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Decolorization of molasses melanoidins and palm oil mill effluent phenolic compounds by fermentative lactic acid bacteria

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Abstract

Lactobacillus plantarum SF5.6 is one of the lactic acid bacteria (LAB) that has the highest ability of molasses melanoidin (MM) decolorization among the 2114 strains of LAB. The strains were isolated from spoilage, pickle fruit and vegetable, soil and sludge from the wastewater treatment system by using technical step of enrichment, primary screening and secondary screening. This LAB strain SF5.6 was identified by 16S rDNA analysis and carbohydrate fermentation (API 50 CH). The top five LAB strains having high MM decolorization (> 55%), namely TBSF5.8-1, TBSF2.1-1, TBSF2.1, FF4A and SF5.6 were selected to determine the optimal condition. It was found that the temperature at 30°C under facultative conditions in GPY-MM medium (0.5% glucose, 0.1% peptone, 0.1% yeast extract, 0.1% sodium acetate, 0.05% $MgSO_4$ and 0.005% $MnCl_2$ in MM solution at pH 6) giving a high microbial growth and MM decolorization for all five strains. It was noticed that the decolorization of MM by LAB strains might be cell growth associated. *L. plantarum* SF5.6 grew rapidly within one day while the other strains took 2–3 days. This *L. plantarum* SF5.6 could rapidly decolorize MM to 60.91% without any lag phase, and it also had the ability to remove 34.00% phenolic compounds and 15.88% color from treated palm oil mill effluent.

Key words: decolorization; *Lactobacillus* sp.; molasses melanoidins; palm oil mill effluent phenolic phenols

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Introduction

As the reserves of oil and gas are being depleted and the price rapidly jumping up, securities of energy could supply the demand towards the establishment of a renewable energy (bioethanol and biodiesel) economy. The commercial production of bioehtanol and biodiesel commonly use molasses and crude palm oil as the raw materials, generating high strength wastewater with a dark brown color. It is estimated that the generation of molasses wastewater and palm oil mill effluent (POME) are approximate 12–16 m^3/m^3 of ethanol production and 2.5–3.0 m^3/ton of crude palm oil. The brownish color of molasses wastewater is mainly from melanoidin pigment, the complex biopolymer occurs via the Millard's reaction. While POME is a brownish liquid waste and has a dense color containing high organic matter of which the composition of substances is not known yet. In this preliminary study, we found POME contains phenolic compounds. Similar to olive mill wastewater (OMW), it has been reported that the dark brown color of OMW is probably from the polymerization of tannins and low molecular weight phenolic compounds (Assas et al., 2002). This waste leads to the reduction of light transparency in water reservoirs resulting in a deterioration of photosynthesis, a reduction of dissolved oxygen and a hazard to micro-and macro-aquatic life (Kumar et al., 1997). In addition, phenolic compounds are phytotoxic and have antibacterial activity (Saez et al., 1992; Capasso et al., 1995; Casa et al., 2003). The dark brown colored substances from molasses melanoidins (MM) are hardly degraded or decolorized by aerobic and anaerobic activated sludge systems (Miyata et al., 2000; Gonzalez et al., 2000) while that from POME phenolic compounds (POMEP) had not been reported on biological treatment.

Nowadays, the primary treatments of molasses wastewater and POME are based on anaerobic systems for reasons of wastewater treatment and biogas production as renewable energy. It can reduce COD with high efficiency but the color still remains in treated wastewater (Satyawali and Balakrishnan, 2008; Zhang et al., 2008a, 2008b). For the decolorization of melanoidin pigment in molasses wastewater, several studies found some high potential aerobic bacterial strains such as *Bacillus* sp., *Pseudomonas* sp., Acetogenic bacteria, *Stenotrophomonas maltohpila* and *Proteus mirabilis* (Nakajima-Kame et al., 1999; Ghosh et al., 2002; Kumar and Chandra, 2006; Mohana et al., 2007). However, aerobic bacteria need energy (oxygen requirement) more than anaerobic bacteria for wastewater

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treatment. Therefore, *Lactobacillus hilgardii* W-NS was first reported to be used in the decolorization of molasses melanoidins under anaerobic condition by Ohmomo et al. (1988a) but the efficiency of decolorization was lower than 30% under optimal conditions. It will be more advantageous to decolorize MM as well as POME phenolic compounds, if we can find a bacterial strain that has a high decolorization activity and can be applied in the anaerobic treatment system.

Therefore, this research has been trying to isolate and screen the higher MM decolorization potential of fermentative lactic acid bacteria (LAB) from various sources of fruit, food, soil and wastewater. Optimization of facultative-anaerobic conditions and temperature were studied for maximum MM decolorization yield of the selected strains. Then, identification of the LAB strain having the highest decolorization was conducted by the genotypic and phenotypic determination. Apart from melanoidins, cane molasses wastewater contains other colorants such as phenolics and caramel (Godshall, 1999). This LAB strain might be able to degrade phenolic compounds contained in wastewater like POME. Finally, the highest MM decolorization strain was applied to test the ability for degradation of phenols in anaerobic treated POME (POMEP) from the crude palm oil factory.

1 Materials and methods

1.1 Preparation of cultivation medium and molasses melanoidins solution

Corn steep liquor containing molasses melanoidins (CSL-MM) was used for a culture medium to enrich the dominant LAB strains having MM decolorizing ability. Glucose peptone yeast extract containing MM (GPY-MM) was used to isolate and determine the MM decolorizing ability of LAB strain in primary and secondary screening.

1.1.1 Molasses melanoidins solution

The MM solution was prepared from the stillage of alcohol fermented cane molasses. The stillage was centrifuged at 10,000 r/min for 15 min and then the supernatant was concentrated by dehydrate four times by volume from the original using a vacuum evaporator (Rotavapor RE-120, Buchi, Switzerland) at a low temperature of 50°C. The concentrated solution was dialyzed in a dialyzing bag having a molecular weight cut off 10,000 Da against deionized water for 12 hr at 4°C. Thus the non-dialyzable molasses pigment solution containing MM was prepared for use. It was diluted with distilled water to a concentration corresponding to an optical density (OD) of 3.5 units at 475 nm and adjusted pH to 6 for this experiment (Sirianuntapiboon et al., 1995).

1.1.2 CSL-MM medium

CSL-MM composed of 2% CSL, 1% glucose, 0.2% yeast extract, 2% glutamic acid, 0.3% NaCl, and 0.02% $MnSO_4$; adjusted to a volume of 100 mL with MM solution at pH 6.

1.1.3 GPY-MM medium

Glucose, peptone and yeast extract containing MM (GPY-MM) composed of 0.5% glucose, 0.1% peptone, 0.1% yeast extract, 0.1% sodium acetate, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.005% $MnCl_2 \cdot 7H_2O$, and adjusted to a volume of 100 mL with MM solution at pH 6.

1.2 Isolating sources and screening of fermentative LAB strains with high ability of MM decolorization

Lactic acid bacteria strains were isolated from pickled fruit and vegetable, spoilage fruit and vegetable, spoilage meat, various sludges from anaerobic wastewater treatment systems, and soil from various places. Three steps for the screening of LAB strains having MM decolorization was used by the step of enrichment, primary screening and secondary screening. The first step of enrichment: 1 g of a sample from each sampling source was suspended in 10 mL of CSL-MM broth. The test tubes were incubated in an anaerobic jar at an ambient temperature (30–35°C) for three days to enrich LAB strains having MM decolorization. The enriched culture broths from various sample sources showing decrease color intensity were collected for the primary screening step. Primary screening: 1 mL aliquots of 10 folds diluted enrichment culture were taken and spread onto GPY-MM agar plates for isolation. After incubation in an anaerobic jar at an ambient temperature for three days, each single colony with clear zone was picked up and streaked on a GPY-MM agar plate again for checking a single pure colony. The isolated LAB strains having the ability to decolorize MM were maintained in GPY-MM agar slants. Finally, in secondary screening the isolated strains from the primary screening were cultivated into 50 mL of GPY-MM broth in a 250 mL Erlenmeyer flask without shaking for anoxic conditions and incubated at an ambient temperature for 3 days. The decolorizing activities of LAB strains from the culture filtrates were compared.

1.3 Effect of facultative-anaerobic conditions and temperature on MM decolorization by LAB

1.3.1 MM decolorization under facultative and anaerobic conditions

The five strains of fermentative LAB having the highest decolorization yield (> 55%) had their microbial ability studied under facultative and anaerobic conditions. The MM decolorization yield of these fives strains were determined within five days from a culture medium of GPY-MM broth under both facultative conditions by being static without shaking and anaerobic conditions by flushing with nitrogen gas for 3 min.

1.3.2 Effect of temperature

The effects of temperature on the selected LAB strains in MM decolorization were studied at 20, 25, 30, 37, 43 and 50°C. These cultures were cultivated in GPY-MM broths at various temperatures for five days. The determination of MM decolorization yield and cell growth were measured at OD 475 and 660 nm, respectively.

1.4 Decolorizing ability of the selected LAB strains in MM solution

The selected five strains of LAB with the highest decolorization yield were cultivated in GPY-MM broth at optimal conditions for five days to investigate growth, medium pH, COD removal and MM decolorization during cultivation. Microbial growth measured the dry weight of cell mass by drying at 105°C for 24 hr against incubation time. COD was measured following a standard method (APHA, 1999). MM decolorization yield was measured the decrease in color density against the initial color density at 475 nm.

1.5 Genotypic and phenotypic identification of selected LAB strains

The highest MM decolorization strain of LAB was identified using genotypic and phenotypic determination techniques.

1.5.1 Genotypic determination

The purified LAB strain was cultured on 100 mL MRS broth with the addition of 0.5% (*W/V*) glycine until log phase growth was obtained. Bacterial pellets were washed by a 10 mL TES buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 6.7% sucrose, pH 8) and resuspended in a 40 mL STET buffer (8% sucrose, 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 5% Triton x-100, pH 8). Then, 1 mL lysozyme (10 mg/mL), 100 μL N-acetylmuramidase (10 mg/mL) and 2.5 mL of 20% sodium dodecyl sulfate (SDS) were added. After that, the mixture extracted DNA using the method of Daud Khaled et al. (1997). The amplification of 16S rDNA was performed with 1 μL of DNA template using 0.5 μL of forward primer EUB 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 0.5 μL of U1492R universal primer (5′-GGTTACCTTGTTACGACTT-3′) in a mixture solution. Amplification conditions consisted of 0.25 μL of Ex Taq polymearase, 5 μL of 10× Ex Taq buffer, 4 μL of dNTP mixture (dATP, dCTP, dGTP and dTTP) and make up volume to 50 μL was carried out. The thermocycler program included an initial denaturing of 98°C for 2 min followed by 30 cycles of denaturing at 98°C for 10 sec, annealing at 46°C for 30 sec, extension at 72°C for 90 sec and finally cooling and stored at 4°C. PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and then introduced into *E. coli* JM 109 using the PTA2 vector (Toyobo Company, Japan). One microgram of purified plasmid was used for sequence analysis of the cloned 16S rDNA fragments. The purified sequencing products were determined by an ALF express automated sequencer. Computer analysis was made using the GENETYX-MAC program (Software Development Co. Ltd., Japan). Sequence data was identified using the tool BLAST programs within the Genbank database.

1.5.2 Phenotypic determination

The morphology of the selected LAB strain was studied. The characteristics of the colonies, gram staining and catalase activity of LAB strain were determined after 48 hr of incubation at 30°C on a GPY-MM plate.

API 50 CH (BioMerieux, France) was used in this study to determine the carbohydrate metabolism of the LAB strain. API 50 CH in conjunction with API 50 CHL medium (BioMerieux, France) was used for the identification of *Lactobacillus* sp. The colonies of LAB strain were picked up and suspended in a turbidity equivalent to 2 McFarland in the API 50 CHL medium. The API 50 CHL medium filled in API 50 CH strip consisted of 50 microtubes was used to study the fermentation of the carbohydrate family and its derivatives (heterosides, polyalcohols, uronic acids) as substrates and an overlay of all microtubes with mineral oil. Then, it was incubated aerobically at 30°C for 48 hr. During incubation, fermentation is revealed by color change (purple to yellow) in the tube, caused by the anaerobic production of acid and detected by pH indicator (Bromocresol Purple) present in the chosen medium. The first tube, no any active ingredient composition, was used as a negative control. The results were compared with *Lactobacillus plantarum* ATCC 14917 as the reference strain.

1.6 Molecular mass distribution using gel filtration chromatography

The supernatant of MM solution before and after treatment by the selected LAB strain was determined by the molecular weight pattern using gel filtration chromatography. Sephadex gel G-50 equilibrated with 0.1 mol/L phosphate buffer. Then, supernatant was placed on the top of a Sephadex G-50 column (2.6 cm × 95 cm) and eluted with 0.1 mol/L phosphate buffer at flow rate of 0.25 mL/min. Effluent was collected at 5 mL/tube. The color intensity in each fraction was measured using spectrophotometer at 475 nm.

1.7 Ability of the selected LAB strain in POME phenolic compounds removal

1.7.1 Treatment of phenolic compounds in treated POME

Laclobacillus plantarum SF5.6, had the highest MM decolorlization, was used for treating phenolic compounds in treated POME (POMEP). The raw POMEP was centrifuged at 5000 r/min for 20 min. Then the supernatant was sterilized by autoclave at 121°C for 20 min. POMEP was supplemented with a GPY medium and adjusted pH to 6. *L. plantarum* SF5.6 was inoculated into 250 mL Erlenmeyer flasks that contained 100 mL of POMEP and incubated at 30°C for five days under facultative conditions without shaking. The dry weight of cell mass was measured by drying at 105°C for 24 hr following a standard method (APHA, 1999). The reduction of phenol in treated POME was detected.

1.7.2 Analysis of phenol in treated POME

Phenolic extracts were prepared following the method by García García et al. (2000). Treated POME was acidified with HCl to pH 2 and then extracted 3 times with ethyl acetate (*V/V*) at ambient temperature. These fractions were combined and dried with anhydrous Na_2SO_4 for 30

min. The extract was concentrated to dryness in a rotary evaporator and re-dissolved with a mixture of methanol and water at ratio of 60:40. For the determination of total phenol content, the method used the Folin-Ciocalteu's phenol reagent (Box, 1983) involving the successive addition of 5 mL sodium carbonate (200 g/L) and 2.5 mL Folin-Ciocalteu's phenol reagent to the 50 mL sample. After 60 min at an ambient temperature, the absorbance was measured at 725 nm against a distilled water and reagent blank.

2 Results and discussion

2.1 Fermentative LAB isolates

The LAB strains having a MM decolorizing ability were isolated from various sources of soil, anaerobic wastewater treatment systems, and fermented and spoilage fruit vegetable and meat in several places from Thailand by enrichment in CSL-MM medium. The enrichment culture was spread on GPY-MM agar plate. The 2114 isolated LAB strains were found and only 1204 LAB strains showing clear zones around colonies in the primary step of isolation.

2.2 Screening of high potential fermentative LAB in MM decolorization

To determine the high potential fermentative LAB in MM decolorization, 1204 LAB strains showing clear zones around colonies were secondary screening by using GPY-MM broth under anaerobic conditions (Table 1). The isolated strains from the various sources showed various yield ranges of MM decolorization. It was noticed that only 35 LAB strains having MM decolorization of more than 55% were screened from 1204 isolated LAB strains of pickling/spoilage fruit and vegetable. Among them, only five strains namely FF4A, TBSF2.1-1, TBSF5.8-1, SF5.6 and TBSF2.1 were found to have higher decolorization yields of about 59.16%, 57.21%, 57.68%, 57.35% and 58.93%, respectively. Small numbers of LAB strains having MM decolorization were found in soil, wastewater and meat.

2.3 Effect of facultative-anaerobic conditions and temperature on MM decolorization

2.3.1 MM decolorization under facultative and anaerobic conditions

To simulate the condition of discharge treated wastewater from anaerobic treatment, five strains of LAB were tested under facultative conditions (no flushing of N_2 gas and static conditions) and anaerobic conditions (flushing of N_2 gas) for MM decolorization of 120 mL vial containing 100 mL GPY-MM medium. These five strains showed the slightly higher MM decolorization yield under the facultative conditions than under anaerobic conditions (Fig. 1). The five strains namely FF4A, TBSF2.1-1, TBSF5.8-1, SF5.6 and TBSF2.1 showed the ability of MM decolorization at 47.68%, 48.66%, 49.88%, 50.85% and 51.10% under anaerobic conditions, respectively and 59.72%, 58.05%, 59.12%, 59.65%, and 57.63% under facultative conditions, respectively. LAB strains tend to be facultatively anaerobic or microaerophilic (Yashima et al., 1970). It appeared to grow well in facultative conditions and provided more activity to decolorize MM. From the above results, the facultative (static) condition was chosen for further study for its higher decolorization yield. Few researchers had reported that the bacterial decolorization of MM under anaerobic conditions by *Lactobacillus hilgardii* W-NS (Ohomomo et al., 1988a) and *Bacillus* sp. (Nakajima-Kame et al., 1999) decolorized 28% and 36% of MM, respectively. By the study of Jain et al. (2002), *Bacillus megaterium* and *Bacillus cereus* were able to decolorize the predigested distillery effluent under aerobic condition for 33% which was slightly lower than that under anaerobic conditions as mention above.

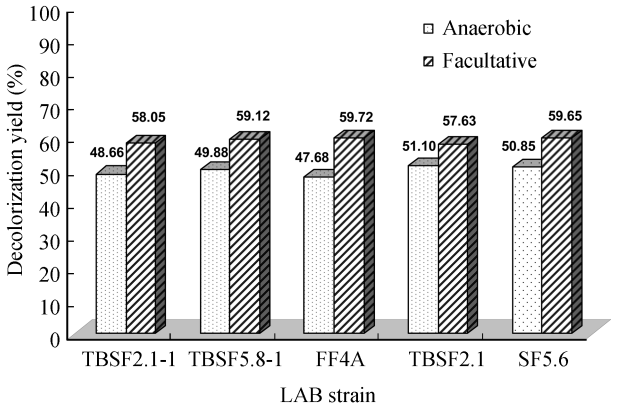

Fig. 1 Decolorization of MM from the five selected LAB strains in medium under facultative and anaerobic conditions.

Table 1 Secondary screening of LAB ability in MM decolorization from various sources

Sample source	Number of strains in each group of decolorization yield				Highest decolorizing ability strain in each group		
	1%–20%	21%–40%	41%–55%	> 55%	Strain	Decolorization yield (%)	Sample source
Soil	–	57	2	–	TBSF5.8-1	57.68	Spoilage *Syzygium jambos*
Wastewater	7	–	15	–	TBSF2.1-1	57.21	Spoilage *Solanum lycopersicum*
Pickling fruit and vegetable	12	43	246	12	TBSF2.1	58.93	Spoilage *Solanum lycopersicum*
Spoilage fruit and vegetable	82	171	522	23	FF4A	59.16	Spoilage *Ocimum tenuiflorum*
Meat	11	–	1	–	SF5.6	57.35	Spoilage *Lansium domesticum*

2.3.2 Optimization of temperature for cell growth and MM decolorization

Various temperatures (20, 25, 30, 37, 43 and 50°C) were studied for the determination of the five selected LAB strains in bacterial growth and MM decolorization. Table 2 shows the optimal temperature (30°C) for cell growth corresponding the maximum MM decolorization yield of FF4A, TBSF2.1-1, TBSF5.8-1, SF5.6 and TBSF2.1 under facultative conditions: 61.16%, 60.21%, 61.31%, 60.91%, and 61.93%, respectively. The profile of cell growth and MM decolorization from each strain at various temperatures slightly increased from 20 to 30°C and slightly decreased at temperatures higher than 30°C. At a thermophilic temperature (> 50°C), cell growth and decolorization were inhibited for these five strains. The study of Mohana et al. (2007) was found the effect of temperature on the treatment of distillery spent wash by a bacterial consortium DMC comprising *Pseudomonas* aeruginosa PAO1, *Stenotrophomonas maltophila* and *Proteus mirabilis*. Increasing temperature from 20 to 37°C affected the increase of the decolorization yield and a further increase in temperature above 40°C adversely affected the decolorization ability of microbial cells.

2.4 Culture profiles of selected LAB strains in MM solution

Culture profiles of five LAB strains are shown in Fig. 2a–e. Five strains of LAB were cultivated in GPY-MM medium at 30°C and under facultative conditions. The decolorizations of MM for the five strains were rapidly high at the beginning of initial pH 6 during 24 hr to 48 hr. Then, the pH in medium of five LAB strains rapidly dropped from 6.00 to 3.68–3.52 by the fast fermentation of glucose (COD) within 24–48 hr and no more MM decolorization at this acidic pH for all five strains. The results were similar to those found by Ohmomo et al.

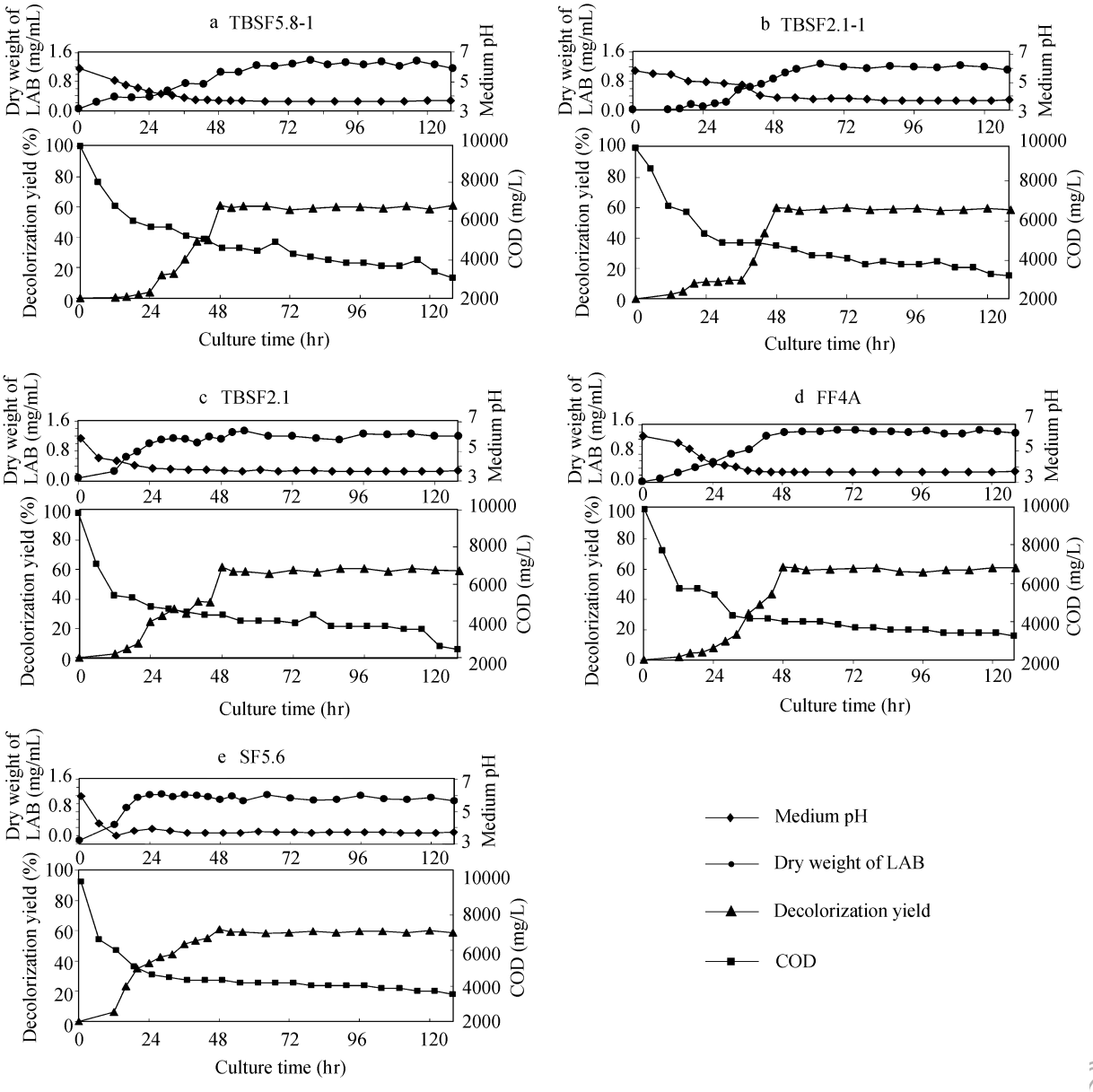

Fig. 2 Culture profile for TBSF5.8-1(a), TBSF2.1-1(b), TBSF2.1(c), FF4A (d), and SF5.6 (e).

Table 2 Effects of temperature on cell density and decolorization yield

LAB strains	OD 660 nm	Decolorization yield (%)	OD 660 nm	Decolorization yield (%)
	20°C		25°C	
TBSF5.8-1	0.737	19.77	0.801	26.16
TBSF2.1-1	0.513	28.84	0.654	43.68
TBSF2.1	0.855	47.14	0.833	42.91
FF4A	0.611	31.87	0.725	39.39
SF5.6	0.612	29.51	0.710	36.55
	30°C		37°C	
TBSF5.8-1	0.897	61.31	0.766	48.66
TBSF2.1-1	0.859	60.21	0.752	51.32
TBSF2.1	0.989	61.93	0.789	53.13
FF4A	0.853	61.16	0.847	51.32
SF5.6	0.751	60.91	0.643	47.86
	43°C		50°C	
TBSF5.8-1	0.210	45.67	0.154	0.00
TBSF2.1-1	0.516	49.53	0.092	0.07
TBSF2.1	0.663	45.40	0.066	0.94
FF4A	0.295	48.11	0.144	0.20
SF5.6	0.446	51.22	0.116	0.33

(1988a) and Mohana et al. (2007) who reported that higher decolorization occurred at pH near neutral as the solubility of melanoidins depended on pH (it is less soluble in acidic pH than in alkaline pH) and pH less than this optimal range led to a decrease in decolorization activity as well as growth rate.

The cell growth of TBSF5.8-1, TBSF2.1-1, TBSF2.1 and FF4A was maximized within 48–72 hr at a concentration of 1.38, 1.28, 1.34 and 1.46 mg/mL, respectively, whereas that of SF5.6 was maximized within 24 hr at a concentration of 1.23 mg/mL. Under facultative conditions obtained more cell mass which LAB strains found to be facultatively anaerobic or microaerophilic and poor growth in the presence of oxygen (Yashima et al., 1970). On the other hand, the maximum MM decolorization by TBSF5.8-1, TBSF2.1-1, TBSF2.1, FF4A and SF5.6 were also found within 48 hr (61.31%, 60.21%, 61.93%, 61.16% and 60.91%, respectively) as well as COD decreasing. LAB strains with their capacity to reduce oxygen pressure, redox potential and pH, offer a new promising approach to bioconversion of MM. It could suggest that the decolorization of MM supplemented with external glucose under facultative conditions indicated sugar oxidases and peroxidases play the major role in MM decolorization (Ohomomo et al., 1988b; Kumar and Chandra, 2006; Sirianuntapiboon and Chairattanawan, 1998; Chaturvedi et al., 2006).

The results show that the MM decolorization of these five LAB strains were associated with LAB growth. In consideration of cell mass to MM decolorization (microbial activity), it was noticed that 1 mg of cell (dry weight) from TBSF5.8-1, TBSF2.1-1, TBSF2.1, FF4A and SF5.6 can decolorize MM and yielded at 44.42%, 47.04%, 46.22%, 41.89 % and 49.52%, respectively. Therefore, LAB strain SF5.6 which showed the highest growth rate and microbial activity in MM decolorization was chosen for further study.

2.5 Identification of strain SF5.6 using genotype and carbohydrate fermentation

Analysis of 16s rDNA was an effective and fast method to identify microorganisms to species level (Henseik et al., 1992). Carbohydrate fermentation was used as a method to confirm test of characteristics with the reference strain. 16S rDNA of strain SF5.6 was the studied and identified species. The amplified 16S rDNA of SF5.6 about 1.5 kb was separated by electrophoresis on a 1% agarose gel in 1× TAE buffer. The gel was stained with ethidium bromide and photo capture under UV light as shown in Fig. 3. One kb fragment obtained from strain SF5.6 was sequenced. The sequence of 1 kb was identified with the *Lactobacillus* group specific sequence in the Genbank database by using the BLAST program. Pair-wise alignments giving a closest match of 98% were identified similarly to *L. plantarum*. In addition to confirm the type of SF5.6, carbohydrate fermentation was used to double check. Forty nine carbohydrates (API 50 CH) were used to examine carbohydrate fermentation of SF5.6 compared to the reference strain. The response of SF5.6 in carbohydrate fermentation and *L. plantarum* ATCC 14917 as reference strain are shown in Table 3. The results of carbohydrate fermentation of *L. plantarum* SF5.6 was closely similar to the reference strain (*L. plantarum* ATCC 14917) (Table 3). The LAB strain SF5.6 was confirmed as *L. plantarum*. The morphology of this strain was milky colonies, rod shaped, Gram-positive

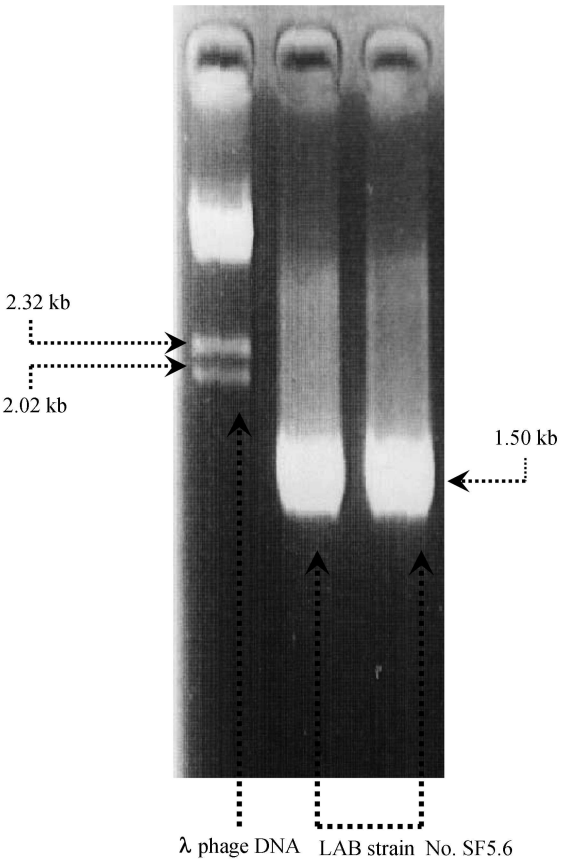

Fig. 3 PCR amplification of Lactobacilli of 16S rDNA-strain SF5.6 compared with α phage DNA marker.

Table 3 Carbohydrate fermentation of strain SF5.6 compared to *L. plantarum* ATCC 14917 as a reference strain

Strip 0–24	SF5.6	Ref. strain	Strip 25–49	SF5.6	Ref. strain
0 Control	–	–	25 Esculin	+	+
1 Glycerol	–	–	26 Salicin	+	+
2 Erythritol	–	–	27 Cellobiose	+	+
3 D-Arabinose	–	–	28 Maltose	+	+
4 L-Arabinose	+	+	29 Lactose	+	+
5 Ribose	+	+	30 Melibiose	+	+
6 D-Xylose	–	–	31 Surcose	+	+
7 L-Xylose	–	–	32 Trehalose	+	+
8 Adonitol	–	–	33 Inulin	–	–
9 b Methyl-D-Xyloside	–	–	34 Melezitose	+	+
10 Galactose	+	+	35 Raffinose	+	+
11 Glucose	+	+	36 Strach	–	–
12 Fructose	+	+	37 Glycogen	–	–
13 Mannose	+	+	38 Xulitol	–	–
14 Sorbose	–	–	39 Gentiobiose	+	+
15 Rhamnose	–	–	40 D-Turanose	+	+
16 Dulcitol	–	–	41 D-Lyxose	–	–
17 Inositol	–	–	42 D-Tagatose	–	–
18 Mannitol	+	+	43 D-Fucose	–	–
19 Sorbitol	–	–	44 L-Fucose	–	–
20 α-Methyl-D-Mannoside	+	+	45 D-Arabitol	–	–
21 α-Methyl-D-Glucoside	–	–	46 L-Arabitol	–	–
22 N-Acetyl-Glucosamine	+	+	47 Gluconate	–	+
23 AMYgdalin	+	+	48 2-Keto-Gluconate	–	–
24 ARButin	+	+	49 5-Keto-Gluconate	–	–

and catalase negative. *L. plantarum* SF5.6 in this study was isolated from spoilage *L. domesticum*. It has been reported that *L. plantarum* is the dominant species often found in vegetable and fruit fermentation (Rodriguez et al., 2009).

2.6 Molasses melanoidins degradation by *L. plantarum*. SF5.6

Figure 4 shows the Sephadex G-50 chromatograms from the elution of melanoidins in MM solution before (fresh) and after treatment by *L. plantarum* SF5.6. Fresh MM solution showed one peak with node of melanoidins. After five days incubation, the Sephadex G-50 chromatograms of melanoidins treated by *L. plantarum* SF5.6 showed the disappearance of the first sharp peak of MM representing high molecular weight fractions of melanoidins and the decrease of the node peak with low molecular weight fractions. The high molecular weight fractions of melanoidins were decolorized by *L. plantarum* SF5.6 while low molecular weight fractions slightly decolorized and remained in MM solution. Similar results were found in the decolorization of molasses wastewater by *A. fumigatus G-2* (Ohmomo et al., 1987) and *Citeromyces* sp. WR-43-6 (Sirianuntapaiboon et al., 2004).

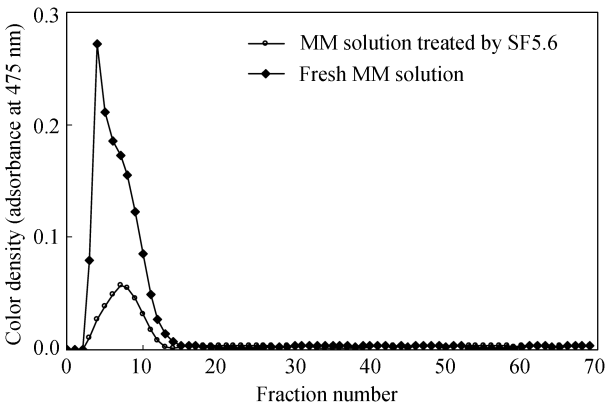

Fig. 4 Sephadex G-50 permeation of fresh MM solution and treated MM solution by SF5.6.

2.7 Ability of *L. plantarum*. SF5.6 in removing POME phenolic compounds

It was found that *L. plantarum* was able to remove recalcitrant phenolic compounds (showing brownish black colorization) in olive mill wastewater (Lamia and Moktar, 2003). Therefore, in this preliminary study *L. plantarum* SF5.6 was applied to test the removal of phenolic compounds in treated brownish POME (POMEP). Table 4 shows results of phenol and color removal by *L. plantarum* SF5.6 incubated at 30°C and initial medium pH 6 for five days under facultative (static) conditions. The initial concentration of total phenol found in anaerobically treated POME was 33.64 mg/L. After treated undiluted POMEP by *L. plantarum* SF5.6, phenol and color were removed by 34.00% and 15.88%, respectively and cell growth up to 1.18 mg dry weight/mL. pH was dropped to 4.19 by the fermentation of *L. plantarum* SF5.6. From the preliminary study, it was shown that *L. plantarum* SF5.6 had a slightly high efficiency in removing phenolic compounds in POME

Table 4 Removal of phenolic compounds and color in POMEP using *L. plantarum* SF5.6

Parameter	Removal (%)	
	Before POMEP treatment	After POMEP treatment
pH	6	4.19
Cell mass (mg dry weight/mL)	0.1	1.18
Phenolic compounds (mg/L)	33.64	22.36
Phenol removal (%)	–	34.00
Decolorization yield (%)	–	15.88

without dilution. Lamia and Moktar (2003) found that phenolic compounds were removed by 46% when olive mill wastewater was diluted ten times. Rodriguez et al. (2009) summarized that a few LAB species such as *L. plantarum* found in the fermented vegetable were well adapted to growth in high phenolic compounds. Landete et al. (2008) studied the degradation of nine phenolic compounds isolated in olive products, it was found that only oleuropein and protocatechuic acid were metabolized by *L. plantarum* strains.

3 Conclusions

Lactic acid bacteria (LAB) showing decolorization of molasses melanoidins (MM) are abundantly present in several sources of pickled and spoilage food and soil and sludge from wastewater treatment systems. *L. plantarum* SF5.6 showed the highest MM decolorization among the 2114 strains of LAB under facultative conditions. The optimum temperature for *L. plantarum* SF5.6 growth and MM decolorization was 30°C. The strain SF5.6 was the fastest growing LAB within 24 hr and had the highest microbial activity in MM decolorization (1 mg of cell mass could decolorize 49.52% of MM) compared to the other LAB strains. pH also affected MM decolorization when decreased from 6 to acidic pH 3.6. In addition, the result of gel filtration chromatogram revealed that the decrease of brownish color in molasses melanoidins occurred from the degradation of high molecular weight fractions on melanoidins. Moreover, *L. plantarum* SF5.6 isolated from spoilage *Lancium domesticum* was applied to remove phenoloic compounds in treated POME (POMEP). The preliminary study found that *L. plantarum* SF5.6 had the ability to remove 34% of phenolic compounds and 15.88% decolorization yield at 30°C under facultative conditions. However, further research should be carried out to optimize conditions for POMEP removal and then scale up in a bioreactor.

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References

APHA (American Public Health Association), AWWA (American Water Works Association), and WEF (Water Environment Federation), 1999. Standard Methods for the Examination of Water and Wastewater (20th ed.). Washington DC, USA.

Assas N, Ayed L, Marouani L, Hamadi M, 2002. Decolorization of fresh and stored-black olive mill wastewater by *Geotrichum Candidum*. *Process Biochemistry*, 38: 361–365.

Box J D, 1983. Investigation of the folin-ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. *Water Research*, 17(5): 511–525.

Capasso R, Evidente A, Schivo L, Orru G, Marcialis M A, Cristinzio G, 1995. Antibacterial polyphenols from olive oil mills waste waters. *Journal of Applied Bacteriology*, 79: 393–398.

Casa R, D'Annibale A, Pieruccetti F, Stazi S R, Giovanorozzi S G, Lo Cascio B, 2003. Reduction of the phenolic components in olive-mill wastewaters by an enzymatic treatment and its impact on durum wheat (*Triticum durum* Desf.) germinability. *Chemosphere*, 50: 959–966.

Chaturvedi S, Chandra R, Rai V, 2006. Isolation and characterization of *Phragmites australis* (L.) rhizosphere bacteria from contaminated site for bioremediation of colored distillery effluent. *Ecological Engineering*, 27: 202–207.

Daud Khaled A K, Neilan B A, Henriksson A, Conway P L, 1997. Identification and phylogenetic analysis of *Lactobacillus* using multiplex RAPD-PCR. *FEMS Microbiology Letters*, 153: 191–197.

García García I, Jiménez Peña P R, Bonilla Venceslada J L, Martín Martín A, Martín Santos M A, Ramos Gmez E, 2000. Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium*, *Aspergillus niger*, *Aspergillus terreus* and *Geotrichum candidum*. *Process Biochemistry*, 35: 751–758.

Gonzalez T, Terron M C, Yague S, Zapico E, Galletti G C, Gonzalez A E, 2000. Pyrolysis/gas chromatography/mass spectrometry monitoring of fungal-biotreated distillery wastewater using *Trametes* sp. I-62 (CECT 20197). *Rapid Communications in Mass Spectrometry*, 14: 1417–1424.

Ghosh M, Ganguli A, Tripathi A K, 2002. Treatment of anaerobically digested distillery spentwash in a two-stage bioreactor using *Pseudomonas putida* and *Aeromonas* sp. *Process Biochemistry*, 37: 857–862.

Godshall M A, 1999. Removal of colorants and polysaccharides and the quality of white sugar. In: Proceedings of Sixth International Symposium organized by Association Andrew van Hook (AvH), Reims, France. 25 March. 28–35.

Henseik R, Krupp G, Stackebrandt E, 1992. Development of diagnostic oligonucleotide probes for four *Lactobacillus* species occurring in the intestinal tract. *Systematic Applied Bacteriology*, 15: 123–128.

Jain N, Minocha A K, Verma C L, 2002. Degradation of predigested distillery effluent by isolated bacterial strains. *Indian Journal of Experimental Biology*, 40: 101–105.

Kumar V, Wait L, Fitzgibbon F, Nigam P, Banat I M, Singh D, 1997. Bioremediation and decolorization of anaerobically digested distillery spent wash. *Biotechnology Letters*, 19: 311–313.

Kumar P, Chandra R, 2006. Decolorisation and detoxification of synthetic molassea melanoidins by individual and mixed cultures of *Bacillus* sp. *Bioresource Technology*, 97: 2097–2102.

Lamia A, Moktar H, 2003. Fermentative decolorization of olive mill wastewater by *Lactobacillus plantarum*. *Process Biochemistry*, 39: 59–65.

Landete J M, Curiel J A, Rodriguez H, de las Rivas B, Munoz R, 2008. Study of the inhibitory activity of phenolic compounds found in olive products and their degradation by *Lactobacillus phantarum* strains. *Food Chemistry*, 107: 320–326.

Miyata N, Mori T, Wahori K I, Fujita M, 2000. Microbial decolorization of melamoidin-containing wastewater: Combined use of activated sludge and the fungus *Coriolus hirsutus*. *Journal of Bioscience and Bioengineering*, 89: 145–150.

Mohana S, Desai C, Madamwar D, 2007. Biodegradation and decolourization of anaerobically treated distillery spent wash by a novel bacterial consortium. *Bioresource Technology*, 98: 333–339.

Nakajima-Kame T, Shimomura M, Nomura N, Chanpornpong T, Nakahava T, 1999. Decolorization of molasses wastewater by *Bacillus* sp. under thermophilic and anaerobic conditions. *Journal of Bioscience and Bioengineering*, 87: 119–121.

Ohmomo S, Kaneko Y, Sirianuntapiboon S, Atthasampunna P, Nakamura I, 1987. Decolorization of molasses wastewater by thermophilic strain *Aspergillus fumigatus* G-2-6. *Agricultural and Biological Chemistry*, 51 (12): 3339–3346.

Ohmomo S, Yoshikawa H, Nozaki K, Nakajima A, Daengsubha W, Nakamura I, 1988a. Continuous decolorization of molasses wastewater using immobilized *Lactobacillus hilgardii* cell. *Agricultural and Biological Chemistry*, 52: 2437–2441.

Ohomomo S, Daengsubha W, Yoshikawa H, Yui M, Nozaki K, Nakajima T et al., 1988b. Screening of anaerobic bacteria with the ability to decolorize molasses melanoidin. *Agricultural and Biological Chemistry*, 51(12): 3339–3346.

Rodriguez H, Curiel J A, Landete J M, de las Rivas B, Felipe F L, Gómez-Cordovés C et al., 2009. Review: Food phenolics and lactic acid bacteria. *International Journal of Food Microbiology*, 132: 79–90.

Saez L, Perez J, Martinez J, 1992. Low molecular weight phenolics attenuation during simulated treatment of wastewaters from olive oil mills in evaporation ponds. *Water Research*, 26: 1261–1266.

Satyawali Y, Balakrishnan M, 2008. Wastewater treatment in molasses-based alcohol distilleries for COD and color removal: A review. *Journal of Environmental Management*, 86: 481–497.

Sirianuntapiboon S, Sihanonth P, Somchai P, Atthasumpunna P, Hayashida S, 1995. An absorption mechanism for the decolorization of melanotdin by *Rhizoctonia* sp. D-90. *Bioscience Biotechnology and Biochemistry*, 59, 1185–1189.

Sirianuntapiboon S, Chairattanawan K, 1998. Some properties of *Coriolus* sp. No. 20 for removal of colour substances from molasses wastewater. *Thammasat International Journal of Science and Technology*, 3: 74–79.

Sirianuntapiboon S, Phothilangka P, Ohmomo S, 2004. Decolorization of molasses wastewater by a strain No. BP103 of acetogenic bacteria. *Bioresource Technology*, 92: 31–39.

Yashima S, Kawal K, Okami Y, Sasaki Y, 1970. Effect of oxygen on glucose dissumilation by heterolactic bacteria. *Journal of Genetic Engineering and Applied Microbiology*, 16: 543–545.

Zhang Y J, Yan L, Qiao X L, Chi L N, Niu X G, Mei Z J et al., 2008a. Integration of biological method and membrane technology in treating palm oil mill effluent. *Journal of Environmental Sciences*, 20(5): 558–564.

Zhang Y J, Yan L, Chi L N, Long X H, Mei Z J, Zhang Z J, 2008b. Startup and operation of anaerobic EGSB reactor treating palm oil mill effluent. *Journal of Environmental Sciences*, 20(6): 658–663.