CHAPTER IV

RESULTS

4.1 Screening of molasses wastewater-decolorizing bacterial isolates

Samples collected from various sources in Thailand, e.g. soils, sediments, and alcoholic distillery effluents, were screened for bacterial isolates capable of decolorizing sugarcane molasses wastewater. Potential bacterial isolates were selected on the basis of rapid molasses decolorization. All isolates were tested for molasses decolorization using the agar plate method, as primary screening, and the shaking culture method, as secondary screening, described as follow.

4.1.1 Primary screening

Five ml of water sample or 5 g of soil samples were added to 50 ml LB broth and incubated at 30°C on rotary shaker 200 rpm for 48 h. Enriched samples were then plated onto MM agar plates and incubated for 48 h at 30°C. All samples were observed for molasses decolorization: a clear zone appeared around the bacterial colony.

After 48 h of incubation, it was found that 90 bacterial isolates showed the clear zone around their colonies on the agar plate (Fig. 4.1). It meant that these isolates showed molasses decolorization and the details of these isolates are shown in Table 4.1. All isolated colonies showing molasses decolorization on MM agar plate were purified by streaking technique and maintained on MM agar slants at 4°C.

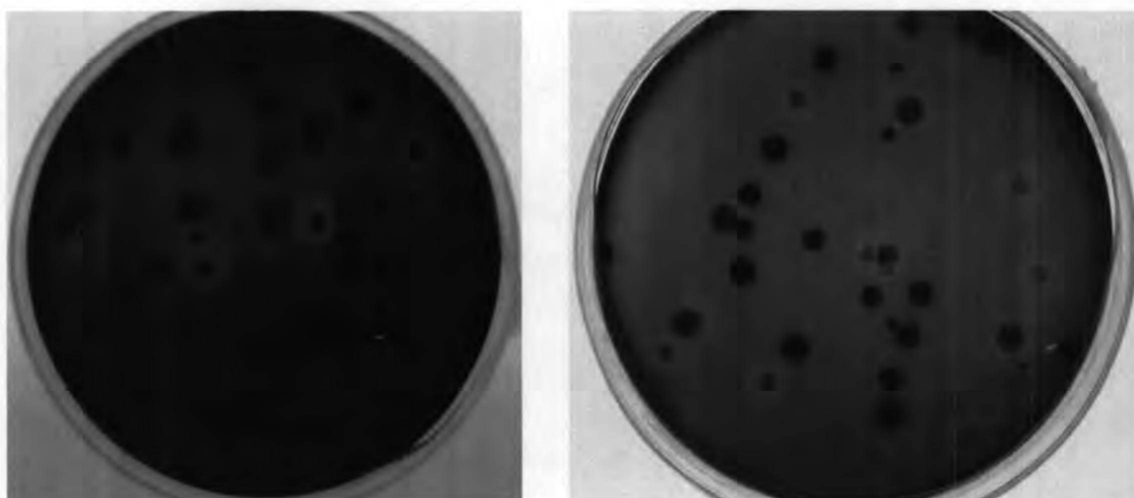

Figure 4.1 Characterization of bacterial colonies on MM agar plates under aerobic incubation for 48 h.

Table 4.1 Primary screening for molasses-decolorizing bacteria.

	Samples	**Number of decolorizing bacteria**	**Isolates**
1	Soil sample 1 from Sangsom Co., Ltd.	4	A1, A2, A5 and A6
2	Soil sample 2 from Sangsom Co., Ltd.	-	-
3	Soil sample 3 from Sangsom Co., Ltd.	3	C2, C3 and C4
4	Slop sample 1 from Sangsom Co., Ltd.	5	D1-D5
5	Slop sample 2 from Sangsom Co., Ltd.	37	E1-E4, E5 and E6-E37
6	Soil sample from Nakonrachasrima Province	4	F1, F2, F3 and F8
7	Soil sample from Srinan national park, Nan Province	4	G1-G4
8	Soil sample 1 from Nan Province	7	H1-H7
9	Soil sample 2 from Nan Province	-	-
10	Soil sample 3 from Nan Province	5	J1-J5
11	Soil sample 4 from Nan Province	4	K1-K4
12	Soil sample 5 from Nan Province	4	L1-L4
13	Soil sample 6 from Nan Province	3	M1-M3
14	Soil sample 7 from Nan Province	-	-
15	Soil sample 8 from Nan Province	-	-
16	Soil sample 9 from Nan Province	6	P1-P6
17	Soil sample from Pukraduang, Leay Province	4	Q1-Q4
Total (isolates)		90	

4.1.2 Secondary screening

In order to select the effective bacterial isolates, the second step of screening was performed. Ninety isolates which showed molasses decolorization in the first step were cultured using the liquid culture method with MM medium. Each isolate was cultured in 50 ml of medium in 250 ml Erlenmeyer flask on rotary shaker at 200 rpm, 30°C for 2 days. Subsequently, the culture broths were collected and centrifuged at 10,000 rpm, 4°C for 10 min. The supernatants were determined for the decolorization by measurement of optical density (OD) of the supernatant at 475 nm. Decolorization of molasses wastewater in MM medium by 90 isolates is shown in Table 4.2.

From the secondary screening, it was found that 26 out of 90 isolates showed molasses decolorization. Only ten isolates i.e. E5, E15, E22, F2, F3, F8, G3, G4, P3 and P4, showed decolorization higher than 10% when compared with control. The isolate E5, which was isolated from slop sample from Sangsom Co.Ltd. alcoholic distillery, showed the highest molasses decolorization at 18.8%. It was selected for further investigations.

Table 4.2 Secondary screening for molasses-decolorizing bacteria

Sample	Decolorization (%)
A1	0.00
A2	0.52
A5	0.00
A6	0.00
C2	0.00
C3	0.00
C4	0.00
D1	0.00
D2	0.00
D3	0.00
D4	0.00
D5	0.00
E1	0.00
E2	2.42
E3	3.87
E4	1.54
E5	**18.80**
E6	0.00
E7	2.59
E8	2.23
E9	4.53
E10	0.64
E11	0.00
E12	1.17
E13	1.59
E14	2.08
E15	**13.64**
E16	0.00
E17	0.00
E18	0.00

Sample	Decolorization (%)
E19	0.00
E20	0.00
E21	0.00
E22	14.24
E23	0.00
E24	0.00
E25	0.00
E26	0.00
E27	0.00
E28	0.00
E29	0.00
E30	0.00
E31	0.00
E32	0.00
E33	0.00
E34	0.00
E35	0.00
E36	0.00
E37	0.00
F1	0.00
F2	16.75
F3	13.38
F8	10.30
G1	0.00
G2	0.00
G3	11.85
G4	12.54
H1	0.00
H2	0.00
H3	0.00

Sampl e	Decolorization (%)
H4	0.00
H5	0.00
H6	0.89
H7	0.89
J1	0.00
J2	0.00
J3	0.00
J4	0.00
J5	0.00
K1	0.00
K2	1.44
K3	1.80
K4	3.72
L1	0.00
L2	0.00
L3	0.00
L4	0.00
M1	0.00
M2	0.00
M3	0.00
P1	0.00
P2	0.00
P3	13.29
P4	13.45
P5	0.00
P6	0.00
Q1	0.00
Q2	0.00
Q3	0.00
Q4	0.00

4.1.3 Investigation of decolorization stability of bacterial isolates E5

Decolorization stability of bacterial isolate E5 was then confirmed through an experiment run in MM broth under shaking conditions. The colony of isolate E5 grown on MM agar plate (Fig. 4.2) was inoculated into MM broth and incubated under aerobic conditions for 48 h. Subsequently, 5 ml aliquots from the bacterial culture were transferred to fresh MM broth and incubated under aerobic conditions for 48 h. After 5 consecutive subcultures, the decreasing decolorization trend of molasses wastewater was observed (Fig. 4.3). The decolorization of molasses wastewater in MM broth by the isolates E5 dropped to 2.3%. In addition, there was observed a slight manifestation of varying decolorization zones around colonies of isolate E5 on MM agar.

Figure 4.2 Bacterial isolate E5 colonies, and clear zones on MM agar

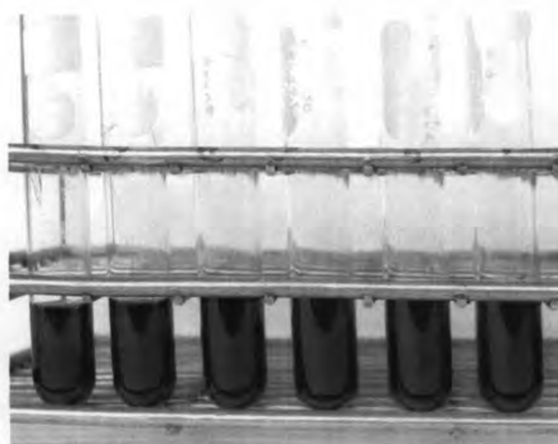

Figure 4.3 MM broth after 5 consecutive subcultures of the bacterial isolates E5.

It demonstrated that pure culture of bacterial isolate E5 displayed limited ability to decolorize molasses wastewater in long-term. Improvement of its culture conditions for further decolorization processes was required. Similarly, it was previously established that bacteria, especially pure culture, displayed limited ability to decolorize melanoidins because of the toxicity of metabolites which were formed and accumulated during molasses pigment decolorization. These metabolites thereby repressed the efficiency of bacterial cells (Raghukumar et al., 2004).

Therefore, the application of bacterial consortium might be a more promising strategy for decolorization molasses wastewater by biological treatment. Since bacterial members of consortium might have different metabolic activities, a bacterial consortium could be highly effective in decolorizing a broad spectrum of structurally diverse melanoidins (Manjinder et al., 2005; Sarayu et al., 2008).

4.2 Screening of molasses wastewater-decolorizing bacterial consortium

The purpose of using a consortium of bacteria as inoculum for molasses decolorization was to increase decolorization and to minimize instability problem of single bacterium by providing synergistic decolorization mechanisms. Therefore, screening of different bacterial consortia was carried out to select the most suitable sample for optimization studies.

Various bacterial consortia from 21 sources were enriched in 50 ml of LB broth and cultivated at $30^{o}C$ on a rotary shaker at 200 rpm. As described in chapter 3, a primary screening was carried out. Enriched bacterial consortia were loaded into holes on MM agar plates. The sterile LB medium was used as control. When decolorization was observed, a clear zone appeared around the holes after incubation at $30^{o}C$ for 48 h under aerobic conditions.

The results of primary screening showed that 9 different bacterial consortia were capable of decolorizing molasses wastewater under aerobic conditions. Clear zones were observed around the holes on MM agar plate (Fig. 4.4). Subsequently, the ability to decolorize molasses of those 9 consortia was re-tested in MM medium with secondary screening. The consortia were cultured in the MM broth at $30^{o}C$ on rotary shaker at 200 rpm for 48 h. The molasses decolorization was determined by measurement of optical density of the supernatant at 475 nm.

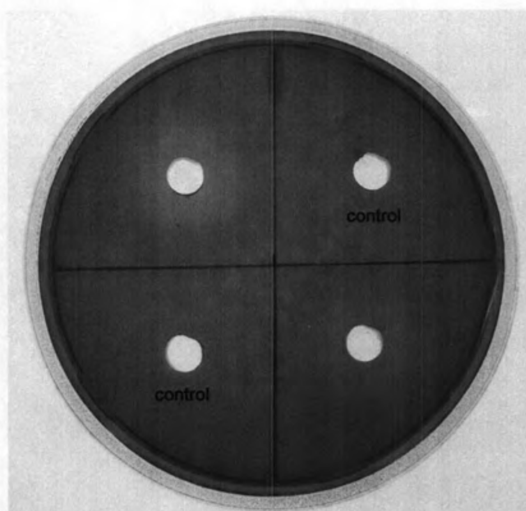

Figure 4.4 Characterization of holes on MM agar plate after incubation of bacterial consortia under aerobic conditions for 48 h.

The overall decolorization profile of the different consortia is shown in the Table 4.3. In comparison with other consortia, maximum decolorization was obtained from CONS8, which was the bacterial consortium enriched from waterfall sediments in Maehongsorn province. This consortium was selected for further study due to its highest decolorization of 20.02% when cultivated in the MM medium under aerobic conditions for 48 h.

Table 4.3 Primary and secondary screening for molasses-decolorizing bacterial consortia

	Samples	Bacterial consortium	Decolorization (%)
1	Soil sample 1 from Sangsom Co.Ltd.	CONS1	0.00
2	Soil sample 2 from Sangsom Co.Ltd.	-	-
3	Soil sample 3 from Sangsom Co.Ltd.	CONS2	0.00
4	Slop sample 1 from Sangsom Co.Ltd.	-	-
5	Slop sample 2 from Sangsom Co.Ltd.	CONS3	17.80
6	Soil sample from PVD, Nakonrachasrima Province	CONS4	11.97
7	Soil sample from Srinan national park, Nan Province	CONS5	5.64
8	Soil sample 1 from Nan Province	-	-
9	Soil sample 2 from Nan Province	-	-
10	Soil sample 3 from Nan Province	-	-
11	Soil sample 4 from Nan Province	CONS6	3.44
12	Soil sample 5 from Nan Province	-	-
13	Soil sample 6 from Nan Province	-	-
14	Soil sample 7 from Nan Province	-	-
15	Soil sample 8 from Nan Province	-	
16	Soil sample 9 from Nan Province	CONS7	13.79
17	Soil sample from Pukraduang, Leay Province	-	-
18	Water sample from Mohpang waterfall, Maehongsorn province	-	-
19	Waterfall sediment sample from Mohpang waterfall, Maehongsorn province	CONS8	20.02
20	Black sand, Chanthaburi Province	CONS9	15.43
21	Sea water of black sand beach, Chanthaburi Province	-	-
	Total	9	

4.3 Optimization of culture conditions for decolorization

Decolorization of the bacterial consortium CONS8 was investigated in the different media and under different culture conditions.

The consortium was cultivated in LB medium under aerobic conditions at 30°C. After 24 h incubation, the medium was then centrifuged at 10,000 rpm, 4°C for 10 min, washed twice and re-suspended in 0.85% NaCl to obtain an OD_{600} of 1. The decolorization experiments were carried out by transferring washed bacterial cells into test tubes containing individual culture medium as follow; molasses wastewater (WW), LB medium with molasses wastewater (LBWW) and modified molasses wastewater (MM), respectively. To examine the effect of initial pH and aeration on the decolorization, culture media (WW, LBWW and MM) were prepared at pH 4, 7 and 9 and the bacterial cells were inoculated and cultivated under different aeration conditions aerobic (agitation at 200 rpm), facultative (without agitation) and anaerobic (CO_2). The schematic diagram of this experiment is shown in Figure 4.5.

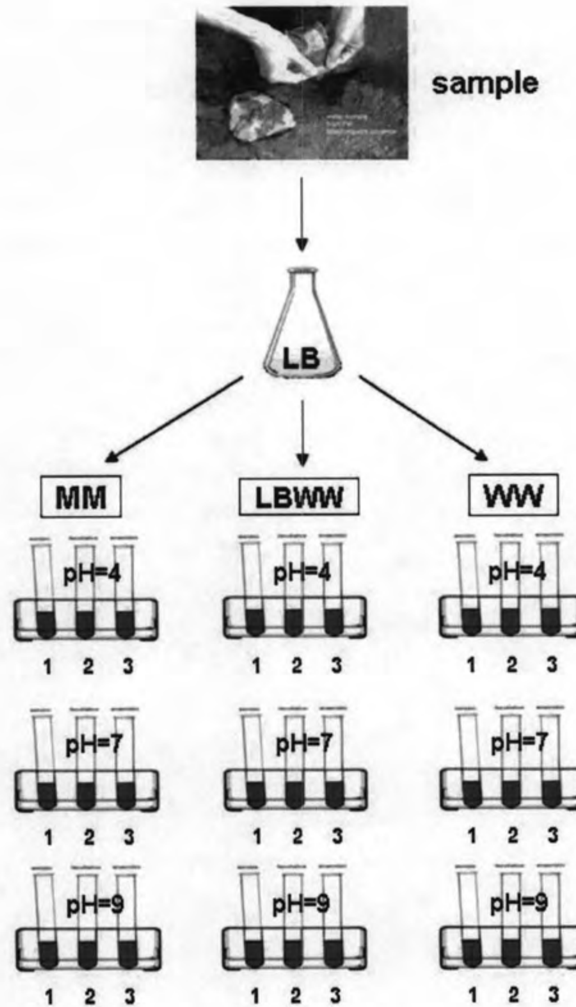

Figure 4.5 Schematic diagram of optimization experiment

Figure 4.6 (A-C) showed the growth of the bacterial consortium after incubation in 3 culture media at pH4 under aerobic, facultative and anaerobic condition. It was shown that bacterial growth was very low in all culture media and culture conditions, especially at pH4 (Fig. 4.6A). The growth of bacterial consortium in MM medium at pH7 under anaerobic condition was higher than any other media and cultured condition (Fig. 4.6B). The maximum bacterial growth in LBWW medium was found under aerobic condition at pH9 (Fig. 4.6C). However, the growth of bacterial consortia in WW medium was very low in all culture conditions.

Figure 4.7 (A-C) showed the molasses-decolorizing activity of the bacterial consortium on different media and different pH. The results showed that the decolorizing activity of bacterial consortium incubated in LBWW medium at pH4 under anaerobic conditions had the highest decolorizing activity approximately 26.5% (Fig. 4.7A). In contrast, only 4.8% was observed using under the same culture conditions in WW medium. Some nutrients and salts necessary for promoting growth and decolorization by the consortium might be lacking in WW medium. It was also found that the decolorization by the consortium incubated in MM medium was higher than other culture media under facultative and aerobic conditions. Decolorization efficiency in MM medium was enhanced up to 25.5% in 48 h under facultative conditions at pH4 whereas cell concentration was very low.

At pH7, the maximum decolorization of 23.8% was also found in MM medium under anaerobic conditions (Fig. 4.7B), whereas the consortium showed only 5% decolorization of MM medium under aerobic conditions at the same pH. At pH9, the highest decolorization of 20.4% was achieved in MM medium under facultative condition (Fig. 4.7C).

The higher color removal at acidic pH observed in this study might be due to the fact that melanoidins responsible for color were less soluble in acidic pH than in alkaline pH (Miranda et al., 1996). Hence, in the acidic pH, the melanoidins might be precipitated and removed more easily.

The difference in decolorization among various culture media might be linked to the fact that molasses wastewater was deficient in carbon content so biodegradation without any extra carbon source was found to be very difficult. Its recalcitrance was also due to presence of melanoidins, brown colored substances, which are formed by Maillard amino carbonyl reaction. Hence, supplementation with labile carbon sources appeared to be necessary for decolorization of molasses wastewater by bacterial consortium. Kambe and his co-workers reported a maximum color removal of molasses (A_{475} = 7.0) of 35.5% by *Bacillus smithii* at 55°C under anaerobic conditions in presence of either peptone or yeast extract as supplemental nutrient while this strain could not use molasses wastewater as sole carbon source (Kambe et al., 1999). Kumar and Chandra have also reported that the addition of 1% glucose as a supplementary carbon source was necessary for molasses decolorization of a modified GPYM medium containing melanoidins (10% volume by volume) by *Bacillus thuringiensis*, *Bacillus brevis*, and *Bacillus* sp. up to 22%, 27.4%, and 27.4%, respectively (Kumar and Chandra, 2006). Acetogenic bacteria strain No.BP103 could also decolorize 73.5% of molasses pigments (A_{475} = 3.5) in molasses wastewater medium supplemented with glucose, yeast extract, and basal

mineral salts whereas the decolorization with this strain was dramatically decreased to only 9.75% in the absence of nutrient supplement (Sirianuntapiboon et al., 2004a). Similar result was also observed on the decolorization by bacterial consortium DMC which achieved a maximum molasses decolorization of 67% using basal medium containing distillery spent wash (A_{475} = 2.8-3) in the presence of 0.5% glucose (Mohana et al., 2007).

In general, several microorganisms that have been shown to degrade melanoidins under anaerobic condition are not suitable for treating effluent from molasses based distilleries. It is possible that they are lack of oxygen, which is necessary for oxidative degradation of melanoidins, in the effluent. However, the results presented in this study showed that color removal under facultative and anaerobic conditions were higher than under aerobic conditions. Hence, the decolorization mechanisms of molasses wastewater by bacterial consortium in this study might result from 2 possible mechanisms. One might be due to the color adsorption by bacterial cell and the other to the metabolism of bacteria under facultative and anaerobic conditions such as fermentation and anaerobic respiration.

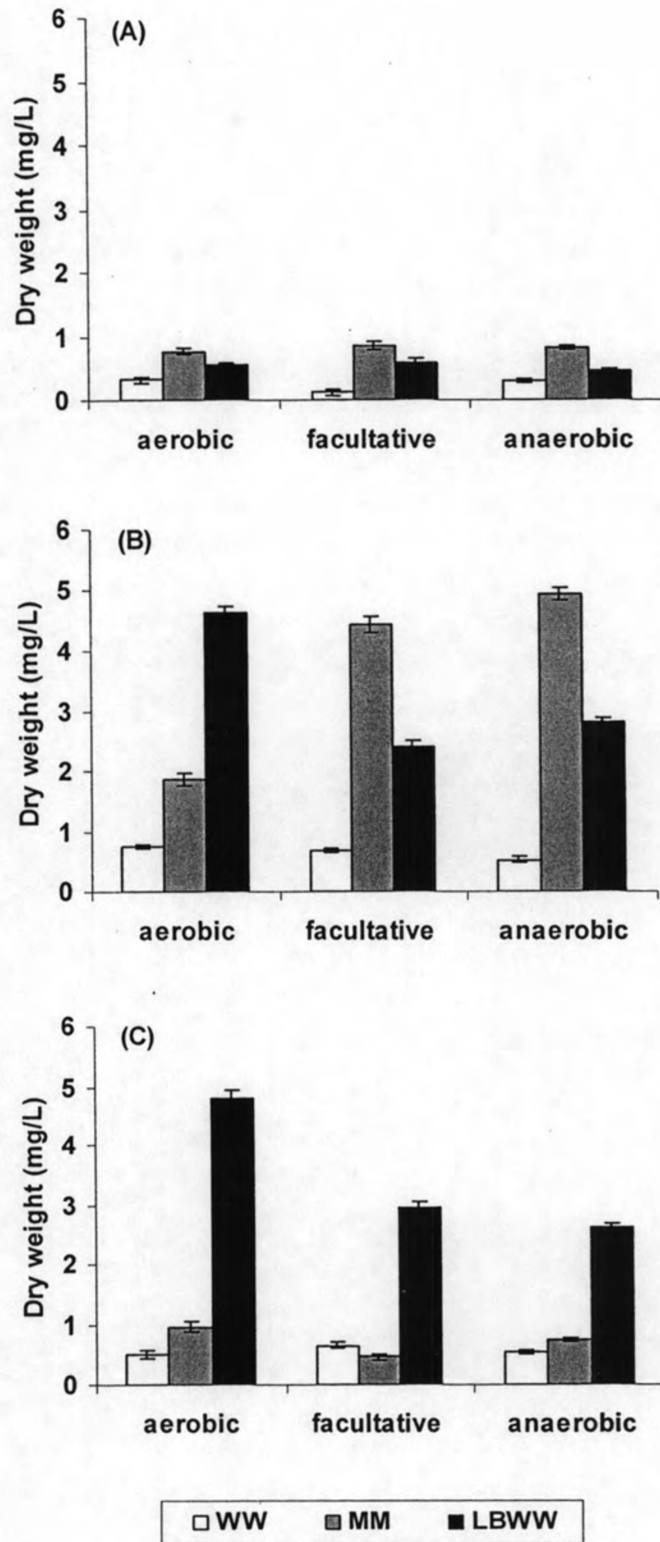

Figure 4.6 Effect of medium composition and culture conditions on growth of bacterial consortium CONS8 at pH4 (A), pH7 (B), and pH9 (C).

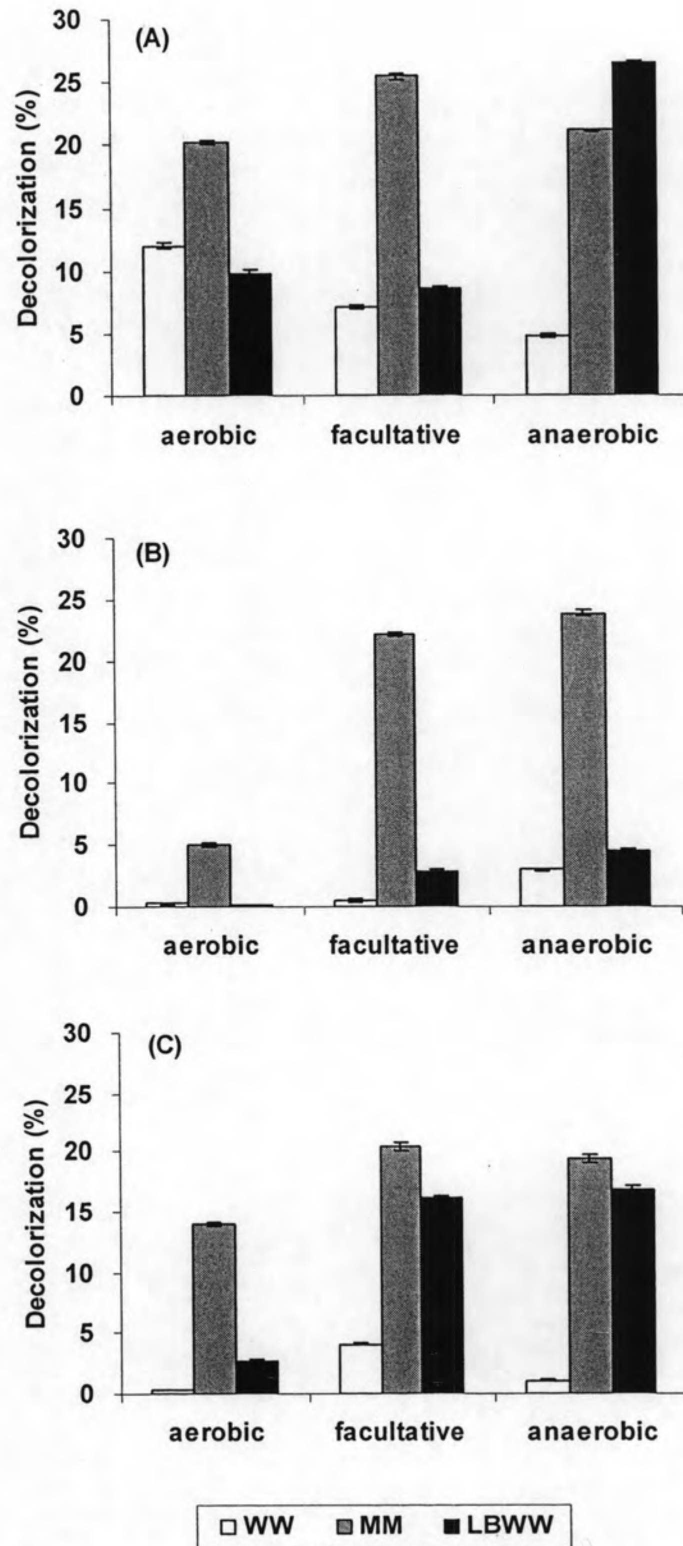

Figure 4.7 Effect of medium compositions and culture conditions on decolorization of molasses wastewater by bacterial consortium CONS8 at pH4 (A), pH7 (B), and pH9 (C).

4.4 Time course of growth and decolorization of the bacterial consortium CONS8

In the study dealing with optimization of culture conditions, our results demonstrated that the bacterial consortium gave the highest decolorizing activity in LBWW medium, pH4 under anaerobic conditions. However, LBWW medium might be never accepted in term of application due to excessive chemical use and high cost. Thus, MM medium was selected for use in the following study. Among MM medium, the bacterial consortium showed the highest decolorization at pH4 under facultative conditions. Unfortunately, facultative conditions are not suitable for application in conventional molasses wastewater treatment, which is mainly aerobic or anaerobic classical process.

Aerobic treatment systems have been demonstrated efficient for the treatment of various kinds of wastewater such as domestic wastewater, dye wastewater and feed wastewater since it could provide removal of high organic load, resistance to organic shock load, odorless, easy operation and maintenance. The aeration in the system promoted growth of microorganisms and conferred uniform population which enhanced the consumption of organic loads (Hammer, 1991; Metcalf & Eddy, 2004). Accordingly, in this study, time course of growth and decolorization of bacterial consortium CONS8 was investigated in MM medium with the initial pH of 4 under aerobic conditions. In order to increase the bacterial biomass, the inoculum was prepared in LB medium prior to transfer into MM medium and cultivated under conditions as indicated above. A typical time course of molasses decolorization in MM medium by a bacterial consortium CONS8 under selected condition is shown in Fig. 4.8. The consortium notabely decolorized MM medium during the first 24 h of culture, and the color removal of 20% was observed after 48 h. The growth of the consortium slightly increased with the increase of cultivation period. In addition, the progression decolorization of molasses wastewater in MM medium by bacterial consortium CONS8 can be observed in the visible spectral sequence presented in Fig. 4.9. The absorbance from 400 to 700 nm decreased along the incubation time.

In this work, although the decolorization with the bacterial consortium CONS8 was lower than those of Basidiomycetes and Deuteromycetes previously reported (Friedrich, 2004; Gonzalez et al., 2000), it still possesses two advantages, i.e. the consortium showed high molasses decolorization under static conditions resulting in saving energy consumption during operation and application of fungi to remove melanoidins in molasses wastewater was a drawback due to their slow growth, spore production and infectivity (Friedrich, 2004). Bacterial decolorization of molasses

wastewater has been reported by many researchers (Dahiya et al., 2001; Murata et al., 1992; Nakajima-Kambe et al., 1999; Sirianuntapiboon et al., 2004a).

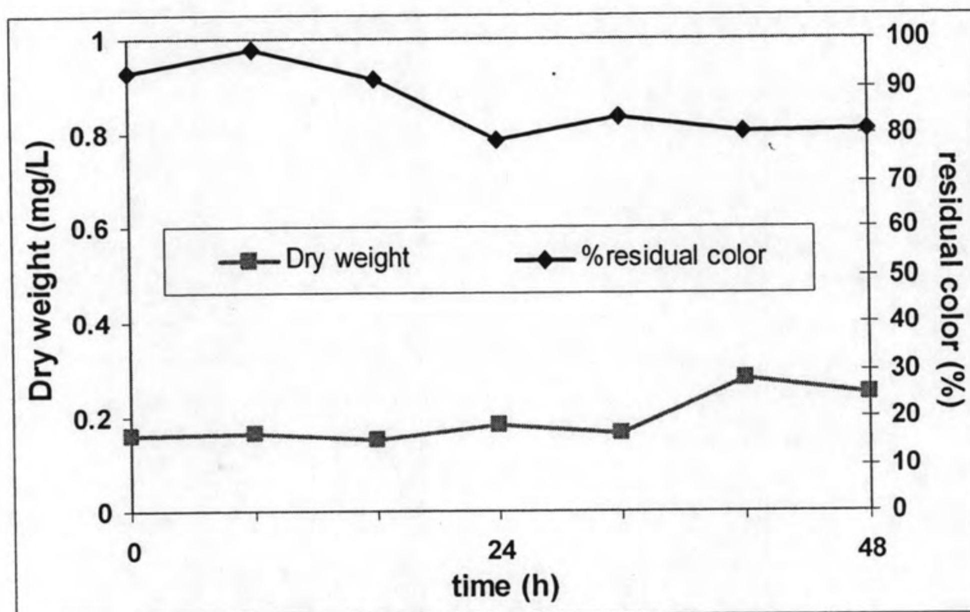

Figure 4.8 Decolorization of molasses wastewater by bacterial consortium CONS8 in MM medium at pH4 versus time. The consortium was grown under aerobic conditions.

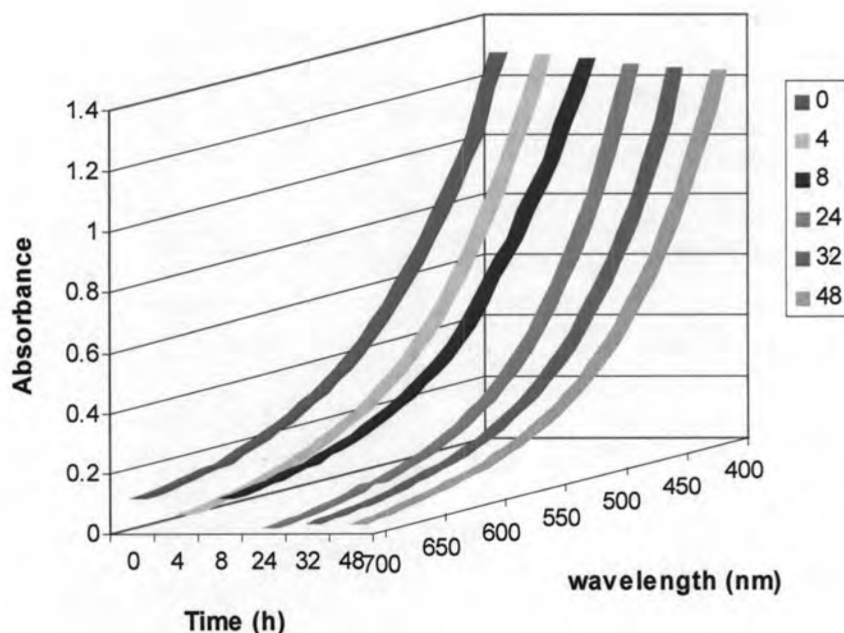

Figure 4.9 Typical visible spectra of supernatant from aerobic decolorization of sugarcane molasses wastewater by bacterial consortium CONS8 under the optimal conditions at various incubation times.

4.5 Identification of bacterial isolates present in the consortium CONS8

The molasses wastewater decolorizing-bacterial consortium CONS8, enriched in LB medium under aerobic conditions, was comprised of 8 (AE1-AE8) bacterial members from different colonies. Each kind of bacteria was identified by BLAST homology based on its partial 16S rDNA sequence as shown in Table 4.4. Some of bacterial strains present in the bacterial consortium CONS8 were previously reported as a molasses decolorizing bacteria. *Pseudomonas*, *Acinetobacter*, *Klebsiella*, and *Bacillus* could decolorize colored components present in molasses wastewater (Kumar and Chandra, 2006; Mohana et al., 2007; Petruccioli et al., 2000). However, the strains AE2 and AE5 had sequence homology of 16S rDNA with other genera deposited as unidentified strains in GENBANK.

Table 4.4 Percent similarity based on the alignments of the partial 16S rDNA sequences of isolated bacteria from consortium CONS8 to their closest bacterial relatives present in the NCBI nucleotide sequence database.

Bacterial strains	Accession no. of the closest strains	Sequences similarity	Closest strains
AE1	DQ226207	96%	*Serratia marcescens*
AE2	DQ816308	99%	Unknown bacterium
AE3	DQ226213	100%	*Acinetobacter* sp.
AE4	AY689030	98%	*Pseudomonas* sp.
AE5	DQ817737	98%	Unknown bacterium
AE6	AF188304	97%	*Comamonas* sp.
AE7	DQ226215	99%	*Klebsiella oxytoca*
AE8	AF529355	97%	Unknown gamma proteobacterium

4.6 Analysis of bacterial community

PCR-DGGE method based on 16S rDNA was used to assess changes in microbial communities (Jensen et al., 1998). In this study, the changes in microbial community were investigated among four culture media at the initial pH of 4 under aerobic conditions. DGGE patterns from bacterial consortium CONS8 with different media in aerobic condition were compared with those of pure cultures (Fig.4.10). Profiles of bacterial consortium CONS8 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 analyses showed significant difference in the bacterial community grown on the different media (Fig 4.10). In addition, DGGE analysis revealed that different bacterial strains were responsible for the molasses decolorization depending on the different culture conditions. Therefore, the selection of aeration condition and medium compositions were factors shaping the bacterial composition in the molasses wastewater-decolorizing consortium.

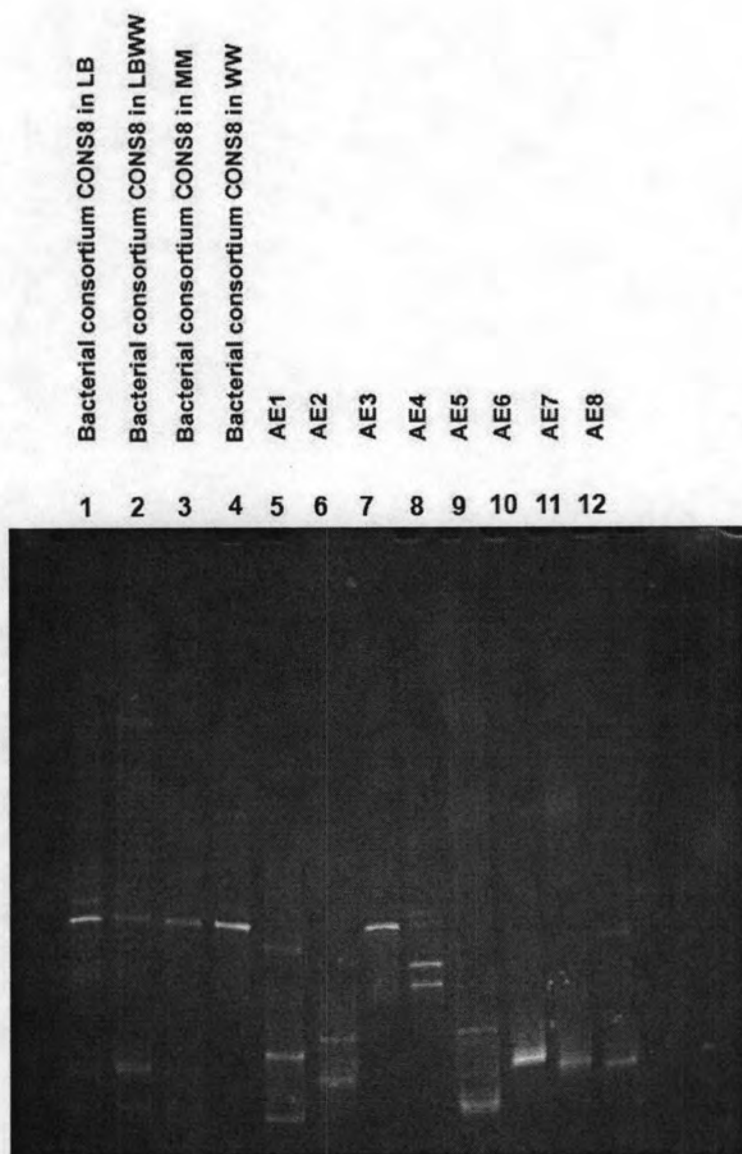

Figure 4.10 DGGE analysis of 16S rDNA sequences amplified from DNA extracted from culture of bacterial consortium CONS8 after enrichment in different culture media under aerobic conditions using PRBA338f and PRUN518r primers. Lanes: 1, Bacterial consortium CONS8 enriched in LB medium; 2, Bacterial consortium CONS8 enriched in LBWW medium; 3, Bacterial consortium CONS8 enriched in MM medium; 4, Bacterial consortium CONS8 enriched in WW medium; 5, AE1 (*Serratia marcescens)*; 6, AE2 (unknown bacterium); 7, AE3 (*Acinetobacter* sp.); 8, AE4 (*Pseudomonas* sp.); 9, AE5 (unknown bacterium); 10, AE6 (*Comamonas* sp.); 11, AE7 (*Klebsiella oxytoca*); and 12, AE8 (unknown gamma proteobacterium).