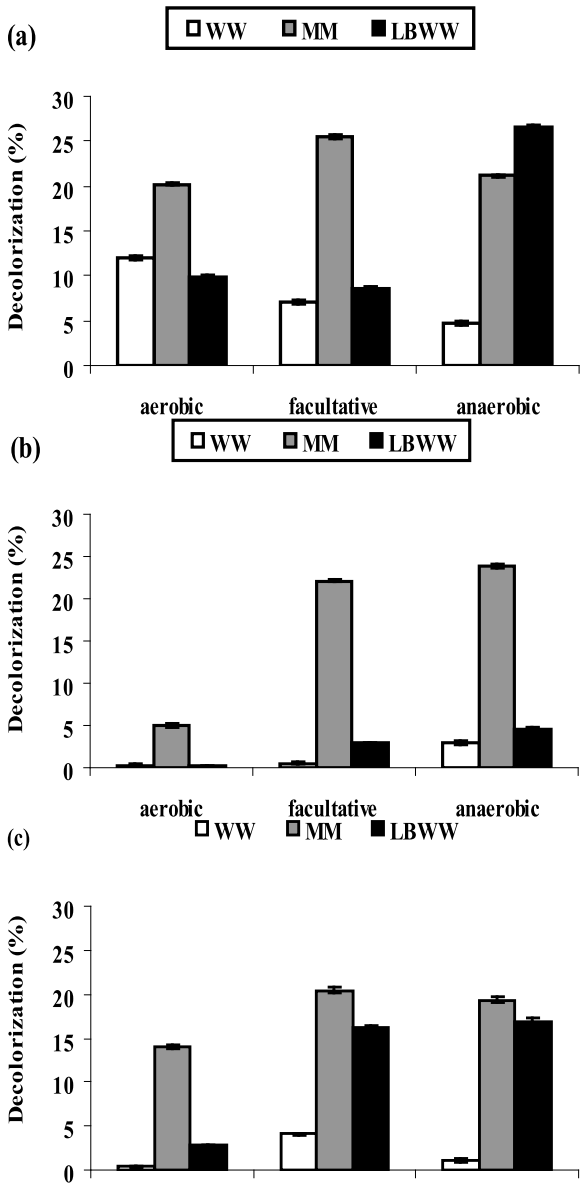

Fig. 1 Effect of medium composition and culture conditions on decolourization of molasses wastewater by a bacterial consortium at (a) pH 4 (b) pH 7 (c) pH 9. Each bar represents the average of three independent experiments and error bars indicate standard deviation.

It is possible that the WW medium lacks some nutrients and salts necessary for promoting growth and decolourization of the consortium. It was also found that the decolourization of a consortium incubated in MM medium was higher than other culture media under aerobic and facultative conditions. However, decolourization in MM medium was highest (25.5%) after 48 h under facultative conditions at pH 4.

At pH 7, the maximum decolourization of 23.8% was found in MM medium under anaerobic conditions (Fig. 1b), whereas the consortium showed only 5% of decolourization in MM medium under aerobic condition at the same pH. At pH 9, the highest decolourization activity of 20.4% was achieved in MM medium under facultative conditions (Fig. 1c).

Fig. 2a shows the growth of the bacterial consortium after being incubated in various culture media at pH 4 under aerobic, facultative and anaerobic conditions. Bacteria had relatively low growth in all culture media and conditions. The growth of the bacterial consortium in MM medium at pH 7 under anaerobic condition was higher than any other media and culture conditions (Fig. 2b). The maximum bacterial growth was found in LBWW medium under aerobic conditions at pH 9 (Fig. 2c). The growth of bacterial consortia in WW medium was very low in all culture conditions.

The difference in decolourization among various culture media might be due to the fact that molasses wastewater was deficient in carbon and biodegradation without any extra carbon source was found to be very difficult. Its recalcitrance was also due to the presence of melanoidins. Hence, supplementation of labile carbon sources appear to be necessary for decolourization of molasses wastewater by the bacterial consortium. Kambe et al[22] reported the maximum colour removal of molasses (A_{475} = 7.0) at 35.5% by *Bacillus smithii* at 55 °C under anaerobic conditions in the presence of either peptone or yeast extract as supplemental nutrient, while this strain could not use molasses wastewater as sole carbon source[22]. Kumar and Chandra[26] have also reported that the addition of 1% glucose as a supplementary carbon source was necessary for molasses decolourization of the modified GPYM medium containing melanoidins (10% v/v) by *Bacillus thuringiensis*, *Bacillus brevis*, and *Bacillus* sp. up to 22%, 27.4%, and 27.4%, respectively[26]. Reportedly, acetogenic bacterium strain No. BP103 could also decolourize 73.5% of molasses pigments (A_{475} = 3.5) in molasses wastewater medium supplemented with glucose, yeast extract, and basal mineral salts whereas the decolourization yields of this strain were dramatically decreased to only 9.75% in the absence of a nutrient supplement[6]. A similar result was observed for the decolourization by bacterial consortium DMC which achieved its maximum molasses decolourization (67%) using basal medium containing distillery spent wash (A_{475} = 2.8–3) in the presence of 0.5% glucose[31].

In general, several microorganisms that have been shown previously to degrade melanoidins are not best

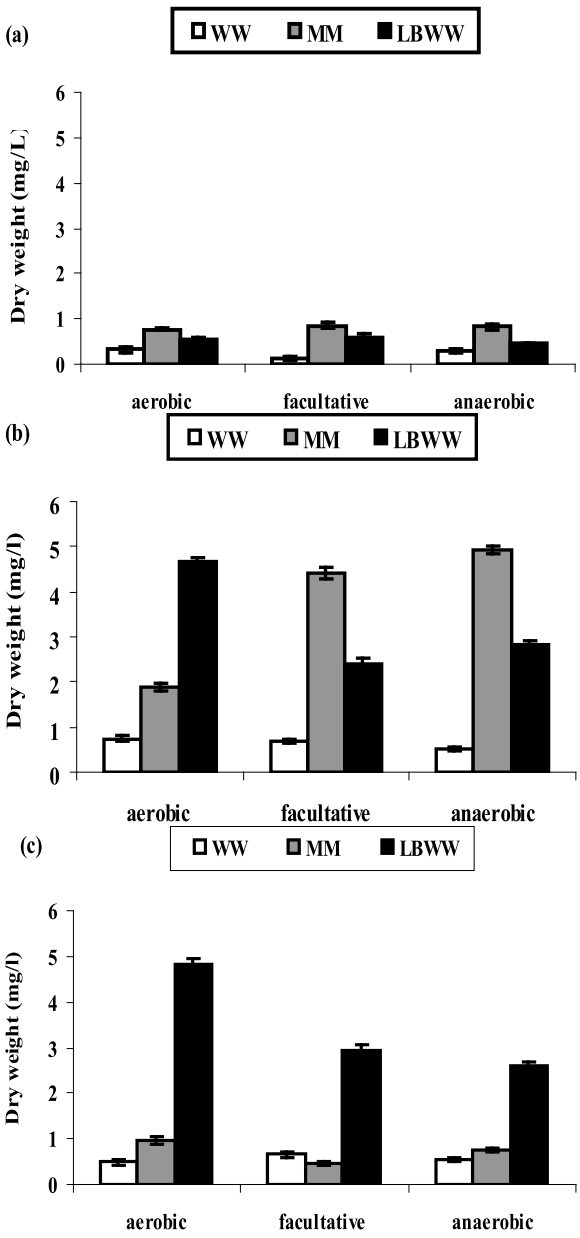

Fig. 2 Effect of medium composition and culture condition on growth of bacterial consortium at (a) pH 4 (b) pH 7 (c) pH 9. Each bar represents the average of three independent experiments and error bars indicate standard deviation.

suited for treating effluent from molasses based distilleries. This is because it is depleted in oxygen which is necessary for oxidative degradation of melanoidins. Interestingly, the results presented in this study show that colour removal of this consortium under facultative and anaerobic conditions were higher than with aerobic conditions in many tested conditions. This might be due to the metabolism of bacterial cell under facultative and anaerobic conditions such as fermentation and anaerobic respiration [19]. Since cell adsorption has been also reported as another mechanism potentially involved in decolourization [20], the experiment on abiotic decolourization was included in order to verify whether the decolourization of molasses wastewater resulted from biological activity or non-biological activity. It was clearly shown that the experiment with the different initial cell concentrations (5–50% v/v) of autoclaved cells, exhibited no decolourization after incubation for 48 h. In addition, to see if there is any adsorption of melanoidins by bacterial cells during incubation, alkaline extraction with 0.1 M NaOH was performed with both living cells and autoclaved cells [20]. The results showed by OD_{475} that NaOH-extractable colour substances were negligible (data not shown). This indicated that decolourization by the bacterial consortia used in this study was due to biological mechanisms.

Time course of growth and decolourization activity of the bacterial consortium

In the study on the optimization of culture conditions, our results demonstrated that the bacterial consortium gave the highest decolourizing activity in LBWW medium, pH 4 under anaerobic conditions. However, LBWW medium might never be accepted for application due to excessive chemical usage and high cost. Thus, MM medium was selected for further study. With MM medium, the bacterial consortium showed the highest decolourization activity at pH 4 under facultative conditions. Unfortunately, molasses wastewater treatments mostly have been operated under aerobic or anaerobic conditions. Anaerobic treatment of molasses wastewater requires lower energy input and nutrients. However, during operation, the inhibitory substances such as phenolic compounds are formed in the system leading to toxicity and an inhibitory effect on the microorganisms responsible for the anaerobic process [19]. Aerobic treatment systems have been also used for the treatment of various kinds of wastewater such as domestic wastewater, dye wastewater, and feed wastewater since they can remove a high organic load, they are resistant to organic shock load and odourless, and the process is easy to maintain. The aeration in the system promotes growth of microorganisms and confers a uniform population which enhances the consumption of organic loads [4, 32].

Accordingly, in this study, the time course of the growth and decolourization of the bacterial consortium was investigated in MM medium with the initial pH of 4 under aerobic conditions. In order to increase the bacterial biomass, the inoculum was

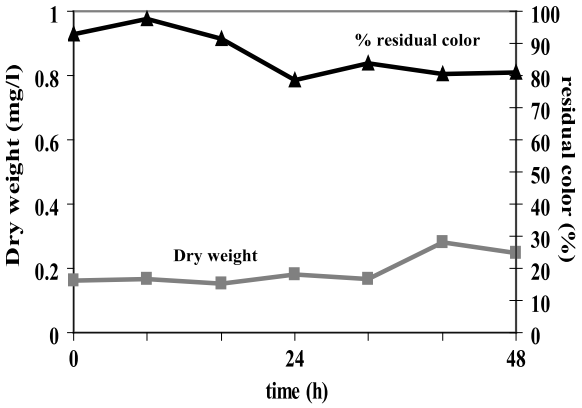

Fig. 3 Time course of decolourization of molasses wastewater by bacterial consortium in modified molasses wastewater medium (MM) at pH 4 under aerobic conditions.

prepared in LB medium under aerobic conditions prior to transferring to MM medium and cultivated under conditions as indicated above. A typical time course of molasses decolourization in MM medium by a bacterial consortium under selected condition is shown in Fig. 3. The consortium decolourized MM medium during the first 24 h of culture, and the colour removal of 20% was achieved after 48 h. The growth of the consortium increased with increasing cultivation period.

The decolourization with this bacterial consortium was lower than those of the fungi *Basidiomycetes* and *Deuteromycetes* previously reported[33,34]. However, the application of fungi to remove melanoidins in molasses wastewater is inconvenient due to their slow growth, spore production and infectivity[34]. To date, bacterial decolourization of molasses wastewater has been reported by many researchers[1,6,21,22]. It was established that bacteria, especially pure culture, displayed a limited capability to decolourize melanoidins because the toxicity of metabolites which were formed and accumulated during decolourization, thereby compromised the efficiency of bacteria[3]. Therefore, the application of bacterial consortium might be a more promising strategy for decolourization of molasses wastewater by biological treatment since each bacterial member of consortium might have different metabolic activities leading to effective decolourization against a broad spectrum of structurally diverse melanoidins[35,36].

Identification of bacterial isolates in the consortium

The molasses wastewater decolourizing bacteria which had been enriched in LB medium under aerobic and anaerobic conditions comprised of 8 aerobic (AE1-AE8) and 8 anaerobic (AN1-AN8) bacteria. Each of the bacterial culture was subjected to 16S rDNA sequencing and compared with those deposited in GenBank using the BLAST program. Bacterial were identified as follows: AE1, *Serratia marcescens*; AE2, unknown bacterium; AE3, *Acinetobacter* sp.; AE4, *Pseudomonas* sp.; AE5, unknown bacterium; AE6, *Comamonas* sp.; AE7, *Klebsiella oxytoca*; AE8, unknown gamma proteobacterium; AN1, *Pseudomonas* sp.; AN2, *Klebsiella oxytoca*; AN3, *Pseudomonas* sp.; AN4, unknown bacterium; AN5, *Bacillus cereus*; AN6, *Citrobacter farmeri*; AN7, mercury-resistant bacterium; and AN8, *Pseudomonas* sp. Some of the bacteria present in this consortium were previously reported as a molasses decolourizing bacteria, e.g., *Pseudomonas*, *Acinetobacter*, *Klebsiella*, and *Bacillus*[26,31,37].

Analysis of microbial community

PCR-DGGE method based on 16S rDNA was used to assess changes in microbial communities[38]. In this study, the changes in microbial community were

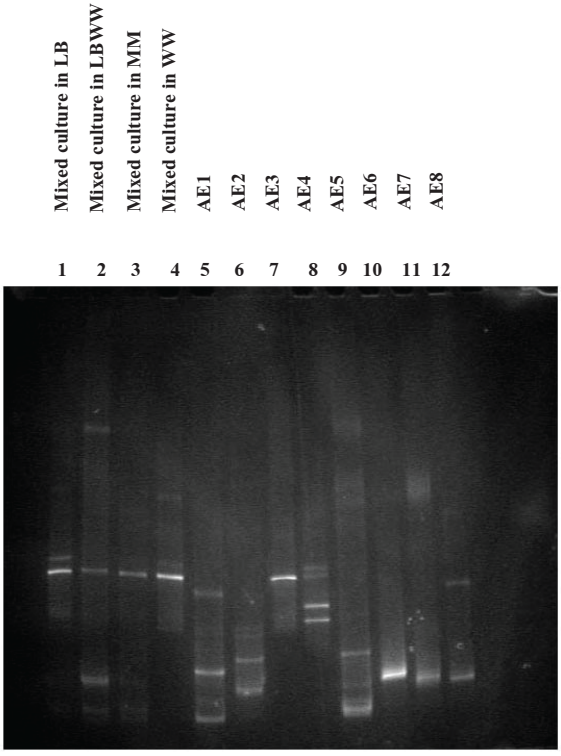

Fig. 4 DGGE pattern constructed from the bacteria present in the enriched bacterial community under aerobic conditions.

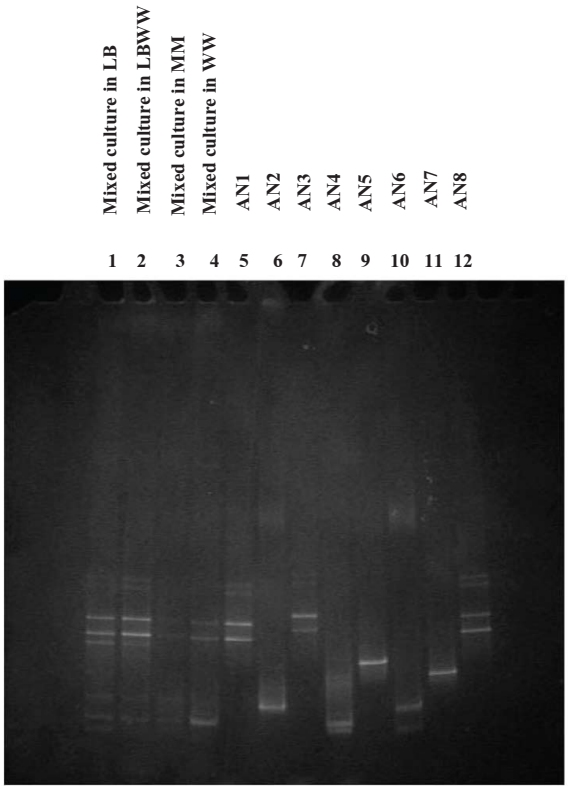

Fig. 5 DGGE pattern constructed from the bacteria present in the enriched bacterial community under anaerobic conditions.

investigated among four culture media at the initial pH of 4 under aerobic and anaerobic conditions (Figs. 4 and 5). DGGE patterns from the bacterial consortium with different media in aerobic condition were compared with those of pure cultures (Fig. 4). Profiles of the bacterial consortium in LB medium (lane 1), LBWW medium (lane 2), MM medium (lane 3), and WW medium (lane 4) are comparable with a common band dominantly detected, potentially corresponding to AE3 (lane 7). DGGE patterns of bacterial consortium and pure cultures in anaerobic conditions were also shown in Fig. 5. Profiles of bacterial consortium in LB medium (lane 1), LBWW medium (lane 2), MM medium (lane 3), and WW medium (lane 4) are similar to common bands detected, potentially corresponding to those of AN1, AN3, and AN8 (lane 5, 7, and 12).

DGGE analyses showed a significant difference in the bacterial community between aerobically and anaerobically enriched consortium (Figs. 4 and 5). Also, DGGE analysis revealed that the different bacteria were responsible for the molasses decolourization in the different culture conditions. Therefore, the selection of both aeration conditions and medium compositions were factors shaping the bacterial composition in the decolourizing consortium.

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