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Full Length Research Paper

Isolation and characterization of rhamnolipid producing *Pseudomonas aeruginosa* strains from waste edible oils

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This study was to screen efficient biosurfactant producing strains from waste edible oils (WEOs). Two strains Y5 and Y17 were screened out by steps of preliminary blood plate screening and secondary flask fermentation screening. The surface tension of the fermentation broth of the two strains diluted by 100 times was lower than 40 mN/m. Preliminary characterization of fermentation products was conducted, which was found to be rhamnolipid ultimately. Y5 strain produced only one kind of glycolipids while the Y17 produced at least three kinds of homologues of glycolipids. Both Y5 and Y17 were identified as *Pseudomonas aeruginosa* strains. This study gave a simple and efficient protocol to screen out biosurfactant producing strains and would pave the way for the production of biosurfactant from WEOs.

Key words: Rhamnolipid, biosurfactant, fermentation, waste edible oils, *Pseudomonas aeruginosa.*

INTRODUCTION

Biosurfactants are amphipathic compounds excreted by microorganisms that exhibit surface activity. Biosurfactants have advantages over their chemical counter-parts in biodegradability, low toxicity, and ecological acceptability and effectiveness at extreme temperature and pH (Qiang et al., 2009). The cost of the substrate will greatly influence the spread of the biosurfactants. Presently, waste edible oils (WEOs) such as drainage oils and frying oils are mostly not utilized efficiently. On the contrary, they become serious pollutants sometimes, or, even more dangerously, are recovered improperly and sold illegally as WEOs to damage the people's health (Guo and Chen, 2009; Yao and Min, 2010). Recently, it was found that WEOs can be utilized to produce biosurfactant (Nitschke et al., 2005), such as rhamnolipid used as emulsifier or de-emulsifier (Yao and Min, 2010) as well as biodiesel (Xiong et al., 2007).

The aims of this study include: (1) to study one simple way to isolate biosurfactant producing strains from WEOs and (2) to evaluate and screen highly efficient strains.

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Abbreviations: ANOVA, Analysis of variance; **BP**, beef-peptone medium; **FT-IR,** fourier transform infrared spectrum; **NA,** nutrient agar medium; **TLC,** thin-layer chromatography; **WEOs,** waste edible oils.

MATERIALS AND METHODS

Samples

Water and soil samples were obtained from the sewage or under cooking stove of the dining-room of Yancheng Teachers University.

Frying oil was salad oil with numerous frying from the dining room of Yancheng Teachers University. It was employed as the sole carbon source in this study.

Culture media

Seed medium (BP) was composed of (g/L) peptone 10, beef extract 5 and NaCl 5. Initial pH value was adjusted to 7, and 15 g/L of agar was added to make plate or slant. The BP was added by 5% defibrinated sheep blood to prepare the blood nutrient agar medium (NA), following (Liu et al., 2006). Fermentation broth was composed of (g/L) $NaNO_3$ 2.5, K_2HPO_4 4.0, KH_2PO_4 4.0, $CaCl_2$ 0.02, $MgSO_4$ 0.2, NaCl 1.0, KCl 1.0 and yeast extract 1.0, initial pH was adjusted to 7.0 after the mineral salt medium was made and 3% (V/V) frying oil (as the sole carbon source) was added separately to individual shaking flasks.

All the chemicals were purchased from Sinopharm Chemical Reagent Co. Ltd in Shanghai, China. All the culture media were autoclaved at 121℃ for 20 min.

Isolation and screening of biosurfactant producing strains

About 5 g soil samples or 5 ml water samples were aseptically added to 45 ml sterilized water and streaked on blood NA plates. Colonies with big and clear hemolytic circles were selected, incubated on slant media, incubated at 30℃, and st ored at 4℃ until further use.

One loopful of culture was inoculated in 20 ml of seed media in a 100 ml flask and incubated on a rotary shaker at 30℃ and 180 rpm for 10 h. Then 2.5 ml (5%) inoculum was transferred to 50 ml biosurfactant producing media in 250 ml shaking flasks. After 3 days of cultivation, the broth supernatant was centrifuged at 9,000 rpm and the biosurfactant producing capacity was measured by oil-spreading method and surface tension measurements. The strains that performed best were selected.

Measurement of biosurfactant production

Oil-spreading method

About 30 ml distilled water was put into Petri dishes, then 50 µl diesel was dropped on the water surface to form oil film, in the midst of which 5 µl fermentation broth (diluted by 10 times with water) was dropped. The diameter of the oil expelling circles was measured by slide caliber (with the degree of accuracy of 0.02 mm). The strains with big circle diameters were selected for surface tension measurement (Plaza et al., 2006; Youssef et al., 2004; Zhang and She, 2005).

Surface tension measurement

The surface tension of the culture broth supernatant (diluted by 100 times) was measured with JYW-200 surface tension instrument (Zhang et al., 2010). Three repetitions were established and ANOVA analysis was conducted.

Characterization of fermentation products

Sulfuric acid-phenol reaction and ultraviolet analysis

Following the reference (Bao et al., 2003), the fermentation broth was diluted by 100 times first, and 2 ml of the diluted broth was put into 15 ml tube, and 1 ml phenol and 5 ml sulfuric acid were added and vortexed. Then, the tubes were heated for 15 min by boiled water bath, cooled to the room temperature, after which ultraviolet scanning was carried out to decide whether there was measured absorption peak at 480 nm.

Thin layer chromatography (TLC) analysis

The supernatant of the biosurfactant-producing fermentation broth was extracted by chloroform : methanol = 2:1 (V/V) and submitted to TLC analysis with the chloroform : methanol : water = 65:15:2 (V/V/V) solution as developing solvent. The chromogenic reagent contained phenol-sulfuric acid reagent in which 3 g phenol and 5 ml sulfuric acid were dissolved into 95 ml ethanol. The brown dot blot will show if the glycolipids exist (Zhang et al., 2010).

Ninhydrin reaction

Exactly 1 ml cell free fermentation broth was put into the tube, and after 3 drops of 0.5% ninhydrin solution added, the tube was put into the boiling water bath for several minutes, to observe the change of color of the reaction mixture.

Characterization of strains

Y5 and Y17 were submitted to plate culture and observation, and gram staining (Garrity et al., 2005). The 16S rRNA PCR and sequence analysis was carried out using modified protocols of reference (Lee et al., 2008). The total DNA was extracted by DNA extraction kit from Hangzhou Sigmens Bioengineering Co. Ltd. (China). Two primers annealing at the 5' and 3' end of the 16 S rRNA gene were:

Forward primer (27F): 5'AGA GTT TGA TCC TGG CTC AG 3'
Reverse primer (1492R): 5' TAC GGY TAC CTT GTT ACG ACT T 3'

The PCR was carried out accordingly. PCR products were sent to Shanghai Sagon Bioengineering Co. Ltd for sequencing. Afterwards, the partial rDNA sequences were analyzed using a BLAST search algorithm to estimate the degree of similarity to other rDNA sequences obtained from the NCBI/GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and the phylogenetic tree was also drawn using strains in the GenBank.

RESULTS AND DISCUSSION

Isolation and screening

Totally, 82 strains were isolated through blood plate assessment. These strains were cultured and stored at slants, then were submitted to screening by protocols, through which S-19, Y-5, and Y-17 were screened out. As shown in Figures 1 and 2, Y5 and Y17 had bigger oil expelling circles (5.5 and 5.8 cm, respectively) and smaller surface tensions (lower than 40 mN/m), showing better performances compared to S19.

Characterization of fermentation products

The sulfuric acid-phenol reaction solution of three strains appeared saffron yellow with the absorbent peak at about 480 nm. According to (Bao et al., 2003), this suggested that glycolipids were produced in the fermentation broth.

The standard curve and the yield of each strain are shown in Figure 3. The yield of glycolipid for Y5 and Y17 was 13.2 and 14.1 g/L, respectively.

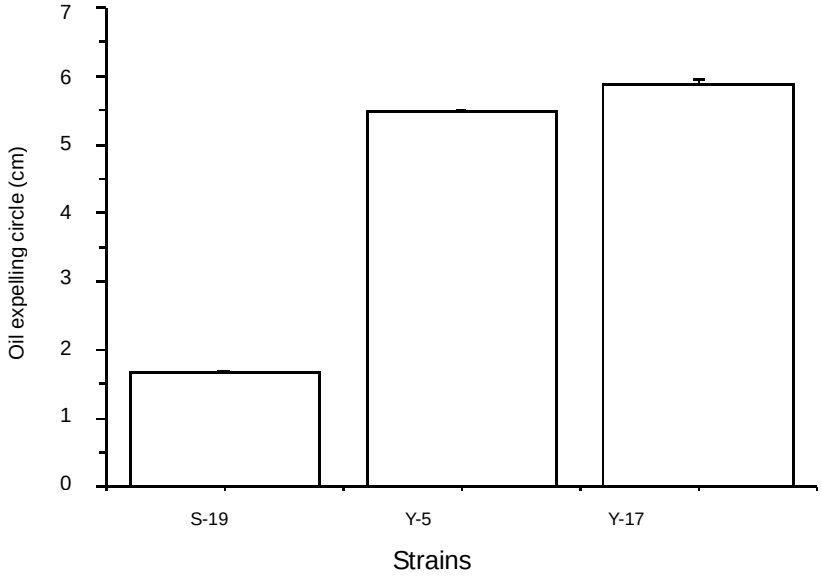

Figure 1. Diameters of oil expelling circles of three strains.

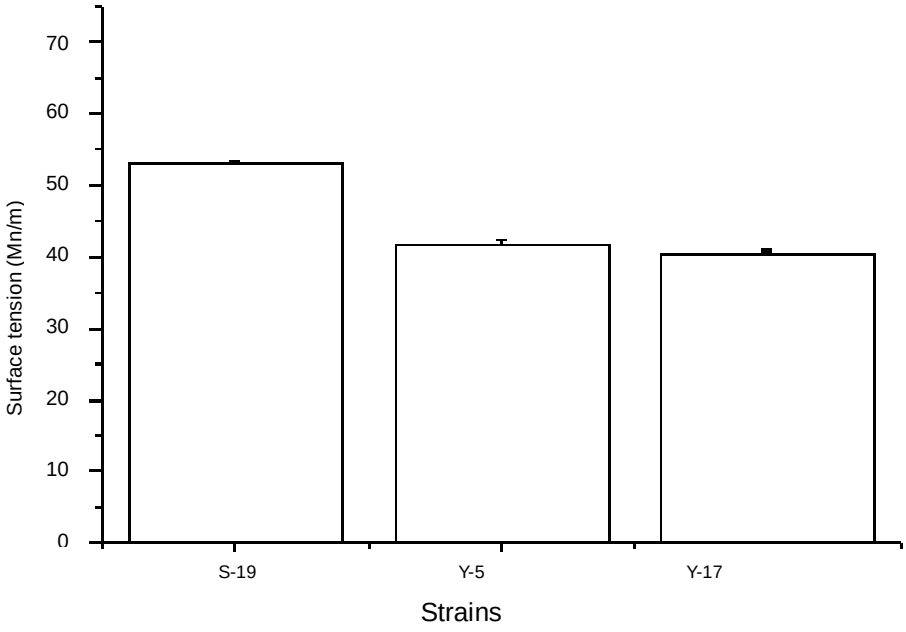

Figure 2. Surface tension values of three strains.

The TLC results are shown in Figure 4. Both Y5 and Y17 produced spots in TLC plates, indicating that both strains could produce glycolipids, and judging from the spots of each strain, Y5 produced more than one kind of glycolipids.

One milliliter extracts by chloroform / methanol extraction solution from Y5 and Y17 fermentation broth was separately added with 3 droplets of ninhydrin solution, and then boiled for reaction. The reaction solutions showed no changes in color, meaning that both strain produced no lipopeptides.

Identification of Y5 and Y17

Y5 and Y17 could form about 3 mm round colonies as well as clear haemolytic circles after cultivation for 2 days on the blood agar plates. Both were Gram negative and

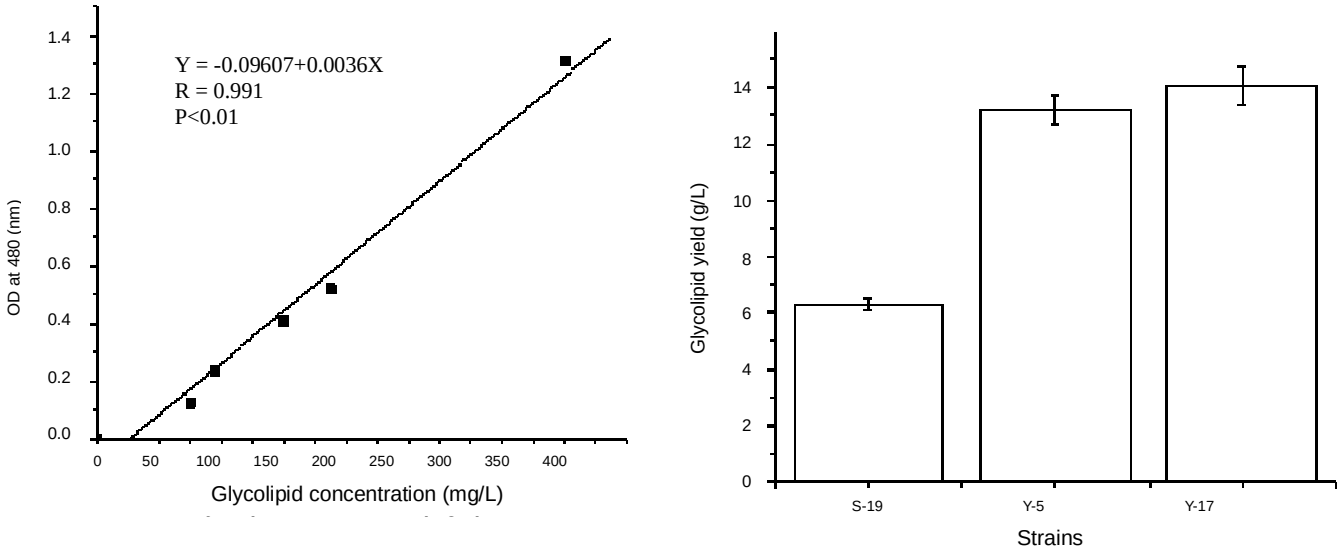

Figure 3. Glycolipid yield of three strains (left is the standard curve between glycolipid concentration and OD value at 480 nm).

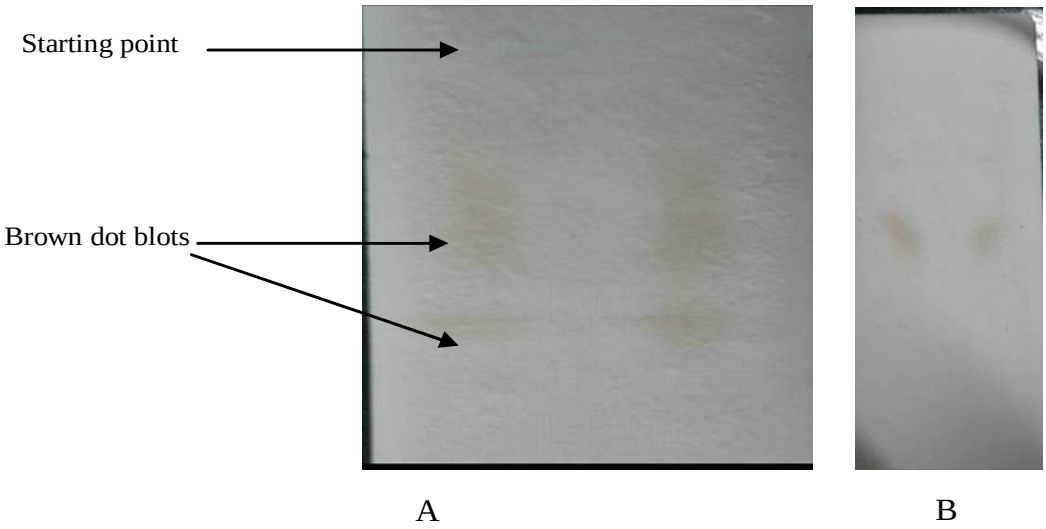

Figure 4. TLC analysis of products of Y5 and Y17 (A：Y-5；B：Y-17, both were set with two repetitions).

rod-shaped bacteria. The sequencing analysis of Y5 and Y17 using the 16 S rRNA gene nucleotide (NT) sequences data showed that both strains had the highest homology (over 99%) with *Pseudomonas aeruginosa* species, and were very close to each other systematically (Figure 5).

Conclusion

As for the carbon source, some researchers consider the oil-containing fructus performed better than waste edible oil from food industry (Huang et al., 2009). However, in actual study and industrialized production, the waste oil is more convenient and more economical to acquire, and that is why we selected the waste edible oil as the sole carbon source when screening was carried out.

Two bacterial strains with high biosurfactant producing efficiency using WEOs were screened out through blood plate and shaking flask fermentation. The preliminary isolation by blood plate and further screening by shaking flask fermentation was an efficient method for screening of glycolipid producing strains. With TLC and ninhydrin analysis, the fermentation products were identified as

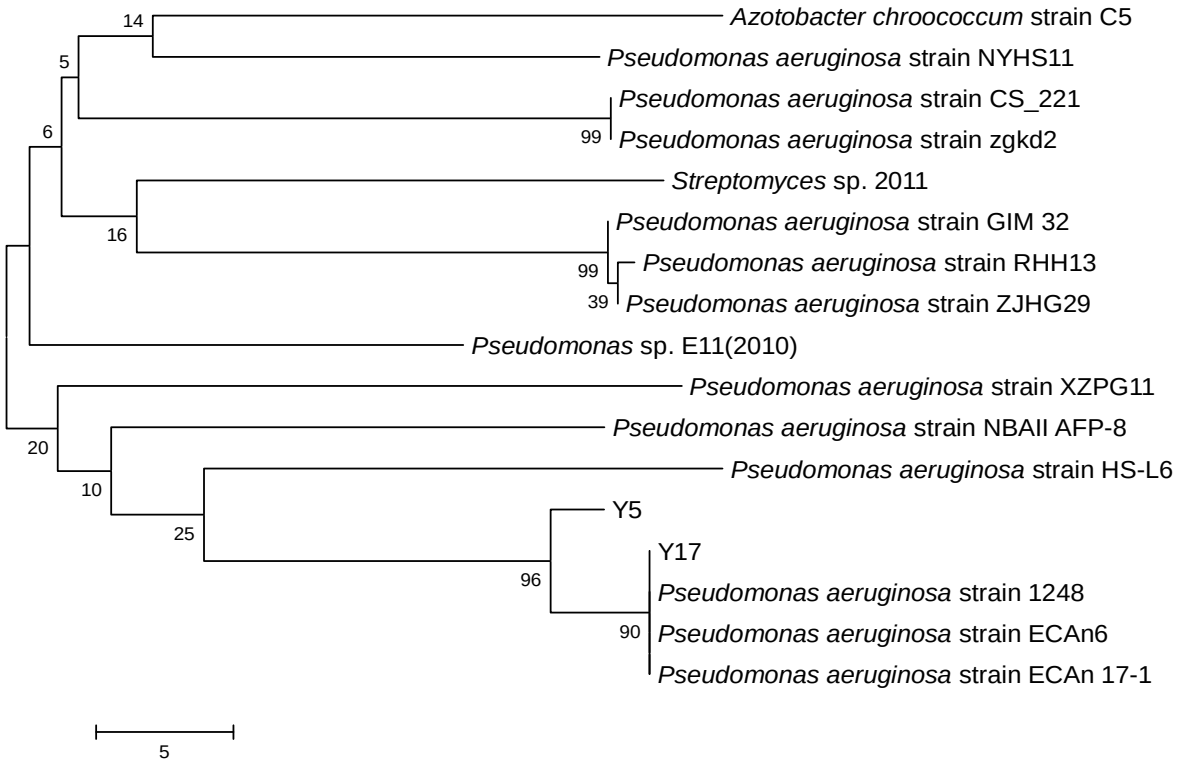

Figure 5. Neighbor-joining trees based on the sequences of 16 S rRNA gene. [Numbers on the tree represent bootstrap value (1000 replications).

glycolipids. Although no further characterization was conducted, since both Y5 and Y17 are *P. aeruginosa* strains, the glycolipids should be rhamnolipid, or isomers of rhamnolipid. Although, both Y5 and Y17 are *P. aeruginosa* systematically, the production of glycolipids was variable.

P. aeruginosa is one of the most popular strains employed for fermentation to produce glycolipid (Lee et al., 2008; Pornsunthorntawee et al., 2008; Huang et al., 2009; Qiang et al., 2009; Yin et al., 2009; Arutchelvi and Doble, 2010; Hazra et al., 2011; Muller et al., 2011; Saikia et al. 2011). The reason might be that *P. aeruginosa* was easier comparatively to isolate and screen, easier to culture (results from this study showed that the growth period to reach exponential phase by batch seed culture was only about 8 to 10 h), and the higher yield of biosurfactant. Meanwhile, *Pseudomonas* genera including *P. aeruginosa* can sometimes be conditional pathogenic bacteria (Hancock and Speert, 2000). In order to screen non-pathogenic *P. aeruginosa* strains with biosurfactant-producing capacity, the test of the existence of pyocyanin might be essential after biosurfactant-producing strains were screened out and before further studies such as characterization of fermentation products and identification of genus or species, since *P. aeruginosa* usually produces pyocyanin which is the main cause of its infections (Kipnis et al., 2006; Lau et al., 2004).

The glycolipid yield of each strain was satisfactory, although it was not so high as some latest references, for instance, Muller's report (2011). Both Y5 and Y17 were efficient glycolipid producers using waste edible oil. With the optimization of fermentation broth compositions and environmental conditions, the yield might increase further.

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