CHAPTER III

MATERIALS AND METHODS

3.1 Molasses wastewater

Molasses wastewater was obtained from Sangsom Co., Ltd., a alcoholic distillery plant from sugarcane molasses in Nakornpathom Province, Thailand. In the laboratory, the effluent samples were stored at 4^{O}C. The effluent was characterized and analyzed for pH, chemical oxygen demand (COD), biological oxygen demand (BOD) based on the Standard Methods for Examination of Water and Wastewater (APHA, 1998). The characteristics of molasses wastewater were indicated in Table 3.1.

Table 3.1 Characteristics of molasses-based distillery wastewaters from Sangsom Co., Ltd

Water Quality index	Slop wastewater
Temperature	95-100^{O}C
pH value	3.8-4.5
TDS	7,600 mg/l
Suspended Solid	15,000 mg/l
Color and odor	Dark brown / bad smell
BOD	35,000 mg/l
COD	100,000 mg/l

3.2 Preparation of molasses wastewater medium

After experiments presented in the chapter 4, three different media were kept for studying the decolorization of molasses wastewater by a bacterial consortium. They were prepared as follow:

- *Molasses wastewater medium (WW)* was made by diluting molasses wastewater from alcoholic distillery with distilled water to 20% (v/v).
- *Molasses wastewater-containing LB medium (LBWW)* was LB medium containing 20% (v/v) of molasses wastewater from alcoholic distillery
- *Modified molasses wastewater medium (MM)* consisted of (20%, v/v) of molasses wastewater from alcoholic distillery, 0.01% (w/v) $NaNO_3$, 0.2% (w/v)

K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.01% (w/v) $MgSO_4 \cdot 12H_2O$, 2% (w/v) glucose and 0.1% (w/v) yeast extract.

3.3 Preparation of different melanoidins-containing wastewater media

Three different media with different sources of melanoidins were prepared. Melanoidins-containing solutions were used including sugarcane molasses wastewater, beet molasses wastewater and Viandox sauce. Other components of the each medium were as follows: 0.01% (w/v) $NaNO_3$, 0.2% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, 0.01% (w/v) $MgSO_4 \cdot 12H_2O$, 2% (w/v) glucose and 0.1% (w/v) yeast extract, and the initial pH was adjusted to 4.

The characteristics of each medium were indicated in Table 3.2. The sugarcane molasses wastewater was obtained from SangSom distillery, Nakhon-Pathom province, Thailand. Beet molasses wastewater and Viandox sauce were obtained from Laboratoire de Génie Chimique, Toulouse, France.

Table 3.2 Characteristics of synthetic melanoidins-containing wastewater

Color substances	Initial concentration (%, v/v)	OD_{475}	COD (g/L)
Viandox	13.5	5.71	22.8
Beet molasses wastewater	41.5	5.90	30.75
Sugarcane molasses wastewater	20	5.71	21.6

3.4 Screening of molasses-decolorizing bacterial consortium

Various bacterial consortia were isolated from natural environments and wastewater treatment plant.

Several samples including soils, sediments and wastewaters were collected from various sources in Thailand to isolate a bacterial consortium which has high molasses-decolorization power. In order to enrich molasses-decolorizing population, five milliliters of each sample were transferred into 50 ml of LB medium in Erlenmeyer flasks and cultivated at 30°C on a rotary shaker at 200 rpm.

For the first step of screening, the inoculum of enriched bacterial consortia, prepared by growing in LB broth under shaking at 200 rpm at 30°C for 24 hours, were loaded into holes drilled with a cork borer (0.7 cm in diameter) on modified molasses wastewater (MM) agar plate. Sterile LB medium was used as control. Decolorization was observed when a clear zone appeared around the holes after incubation at room temperature for 48 h under either aerobic. Bacterial consortia which showed high molasses decolorization in the primary screening were cultured in Erlenmeyer flask containing modified molasses wastewater medium (MM) at 30°C on rotary shaker at 200 rpm.

The bacterial consortium was chosen for its ability to decolorize the medium. It exhibited the highest melanoidin decolorization efficiency (20% color reduction) within 48 h under aerobic conditions and this consortium was selected for further study. The bacterial consortium samples were stored at 0°C in LB-broth supplemented with (15%, v/v) of glycerol until used in the experiments.

3.5 Construction of bacterial consortia for optimal decolorization

In order to find a bacterial consortium efficient for the color removal in mixed cultures, experiments were performed with various combinations of bacteria, namely *Klebsiella oxytoca* (T1), *Serratia mercescens* (T2), *Citrobacter* sp. (T3) and unknown bacterium DQ817737 (T4).

For construction of the active bacterial consortia, a loopful of each bacterium (T1, T2, T3 and T4) from LB plate was precultured in 50 ml LB at 30°C under shaking at 200 rpm. After 24 h, bacterial cells of each strain were harvested by centrifugation at 10,000 rpm at 4°C for 10 min then washed with sterile normal saline solution. Washed bacterial cells at appropriate volume were subsequenctly inoculated into fresh synthetic melanoidins-containing wastewater media to obtain an initial OD_{600} of 0.2. Several consortia comprising of different bacterial compositions were constructed at the same initial cell density.

3.6 Inocula preparation

3.6.1 Decolorization of different melanoidins-containing wastewater media

For decolorization of the different melanoidins-containing wastewaters, the inoculum was prepared by transferring the bacterial consortium into a flask containing 50 ml LB medium and incubated for 24 h under shaking (200 rpm) at

30°C. 10% inoculum was transferred into shake flasks containing 250 ml of melanoidins-containing wastewater medium, each flask containing one different colored substance. The consortium was then incubated under shaking conditions (200 rpm) at pH 4, 30°C.

3.6.2 Study of the optimal decolorization

The bacterial consortium was transferred into 250 ml Erlenmeyer flasks containing 50 ml synthetic melanoidins-containing wastewater medium, using 2% (v/v) Viandox as color substance. Incubation was carried out under shaking at 200 rpm, 30°C for 48 h. Bacterial cells were harvested with 3 h intervals by centrifugation at 10,000 rpm, 4°C for 10 min.

3.6.3 Study on limitation of decolorization

The bacterial consortium was inoculated into melanoidins-containing wastewater medium and cultivated under shaking (200 rpm) at 30°C for 48 h. Cells were harvested by centrifugation (10,000 rpm, 10 min, 4°C) and washed three times successively with sterile normal saline solution in order to eliminate the residual culture medium. Washed bacterial cells were resuspended in the fresh culture medium of the same volume and cultivated under condition as described above.

Meanwhile, the used culture medium was centrifuged again at 10,000 rpm for 10 min at 4°C to completely remove the bacterial cells, then inoculated with fresh bacterial cells (10% w/v) and cultivated under the same condition as described above.

3.7 Identification of effluent decolorizing consortium

3.7.1 Bacterial identification by 16s rDNA sequence

On the basis of morphological study, the bacterial strains present in enriched bacterial consortium were isolated into pure culture. A loopful of each strain taken from an isolated colony on LB plate was precultured in 50-ml Bacto LB broth (in g l^{-1}; 10 tryptone, 5 yeast extract, and 10 NaCl) for 12 hours. The culture broths were centrifuged at 10,000 rpm for 2-5 min and the cell pellets 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.

DNA solutions were separated by centrifugation at 10,000-12,000 rpm for 5-10 min and used as DNA template for 16S rDNA amplification. Extracted DNA was

amplified by using PCR and the universal 16S rDNA primers 20F (5'-GAG TTT GAT CCT GGC TCA G-3') and 802R (5'-TAC CAG GGT ATC TAA TCC-3'). The PCR reaction was run with PCR Buffer pH 8.8 (Biolab containing: 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_2$ and 0.1% Triton X-100), 0.4 µM of deoxynucleotide triphosphate (dNTP), 0.4 µM of each primer and 1 U of *Taq* polymerase (Biolab). The initial cycle consisted of 5 min at 95°C and was followed by 25-30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The final cycle for extension step consisted of 72°C for 3 min. The PCR products from 20F and 802R primers were used as template for PCR based DNA sequencing. For increasing the specificity of DNA product, UFUL primer (5'-GCCTAACACATGCAAGTCGA-3') was used in DNA sequencing reaction (Nilsson et al., 2003). The DNA sequencing reaction solution contained Bigdye Termination v3.1 cycle sequencing kit (Master Mix) and 3.2 pM of UFUL primer. The initial cycle consisted of 5 min at 95°C and followed by 30 cycles of 95°C for 30 s, 50°C for 10 s, and 60°C for 4 min. The final cycle for extension step consisted of 60°C for 4 min. 62.5 µl of absolute ethanol, 3.0 µl of 3M sodium acetate pH 4.6 and 14.5 µl of distilled water were added to resulting amplicons and they were incubated at room temperature for 15 min. Then amplicons were centrifuged at 12,000 rpm, room temperature for 15 min and washed with 70% ethanol. The amplicons were dried at 95°C for 2-3 min and resuspened with 10-15 µl of Hi-Di Formamide. Samples were boiled at 95°C for 2 min and cooled down for 5 min. Samples were sequenced by Automated DNA Sequencer. 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 Center for Biotechnology Information.

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

DNA was directly extracted from bacterial culture with bead-beating instrument and UltraCleanTM Soil DNA Kit (MO BIO Laboratories, Inc).

Then, each DNA sample was amplified by 16S rDNA PCR technique using PRBA 338F+CG clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3') and PRUN518R (5'-ATT ACC GCG GCT GCT GG-3') primers. The initial cycle consisted of 5 min at 94°C and followed by 20 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. After that, each sample went trough 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The final cycle for extension step was at 72°C for 10 min.

PCR product was subsequently run on 8% polyacrylamide gel with a denaturing gradient of urea and formamide denaturant ranging from 25-60% for 5 hr

at 130 volts with 1xTAE. DGGE gel was stained in 50 µg per ml of ethidium bromide for 20 minutes.

DNA band profiles can be detected under the UV transilluminater (Cindy et al., 2000; Edenborn and Sexstone, 2007).

3.8 Device for the decolorization in membrane bioreactor

The molasses wastewater treatment by bacterial consortium was run continuously in membrane bioreactor. The schematic diagram of membrane bioreactor was illustrated in Figure 3.1. A cross flow membrane filtration set with a total area of 0.013 m^2 was installed in the treatment system. The membrane bioreactor process was carried out in 2L of reactor with working volume of 1.6L at 30^{o}C with sludge retention time (SRT) of 50 days and hydraulic retention time (HRT) at 20 and 40 h, respectively. The aeration was maintained at 0.1 vvm with agitation speed at 150 rpm.

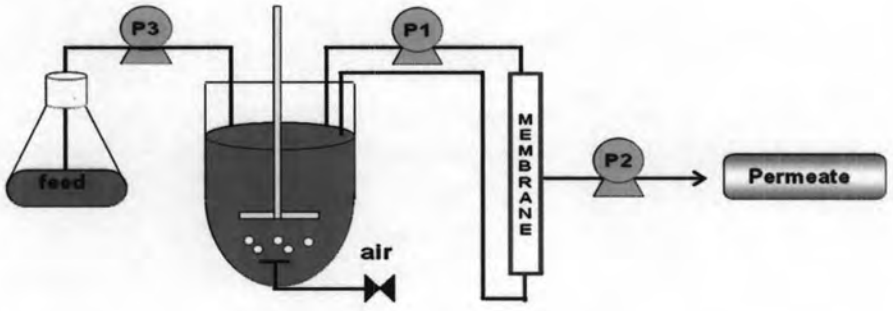

Figure 3.1 Schematic diagram of wastewater treatment using MBR.

3.9 Analytical methods

3.9.1 Measurement of bacterial growth

Bacterial growth was determined by direct and indirect methods depending on available equipment in Thai or French laboratory.

1. **Viable plate count method**

Sample of cell suspension was diluted and spread (volume to be specified) over the surface of a growth medium, then, the plates were incubated at 30^{o}C. The

colonies were counted after 24h. Total number of colonies is determined. Each plate should have 30-300 colonies at least.

2. Spectrophotometry

Optical density (OD) was also used to monitor the bacterial growth in this study. The absorbance was measured at 600 nm by using Hitachi spectrophotometer, model U-2000. The bacterial density (C*) was calculated using Equation; $C^* = C_1 - C_O$; where C_1 is the OD value of the culture broth; and C_O is the OD value of supernatant obtained after centrifugation of the culture broth.

3. Dry weight technique.

Cells in suspension were collected by centrifugation (10,000 rpm for 10 min at 4^oC), washed with distilled water, and dried in an oven at 80^oC until getting a constant dry weight.

3.9.2 Melanoidins color determination

The color intensity of melanoidins-containing wastewater was determined by measuring optical density (OD) at 475 nm of the supernatant obtained upon centrifugation (10,000 rpm for 10 min at 4^oC) of 5 ml culture by using Hitachi spectrophotometer, model U-2000.

3.9.3 Chemical oxygen demand (COD) analysis

The COD content was determined by a spectrophotometric method using Hach COD reagent test kit (HACH Company, USA). See appendix 1 for details.

3.9.4 Total nitrogen analysis

The total nitrogen was determined by a spectrophotometric method using Hach Total nitrogen reagent test kit (HACH Company, USA). See appendix 1 for details