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Final Report

Bio-Engineering High Performance Microbial Strains for MEOR by Directed Protein Evolution Technology

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Project Summary Page

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Abstract

The main objectives of this three-year research project are: 1) to employ the latest advances in genetics and bioengineering, especially Directed Protein Evolution technology, to improve the effectiveness of the microbial enhanced oil recovery (MEOR) process. 2) to improve the surfactant activity and the thermal stability of bio-surfactant systems for MEOR; and 3) to develop improved laboratory methods and tools that screen quickly candidate bio-systems for EOR.

Biosurfactants have been receiving increasing attention as Enhanced Oil Recovery (EOR) agents because of their unique properties (i.e., mild production conditions, lower toxicity, and higher biodegradability) compared to their synthetic chemical counterparts. Rhamnolipid as a potent natural biosurfactant has a wide range of potential applications, including EOR and bioremediation. During the three-year of the project period, we have successfully cloned the genes involved in the rhamnolipid bio-synthesis. And by using the Transposon containing Rhamnosyltransferase gene rhlAB, we engineered the new mutant strains *P. aeruginosa* PEER02 and *E. coli* TnERAB so they can produce rhamnolipid biosurfactans. We were able to produce rhamnolipds in both *P. aeroginosa* PAO1-RhlA$^-$ strain and *P. fluorescens* ATCC15453 strain, with the increase of 55 to 175 fold in rhamnolipid production comparing with wild type bacteria strain. We have also completed the first round direct evolution studies using Error-prone PCR technique and have constructed the library of RhlAB-containing Transposon to express mutant gene in heterologous hosts. Several methods, such as colorimetric agar plate assay, colorimetric spectrophotometer assay, bioactive assay and oil spreading assay have been established to detect and screen rhamnolipid production.

Our engineered *P. aeruginosa* PEER02 strain can produce rhamnolipids with different carbon sources as substrate. Interfacial tension analysis (IFT) showed that different rhamnolipids from different substrates gave different performance. Those rhamnolipids with plant oil as substrate showed as low an IFT as 0.05mN/m in the buffer solution with pH5.0 and 2% NaCl. Core flooding tests showed that rhamnolipids produced by our engineered bacteria are effective agents for EOR. At 250ppm rhamnolipid concentration from *P. aeruginosa* PEER02, 42% of the remaining oil after waterflood was recovered. These results were therefore significant towards considering the exploration of the studied rhamnolipids as EOR agents.

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LIST OF ACRONYMS AND ABBREVIATIONS

EOR	Enhanced Oil Recovery
HPLC	High Pressure Liquid Chromatography
IFT	Interfacial Tension
LB	Luria-Bertani
LC-MS	Liquid Chromatography and Mass Spectrometry
MEOR	Microbial Enhanced Oil Recovery
PV	Pore Volumes
RC	Reservoir Cleaning

SPE	Society of Petroleum Engineers
TLC	Thin Layer Chromatography
WR	Water Removal

Acknowledgements

We would like to thank DOE for their financial support and guidance throughout this project.

4. EXECUTIVE SUMMARY

This report summarizes the research work performed and results of this 3-year project. The main objectives are to employ the latest bioengineering technologies to develop manufacturing methods at the surface that can create more cost-effective surfactants for the chemical flood EOR process; and to improve the surfactant activity and the thermal stability of bio-surfactant systems deployed subsurface for MEOR. Other objectives are to develop improved laboratory methods and tools that screen quickly candidate bio-systems for EOR. These detailed laboratory procedures to identify effectively and quickly mutated genes for their bio-surfactant activity and thermal stability is a substantial effort. Documenting these procedures will accelerate greatly the development of yet newer bio-systems for improved oil recovery applications in similar, future studies.

During the three-year project period,

1. We have successfully cloned the genes involved in the rhamnolipid bio-synthesis. And by using the Transposon containing Rhamnosyltransferase gene rhlAB, we have successfully produced rhamnolipds in both *P. aeroginosa* PAO1-RhlA$^-$ cells and *E.coli* cells, with the increase of 55 to 175 fold in rhamnolipid production compared with wild type bacteria strain.
2. We have completed the several round direct evolution studies using Error-prone PCR technique and have constructed the library of RhlAB-containing Transposon to express mutant genes in heterologous hosts.
3. We successfully engineered the new mutant strains *P. aeruginosa* PEER02 and *E. coli* TnERAB so they can produce rhamnolipid biosurfactans. LC-MS spectrum showed the structure of purified rhamnolipids from *P. aeruginosa* PEER02 was similar to those from other *P. aeruginosa* strains, but have different percentage for each component. The main component of purified rhamnolipids from *E. coli* TnERAB is C10-C10 with monorhamnose (2 alkyl chains, each with 10 carbons, and a single rhamnose head group).
4. Core flooding tests showed that rhamnolipids produced by our engineered bacteria are effective agents for EOR. At 250ppm rhamnolipid concentration from *P. aeruginosa* PEER02, 42% of the remaining oil after waterflood was recovered. These results were therefore important for considering the exploration of the studied rhamnolipids as EOR agents.

5. Our engineered *P. aeruginosa* PEER02 strain can produce rhamnolipids with different carbon sources as substrate. Interfacial tension analysis (IFT) showed that different rhamnolipids from different substrates gave different performance. Those rhamnolipids with plant oil as substrate showed as low an IFT as 0.05mN/m in the buffer solution with pH5.0 and 2% NaCl. Fed-batch fermentation with soybean oil as substrate greatly enhanced the production of rhamnolipids, and the yield reached about 25g/L.These results showed some potential for producing high-performance rhamnolipids with high yield from low-cost renewable resources.

6. Through the methodology of synthetic biology and metabolic engineering, we engineered *E. coli* strains harboring various gene combinations from *P. aeruginosa* and successfully produced either mono-rhamnolipids or di-rhamnolipids (one or two head groups). Engineered rhamnolipids showed different performance in interfacial tension and antimicrobial activity. LC-MS analysis confirmed they mainly contained C_{10}-C_{10} (2 alkyl chains, each with 10 carbons) and C_{10}-C_8 (2 alkyl chains, one with 10 carbons and the other with 8 carbons) carbon chain as well as carbon chains with other lengths in small percentages.

7. A mutant with different product selectivity was found via directed evolution and subsequent high-throughput screening. Compared with the wild-type strain, the mutant almost produced rhamnolipids with only C_{10}-C_{10} carbon chain.

We have applied the latest scientific methods in directed protein evolution technology and microbiology to create superior bio-systems that will be highly effective in producing bio-surfactant. We reached the following scientific breakthroughs:

- A first application of genetic bioengineering to create a chemical product for oil and gas production operations.
- Advancement of our understanding of the mechanisms of the bacteria responsible for the manufacture of bio-surfactants of interest to industry

Engineering-based breakthroughs include:

- New bio-systems to produce surfactants that could reduce the cost of conventional surfactant flooding.
- New bio-systems for MEOR applications that generate greater amounts of bio-surfactant and could thrive in more severe reservoir conditions.

- A proven methodology to evaluate rapidly other candidate bio-systems to create other bio-surfactants.
- By demonstrating success here with this general approach of genetic engineering, it will promote bio-production of yet other types of oilfield chemicals.

5. INTRODUCTION

This report provides a summary of the entire 3-year project. The main objective is to employ the latest advances in genetics and bioengineering, especially the Directed Protein Evolution technology, to improve the effectiveness of the MEOR process.

5.1 Background

As we all know, there is a finite limit to the amount of fossil fuel. After more than a century of exploration, it is more and more difficult to find new hydrocarbon reservoirs, and from the existing reservoirs, less and less reserves are available. Therefore, in order to maintain the current rate of production and to provide adequate supply to the US consumers and industry for increasing demand, we must find new ways to increase hydrocarbon production while the amount of reserves keeps dwindling. This is truly a paradox, and a scientific topic of immense economic and national security implications. For example, in US we now import well over half of the oil consumed in order to meet current demand.

Most oil is produced in two phases: primary and secondary recovery. During primary recovery, the natural pressure of the reservoir drives oil into the wellbore, and artificial lift techniques (such as pumps) bring the oil to the surface. Only about 10 percent of a reservoir's original oil is typically produced during primary recovery. For over 50 years producers have employed secondary recovery techniques, usually by injecting water to displace oil, to extend the productive life of oil fields, often increasing ultimate recovery to 20 – 40 percent. Regarding natural gas, the US has maintained sufficient production for domestic use, but this has been due to a spike in the prices that can support increased drilling activity, plus a significant increase in producing now "unconventional" gas resources such as from "tight' sandstones, and shale formations, and coal beds.

Hydrocarbon producers have aggressive programs in place to drill more wells, plus conduct well stimulation operations (e.g. fracturing, acid and solvents to clean wells, etc.) designed to remove material impeding oil and gas flow to the surface, and provide flow channels of high permeability in order to extract more hydrocarbons faster. Chemicals (e.g. surfactants and polymers) added to these stimulation fluids are a key to having successful results at treated wells in these EOR technologies by these additives controlling fluid properties (e.g. surface tension) and solid/mineral interactions (e.g. fluid wetting). Schlumberger, Halliburton, and BJ Services, for example, are major corporations that provide these well treatment technologies to the oil and gas producers. The market in oil field chemistry to support EOR and other routine oil field activities is about $4 billion/yr and growing.

If bio-surfactants can be manufactured at a low cost, then this offers us the capability of creating a new generation of high performance surfactant formulations for immediate use in oil and gas production operations for MEOR. Such bio-based surfactant formulations would have several potential advantages over using the current generation of synthetic surfactants: 1) lower cost, 2) customized chemical structure for better performance characteristics for target EOR applications, 3) created from renewable resources instead of petroleum, 4) inherently more environmentally friendly.

Beyond the immediate need and growing opportunity to make the current hydrocarbon production operations more efficient by deploying low cost bio-surfactants, an enormous, but future "step change" opportunity is so-called tertiary or EOR. Even with using the best practices in conventional primary and secondary recovery operations, there is usually left two-thirds of the oil in the reservoir. In the United States, the resource of crude oil totals 649 billion barrels (NOTE: 1 barrel = 42 gallons). This resource consists of 183 billion barrels of cumulative production, 22 billion barrels of proven reserves, 67 billion barrels of undiscovered reserves and 377 billion barrels are still remaining in discovered reserves (Figure 5-1-1). The 377 billion barrels, represents 2/3 of the crude oil that is trapped in already discovered oil reserves after primary and secondary conventional productions have been completed. However, further recovery of this trapped oil is challenging because the remaining oil is often located in regions of the reservoir that

are difficult to access, and the oil is trapped in the micron-size pores of reservoir rock by high capillary forces.

Different EOR methods to mobilize remaining oil such as injection of steam, carbon dioxide, and surfactant/polymer chemicals have the capability of recovering perhaps another 20 percent of the oil that was originally in place. The fluid volumes to conduct an EOR operation are immense because they must displace ("flood") an entire reservoir (millions of barrels). For example, to process a medium size reservoir to recover 100 million barrels of oil might require injection of 500 million pounds of synthetic surfactant spread throughout 300 million barrels of fluid.

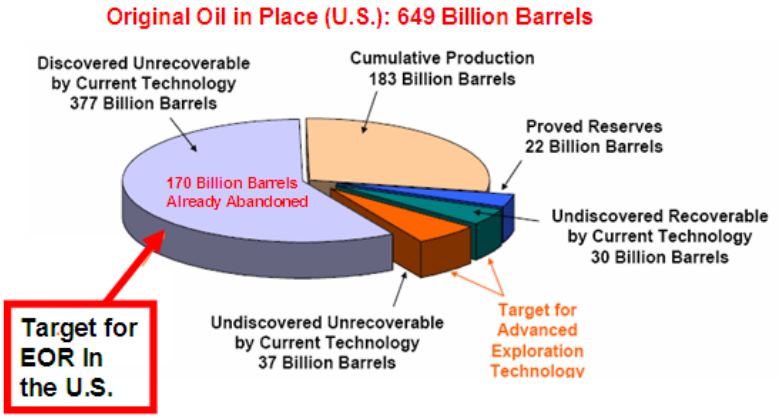


Figure 5-1-1. US oil reserves that are target for Enhanced Oil Recovery

Surfactant based EOR has potential to address a significant fraction of this future 377 billion bbl target, and more attractively, this method targets premium quality light crude oil. Worldwide, the Society of Petroleum Engineers (SPE) estimate the potential resource for EOR is 2 trillion barrels (Thomas, 2006), and that much of that is recoverable by chemical methods.

The chemical material cost is perhaps the main factor controlling the profitability (and main barrier to widespread commercial implementation, Wu 1996, Taber 1996). An inherent potential advantage of a biological approach is that we can make surfactants whose costs are decoupled from the price of crude oil. The nutrients and other raw materials to create bio-surfactants do not have to be petroleum-based, and in fact potentially may come from waste streams. Commercial synthetic surfactants invariably are more expensive than the crude oil from which they are derived.

Even with the current, high price of synthetic surfactants, there is a huge potential global demand from conventional chemical flooding. For example, in China alone, it is estimated that roughly 30 billion pounds of surfactant could be required to meet their future EOR objective of several billion barrels of incremental oil production (Liu, 2001). Also, Chevron is planning an expanded field test of surfactant EOR in their Minas Field, which would require over a billion pounds of surfactant at full commercial scale (Bou-Mikael, 2000). So capturing even a small fraction of this potential market with low cost bio-surfactants would represent a new billion pound chemical product.

Finally, an even another longer-range option for us is to supply customized bacteria for MEOR as another EOR technology. In this case active bacteria are injected directly into an oil reservoir, and these bacteria in turn generate in-situ surfactants, polymers, and gases that can induce additional oil recovery. It is estimated by DOE that 46 percent of the US domestic reservoirs with original oil in place that exceeds 20 billion barrels could be candidates for MEOR. Several studies have had promising results, with bio-systems in MEOR laboratory tests recovering typically of 10 – 20% of water flood incremental oil (Yonebayashi, 1997).

For EOR applications, chemical surfactants cost in range of $1 to $3 per pound, whereas lignin-based sulfonates are cheaper. But the current cost of bio-surfactants may be about 3 to 10 times higher using current technology (Desai and Banat, 1997). Attempts have been made to improve overall process economics in bio-surfactant production. Those efforts have been focused on searching for cheap substrates, increasing the productivity by manipulating physiological conditions, mixing multiple microbial cultures, and modifying the downstream recovery processes. Potential overall improvements via these strategies are limited.

Technical Barriers and Need of our Research

The fermentative (microbiological) production of value added chemicals from agriculture biomass-derived substrates is the historical essence of industrial biotechnology. One such value added product that can be produced by microorganisms is a structurally diverse group of surface-active molecules named biosurfactant. These molecules reduce surface and interfacial tensions in both

aqueous solutions and hydrocarbon mixtures, which makes them good candidates for our target EOR use and other oil field applications. In the past few decades, biosurfactants have gained more and more attention because they have many advantages over the chemical surfactants, such as biodegradability; lower toxicity; better environmental compatibility; higher selectivity and specific activity at extreme temperatures, pH and salinity; and the ability to be synthesized from renewable feedstocks. However, the widespread use of biosurfactants has yet to be seen because of the several technological barriers that have limited the large scale production of the biosurfactants and the lack of state-of-the-art applications that utilize biosurfactants.

Barrier 1. High manufacturing costs of biosurfactants

For EOR applications, chemical surfactants cost in range of $1 to $3 per pound, whereas lignin-based sulfonates are cheaper. But the current cost of bio-surfactants may be about 3 to 10 times higher using current technology (Desai and Banat, 1997). Attempts have been made to improve overall process economics in bio-surfactant production. Those efforts have been focused on searching for cheap substrates, increasing the productivity by manipulating physiological conditions, mixing multiple microbial cultures, and modifying the downstream recovery processes. Potential overall improvements via these strategies are quite limited. Recent developments in the biotechnology area have provided us new ways to target this problem.

Barrier 2. Lack of high performance biosurfactants that could functionally replace and surpass the conventional surfactants for oilfield applications.

Developing effective EOR applications can be very challenging because of the complexity of the oil reservoirs environment, such as big differences in temperature, pH and salinity, and because of the complexity of the chemical and physical property of the crude oils, such as huge viscosity differences. There has been a need for varieties of surfactants for different EOR applications, and many special performance synthetic surfactants have been created for this purpose. Obviously, in order for biosurfactants to be used in widespread EOR applications, we also need to produce biosurfactants with diverse physical properties, especially superior functionalities over their

synthetic counterparts. However, relying on discover of different naturally existing bacteria strains to produce biosurfactants with diverse functions is slow and insufficient. Again, modern biotechnology, such as Directed Evolution Technology, has provided us a solution.

The rhamnolipid biosurfactants have been studied extensively (Liang and Wullbrandt, 1999). Rhamnolipid is produced as mixtures in various proportions, including one or two rhamnoses attached to β-hydroxyalkanoic acid (Soberon-Chavez et al., 2005). The lengths of the fatty acid chains of rhamnolipid vary significantly, resulting in a multitude of different rhamnolipid congeners. This includes fatty acyl chains with lengths of 8, 10, 12, and 14 carbons, as well as of 12- or 14-carbon chains with a single double-bond (Figure 5-1-2). The type of rhamnolipid produced depends on the bacterial strain, the carbon source used, and the process strategy (Robert et al., 1989; Mulligan et al., 1993). Rhamnolipid induces a remarkably larger reduction in the surface tension of water from 72 to values below 30mN/m and it reduces the interfacial tension of water/oil systems from 43mN/m to values below 1mN/m. Rhamnolipid also has an excellent emulsifying power with a variety of hydrocarbons and vegetable oils (Abalos et al., 2001).

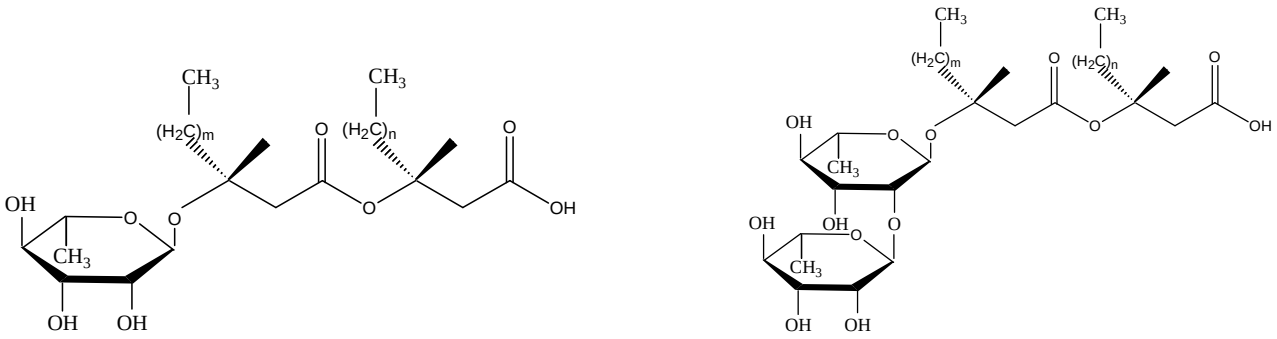


Figure 5-1-2. Structure of rhamnolipids produced by *P. aeruginosa*. Left: mono-rhamnolipid; Right: di-rhamnolipid. Alkyl chain length may vary. (In shorthand, mono-rhamnolipid: Rha-C_{m+4}-C_{n+4}, di-rhamnolipid: Rha- Rha-C_{m+4}-C_{n+4}, m, n=4-8)

The increasing ecological concern with using synthetic chemical surfactants has led us to propose rhamnolipid as environmentally benign substitute, although it will be necessary to reduce production costs. The use of renewable low-cost substrates, such as plant oil and grain starch, and even lignocellulosic biomass, could dramatically increase the economics of rhamnolipid production (Nitschke et al., 2005; Mukherjee et al., 2006). Many bacteria, especially Pseudomonads, can utilize efficiently renewable low-cost substrates, but they either lack the ability to biosynthesize the

rhamnolipid, or only have very low yield of rhamnolipid. Therefore, we could use advanced molecular biotechniques to bioengineer renewable substrate-consuming bacteria that produce inexpensive rhamnolipid with high yield.

The main purpose of our research is to employ advanced bioengineering methods to engineer bacteria with the capability to produce biosurfactant with much greater activity than at present. And because these biosurfactants materials are to be used in the oilfield, there is much less concern with the purity, color, etc. of the produced product. We can save on much of the expense normally associated with product separation and purification by accepting a less refined product for oilfield use. Therefore, the biosurfactant manufacture can be established at or near a target oil field, then the supernatant solution containing the chemical may be a sufficiently concentrated product as is (there is little or no transportation cost, hence excess water in the product is not a factor for this strategy.) Furthermore, by optimizing the engineered the bacteria using cheap agriculture substrates, such as plant oils, as nutrients to produce biosurfactants, it could further drive down the cost of manufacturing biosurfactants, making them more competitive with the conventional surfactants.

6. Experimental Procedures and Methods

Bacterial strain, media and chemicals

The strains and plasmids used in this study were summarized in Table S1 (see supplemental materials). Briefly, *P. aeroginosa* PAO1-RhlA$^-$ (Rahim et al, 2001) and *E. coli* BL21(DE3) were the parental strains for engineering rhamnolipid production. *E. coli* DH5 was the host strain for constructing various recombinant plasmids. The *E. coli* strains were commercially available. Except for rhamnolipid fermentation, all these bacteria were grown on LB (Luria-Bertani) media containing suitable antibiotics at 37 ºC (Sambrook and Russell, 2001). Unless noted otherwise, antibiotics were used at the following concentration: chloramphenicol, 100 mg/mL for *P. aeruginosa* and 25 mg/mL for *E. coli*; ampicillin, 50 mg/mL for *E. coli*; kanamycin, 40 mg/mL for

E. coli. All enzymes used for DNA manipulation were purchased from New England Biolabs (Beverly, MA). EZ::TN™ Transposase was purchased from EPICENTRE Biotechnologies (Madison, WI).

Fermentation media and conditions

Nutrient broth from BD (Becton, Dickinson and Company, Franklin Lakes, NJ) was used for seed culture of *P. aeruginosa* and LB for seed culture of *E. coli*. The mineral salt (MS) medium supplemented 0.4% or 2% glucose or 2% soybean oil as carbon source was the rhamnolipid fermentation medium. The MS medium contained (per L): 15 g $NaNO_3$; 1.1 g KCl; 1.1 g NaCl; 0.00028 g $FeSO_4 \cdot 7H_2O$; 3.4 g KH_2PO_4; 4.4 g K_2HPO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.5 g yeast extract; and 5 ml of a trace element solution containing (per L): 0.29 g $ZnSO_4 \cdot 7H_2O$; 0.24 g $CaCl_2 \cdot 4H_2O$; 0.25 g $CuSO_4 \cdot 5H_2O$; and 0.17 g $MnSO_4 \cdot H_2O$, (Lindhardt et al, 1989). The trace element solution was filter-sterilized through a 0.2-µm membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool.

P. aeruginosa wild-type and mutant were first grown in nutrient broth for 24 h at 30ºC with shaking and then diluted 1:10 into MS medium plus 2% glucose or soybean oil and incubated for 4 days. *E. coli* wild-type and mutant were first grown in LB for 24h at 30ºC with shaking and then diluted 1:10 into MS or LB media plus 0.4% glucose supplemented 50mM IPTG as inducer and incubated for 24 h. Incubation was carried out in 250-mL Erlenmeyer flask with 25 mL medium and at 30ºC with orbital shaking at 250 rpm.

Plasmid and strain construction

The pMOD-2C derived from pMOD-2 (EPICENTRE Biotechnologies Madison, WI) was for constructing artificial transposon. The pACYC184 was digested with *Xba*I and *Sty*I to produce a

chloramphenicol resistant cassette. After being blunted with T4 DNA polymerase, this chloramphenicol resistant cassette was inserted into the *Sma*I site of pMOD2 to produce pMOD-2C. Rhamnosyltransferase 1 complex gene RhlAB with native operon promoter was amplified with primers RhlAB-1a (5'- CCCAATCTCTAGATGCCTTTTCCGCCAACCCCTCGCTG-3') and RhlAB-2 (5'-AACCAAGCT TTCAGGACGCAGCCTTCAGCCATCG-3') and *P. aeruginosa* PA01 genomic DNA (purchased from American Type Culture Collection) as template; PCR product of RhlAB was digested with *Xba*I and *Hind*III and cloned into pMOD-2C to produce the recombinant plasmid pMOD-2CRABa. 3.5kb chimeric transposon TnRABa with native operon promoter was produced by digesting pMOD-2CRABa with *Psh*AI. Coding sequence of rhamnosyltransferase 1 complex RhlAB was amplified with primers RhlAB-1b (5'-AGTTGGTACCATG CGGCGCGAAAGTCTGTTGG-3') and RhlAB-2 (5'-AACCAAGCTTTCAGGACGCAGCCTTCA GCCATCG-3'); PCR product was digested with *Kpn*I and *Hind*III and cloned into pET30a(+) to produce the recombinant plasmid pETRAB. 2.4kb *Sph*I-*Hind*III fragment from pETRAB was cloned into pMOD-2C to produce the recombinant plasmid pMOD-2CRABb (In this case, *Sph*I in the fragment and *Xba*I in pMOD-2C were blunted first, then ligated). Chimeric transposon TnRABb with T7 promoter was produced by digesting pMOD-2CRABb with *Psh*AI (Figure 6-1-1). Transponon TnRABa or TnRABb was incubated with EZ::TN™ Transposase in the absence of Mg^{2+} to produce transposome TnRABasome or TnRABbsome (Figure 6-1-2) that was transformed into *P. aeruginosa* PAO1-RhlA^{-} or *E. coili* BL21(DE3) by electroporation (Smith and Iglewski, 1989), respectively (Figure 6-1-3). After genotypic and phenotypic analysis, engineered strains *P. aeruginosa* PEER02 and *E. coli* TnRAB were constructed and ready for rhamnolipid production.

Mapping of transposon insertion site

Five micrograms of transposant (*P. aeruginosa* PEER02 or *E. coli* TnERAB) genomic DNA was digested with *Taq*I and then circularized in a ligation reaction using T4 DNA ligase at a DNA concentration of 5 ng/μL. Ligation products were purified with UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA) and resuspended in water at 10 ng/μL. Inverse-PCR was performed on 100 ng of ligated DNA using the primers TnAB1(5'-CATAATGAAATAAGATCACTACCGGGC-3') and TnAB2 (5'-GTGGTCGAACGTTGTCATAGGGA-3'), which face outward from the transposon sequence. The Inverse-PCR products were purified using the QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA) and sequenced using the primers of TnAB1 and TnAB2. The resultant sequences were aligned with the complete genome of *P. aeruginosa* PAO1 (Stover et al., 2000) or *E. coli* K-12 (Blattner et al., 1997) to confirm the insertion site of transposon.

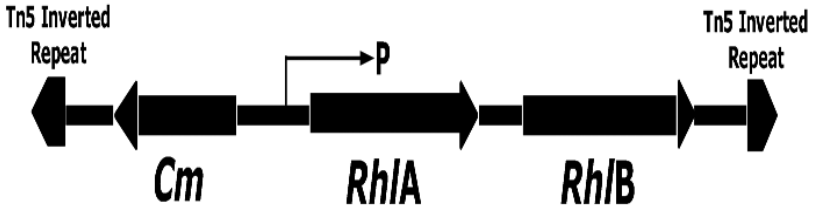


Figure 6-1-1. Structure of rhamnolipid biosynthetic gene RhlAB-containing Transposon (TnRABa or TnRABb); Cm:chloramphenicol resistant cassette; P: RhlAB native promoter or T7 promoter; TnRABa with the native promoter of RhlAB; TnRABb with T7 promoter fused with RhlAB.

Figure 6-1-2. Transposome TnRABasome or TnRABbsome construction

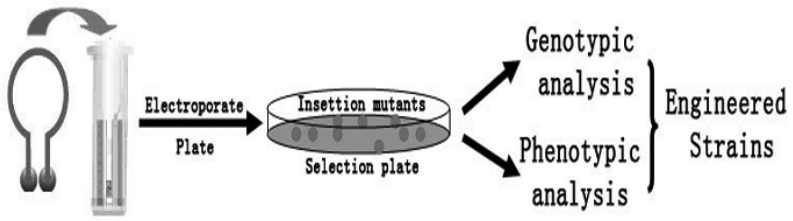


Figure 6-1-3. Transposome electroporation and mutant selection.

Rhamnolipid quantification

Rhamnolipid was quantified in triplicate by the colorimetric determination of sugars with orcinol (Candrasekaran and Bemiller 1980). Rhamnolipid was purified by first separating the cells from supernatant by centrifugation (10,000×g). The supernatant was then extracted with chloroform and ethanol. The 0.5 mL rhamnolipid sample was extracted with 1 mL chloroform:ethanol (2:1, v/v). The organic phase was evaporated to dryness and 0.5 mL of H_2O was added. To 0.1 mL of each sample with suitable dilution, 0.9 mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80ºC the samples were cooled at room temperature and the OD_{421} was measured. The rhamnolipid concentration was calculated from standard curves prepared with L-rhamnose (0-50 mg/L) and expressed as rhamnose equivalent.

Rhamnolipid purification, TLC and HPLC/MS analysis

The rhamnolipid was precipitated by acidifying culture supernatant to pH 2 with concentrated HCl and kept at 4°C overnight, then recovered by centrifugation at 10,000×g for 1 h and dissolved in deionized water. 2 volumes of chloroform:ethanol (2:1, v/v) were added to rhamnolipid solution and shaken 30 min for extraction. The organic phase was picked and evaporated to dryness. Finally the rhamnolipid residue was dissolved in deionized water to 500 mg/L.

The purified rhamnolipid was separated, visualized, and compared with known rhamnolipid sample (JBR425, Jeneil Biosurfactant Co., LLC) using TLC (silica gel 60 plate). A 10 μL sample (500 mg/L) was loaded into a silica gel 60 plate. After drying at room temperature, the silica gel was developed with a solution of chloroform-methanol-water (65:15:2 by volume), then visualized using a 50:1:0.05 (by volume) mixture of the solution glacial acetic acid-sulfuric acid-anisaldehyde at 90°C for 30 min. The HPLC/MS analyses were performed according to Schenk's method with minor modification (Schenk et al., 1995). Briefly, a Gemini C18 column (2×50 mm, 5μm particle) from Phenomenex and a ThermoFinnigan LCQ Classic ion trap mass spectrometer were used. The HPLC gradient was: starting at 8% solvent B and holding for 1 minute, then ramping to 75% solvent B in 20 minutes, holding at 75% solvent B for 10 minutes, backing to 8% solvent B in 1 minute and holding at 8% solvent B for 5 minutes. Solvent A is 98:2 (v/v) water:acetonitrile with 0.1% acetic acid, and solvent B is 10:90 water:acetonitrile with 0.1% acetic acid. A 10μL sample of 50 mg/L rhamnolipid was applied for HPLC/MS analysis. The mass spectrometer was operated in the negative ion mode scanning 250-950 m/z range.

IFT characteristics of rhamnolipid

The IFT was determined by using a spinning drop tensiometer (Temco Inc.) as detailed by Cayais (1975). Aqueous rhamnolipid solution was loaded into glass tube (5×100 mm), followed by injection of 1.5 μL n-octane. The glass tube with solution was spun in the tensiometer, and IFT was determined from the n-octane drop geometry. After spinning for 10 min, the data were collected for analysis. In these studies, we measured the "fresh" IFT values without pre-mixing rhamnolipid solution and n-octane together to reach phase equilibrium. To adjust the pH of rhamnolipid

solution, Citrate-Na_2HPO_4 buffer (pH 3-8) and Boric acid-KCl-NaOH buffer (pH 8-10) were used. Unless noted otherwise, IFT was measured at 30°C.

Sand pack core flooding test

A sand-pack core flooding test was performed at room temperature according to Shuler's method with some modifications (Shuler et al., 1989). Dried sand (28-60 mesh) manufactured by Paragon Building Products Inc (Norco, CA) was packed into a stainless steel tube (2×60cm) to create an artificial core. This sand pack was placed horizontally and the air evacuated from it with a vacuum pump. Next, a buffer-brine solution (Citrate- Na_2HPO_4 buffer, with 2 wt% NaCl added) adjusted to pH 5.0 was introduced at 1 mL/min into the pack so that this aqueous phase now occupies all of the pore space. This particular buffer-brine combination was selected because, based on the IFT measurements, rhamnolipid had lowest IFT under this condition. The pore volume (PV) of this pack was calculated as the volume of brine that is imbibed (calculated by weighing the sand-packed core before and after being saturated with this brine of known density.) The porosity of the sand pack is the pore volume divided by its total volume. The brine permeability of the pack is calculated from Darcy's Law by injecting the buffer-brine at a constant flow rate and measuring the pressure gradient. For the sand pack experiment reported here, the pore volume, porosity, and permeability is 85 ml, 45%, and 17.8 Darcies, respectively. Next, n-octane (selected to be the oil phase) displaced the brine in the sand pack until no more water came out . The oil-saturated core was aged for 24 h. Then the buffer-brine was injected into the aged oil-saturated core until no more oil came out.

So at this point, the sand pack core with trapped oil was ready for flooding with rhamnolipid solution. About 3 PV of 250 mg/L rhamnolipid solution (made up in Citrate- Na_2HPO_4 buffer, 2% NaCl, pH 5.0) were injected at 1mL/min. This same flow rate is used in all the flow steps associated with the sand pack experiment. Next, the core was shut in and incubated overnight at room temperature. The other 3 PV of 250 mg/L rhamnolipid solution was injected at 1mL/min during the second day. After concluding rhamnolipid flooding, 6 PV of buffer-brine were injected. The produced fraction was collected in a titration tube with 0.1mL accuracy. After keeping few minutes, the oil phase and water phase would separate naturally, and the oil volume was measured according the graduation in the titration tube with 0.1 mL accuracy. The oil recovery and water cut (percentage of oil- and non-oil phase in each elution fraction of approximately 10 mL were recorded during the rhamnolipid solution and subsequent buffer-brine injection steps, as well as the cumulative oil recovery as a percent of the trapped oil before rhamnolipid flooding. The IFT and the rhamnolipid concentration for some of the eluted fractions during the rhamnolipid and the buffer-brine flush were measured with the methods mentioned above.

7. RESULTS AND DISCUSSION

7.1 Summary of the Tasks Performed

Task 1.0 Directed evolution studies for *P. aeruginosa* (24 months)

These studies have identified several "super-mutants" with one or more desirable characteristics, 1) "super-activity", 2) thermal stability, or 3) use of cheap substrates. We have written a paper documenting in particular the detailed laboratory procedures to arrive at the successful mutant candidates.

Subtask 1.1 (8 months) -- Develop screening methods to carry out an evaluation of the

performance of candidate mutants, based on one of three approaches: thin-layer chromatography, bioactivity assay or colormetric analysis. Conduct the first round mutagenesis tests via Error-prone PCR. Perform "base-case" fermentation with the natural, wild form of the two study bacteria.

Subtask 1.2 (14 months) -- Second round mutation studies to obtain to a smaller number of better candidate mutants. Perform batch fermentation on selected mutated strains to demonstrate achieve a desired effect.

Subtask 1.3 (12 months) -- Further generation of improved mutations. Perform batch fermentation on selected mutated strains to quantify changes in bio-surfactant production.

Task 2.0 Genetic cloning of "supermutants" into thermophilic oilfield and other host bacteria strains (29 months)

The purpose of these studies is to clone the mutants with high activity and thermal stability into microbes adaptable to in-situ, oilfield reservoir conditions. Part of this task is to document the detailed laboratory procedures and results in our efforts to create a robust MEOR microbe.

Subtask 2.1 (6 months) -- Literature review of thermophilic microbes that are candidates for oil reservoirs.

Subtask 2.2 (14 months) -- Develop laboratory methods to add bio-surfactant function to these target microbes. Initially use wild, natural versions of *P. aeruginosa* as the genome source.

Subtask 2.3 (9 months) -- Clone the mutant with the best features for in-situ MEOR (high activity and thermal stability) into the *E.Coli* cells.

Task 3.0 Evaluation of Bio-Surfactants and Bio-Systems for EOR (21 months)

The purpose of these studies is to 1) characterize and optimize the target bio-surfactants (rhamnolipids and surfactin) as candidate chemicals for conventional chemical EOR. One activity documents the surfactant performance screening procedures and results in a report.

Subtask 3.1 (5 months) -- Micro-scale tests with purified rhamnolipid. Develop analytical methods to measure bio-surfactant concentrations in solution. Using whole cell lysate generated from the wild bacteria strains, determine phase behavior and IFT versus a series of n-alkanes to characterize the behavior of the 2 bio-surfactants as EOR agents. Also conduct screening tests for bio-surfactant adsorption onto reservoir materials such as kaolinite.

Subtask 3.2 (9 months) -- Use whole cell lysate from fermentation tests from the best "supermutants" to spot check the bio-surfactant phase behavior and IFT versus a series of n-alkanes, plus re-test the solid adsorption behavior (expect same results as in Subtask 3.1). Conduct detailed tertiary oil displacement performance tests of generated rhamnolipid and surfactin in Berea (and perhaps also in carbonate) core floods. Optimize oil-displacement performance for at least one of the two candidate bio-surfactant systems for an example light crude oil system. Compare and contrast with a synthetic alkyl aryl sulfonate surfactant system.

Subtask 3.3 (7 months) -- Conduct laboratory core flood tests of genetically engineered versus wild strain microorganisms for MEOR. Evaluate in a series coreflood experiments the ability of test bio-systems to displace waterflood residual oil.

7.2 Directed evolution studies for P. aeruginosa and cloning of mutants into host bacteria strains

7.2.1 Overview the molecular mechanism of the rhamnolipid bio-synthesis

The goal of the studies in this Task is to identify the "super-mutant-genes" involved in the rhamnolipid or surfactin biosynthesis with one or more desirable characteristics, 1) "super-activity", 2) thermal stability, or 3) use of cheap substrates.

P. aeruginosa produces extracellular glycolipids named rhamnolipid which is composed of L-rhamnose and 3-hydroxyalkanoic acid. In liquid cultures, they are produced as a complex mixture of congeners containing one or two 3-hydroxy fatty acids of various lengths, linked to a mono- or dirhamnose moiety. In general, the two more abundant rhamnolipids are L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate. According to the biosynthetic pathway proposed by Burger et al, rhamnolipid synthesis proceeds by two sequential glycosyl transfer reactions, each catalyzed by a different rhamnosyltransferase. The first rhamnosyltransferase, which catalyses the transfer of TDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA), is encoded by the rhlAB operon (Figure 7-2-1). Both genes, co-expressed from the same promoter, are essential for rhamnolipid

synthesis. Environmental factors, especially nutritional conditions, influence rhamnolipid production. This quorum sensing system is composed of rhlI, the N-butyrylhomoserine lactone autoinducer synthase gene, and rhlR, which encodes the transcriptional activator. The second rhamnosyltransferase, encoded by rhlC, has been characterized and its expression shown to be co-coordinately regulated with rhlAB by the same quorum sensing system.

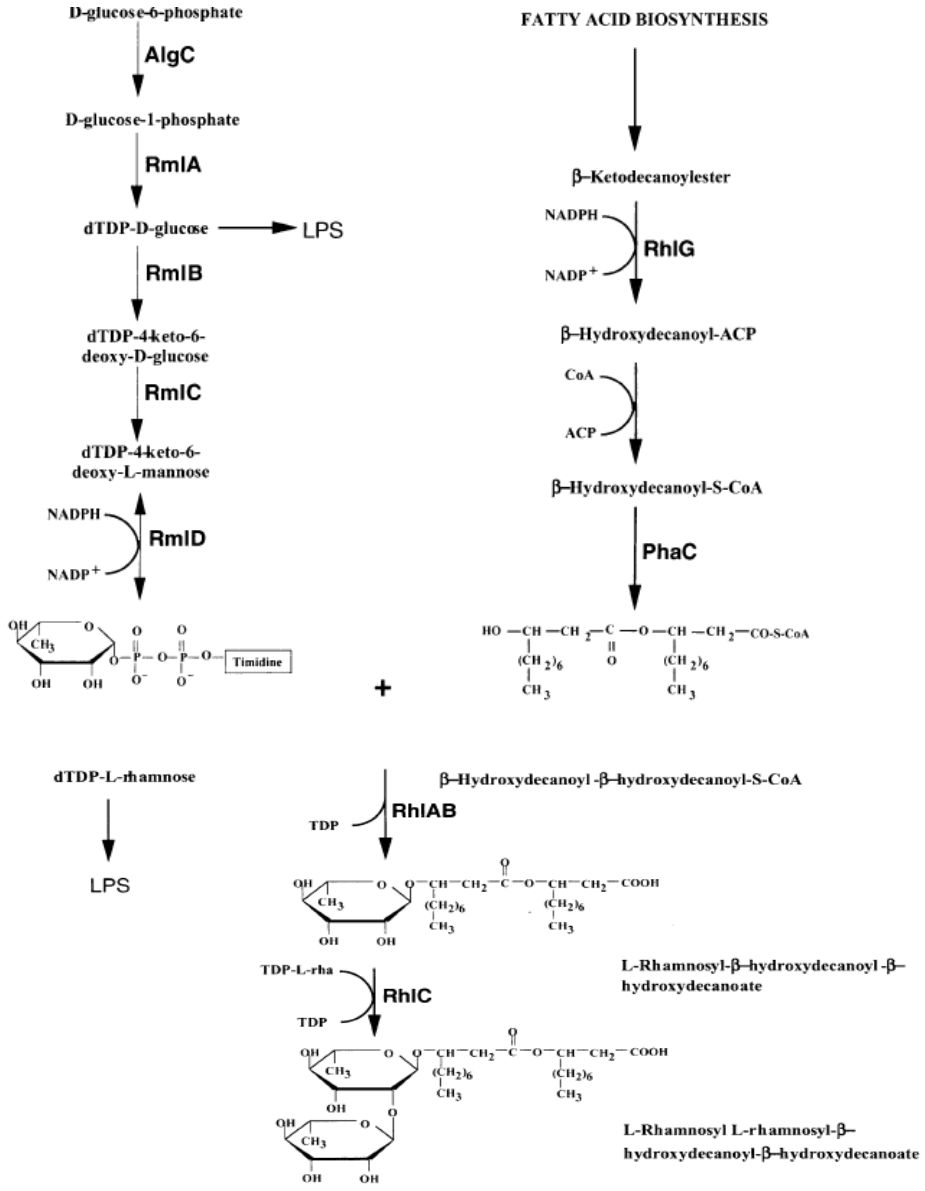


Figure 7-2-1. Putative synthetic pathway of rhamnolipids in P. aeruginosa

7.2.2 Vector preparation for gene cloning

1) The following commercial vectors were used in our rhamnosyltransferase gene cloning study.

a) pUC19

pUC19 (Invitrogen) is a small, high-copy number *E. coli* plasmid cloning vector containing multiple cloning site and Ampicillin resistance gene (Figure 7-2-2a).

b) pACYC177

pACYC177 (New England Biolabs) is an *E. coli* plasmid cloning vector containing double drug resistance gene (amicillin and kanamycin).(Figure 7-2-2b).

c) pACYC184

pACYC184 (New England Biolabs) is an *E. coli* plasmid cloning vector containing double drug resistance gene (chloramphenicol and tetracycline).(Figure 7-2-2c).

d) pBluescript SK(+) II

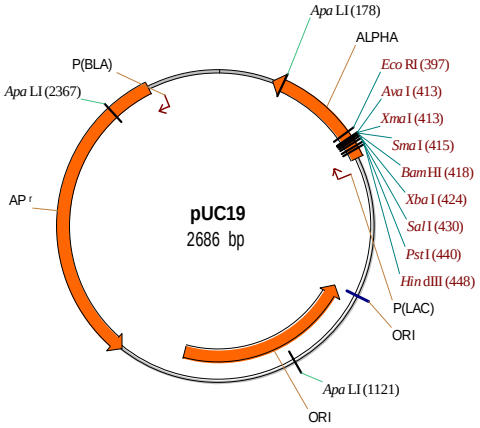
pBluescript SK(+) (Stratagene) II is a small, high-copy number *E. coli* phagemid cloning vector containing multiple cloning site and ampicillin resistance gene (Figure 7-2-2d).

e) pET30a(+)

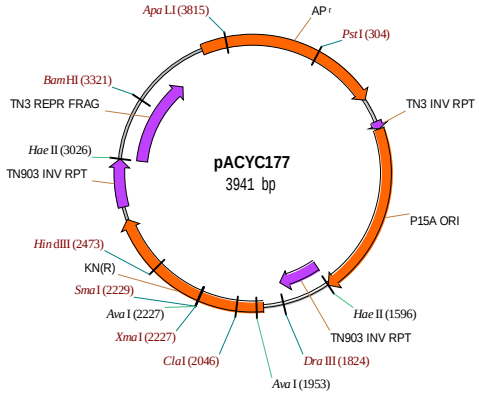
pET30a(+) (Novagen) is an *E. coli* plasmid expression vector containing multiple cloning site and kanamycin resistance gene (Figure 7-2-2e).

f) pMOD-2 Transposon Construction Vector

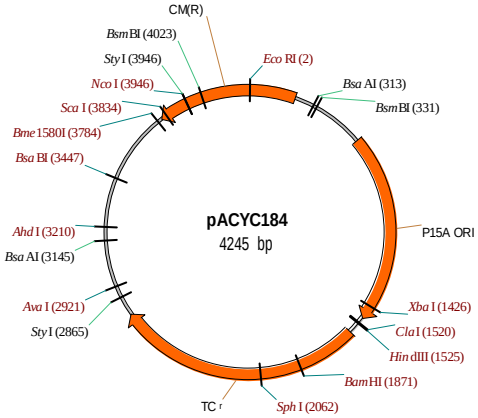
pMOD-2 Transposon Construction Vector (Takara) is a pUC-based vector containing multiple cloning site and ampicillin resistance gene (Figure 7-2-2f). After *Pvu*II or *Psh*AI digestion, a Tn5 transposon was produced.



(a)



(b)



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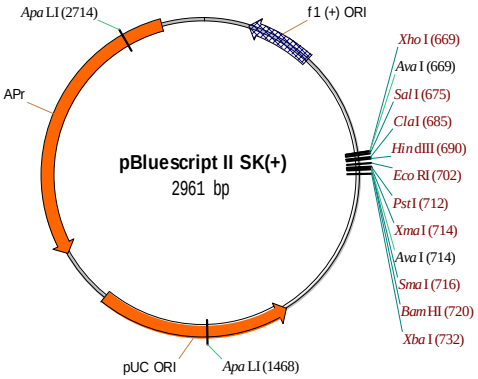

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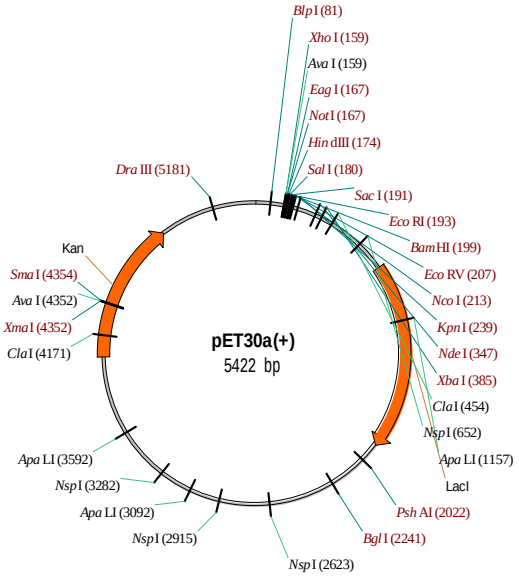
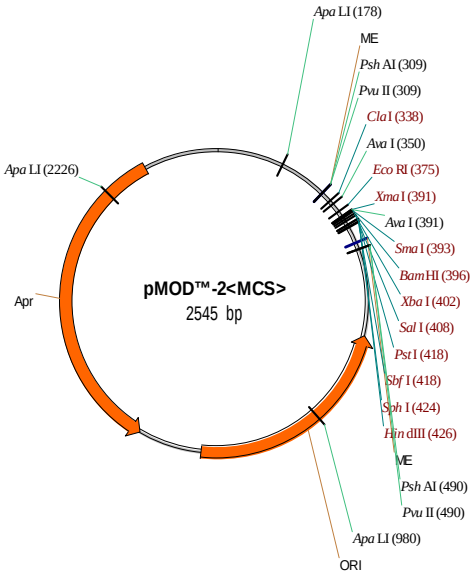


(e)



(f)

Figure 7-2-2. Maps of commercial vectors used in our gene-cloning study

2) Vectors specially constructed for our rhamnosyltransferase gene cloning study

a) pUC19C

pUC19 was digested with *Aat*II and *Adh*I and then treated with T4 DNA polymerase to produce a 1.7kb double blunted-end fragment (Figure 7-2-2a). pACYC184 was digested *Bsa*AI and *Bme*1580I and then treated with T4 DNA polymerase to produce a 750bp double blunted-end fragment which contains chloramphenicol resistance cassette (Figure 7-2-2c). These two fragments were ligated with T4 DNA ligase and transformed into *E. coli* to produce recombinant plasmid pUC19C (Figure 7-2-3a).

b) pUC19K

pUC19 was digested with *Aat*II and *Adh*I and then treated with T4 DNA polymerase to produce a 1.7kb double blunted-end fragment (Figure 7-2-2a). pACYC177 was digested *Dra*III and *Hae*II and then treated with T4 DNA polymerase to produce a 1.2kp double blunted-end fragment which contains kanamycin resistance cassette (Figure 7-2-2b). These two fragments were ligated with T4 DNA ligase and transformed into *E. coli* to produce recombinant plasmid pUC19K (Figure 7-2-3b).

c) pMOD-2C

pMOD-2 Transposon Construction Vector was digested with *Sma*I and then treated with alkaline phosphatase to produce linear fragment. pACYC184 was digested *Bsa*AI and *Bme*1580I and then treated with T4 DNA polymerase to produce a 750bp double blunted-end fragment which contains chloramphenicol resistance cassette (Figure 7-2-2c). These two fragments were ligated with T4 DNA ligase and transformed into *E. coli* to produce recombinant plasmid pMOD-2C (Figure 7-2-3c). Thus, the recombinant plasmid pMOD-2C can produce Tn5-derived tranposon which containing chloramphenicol resistance gene after *Pvu*II or *Psh*AI digestion.

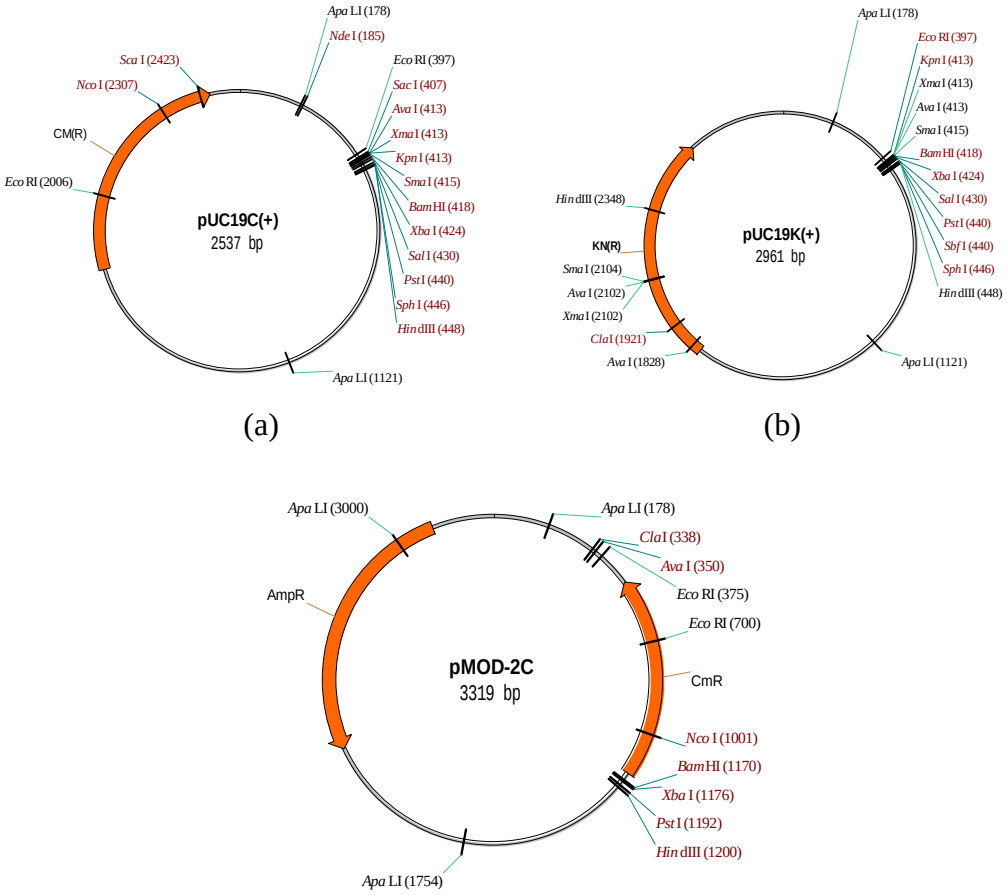


Figure 7-2-3. Vectors specially constructed in the gene-cloning study

7.2.3 Cloning Genes Involved in Rhamnolipid Biosynthesis

1) Cloning and Identification of Rhamnolipid Biosynthesis Gene Cluster from *Pesudomonas aeruginosa* PAO1

Gene Cloning by PCR

a) PCR product of RhlABRI was digested with *Xba*I and *Hind*III and cloned into pUC19 and pBluescript II SK(+), respectively. The recombinant plasmid pUCR01 and pSKR01 were produced, respectively (Figure 7-2-4a and Figure 7-2-4b).

b) PCR product of RhlAB was digested with *Xba*I and *Eco*RI and cloned into pUC19 and pBluescript II SK(+), respectively. The recombinant plasmid pUCRPAB and pSKRPAB were produced, respectively (Figure 7-2-4c and Figure 7-2-4d).

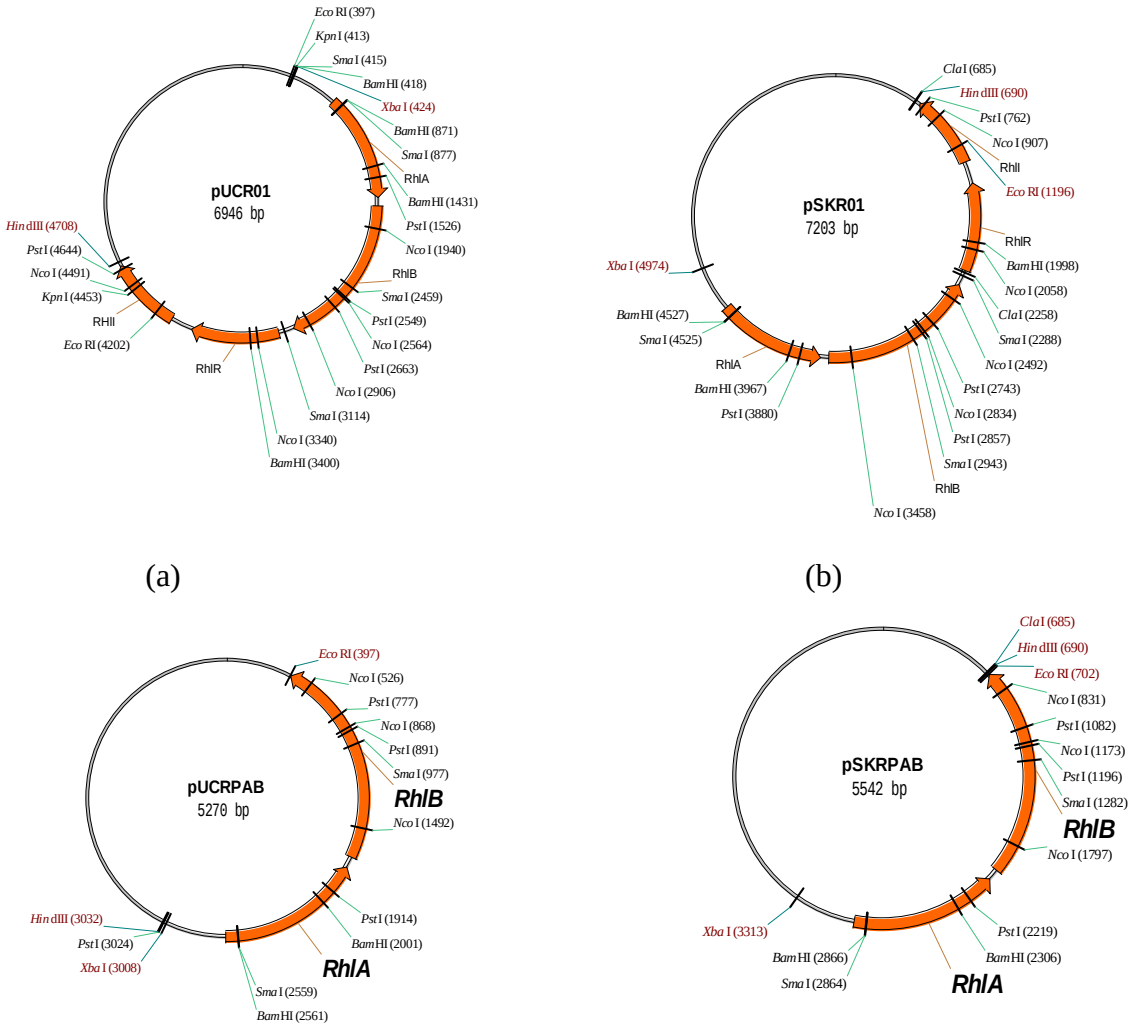


Figure 7-2-4. Recombinant plasmids containing rhamnolipid biosynthesis genes

7.2.4 Cloning and Identification of dTDP-L-rhamnose Biosynthetic Gene Cluster from *Pesudomonas aeruginosa* PAO1

Whole dTDP-L-rhamnose Biosynthetic Gene Cluster RmlABCD with native operon promoter (containing upstream 300bp sequence) was amplified with primer PaRml-1 and PaRml-2 and *P. aeruginosa* PA01genomic DNA (from ATCC) as template; PCR product of RmlABCD was digested with *Xba*I and *Hind*III and cloned into pUC19C. The recombinant plasmid pUCCPaRml was produced and identified by restriction digestion analysis (Figure 7-2-5).

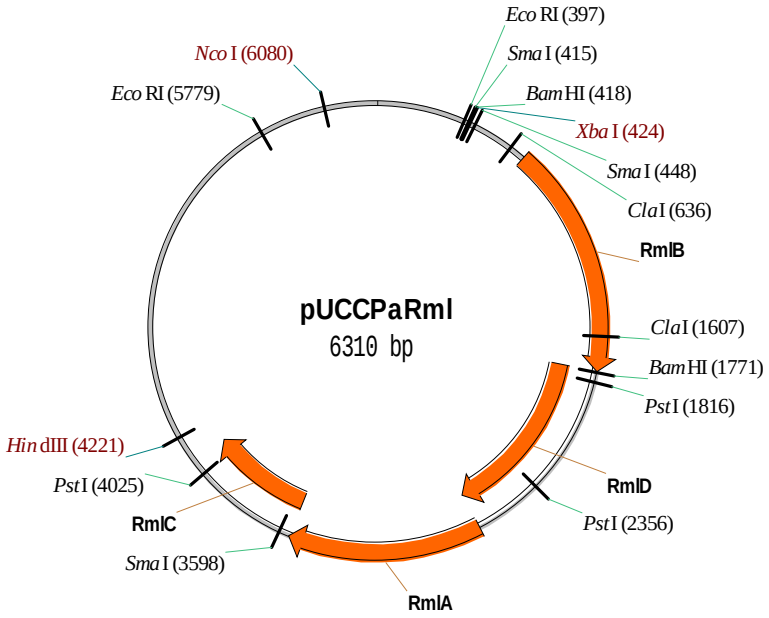


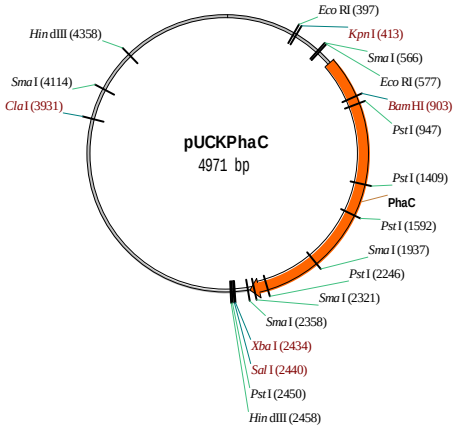
Figure 7-2-5. Recombinant plasmids containing dTDP-L-rhamnose Biosynthetic Gene Cluster

7.2.5 Cloning and Identification of polyhydroxyalcanoate synthase PhaC and ketoacyl reductase RhlG from *Pesudomonas aeruginosa* PAO1

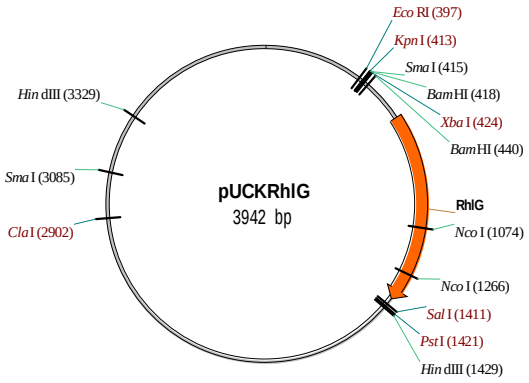
a) Polyhydroxyalcanoate synthase PhaC with native operon promoter (containing upstream 300bp sequence) was amplified with primer PaPhaC-1 and PaPhaC-2 and *P. aeruginosa* PA01genomic DNA (from ATCC) as template; PCR product of PhaC was digested with *Kpn*I and *Xba*I and cloned into pUC19K. The recombinant plasmid pUCKPaPhaC was produced and identified by restriction digestion analysis (Figure 7-2-6a).

b) Ketoacyl reductase RhlG with native operon promoter (containing upstream 300bp sequence) was amplified with primer PaRhlG-1 and PaRhlG-2 and *P. aeruginosa* PA01genomic DNA (from ATCC) as template; PCR product of RhlG was digested with *Xba*I and *Sal*I and cloned into pUC19K. The recombinant plasmid pUCKPaRhlG was produced and identified by restriction digestion analysis (Figure 7-2-6b).

c) PCR product of RhlG after *Xba*I and *Sal*I digestion was also cloned into pUCKPaPhaC. The recombinant plasmid pUCKPaPhaCRhlG was produced and identified by restriction digestion analysis (Figure 7-2-6c).



(a)



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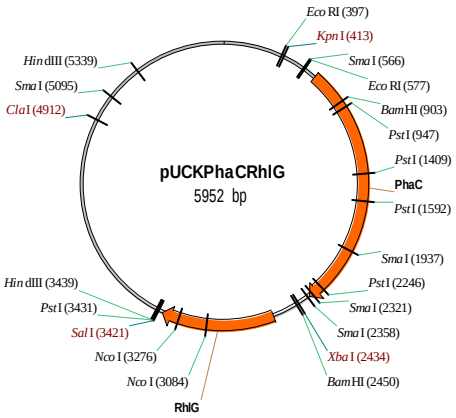


(c)

Figure 7-2-6. Recombinant plasmids containing Polyhydroxyalcanoate synthase and Ketoacyl reductase

7.2.6 Construction of Engineered *E. coli* for producing rhamnolipids

The different combinations of plasmids that contain rhamnolipid biosynthetic genes were transformed into *E. coli* DH5α to obtain the following engineered strains as listed in Table 1.

Table 1 Engineered *E. coli* for producing rhamnolipids

Strain	Phenotype
E. coli ABMSR01	containing pSKR01; Amp
E. coli ABMUR01	containing pUCR01; Amp
E. coli ABMSRAB	containing pSKRAB; Amp
E. coli ABMURAB	containing pUCRAB; Amp
E. coli ABMR71	containing pUCRAB and pUCCRml; Amp, Cm
E. coli ABMR701	containing pUCRAB, pUCCRml and pUCKPhaC; Amp, Cm, and Kan
E. coli ABMR702	containing pUCRAB, pUCCRml and pUCKRhlG; Amp, Cm, and Kan
E. coli ABMR703	containing pUCRAB, pUCCRml and pUCKPhaCRhlG; Amp, Cm, and Kan

Amp: ampicillin resistance; Cm: Chloramphenicol resistance; Kan: kanamycin resistance.

7.2.7 Construction of RhlAB-containing Transposon insertion mutants

Rhamnosyltransferase 1 complex RhlAB with native operon promoter (containing upstream 400bp sequence) was amplified with primer RhlABRL-1(5'-CCCAATCTCTAGATGCCTTTTCCGCCAACCCCTCGCTG-3') and RhlB-2 (5' - AAC CAA GCT TTC AGG ACG CAG CCT TCA GCC ATC G - 3') and *P. aeruginosa* PA01genomic DNA as template; PCR product of RhlAB was digested with XbaI and HindIII and cloned into pMOD-2C to produce the recombinant plasmid pMOD-2CRAB (Figure 7-2-7). 3.5kb RhlAB-containing Transposon was produced by digesting pMOD-2CRAB with *Psh*AI. RhlAB-containing Transposon (TnCRPAB) was transformed into transposome after adding transposase (Figure 7-2-8).

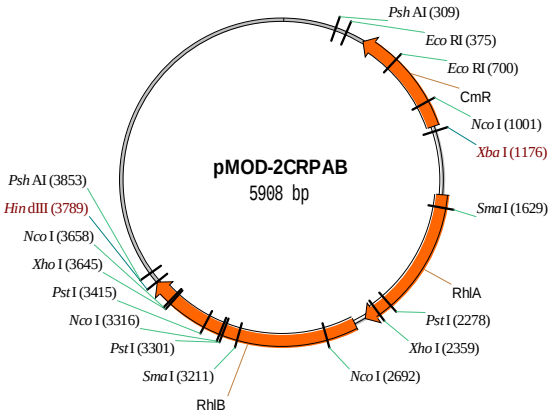
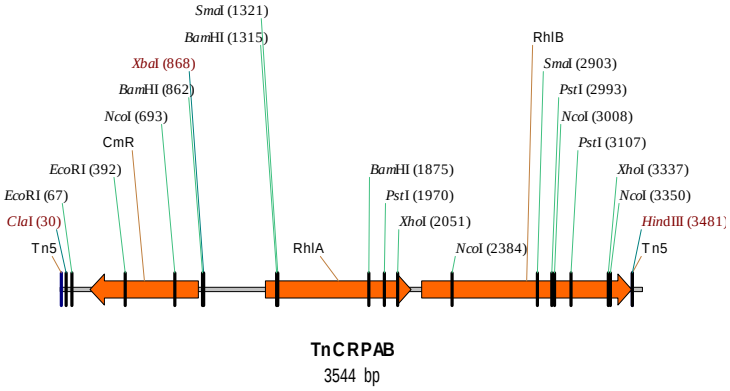


Figure 7-2-7 Recombinant plasmids with RhlAB-containing Transposon



(a)

(b)

Figure 7-2-8 Structure of RhlAB-containing Transposon (a) and diagram of transposome construction (b)

RhlAB-containing Transposome then was transformed into *P. aeroginosa* PAO1RhlA- (a gift from Dr. Joseph S. Lam) and *P. fluorescens* ATCC15453 by electroporation. Insertion mutants was selected by antibiotics resistance and confirmed by PCR (Figure 7-2-9).

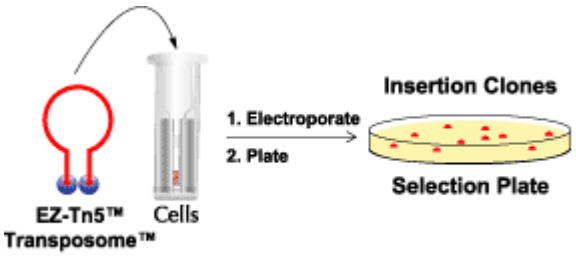


Figure 7-2-9 Diagram of Insertion mutant construction and selection

7.2.8 Error-prone PCR of Rhamnosyltransferase complex RhlAB

Error-prone PCR of Rhamnosyltransferase 1 complex RhlAB was conducted with GeneMorph II Random Mutagenesis Kit (Stratagene) according to instruction manual. The primers for PCR were RhlABRL-1 (5'- CCCAATCTCTAGATGCCTTTTCCGCCAACCCCTCGCTG-3') and RhlB-2 (5' - AAC CAA GCT TTC AGG ACG CAG CCT TCA GCC ATC G - 3'). Plamid pUCRPAB (Figure 7-2-2-1c) was as DNA template.

7.2.9 Library construction of RhlAB-containing Transposon insertion mutants of P. aeroginosa

Error-prone PCR product of RhlAB was ligated with 3.3kb XbaI-HindIII fragment from plasmid pMOD-2C (Figure 7-2-2c) with T4 DNA liagse. Ligation product was digested with *Psh*AI and a 3.5kb *Psh*AI-fragment which is RhlAB-containing transposon was purified from digestion product. RhlAB-containing transposon was transformed into *P. aeroginosa* PAO1-RhlA$^-$ by electroporation. Electroporated *P. aeroginosa* PAO1-RhlA$^-$ was plated in LB plus chloramphenicol (50μg/L) and incubated at 30ºC for 3 days. Those colonies appeared on LB agar generally were RhlAB-containing Transposon insertion mutants. We finally construct a library of 3500 mutants for further screening for rhamnolipid production and structure analysis by HPLC-MS.

7.2.10 Develop screening methods to carry out an evaluation of the performance of candidate mutants

Analytical method for rhamnolipid (Oricinol Method)

1) Rhamnolipids were purified by first separating the cells from supernatant by centrifugation (10000 ×g);
2) The supernatant was then extracted with chloroform and ethanol. 0.5mL rhamnolipid sample was extracted with 1mL chloroform:ethanol (2:1, v/v);
3) The organic phase was evaporated to dryness and 0.2mL of H_2O was added;
4) To 0.1mL of each sample 0.9mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added;
5) After heating for 30min at 80ºC the samples were cooled at room temperature (15-20min) and the OD_{421} was measured;
6) The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose (0-50mg/L) and expressed as rhamnose equivalents.

Results

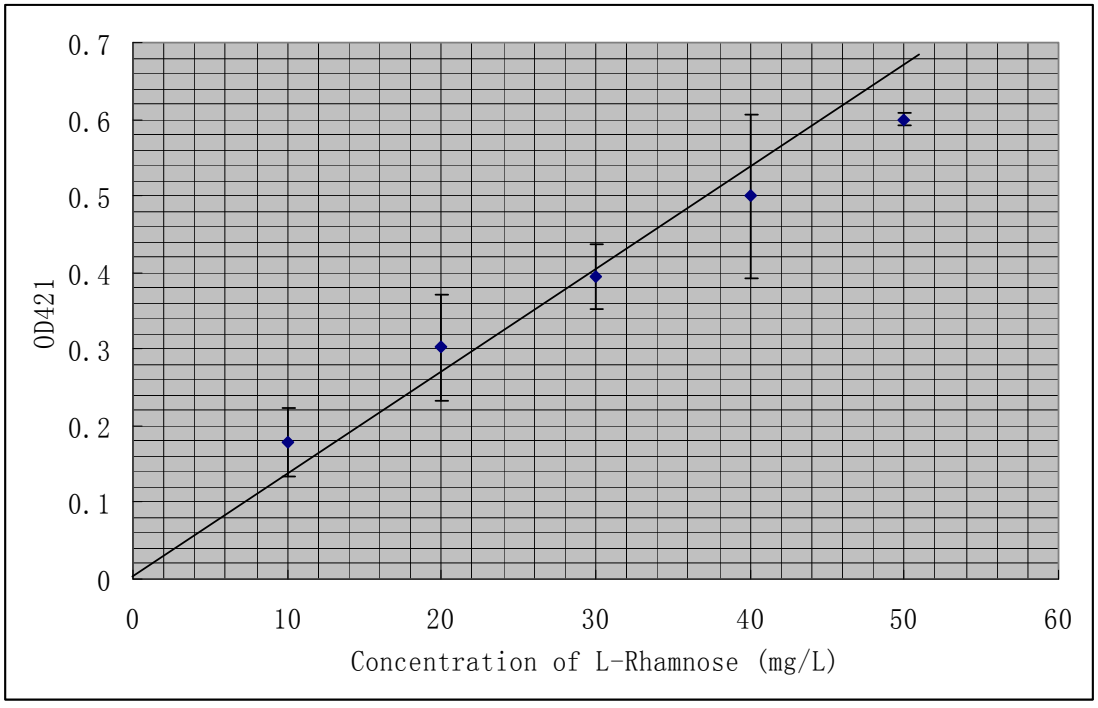


Figure 7-2-10. Standard Curve of L-Rhamnose assay by Orcinol method

semi-quantitative agar plate assay to detect the extracellular rhamnolipids.

On the light blue agar that contains CTAB and basic dye methylene blue, rhamnolipid- productive pseudomonas colonies are surrounded by dark blue halos. And the diameter of the dark blue halo is proportional to the rhamnolipid concentration. The Figure 7-2-11 illustrates the blue colonies that produce rhamnolipids.

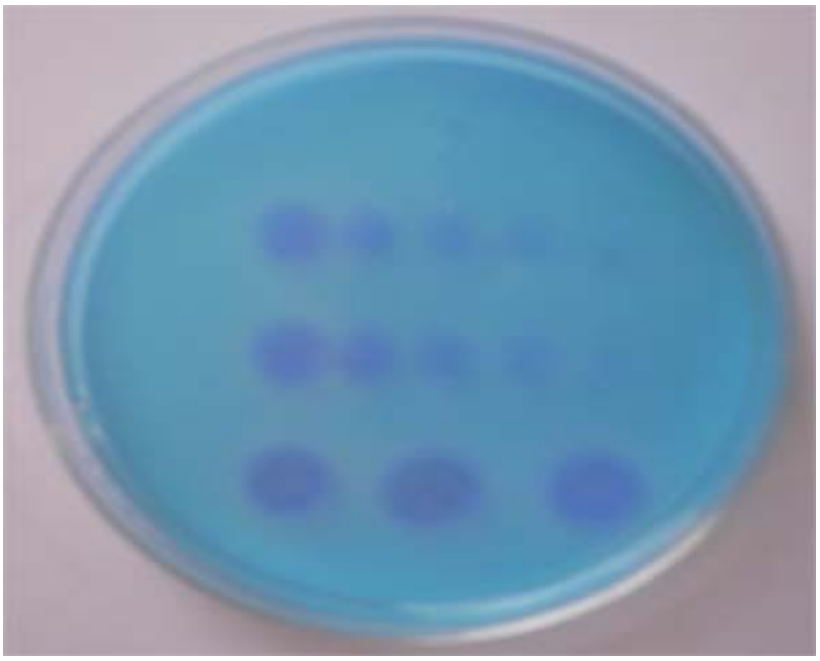
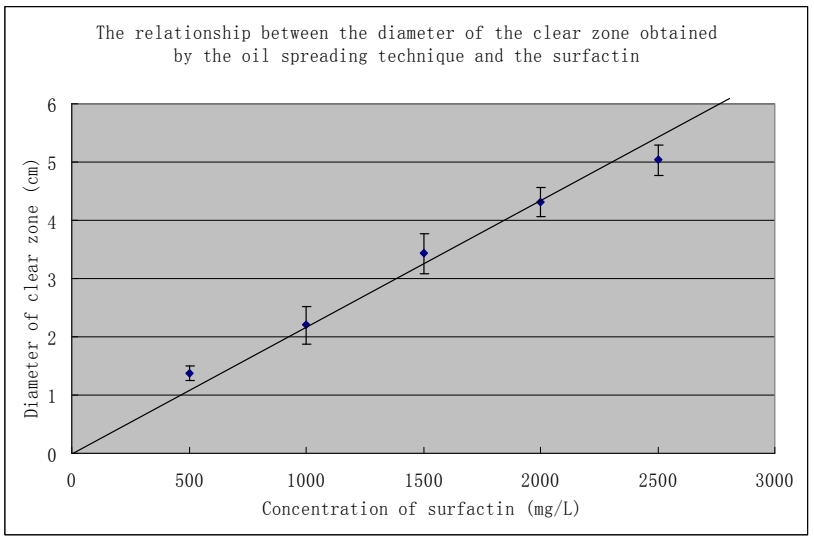


Figure 7-2-11. Rhamnolipids form blue halo on agar that contains CTAB and basic dye methylene blue

Quantification of biosurfactant by Oil Spreading Technique

1) Add 50mL of distilled water to a large Petri dish (15cm diameter);
2) Add 50μL of petroleum crude oil (CRC53352-1, McElroy field, Chevron Texaco) to the surface of the water;
3) Add 10μL (for different biosurfactant, there is different concentration range) to the surface of oil;
4) Measure the diameter of the clear zone on the oil surface after 2 minutes;
5) Make plot of standard curve. The diameters of triplicate samples were determined (Figure 7-2-12).



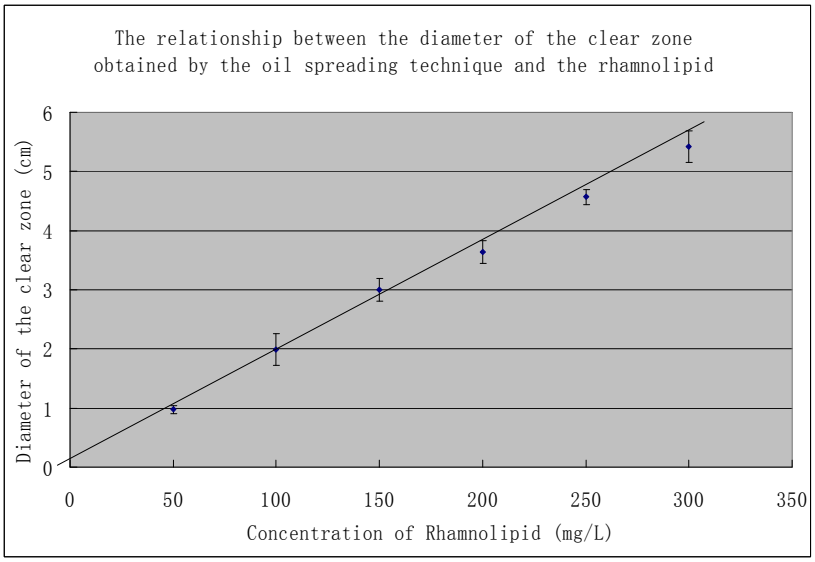


Figure 7-2-12. The relationship between the diameter of the clear zone obtained by the oil spreading technique and the concentration of biosurfactant. Error bars indicate the standard deviation of the three independent measurements. Surfactin was from Sigma (St. Louis, MO). Rhamnolipid was from bench-top fermentation by *Pseudonomas fluroscrens* PFAB01 and the concentration was quantified by Orcinol method.

7.3 Production and characterization of rhamnolipid

7.3.1 Rhamnolipids production and characterization by engineered P. aeruginosa and E. coli

RhlAB is the key enzyme of rhamnolipids biosynthesis, but this biosynthesis was modulated by the complex transcriptional regulatory network in *P. aeruginosa* (Soberon-Chavez and Aguirre-Ramirez, 2005). However, to achieve the rhamnolipids production in the strains which can not

produce rhamnolipids, integrating RhlAB is indispensable. In this circumstance, convenient molecular biotechniques would give great insights. Here, we show the state-of-the-art transposome-mediated chromosomal integration (Figure 7-2-8) to modify metabolic pathway of rhamnolipid biosynthesis. Briefly, RhlAB with native promoter and selection maker gene Cm (chloramphenicol resistance gene) were cloned into Tn5 derived transposon plamid pMOD-2 (Epcentre Biotechnologies, Madison, WI) to produce the recombinant plasmid pMOD-2CRAB. pMOD-2CRAB was digested with PshAI to obtain chimeric transposon TnRAB (Figure 7-2-7) which can bind transposase to produce transposome TnRABsome. TnRABsome was electroporated into *P. aeruginosa* PAO1-rhlA$^-$ wild-type strain. Consequently, we successfully constructed rhamnolipid-producing *P. aeruginosa* from wild-type strain (which was absent the ability for rhamnolipid biosynthesis) by transposome-mediated chromosomal integration of RhlAB after genotypic and phenotypic analysis. RhlAB with native promoter was inserted into chromosome of rhamnolipid-deficient strain (*P. areuginosa* PAO1-rhlA$^-$), and mutants strain *P. areuginosa* PEER02 which was confirmed to have RhlAB by PCR amplification and subsequent sequencing (data not shown) can effectively produce rhamnolipid in saline media with either glucose, or soybean oil as substrate. As showed in Table 2, soybean oil was better substrate for rhamnolipid production than glucose, and the yield of rhamnolipid in soybean oil was two-fold more than in glucose.

Table 2 Rhamnolipid fermentation by *Pseudonomas aeruginosa* mutant

Stains	Rhamnolipid (mg/L)	
	Glucose	Soybean oil
P. areuginosa PAO1-RhlA$^-$	ND	ND
P. areuginosa PEER02	785.4	1819.1

ND: none detected

In addition, to overcome the complex environmental regulation of rhamnolipid biosynthesis in *P. aeruginosa*, we attempted to achieve rhamnolipd formation in *E. coli* by same transposome-mediated chromosomal integration of RhlAB. T7 promoter replace native promoter of RhlAB in the transposon TnRAB to produce another chimeric transposon TnRAB7. This new transposon was

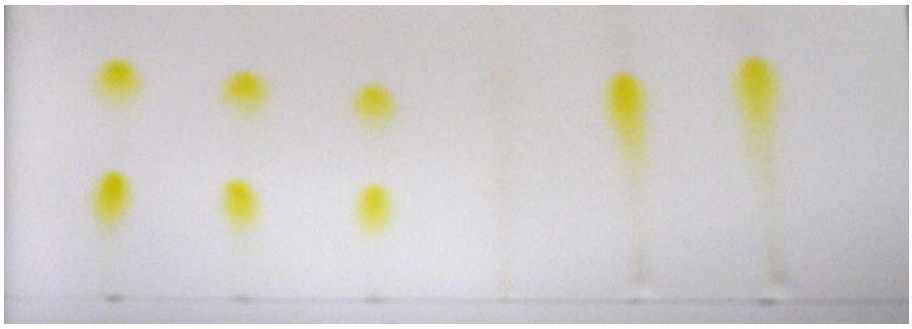
inserted into the chromosome of *E. coli* BL21(DE3) by transposome-mediated integration to engineer a strain *E. col*i TnERAB which was also confirmed by PCR (data not shown). With IPTG induction, *E. col*i TnERAB can produce rhamnolipid both in rich (LB) and minimal (MS) media and glucose as substrate. Because *E. coli* can grow better in LB media than minimal media, rhamnolipid yield is higher in LB media (Table 3).

Table 3 Rhamnolipid fermentation by engineered *E. coli* in rich or minimal media

Stains	Rhamnolipid (mg/L)	
	LB plus 0.4% glucose	MS plus 0.4% glucose
E. coli BL21(DE3)	ND	ND
*E. col*i TnERAB	175.3	75.6

ND: none detected

Rhamnolipids, as the best known biosurfactants, consist of one or two units of rhamnose linked to one or two fatty acid chains with C8-C12 carbon atoms, which may or may not be saturated (Figure 7-3-1). TLC results suggested that the isolated surface-active products from *P. aeruginosa* PAAB and E. coli TnERAB were composed of rhamnolipids (Figure 7-3-1). The products were separated on TLC plates alongside a sample of a commercially available purified rhamnolipid (JBR425, Jeneil Biosurfactant Company). When these samples were visualized, the products from *P. aeruginosa* PEER02 have two spots which were similar to the commercial product. The lower spot consisted of di-rhamnolipids, while the higher spot consisted of mono-rhamnolipids. The products from *E. coli* TnERAB only have the higher spots which are mono-rhamnolipids. Thus, we also proved that RhlAB only synthezied mono-rhamnolipids. Di-rhamnolipids biosynthesis needs help from other gene(s). *P. aeruginosa* PEER02 can produce di-rhamnolipids because it contain gene RhlC which was confirmed by PCR and sequencing (data not shown).

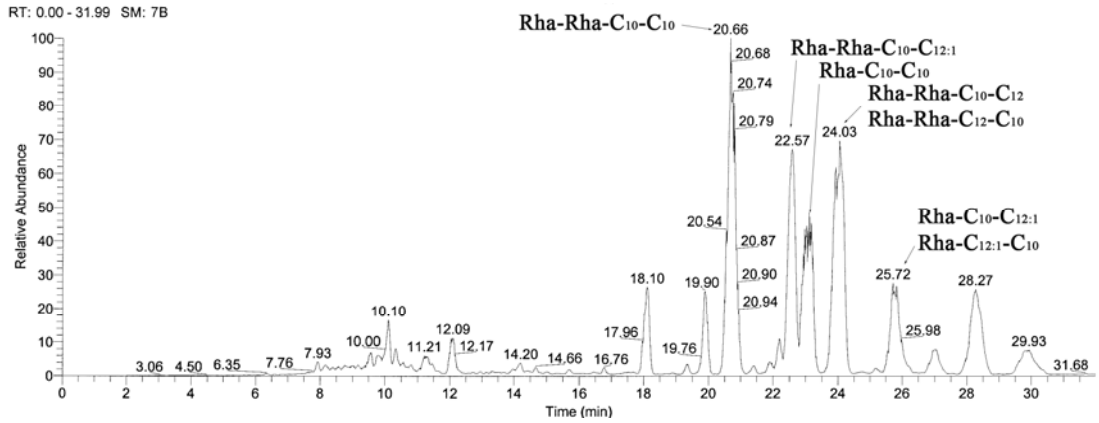


(a) (b) (c) (d) (e)

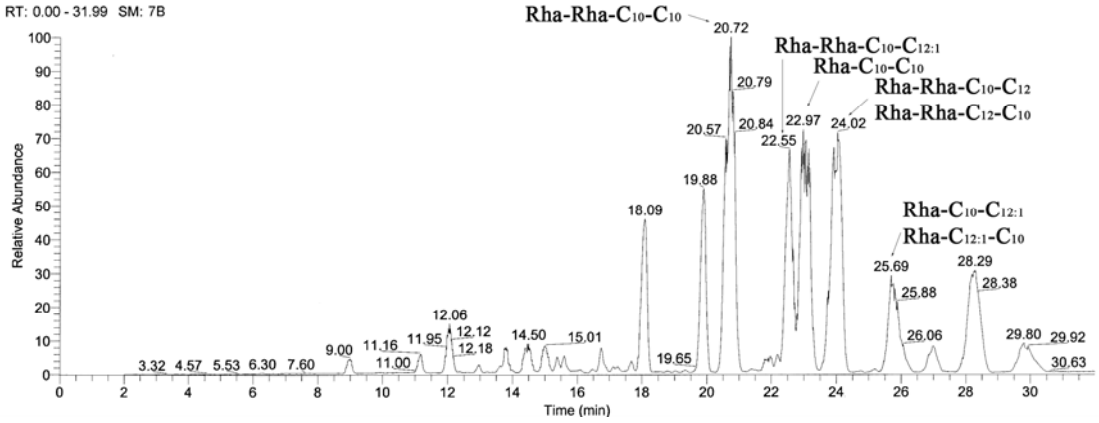
Figure 7-3-1 TLC analysis of rhamnolipids from engineered strains. (a) Commercial rhamnolipids JBR425; (b) Rhamnolipids produced by *P. aeruginosa* PEER02 with soybean oil as glucose; (c) Rhamnolipids produced by *P. aeruginosa* PEER02 with soybean oil as substrate; (d) Rhamnolipids produced by *E. coli* TnERAB in LB plus glucose media; (e) Rhamnolipids produced by *E. coli* TnERAB in MS plus glucose media; (10 μL sample of 500mg/L for each lane).

Then, the products from our engineered strains were next submitted to HPLC/MS analysis to further confirm the presence of rhmanolipids. Rhamnolipids in crude fermentative broth was precipitated after acidifying with concentrated HCl, and extracted with chloroform : ethanol (2:1,v/v). Organic phase was collected and air dried. After drying, the yellow oil paste was suspended in water. The sample was ready for LC-MS analysis as well as for IFT analysis. Rhamnolipid structural information was obtained through the use of mass detector equipment with electrospray ionization (ESI) probe. Figure 7-3-2 presents the base peak ion intensity chromatograms for the rhamnolipid samples produced by our engineered strains with soybean oil or glucose as carbon source. The profiles of these chromatograms differ mostly in the late eluting region which is composed mainly of rhamnolipids containing only one fatty acid moiety. The proportions of the various rhamnolipids listed in Table 4 were obtained from the relative intensities of their corresponding pseudomolecular ions. For rhamnolipids of the same molecular weight which were chromatographically resolved, this was simply performed by integration of the intensities of their pseudomolecular ion. For isomers that were not chromatographically resolved, their relative proportion was determined by the relative intensities of different fragments ions produced by cleavage of the two molecules at the same position. For example, the rhamnolipid Rha-C8-C10 was not sufficiently separated from Rha-C10-C8 to allow direct quantification on the basis of the

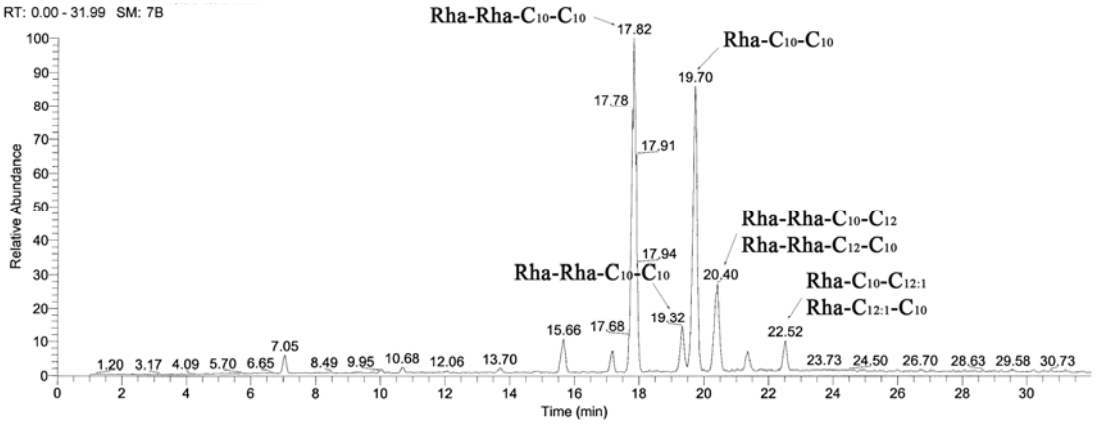
intensity of their pseudomolecular ion at m/z 475. However, their mass spectra differ by the presence of an m/z 305 ion for the former and an m/z ion 333 for the latter. These two ions arise from cleavage at the 3-carbon-oxygen bond in both molecules. The relative intensities of the two isomers were calculated by measuring the relative intensities of both ions, in an averaged spectrum obtained from all the spectra presenting the proper pseudomolecular ion, and multiplying these two values with the intensity of their common pseudomolecular. This method allows the analysis of very closely related rhamnolipids without resorting to long and difficult chromatographic separation.



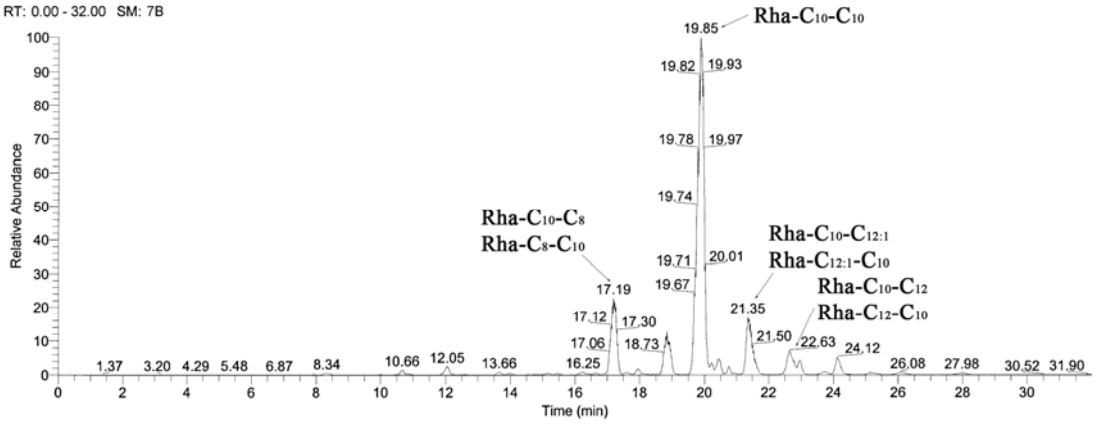
(a)



(b)



(c)



(d)

Figure 7-3-2. Base peak ion intensity chromatograms of rhamnolipids from various sources. (a) rhamnolipids produced from minimal media supplemented soybean oil by *P. aeruginosa* PEER02; (b) rhamnolipids produced from minimal media supplemented glucose by *P. aeruginosa* PEER02; (c) commercial rhamnolipids JBR425; (d) rhamnolipids produced from minimal media supplemented glucose by *E. coli* TnERAB;

In the ion intensity chromatograms (Figure 7-3-2), the main structural composition of rhamnolipids from each samples were labeled. For the samples from *E. coli*, the main composition is Rha-C10-C10 which almost occupied 40~60% of total amount according the samples from different media. The main compositions of the samples from engineered *P. aeruginosa* also have C10-C10 carbon chain: Rha-C10-C10 and Rha-Rha-C10-C10. The percentage of these compositions is more than 30%. Another abundant structure composition of rhamnolipids from P. aeruginosa is Rha-Rha-C10-C12 or Rha-Rha-C12-C10. They have same pseudomolecular ion and total amount is more than 10%.

Table 4. Structure and relative abundance of the rhamnolipids from various sources

Structure	Pseudomolecular Ion (m/z)	Relative abundance (%)			
		JBR425	PEER02/G	PEER02/S	TnERAB
Rha-Rha-C_8-C_{10}	621	2.06	2.53	3.77	ND
Rha-Rha-C_{10}-C_8	621	1.37	3.51	0.30	ND
Rha-C_8-C_{10}	475	1.41	4.73	ND	5.65
Rha-C_{10}-C_8	475	0.52	2.21	3.35	5.65
Rha-Rha-C_{10}-C_{10}	649	36.45	19.37	20.20	ND
Rha-Rha-C_{10}-$C_{12:1}$	675	4.08	9.70	13.24	ND
Rha-Rha- $C_{12:1}$-C_{10}	675	ND*	ND	ND	ND
Rha-C_{10}-C_{10}	503	28.13	15.33	12.07	57.08
Rha-Rha-C_{10}-C_{12}	677	8.13	10.37	11.59	ND
Rha-Rha-C_{12}-C_{10}	677	2.18	5.76	6.62	ND
Rha-C10- $C_{12:1}$	529	1.15	0.37	6.33	0.14
Rha- $C_{12:1}$-C_{10}	529	0.78	5.25	ND	9.05
Rha-Rha- $C_{12:1}$-C_{12}	703	ND	ND	ND	ND
Rha-Rha-C_{12}- $C_{12:1}$	703	ND	0.87	ND	ND
Rha-Rha-C_{10}-$C_{14:1}$	703	ND	0.16	1.07	ND
Rha-Rha-$C_{14:1}$-C_{10}	703	ND	0.44	0.68	ND
Rha-C_{10}-C_{12}	531	2.15	4.46	3.96	1.50
Rha-C_{12}-C_{10}	531	0.42	2.35	2.64	2.25
Rha-Rha-C_{12}-C_{12}	705	ND	2.15	2.14	ND

JBR425: commercial rhamnolipids; PEER02/G: rhamnolipids from *P. aeruginosa* PEER02 with glucose as substrate in MS media; PEER02/S: rhamnolipids from *P. aeruginosa* PEER02 with soybean oil as substrate in MS media; TnERAB: rhamnolipids from *E. coli* TnERAB with glucose as substrate in MS media and IPTG induction; * ND: none detected

7.3.2 Rhamnolipids production and characterization from various substrates by engineered *Pseudomona aeruginosa*

Rhamnolipids, as one of main type of biosurfactants, have advantages over their chemicals counterparts because they are biodegradable, have low toxicity, are effective at extreme temperatures or pH values (Cameotra and Makkar, 1998; Liang et al., 1999) and show better environmental compatibility (Georgiou *et al.*, 1990). Nevertheless, from an economic standpoint, biosurfactants are not yet competitive with the synthetics. Rhamnolipids can only replace synthetic surfactants if the cost of the raw material and the process costs are reduced. So far, several renewable substrates from various sources, especially from industrial wastes have been intensively

studied for microorganism cultivation and surfactant production at an experimental scale. Here we make efforts to use alternate low-cost substrates to facilitate industrial development of rhamnolipids production by engineered *Pseudomonas aeruginosa* which was constructed in our lab.

Fermentation experiments showed *P. aeruginosa* PEER02 can produce rhamnolipids in minimal salts media containing various carbon sources, including glucose, fructose, glycerol, ethanol, soybean oil, corn oil, frying oil, palm oil, peanut oil, coconut oil, olive oil, grape seed oil, etc. The results of preliminary carbon source test are shown below in Table 5.

Table 5. Effect of various carbon sources on rhamnolipids production by *P. aeruginosa* PEER02 in minimal media

Substrate	Fermentation time (day)								
	2			4			6		
	Growth	RL (g/L)	Conversion	Growth	RL (g/L)	Conversion	Growth	RL (g/L)	Conversion
Glucose	3.86	3.277	0.164	2.32	2.532	0.127	1.73	2.379	0.119
Fructose	1.39	0.156	0.008	2.84	1.239	0.062	4.04	4.221	0.211
Xylose	0.49	0.162	0.008	0.62	0.264	0.013	0.47	0.345	0.017
Lactose	NG**	ND***	ND	NG	ND	ND	NG	ND	ND
Sucrose	NG	ND	ND	NG	ND	ND	NG	ND	ND
Maltose	NG	ND	ND	NG	ND	ND	NG	ND	ND
Soluble starch	NG	ND	ND	NG	ND	ND	NG	ND	ND
Ethanol	0.89	0.060	0.003	2.33	1.233	0.062	2.43	1.989	0.099
Glycerol	3.89	2.472	0.124	4.73	9.969	0.498	4.97	7.296	0.365
Soybean oil	4.88	5.049	0.252	4.09	7.095	0.355	1.70	3.648	0.182
Corn oil	5.21	5.112	0.256	4.22	8.166	0.408	2.10	4.797	0.240
Peanut oil	5.16	5.523	0.276	4.39	6.723	0.336	2.68	6.708	0.335
Palm oil	5.71	4.233	0.212	2.49	4.362	0.218	2.37	4.071	0.204
Coconut oil	5.71	6.099	0.305	3.82	5.448	0.272	2.23	6.363	0.318
Grapeseed oil	5.51	5.166	0.258	1.85	4.152	0.208	0.94	4.863	0.243
Olive oil	5.66	3.489	0.174	4.74	4.668	0.233	3.00	5.583	0.279
Frying oil	5.49	4.881	0.244	4.00	7.347	0.367	2.05	4.230	0.212
n-Octane	NG	ND	ND	NG	ND	ND	NG	ND	ND
n-Decane	NG	ND	ND	NG	ND	ND	NG	ND	ND
n-Dodecane	NG	ND	ND	NG	ND	ND	NG	ND	ND
n-Tetradecane	NG	ND	ND	NG	ND	ND	NG	ND	ND

n-Hexadecane	NG	ND	ND	NG	ND	ND	NG	ND	ND
Crude oil	NG	ND	ND	NG	ND	ND	NG	ND	ND
Mineral oil	NG	ND	ND	NG	ND	ND	NG	ND	ND

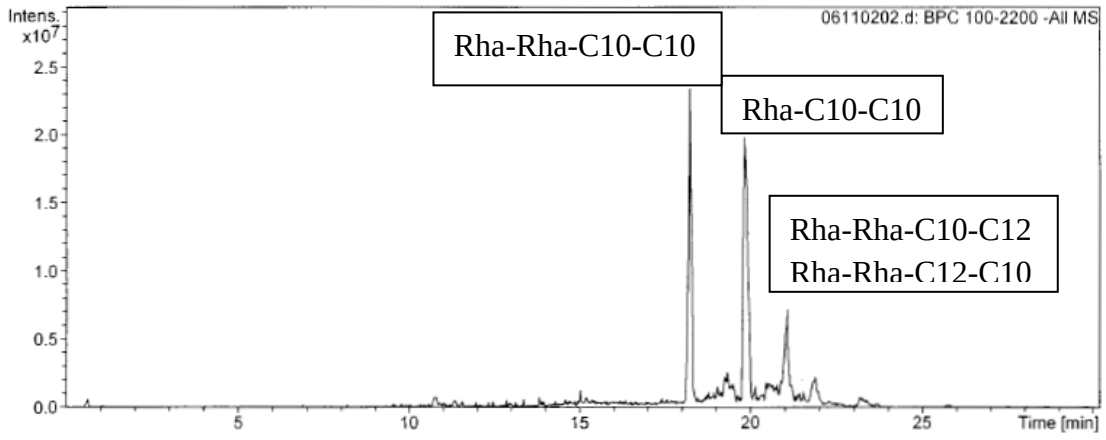
* For liquid substrate, the conversion is g/mL, and for solid substrate, g/g;

** NG: no growth; *** ND: none detected

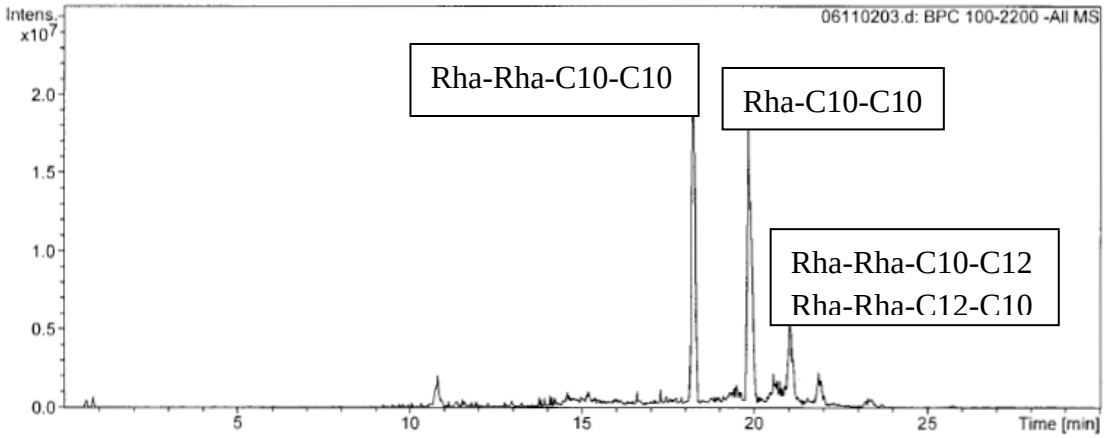
P. aeruginosa PEER02 can not grow in sucrose, lactose, maltose as well as all hydrocarbons tested. Thus, this engineered strain can not produce rhamnolipids with these substrates. Compared with the substrates of various plant oils, *P. aeruginosa* PEER02 produce less rhamnolipids with sugars as substrates. In all plants oils, corn oil is the best substrate for rhamnolipids production by *P. aeruginosa* PEER02. The yield is about 8g/L, and the productivity (conversion rate) is about 34%. However, in our investigation, glycerol will give the highest yield and productivity. Its yield and productivity is about 10g/L and 50%, respectively.

Rhamnolipids characterization from different substrates

Rhamnolipids were produced with *P. aeruginosa* PEER02 from 12 different substrates, including 8 plant oil, 2 sugars, two others, glycerol and ethanol. Rhamnolipids from different substrates were purified for HPLC/MS analysis (Figure 7-3-3). From base peak ion intensity chromatograms of rhamnolipids from various sources, the main compositions of each samples and their percentage were summarized in Table 3 The purified samples were also applied for interfacial tension analysis agains n-Octane (Table 4). Geneally, the higher the percentage of di-rhamnolipids with longer carbon chain was, the lower its IFT was.



(a) Soybean oil



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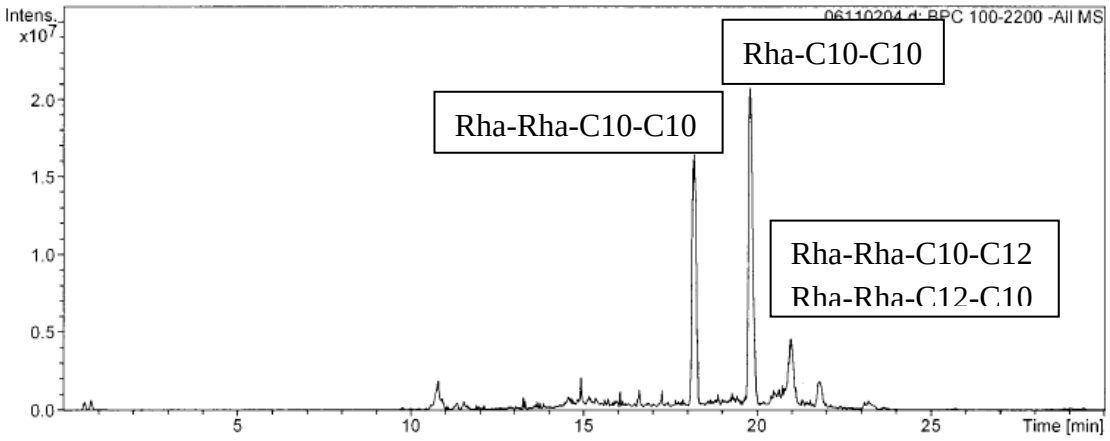
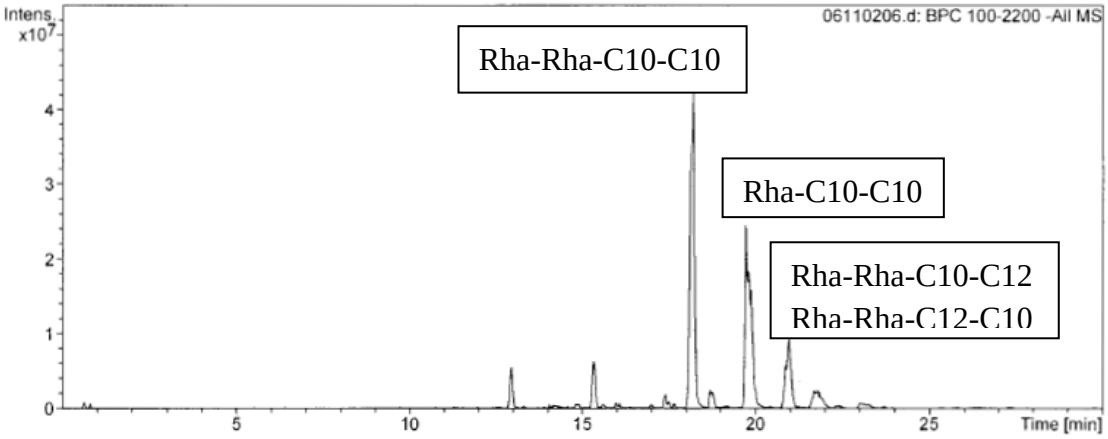
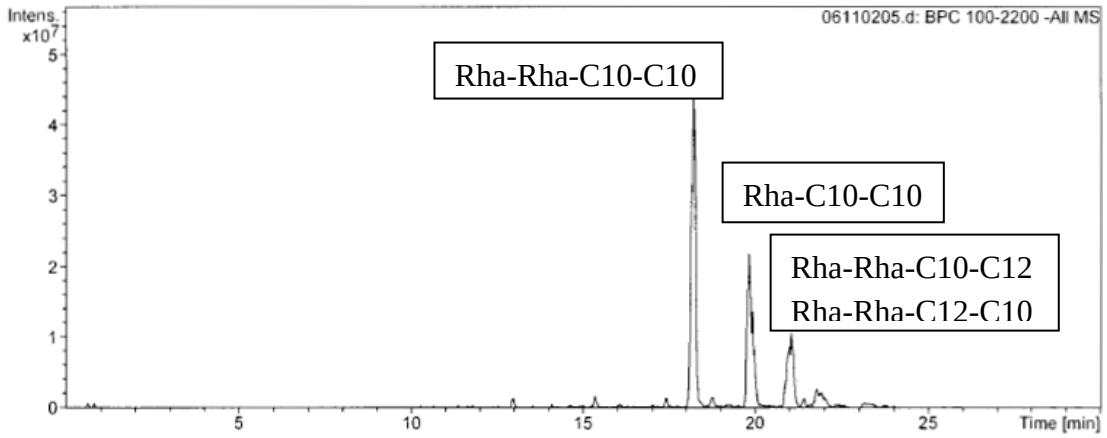


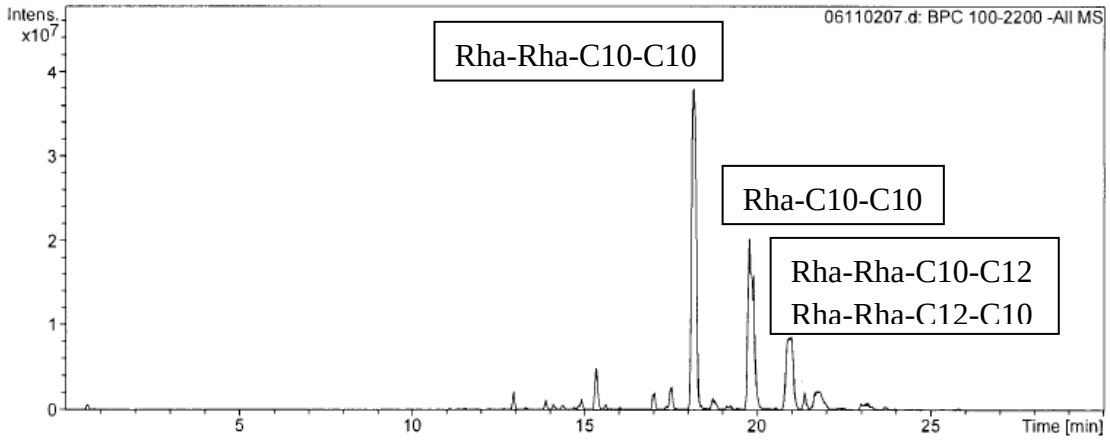
(c) Frying oil



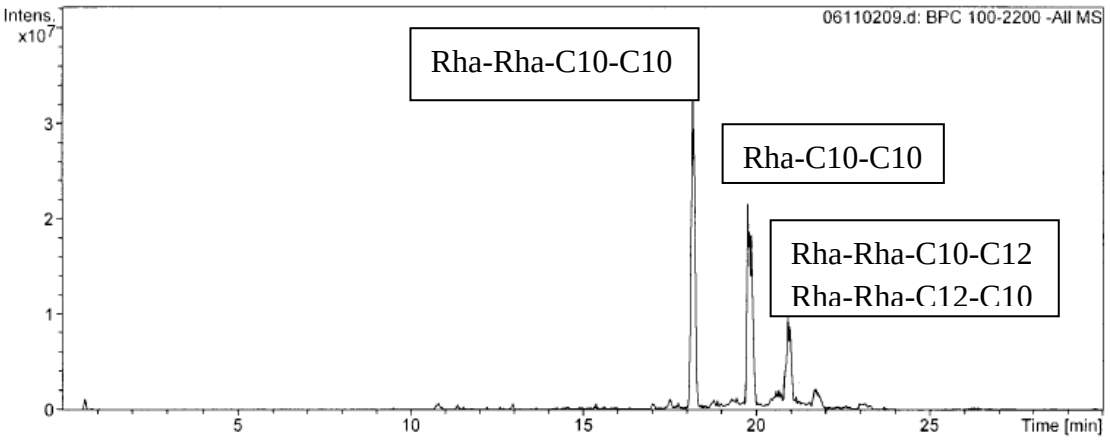
(d) Palm oil



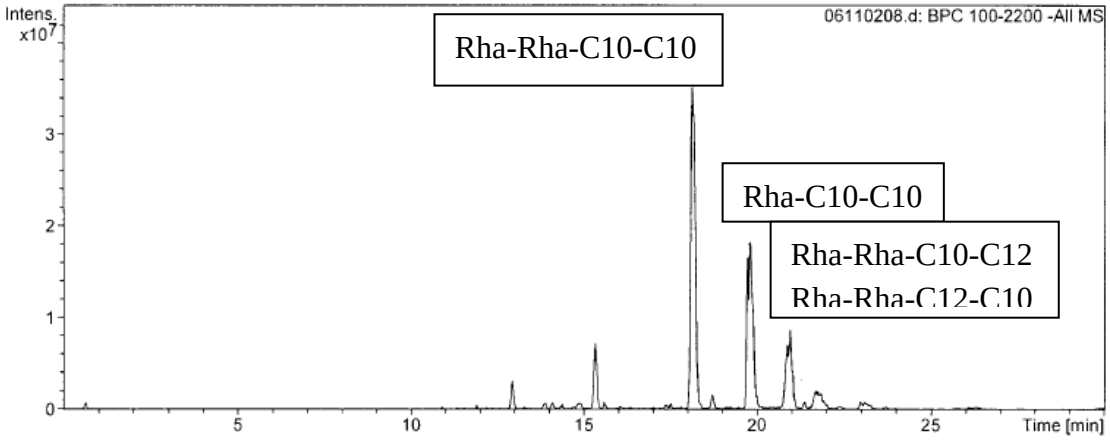
(e) Coconut oil



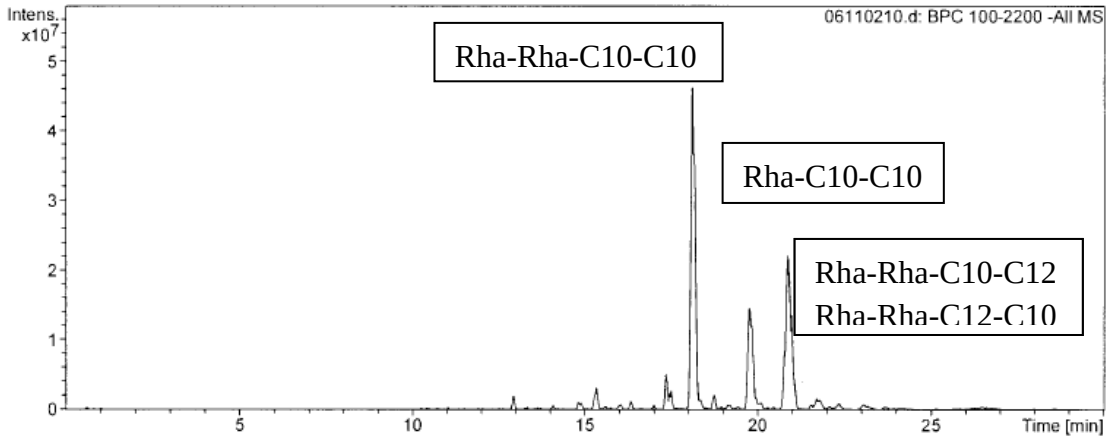
(f) Peanut oil



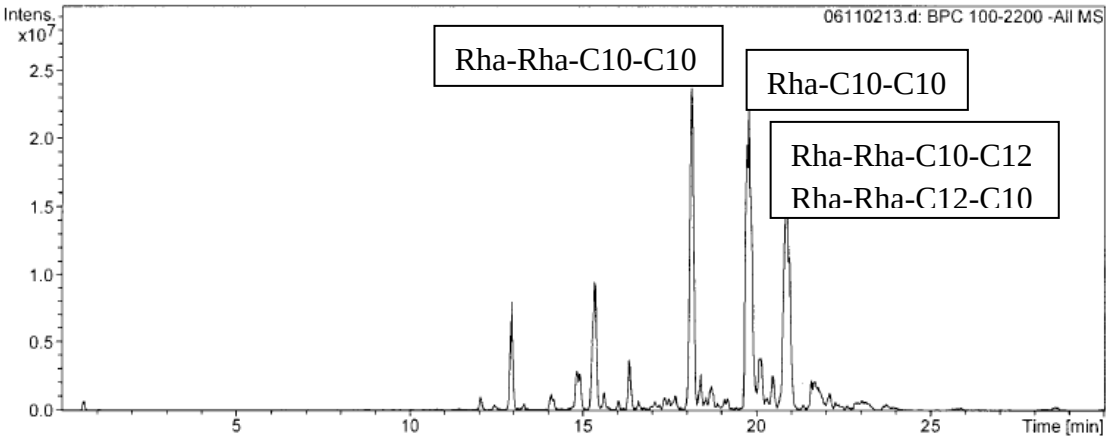
(g) Olive oil



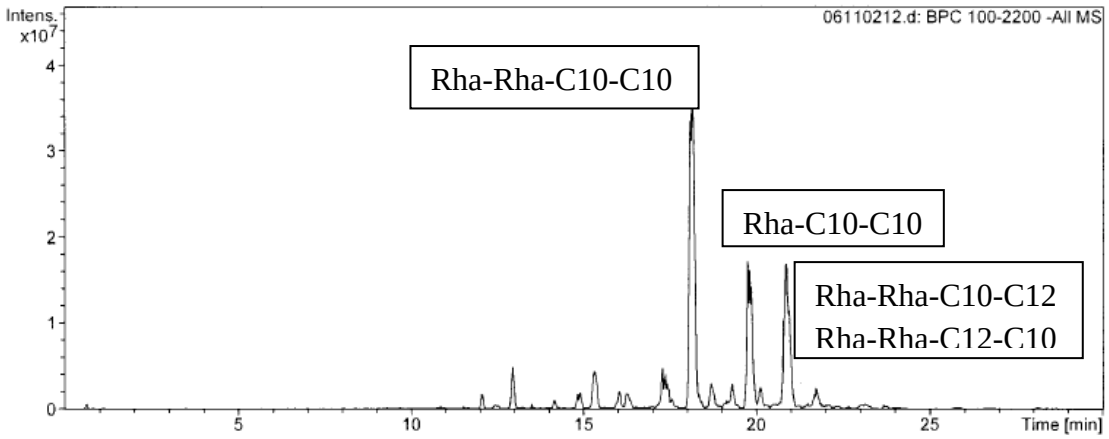
(h) Grapeseed oil



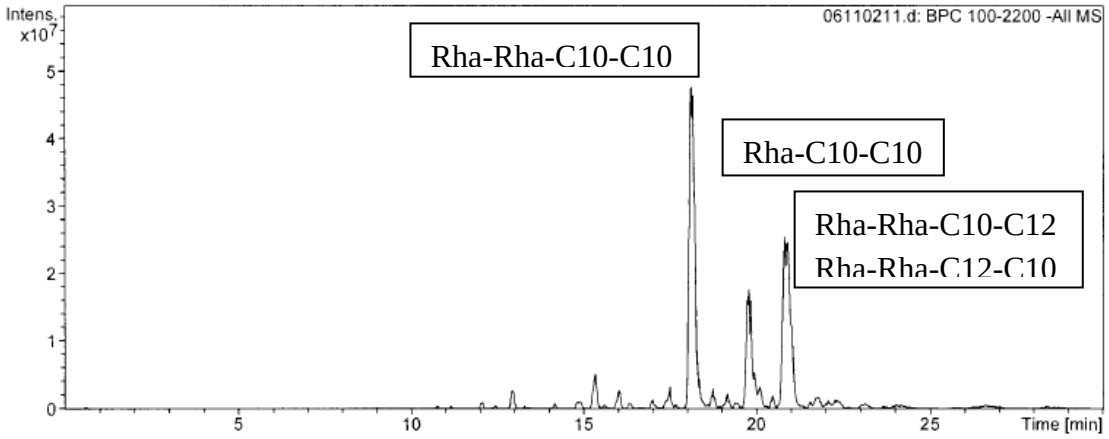
(i) Glucose



(j) Glycerol



(k) Ethanol



(l) Fructose

Figure 7-3-3. Base peak ion intensity chromatograms of rhamnolipids produced from different carbon sources. (a) soybean oil; (b) corn oil; (c) frying oil; (d) palm oil; (e) coconut oil; (f) peanut oil; (g) olive oil; (h) grapeseed oil; (i) glucose; (j) glycerol; (k) ethanol; (l) fructose. The main compositions are labeled in each chromatogram.

Table 6. Structure and relative abundance of the rhamnolipids from various sources

No.	Substrate	Rha-Rha-C10-C10 m/z 649	Rha-C10-C10 m/z 503	Rha-Rha-C10-C12 (or C12-C10) m/z 677
1	Soybean oil	37.4	47.7	14.9
2	Corn oil	43.9	43.5	12.6
3	Frying oil	36.8	48.8	14.4

4	Palm oil	51.3	31.3	17.4
5	Coconut oil	47.7	33.6	12.4
6	Peanut oil	49.6	32.6	17.8
7	Olive oil	47.6	31.5	14.6
8	Grapeseed oil	44.3	38.5	17.2
9	Glucose	49.5	18.4	32.1
10	Glycerol	52.5	22.2	34.6
11	Ethanol	23.3	33.2	25.2
12	Fructose	47.9	17.5	24.4

7.3.2 Fed-batch fermentation of Rhamnolipids production by *P. aeruginosa* PEER02

Fed-batch fermentations of rhamnolipids production with starting 2% (S2) and 4% (S4) soybean oil in fermentative media, respectively, were investigated. For S2, an additional 2% soybean oil was supplemented into media every day of first 4days. For S4, an additional 3% soybean oil was supplemented at second and fourth day, respectively. The final concentration of soybean oil was 10% for both fed-batch fermentations.

From Figure7-3-4, fed-batch fermentation greatly enhanced the production of rhamnolipids, and the yield reached 23~25g/L. Generally speaking it is more efficient to add the substrate step wise, rather than all at the start of the fermentation process. It could be expected that the surfactant production and percent conversion could be improved from this result when optimized as a commercial process.

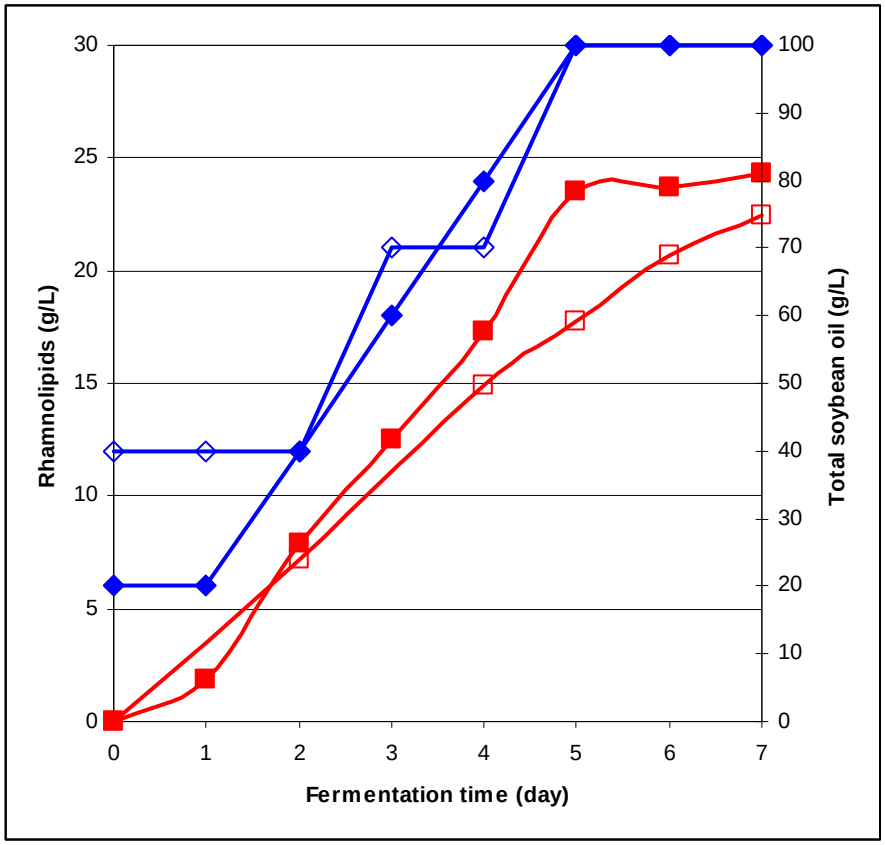


Figure 7-3-4 Fed-batch fermentation of Rhamnolipids production by *P.aeruginosa* PEER02. Red line with solid square: rhamnolipids production curve of S2; Red line with unfilled square: rhamnolipids production curve of S4; Blue line with solid diamond: fed-batch line of S2; Blue line with unfilled diamond: fed-batch line of S4.

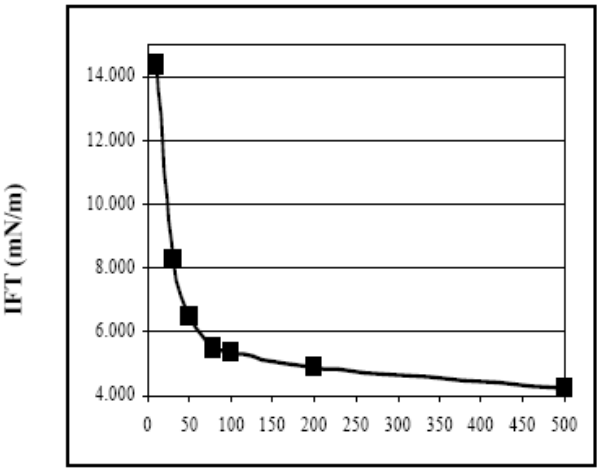
7.4 Interfacial tension (IFT) analysis of Rhamnolipids

7.4.1 IFT analysis of rhamnolipids produced by PEER02 strain.

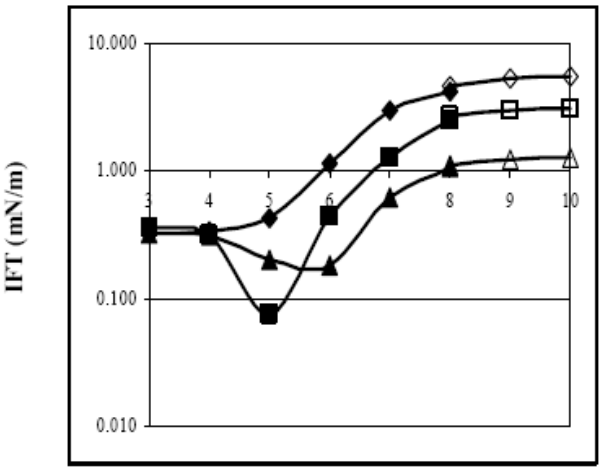
Like their chemical counterpart, biosurfactants also reduce interfacial tension between oil and water, thus decreasing the energy required to extract trapped oil in the porous matrix and displace this into the mobile liquid phase. But under what conditions can biosurfactants play a significant role in this process? How does one know when to expect biosurfactants to be a significant factor in some system under investigation? Here, rhamnolipids produced by *P. aeruginosa* PEER02 with soybean oil as carbon source was used to examine one of interfacial phenomena of biosurfactants -

how rhamnolipids reduce interfacial tension and the effects of altering salt concentration, pH and temperature on rhamnolipids activity.

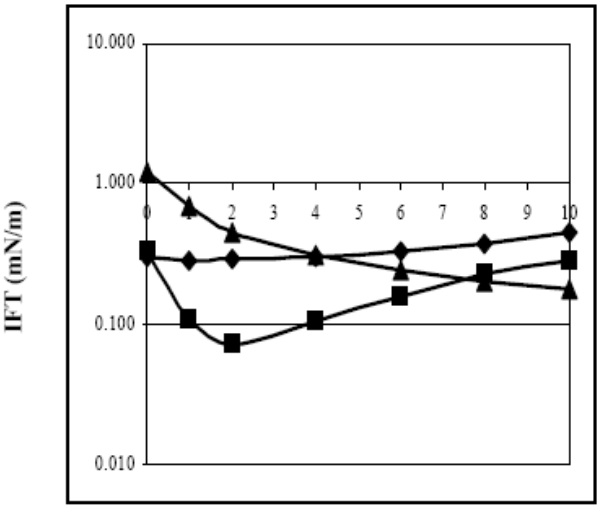
First, we investigated the effects of different concentrations of rhamnolipids on IFT to find a suitable concentration for analyzing IFT of rhamnolipid in various conditions. All of these tests used n-octane as the hydrocarbon phase. As shown in Figure 7-4-1a, the IFT of rhamnolipid solution rapidly decreases with an increase of rhamnolipid concentration. When the concentration exceeds the critical micelle concentration (CMC), the change of IFT becomes small. According to the results of Figure7-4-1a, the CMC of our product is about 75mg/L. Hence, we selected 100ppm rhamnolipids for our subsequent IFT condition test.



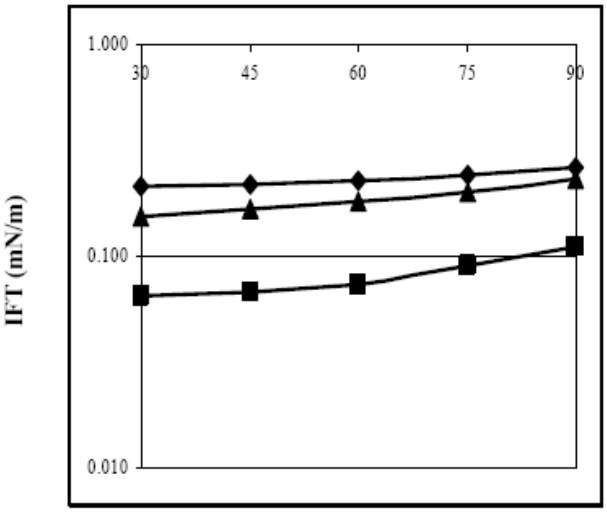
(a)



(b)



(c)



(d)

Figure 7-4-1. IFT analysis of PEER02 rhamnolipids in various conditions. (a) Profile of IFT of different concentration of rhamnolipids in water; (b) Effects of Salinity on IFT of rhamnolipids, Diamonds - 1% NaCl, Squares – 2% NaCl and Triangles – 8% NaCl; (c) Effects of pH on IFT of rhamnolipids, Triangles – pH4, Squares – pH5 and Diamonds - pH6; (d) Effects of temperature on IFT of rhamnolipids, Diamonds – pH4, Squares – pH5 and Triangles - pH6.

From Figure7-4-1b, we know that in the absence of additional salinity, rhamnolipids showed low IFT in the low pH buffer system, and in the range of pH 3-5, rhamnolipids showed the lowest IFT of about 0.3. If there is any additional salinity, the IFT profile is sensitive to pH. For example, when 2% NaCl was added into rhamnolipids with pH5, the IFT of this solution decrease dramatically almost 5 fold. However, with the increase of salinity, not only the pH with lowest IFT will increase, but the absolute value of IFT also increases. In addition, IFT profile of rhamnolipid solution with same pH but different salinity will be greatly different. For pH6, the IFT will decrease with the increase of salinity. But for pH4, the behavior is the reverse. Increasing salinity resulted in IFT increase in the range of 0~10% NaCl. However, in pH5, there is an optimal salinity. Adding 2% NaCl would show the lowest IFT for pH5 rhamnolipid solution (Figure 7-4-1c).

Temperature is an important parameter for oil reservoir. Hence, temperature performance of rhamnolipid is indispensable when evaluating a product if you want to use it for enhancing oil recovery. Our experiments showed that IFT only changed slightly (increased slightly) in the range of 30-90°C (Figure 7-4-1d).

7.4.2 IFT analysis of the rhamnolipids produced from different substrates

Rhamnolipids were produced with *P. aeruginosa* PEER02 from 12 different substrates, including 8 plant oil, 2 sugars, two others, glycerol and ethanol. The purified samples were applied for interfacial tension analysis agains n-Octane (Table 7). Geneally, the higher the percentage of di-rhamnolipids with longer carbon chain was, the lower its IFT was.

Table 7 IFT analysis of Rhamnolipids produced from various substrates

No.	Substrate	(a) 6000rpm/30°C		(b) 3000rpm/30°C		
		mN/m @5min	mN/m @10min	mN/m @1min	mN/m @5min	mN/m @10min
1	Soybean oil	5.486	4.989	0.280	0.149	0.077

2	Corn oil	6.321	6.052	0.412	0.232	0.174
3	Frying oil	6.367	6.190	0.520	0.331	0.253
4	Palm oil	5.947	5.928	0.078	0.064	0.055
5	Coconut oil	5.292	5.233	0.033	0.130	0.012
6	Peanut oil	4.244	3.341	0.036	0.014	0.015
7	Olive oil	5.021	5.007	0.088	0.053	0.037
8	Grapeseed oil	5.050	5.012	0.181	0.111	0.061
9	Glucose	5.737	5.955	0.138	0.140	0.128
10	Glycerol	5.049	4.981	0.188	0.168	0.151
11	Ethanol	2.498	2.439	0.040	0.022	0.011
12	Fructose	5.942	4.976	0.430	0.418	0.413

(a) dilute supernatant of fermentation to 100 ppm with distilled water

(b) dilute supernatant of fermentation to 100 ppm buffer pH5 and 2% added NaCl

7.4.3 Rhamnolipids produced by six Engineered strains Displayed Different Optimal IFT Values in Response to Different pH Conditions

Rhamnolipid biosurfactants were fermented and adjusted to final concentration of 250ppm by oil spreading assay. Then the pH values were adjusted from 5 to 12 using two different buffer systems – citric acid wide range buffer system and boric acid universal buffer system. IFT values were then measured under each different pH conditions and plotted in Figure 7-4-2 and 7-4-3. In the citric acid based buffer system, the lowest IFT were obtained at PH 8 with the products of strain ETRA and ETRAC, white the lowest IFT of the products of other 4 strains were obtained at pH 7. But in the Boric acid buffer system, the pH values of the lowest IFT were shifted downwards, with products of strain ETRA and ETRAC obtaining lowest IF at pH 7; and products of strain ETRhl, ETRABC and ETRhl-RC obtaining lowest IF at pH 7. The only exception was the product of strain ETRAB, which obtaining lowest IFT at pH7 in both buffer systems. Figure 7-4-4 showed the effects of the salinity at pH7. The lowest IFT values for all six strains were obtained when 2% NaCl was added to the buffer. What surprised us was that even there were no detectable rhamnolipids produced by strain ETRA and ETRAC, their products produced very low IFT values.

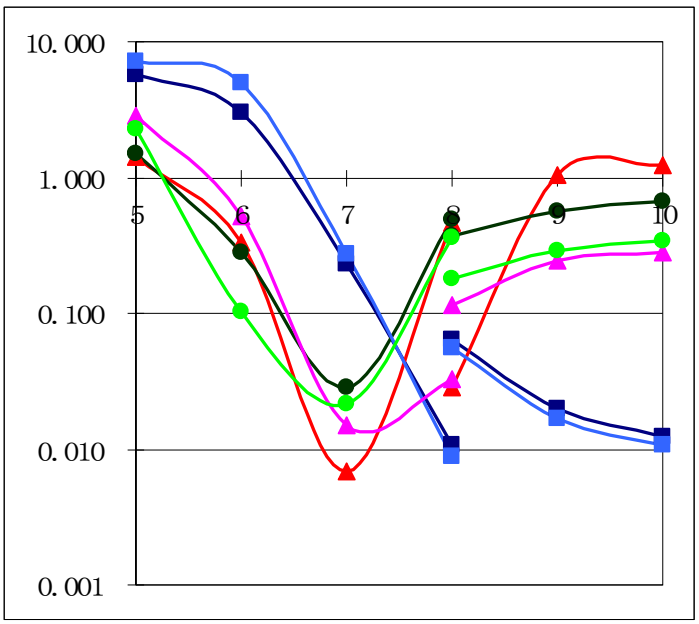


Figure 7-4-2 IFT of 250ppm rhamnolipids produced by Engineered E.coli strains in the Citric acid buffer system. Dark blue squares: ETRA; Red triangles: ETRAB; Dark green circles: ETRhl; Light Bblue squares: ETRAC; Pink Triangles: ETRABC; Light green circles: ETRhl-RC; Open symbol : 40mM Citric acid-Na2HPO4 wide range buffer (pH2.6-8.0); Solid symbol: 40mM Boric acid-KCl-NaOH buffer (pH8.0-10.0);

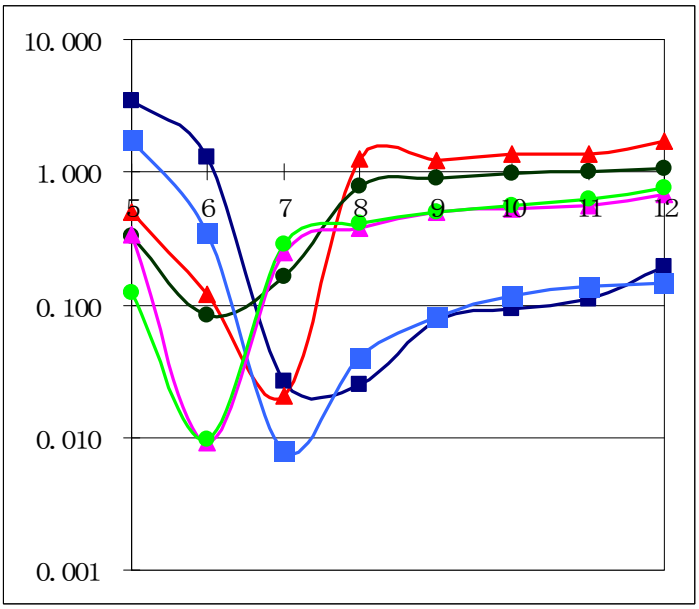


Figure 7-4-3. IFT of 250ppm rhamnolipids produced by Engineered E.coli strains in the C Boric acid approximate universal buffer system. Dark blue squares: ETRA; Red triangles: ETRAB; Dark green circles: ETRhl; Light Bblue squares: ETRAC; Pink Triangles: ETRABC; Light green circles: ETRhl-RC.

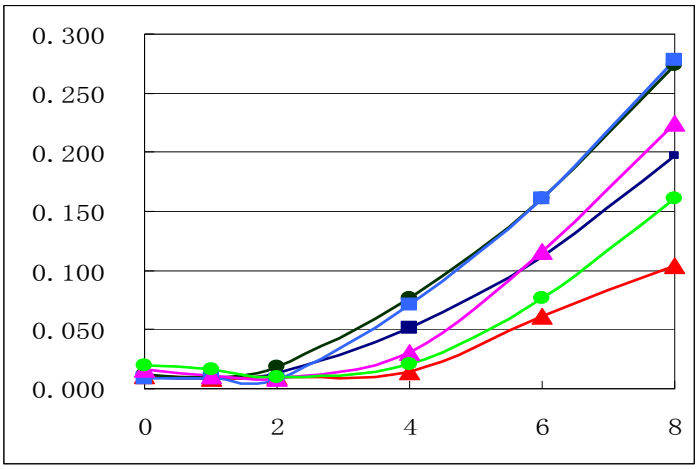


Figure 7-4-4. IFT of the rhamnolipids produced by six E.coli strains under different salinities. Dark blue squares: ETRA in pH8 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer; Red triangles: ETRAB in pH7 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer; Dark green circles: ETRhl in pH7 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer Light blue squares: ETRAC in pH8 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer; Pink triangles: ETRABC in pH7 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer; Light green circles: ETRhl-RC in pH7 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer

7.5 Rhamnolipid production and divergent evolution in *E.coli*

Rhamnolipids, as the entensively studied biosurfactants, were mainly produced from *Pseudomonas aerugina* or other Pseudomonads. Rhamnolipids production in Pseudomonads is a part of quorom sensing process, which has complex regulation. Thus, optimizing production of rhamnolipids in Pseudomonads is quite difficult. In addition, many *P. aeruginosa* strains are pathogenic strains that are limited or not suitable for industrial applicatiion. *E. coli*, as generally recoginzed as safe (GRAS) microorgainsm, was comprehensively applied as engineered host strains for many bioproducts production, including proteins, enzymes, metabolites and other pharmaceuticals.

The type of rhamnolipid produced depends on the bacterial strain, the carbon source used, and the process strategy (Mulligan *et al*., 1993). The lengths of the fatty acid chains of rhamnolipids can vary significantly, resulting in a multitude of different rhamnolipd compositions. Fatty acyl chains composed of 8, 10, 12, and 14 carbons in length, as well as 12- or 14-carbon chains with double bonds. The different structures of rhamnolipids resulted in different properties with respect to interfacial tension, solubility, and charge (Zhang and Miller, 1995). For example, the interfacial tension between hexadecane and water was decreased to <0.1 mN/m by the dR-Me (a methyl ester form of rhamnolipids) but was only decreased to 5 mN/m by the dR-A (the acid form of rhamnolipids). Solubilization and biodegradation of two alkanes in different physical states, liquid and solid, were determined at dirhamnolipid concentrations ranging from 0.01 to 0.1 mM (7 to 70

mg/liter). The low product selectivity of rhamnolipids biosynthesis in bacteria showed that the key biosynthetic enzyme, here RhlAB, would function promiscuously (Khersonsky et al., 2006; James and Tawfik 2001). Many promiscuous functions of enzymes were proved to be susceptible to directed evolution. Here, in to order to improve the performance of rhamnolipids from *E coli* directed evolution of RhlAB was investigated.

From the biosynthetic pathway, the synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyltransferase (rhamnosyltransferase 1 or 2) with TDP-rhamnose acting as a rhamnosyl donor and 3-hydroxyalkanoyl-3-hydroxyalkanoate acting as acceptor. L-rhamnosyl-3- hydroxyalkanoyl-3-hydroxyalkanoate (Rha-C_m-C_n, m and n: 8, 10, 12, or 14) and L-Rhamnosyl-L- rhamnosyl-3-hydroxyalkanoyl-3-hydroxyalkanoate (Rha-Rha-C_m-C_n) were referred to as rhamnolipids 1 (mono-rhamnolipids) and 2 (di-rhamnolipds), respectively. Rhamnosyltransferase 1 is encoded by the RhlA and B (or RhlAB) genes, which are organized in an operon and responsible for biosynthesis of mono-rhamnolipds. The active enzyme complex is located in the cytoplasmic membrane, with the RhlA protein being localized in the periplasm and the catalytically active RhlB component crossing the membrane. Rhamnosyltransferase 2 is encoded by the RhlC gene that is located in an operon with an upstream gene (PA1131) of unknown in *P. aeruginosa* PAO1 and not organized with RhlAB.

According to the metabolic engineering methodology for engineering rhamnolipid-producing *E. coli* (Figure 7-5-1), various gene combinations were constructed (Figure 7-5-2) and transformed into *E. coli* BL21(DE3) to obtain the corresponding engineered strains which can produce either mono-rhamnolipids or dirhamnolipids. The corresponding engineered strains and control strain were designated *E. coli* ETRA, ETRAC, ETRAB, ETRABC, ETRhl, ETRhl-RC and ET30, respectively.

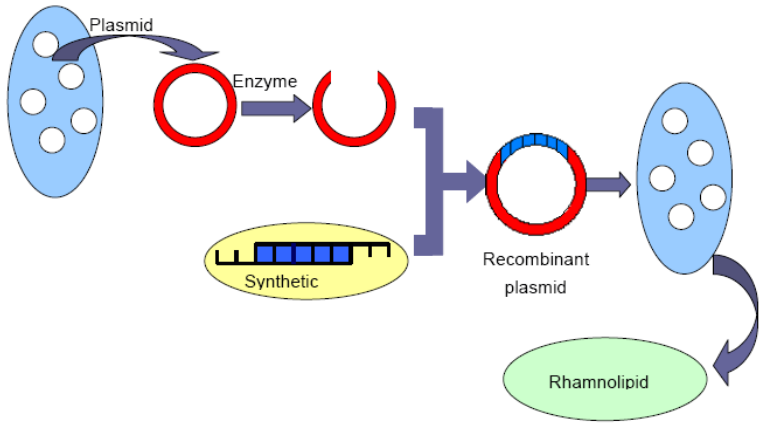
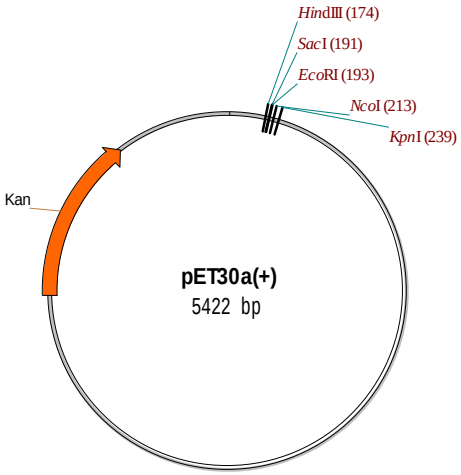


Figure 7-5-1. Diagram for Metabolic Engineering Methodology for Engineering Rhamnolipid-producing *E. coli*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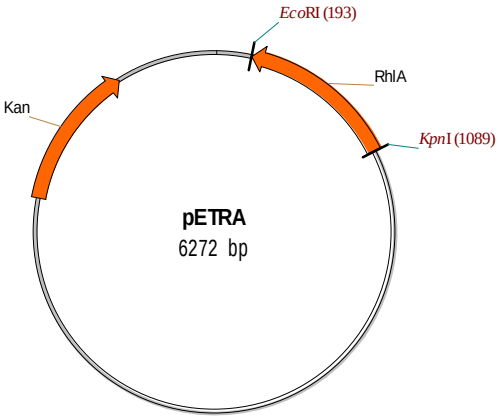
SacI (191)
EcoRI (193)
NcoI (213)
KpnI (239)
Kan
pET30a(+)
5422 bp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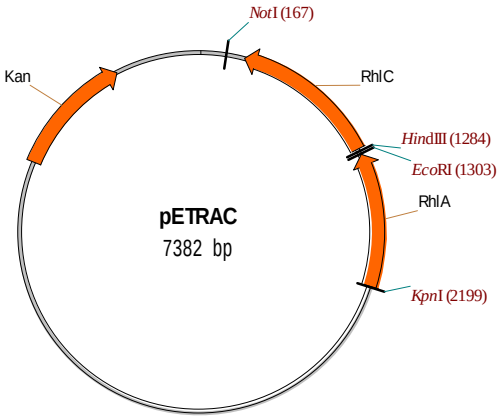
RhlA
Kan
KpnI (1089)
pETRA
6272 bp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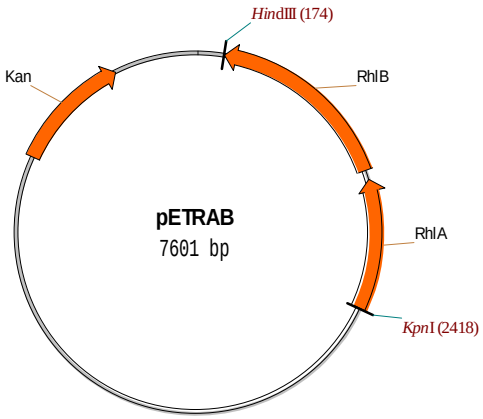
RhlC
Kan
HindIII (1284)
EcoRI (1303)
RhlA
pETRAC
7382 bp
KpnI (2199)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Kan
RhlB
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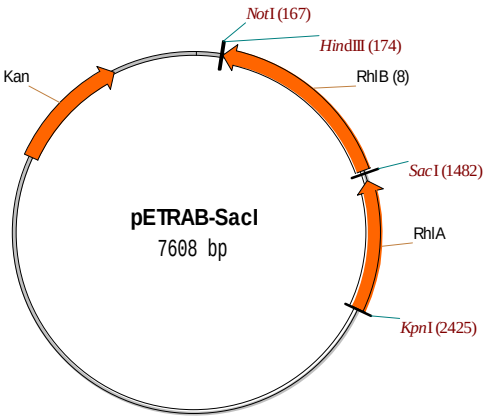
RhlA
KpnI (2418)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Kan
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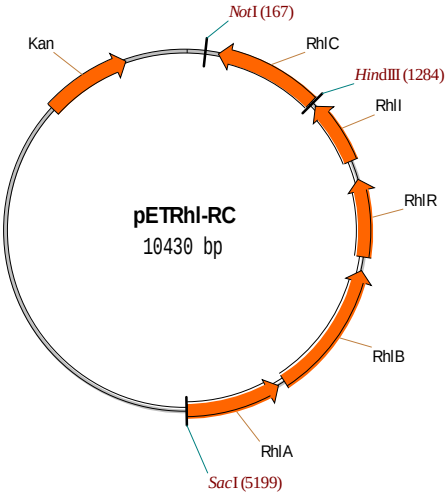
7608 bp
RhlA
KpnI (2425)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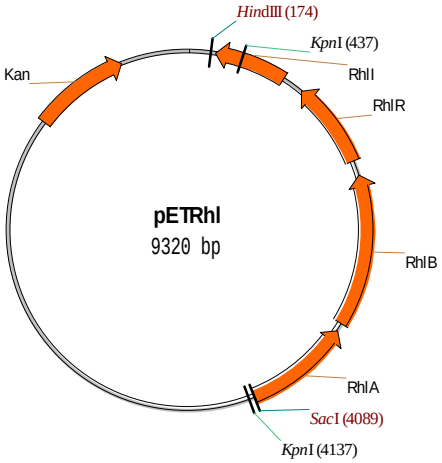
Kan
RhlC
HindIII (1284)
RhlI
RhlR
pETRhl-RC
10430 bp
RhlB
RhlA
SacI (5199)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KpnI (437)
Kan
RhlI
RhlR
pETRhl
9320 bp
RhlB
RhlA
SacI (4089)
KpnI (4137)

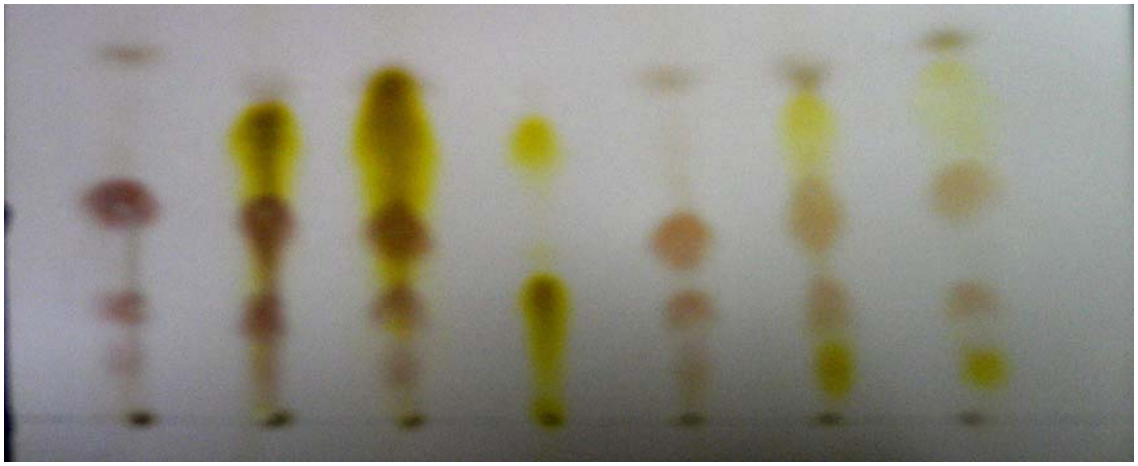
Figure 7-5-2 Plasmid map for engineering rhamnolipid-producing *E. coli.* Parental plasmids pET30a(+) and Recombinant expression plasmids

Preliminary fermentation of rhamnolids by engineered *E. coli* in rich (LB) or minimal (MS) media plus 0.4% glucose was carried out. The rhamnolipids yield and the IFT of their fermentative broth were shown in Table 8.

Table 8. Preliminary fermentation of rhamnolids by engineered *E. coli* in rich (LB) or minimal (MS) media

Strain	MS media		LB media	
	IFT(mN/m)	Rhamnolipid (mg/L)	IFT(mN/m)	Rhamnolipid (mg/L)
E. coli ET30	40.1	<2	35.0	<5
*E. col*i ETRA	5.8	75.0	0.015	190.0
*E. col*i ETRAB	0.49	115.0	0.24	199.5
*E. col*i ETRhl	0.76	125.0	0.30	231.5
*E. col*i ETRAC	5.2005	83.3	0.013	253.8
*E. col*i ETRABC	0.35	213.3	0.22	230.0
*E. col*i ETRhl-RC	0.39	185.0	0.32105	235.0

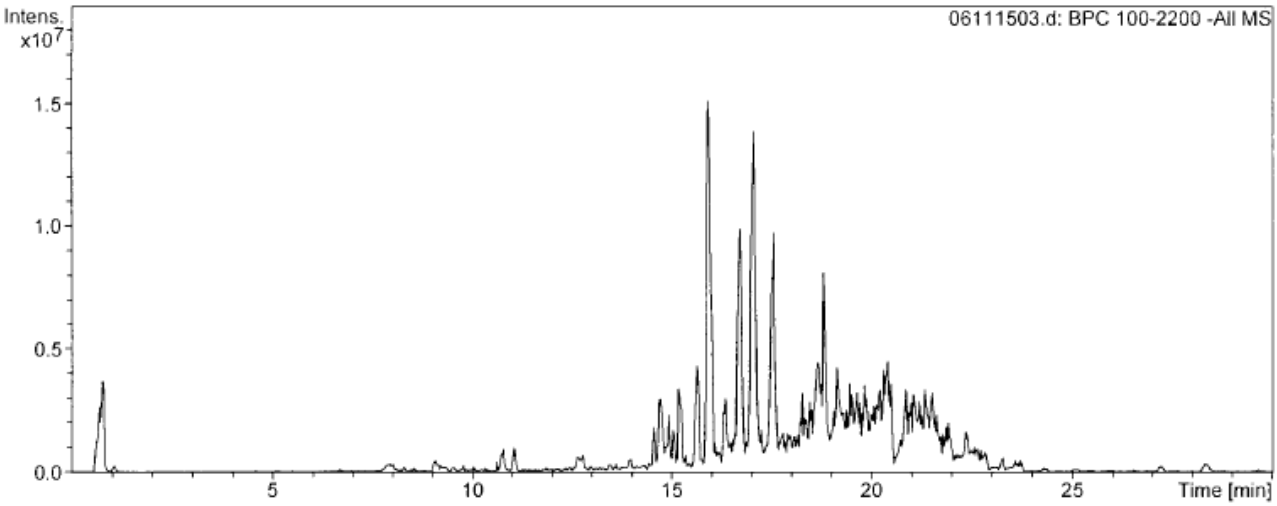
The IFT analysis and oil-spreading test indicate *E. coli* ETRA and *E. coli* ETRAC really produced some kind of biosurfactant, but TLC analysis showed (Fig 7-5-3) that they did not produce mono-rhamnolipids and/or di-rhamnolipids (yellow dots). However, they do produce other kinds of compounds (purple dots) which need to be confirmed, e.g., LC-MS. *E. coli* ETRAB and ETRhl produced mono-rhamnolipids, and *E. coli* ETRABC and ETRhl-RC produced mono-rhamnolipids and di-rhamnolipids (Figure 7-5-4). The ratio of di/mono-rhamnolipids were approximately 2:1~3:1.



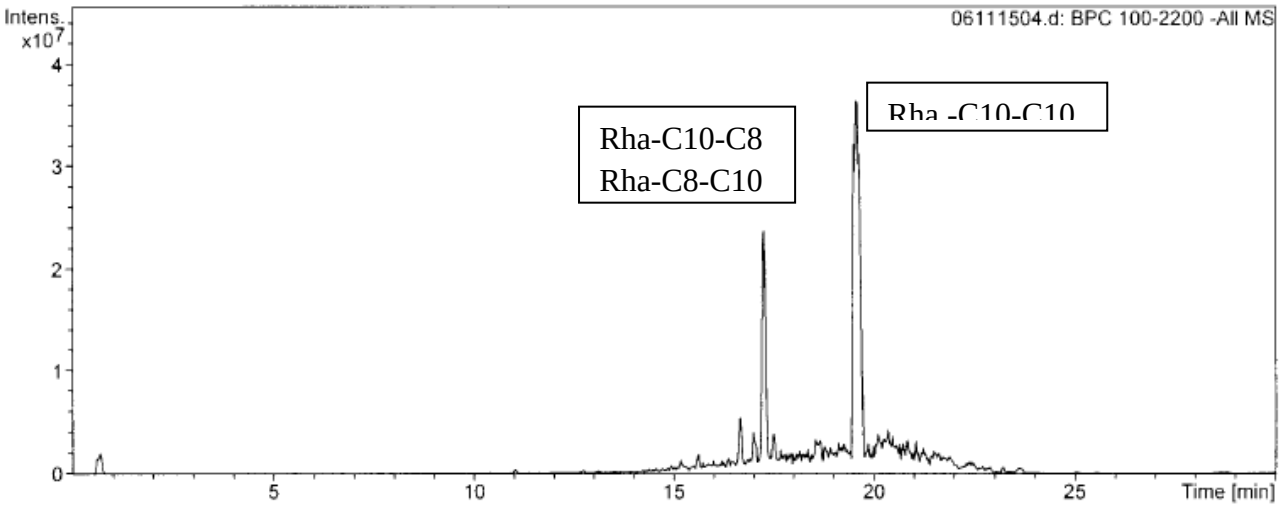
(a) (b) (c) (d) (e) (f) (g)

Figure 7-5-3. Thin layer chromatography (TLC) of rhamnolipids from engineered *E coli*. (a) ETRA; (b) ETRAB; (c) ETRhl; (d) Commercial rhamnolipids (as standard); (e) ETRAC; (f) ETRABC; (g) ETRhl-RC.

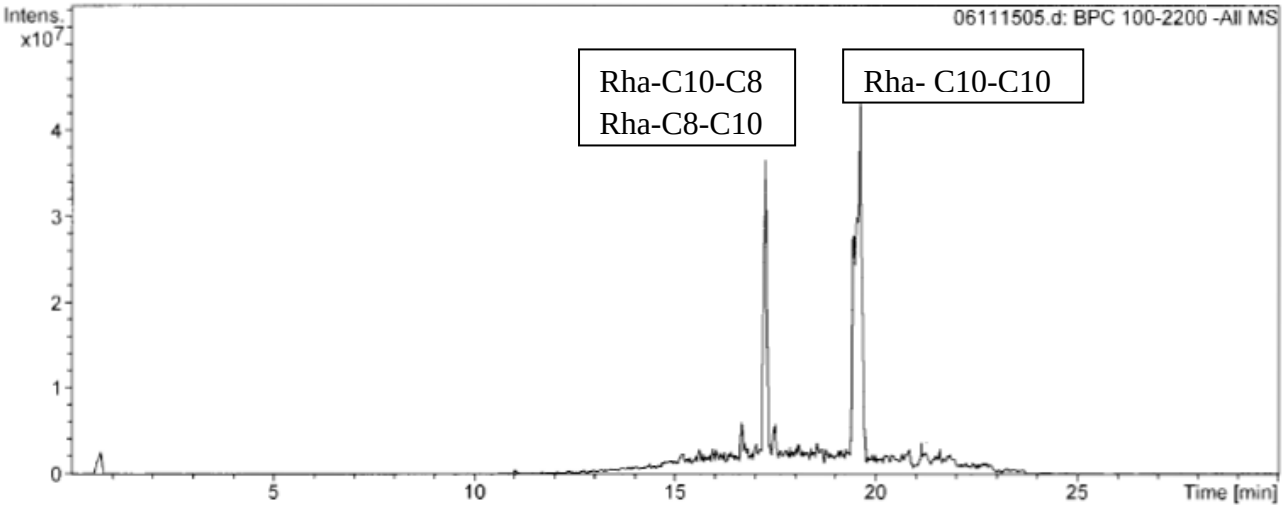
HPLC-MS analysis of these rhamnolipids further confirmed the structures of various surfactants from engineered strains (Figure 7-5-4). Note for the ETRA and the ETRAC that the peaks are much lower than the other 4 samples, plus their elution times are shorter. This further confirms that these 2 strains did not make rhamnolipid surfactants.



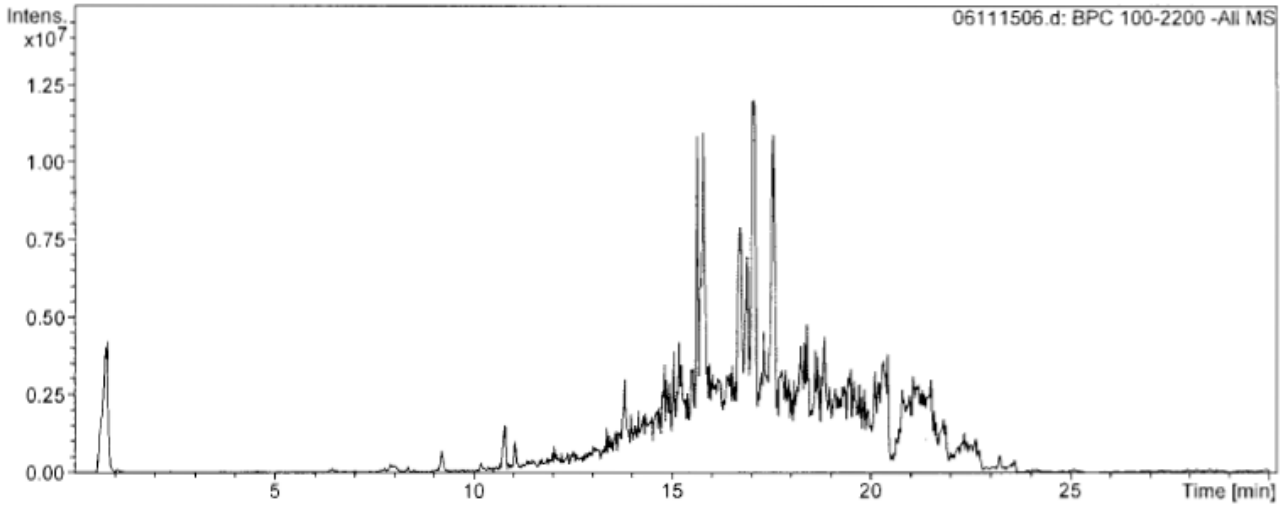
(a)


Intens.
x10^7
06111504.d: BPC 100-2200 -All MS
Rha-C10-C8
Rha-C8-C10
Rha -C10-C10
Time [min]

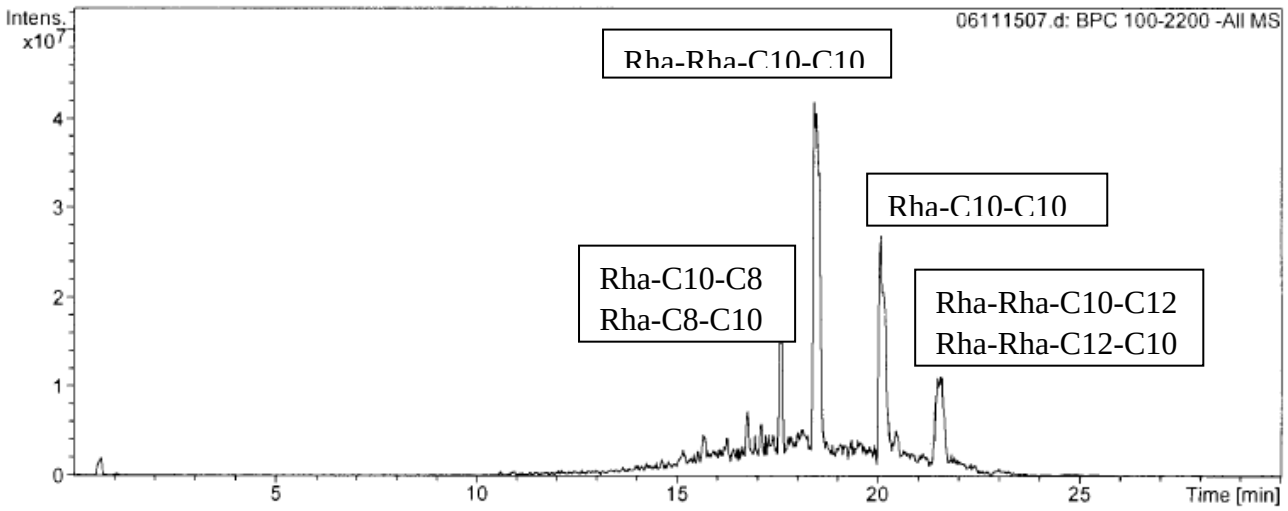
(b)


Intens.
x10^7
06111505.d: BPC 100-2200 -All MS
Rha-C10-C8
Rha-C8-C10
Rha- C10-C10
Time [min]

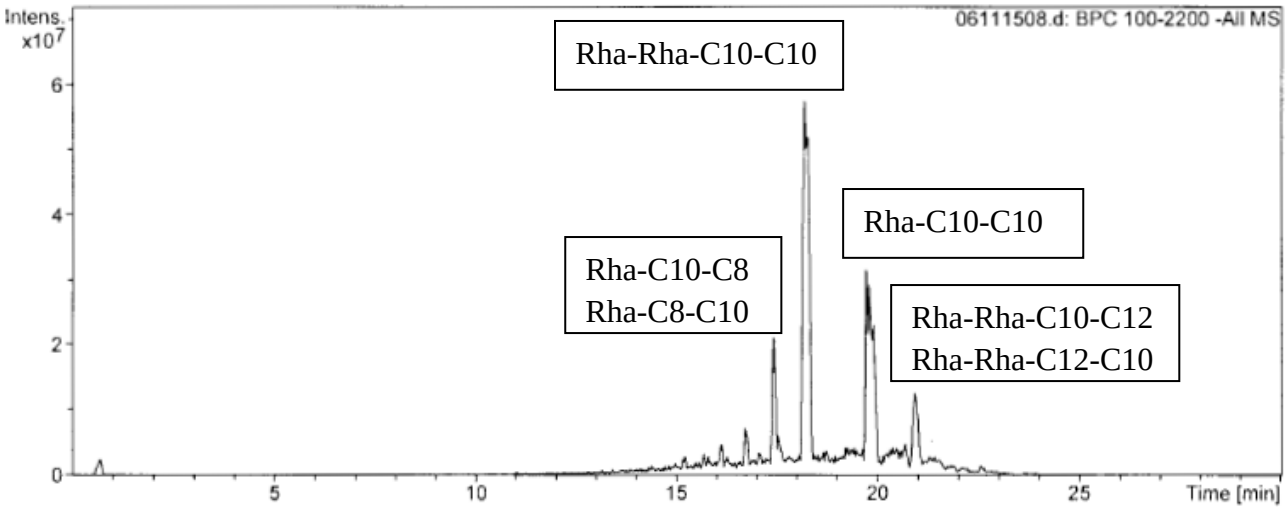
(c)


Intens.
x10^7
06111506.d: BPC 100-2200 -All MS
Time [min]

(d)



(e)



(f)

Figure 7-5-4. Base peak ion intensity chromatograms of rhamnolipids from various engineered *E. coli* strains. (a) ETRA; (b) ETRAB; (c) ETRhl; (d) ETRAC; (e) ETRABC; (f) ETRhl-RC. The main compositions are labeled in each chromatogram.

Table 9. Structure and relative abundance of the rhamnolipids from various engineered *E. coli*

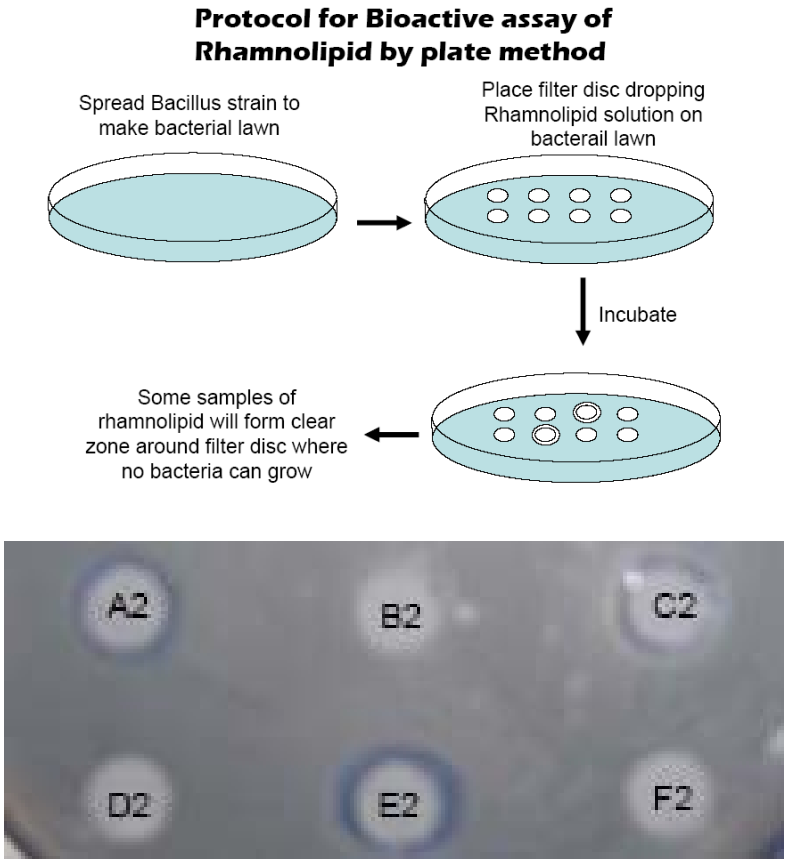
Strains	Rha-Rha-C10-C10 m/z 649	Rha-C10-C10 m/z 503	Rha-Rha-C10-C12 (or C12-C10) m/z 677	Rha-C8-C10 (or C10-C8) m/z 475
E. coli ETRA	-	-	-	-
E. coli ETRAB	-	73.1	-	26.9
E. coli ETRhl	-	69.7	-	30.3
E. coli ETRAC	-	-	-	-
E. coli ETRABC	45.2	28.7	15.0	6.0
E. coli ETRhl-RC	45.8	30.2	8.9	8.5

- denotes that it has no evidence of that chemical structure

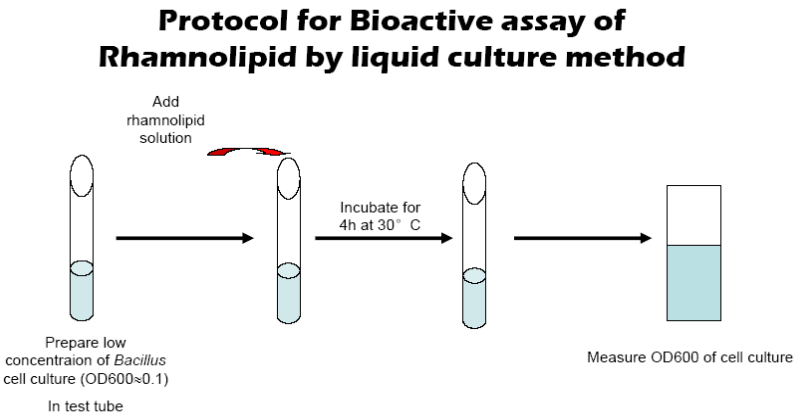
Directed evolution of RhlAB to improve rhamnolipids production in *E. coli*

Mutant RhlA and RhlB genes were produced by error-prone PCR with RhlA-21 (5'-AGTTGGTACCATGCGGCGCGAAAGTCTGTTGG -3') and RhlA-22 (5' TCAGGAGCTCTTATGCAACCGCAAAGCCCG-3'), and RhlB21 (5'-GACGGAGCTCAAG***AAGGAG***ATATACATATGCACGCCATCCTCATCGCCATC-3') and RhlB-22 (5' -AACCAAGCTTTCAGGACGCAGCCTTCAGCCATCG - 3'). Muant RhlA or RhlB PCR products were digested with the corresponding restrictione enzymes (KpnI and SacI for RhlA; SacI and HindIII for RhlB) and cloned into pETRAB-SacI to replace wild-type genes of RhlA or RhlB. The mutants plamids were transformed into *E. coli* BL21(DE3) to produce a mutant library.

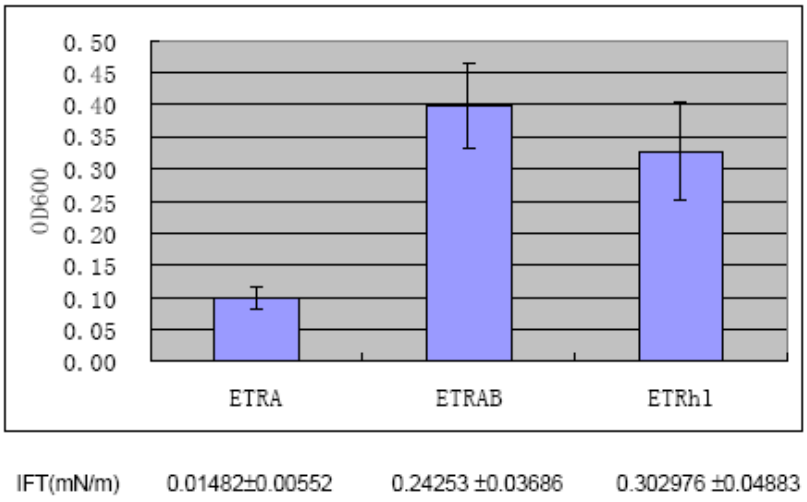
To screen the mutant strains which have desire properties, a robust high throughput screening method should be developed. Here, we focused on panning the mutants which have high performance of interfacial tension from *E. coli* mutant library. First, rhamnolipids from *E. coli* showed bioactivity according to the results of plate methods (Figure 7-5-5a). Filter disc containing rhamnolipids could effectively inhibit the growth of *Bacillus subtilis* cell and rhamnolipids with low interfacial tension have a larger clearing zone (Figure 7-5-5a, E2). Then, using a liquid culture method (Figure 7-5-5b, adding different rhamnolipids to diluted *Bacillus subtilis* cell culture), different rhamnolipids showed different the ability of inhibition (Figure 7-5-5c).


Protocol for Bioactive assay of
Rhamnolipid by plate method
Spread Bacillus strain to
make bacterial lawn
Place filter disc dropping
Rhamnolipid solution on
bacterail lawn
Incubate
Some samples of
rhamnolipid will form clear
zone around filter disc where
no bacteria can grow
A2
B2
C2
D2
E2
F2

(a)


Protocol for Bioactive assay of
Rhamnolipid by liquid culture method
Add
rhamnolipid
solution
Incubate for
4h at 30° C
Prepare low
concentraion of Bacillus
cell culture (OD600≈0.1)
In test tube
Measure OD600 of cell culture

(b)



IFT(mN/m)	0.01482±0.00552	0.24253 ±0.03686	0.302976 ±0.04883

(c)

Figure 7-5-5. Bioactive assay of rhamnolipids from *E. coli*. (a) Plate method; (b) Liquid culture method, and (c) results. This suggests a correlation of the plate results and IFT.

Based on the results from the plate method and liquid culture method of bioactive assay of rhamnolipids, a high throughput screening method for primary screening mutants which can produce rhamnolipids with high performance of interfacial tension was developed (Fig 7-5-6).

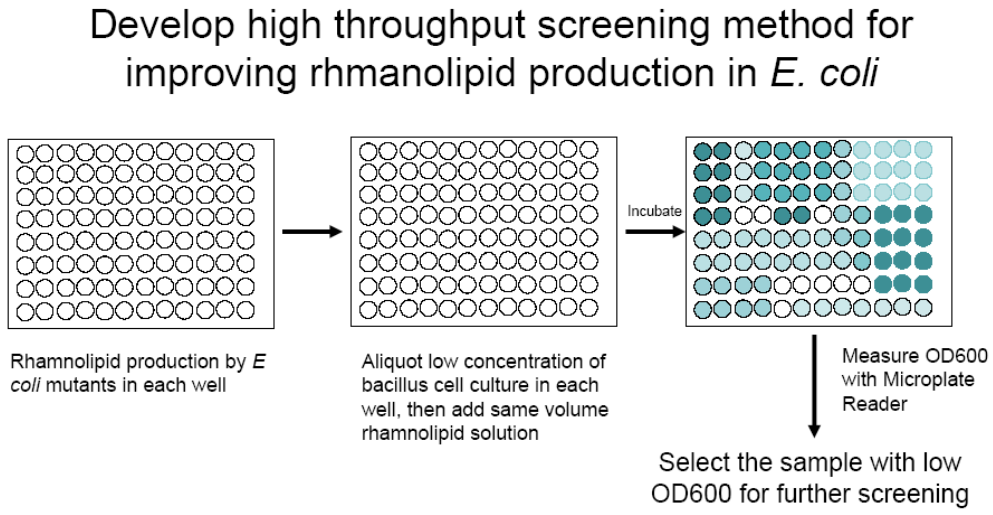


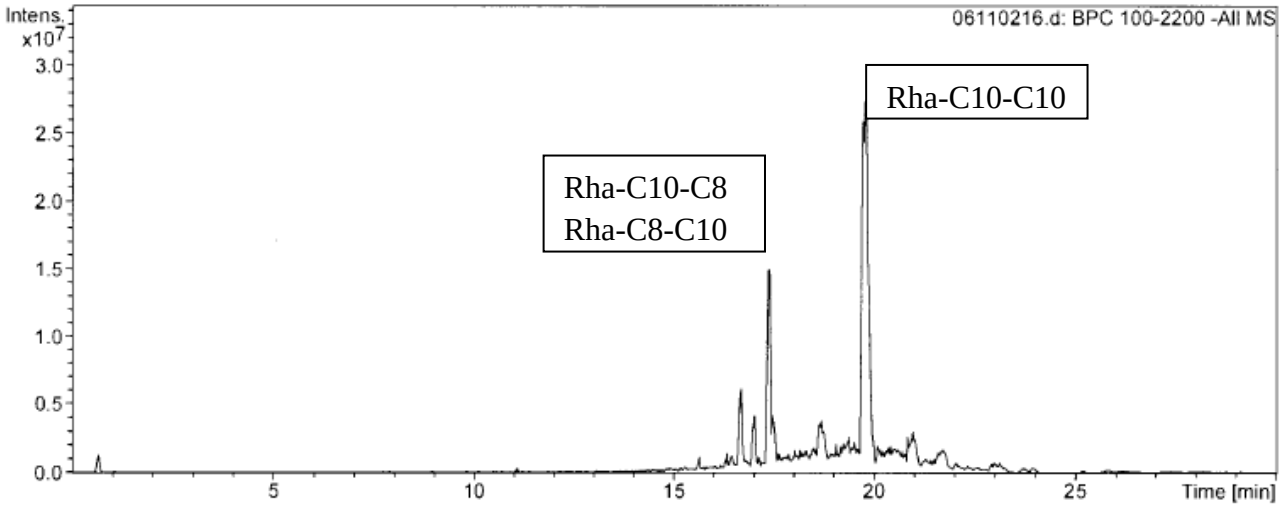
Figure 7-5-6. Developing high throughput screening method to identify candidates with improved rhamnolipids production in *E coli*.

Mutant rhamnolipids characterization

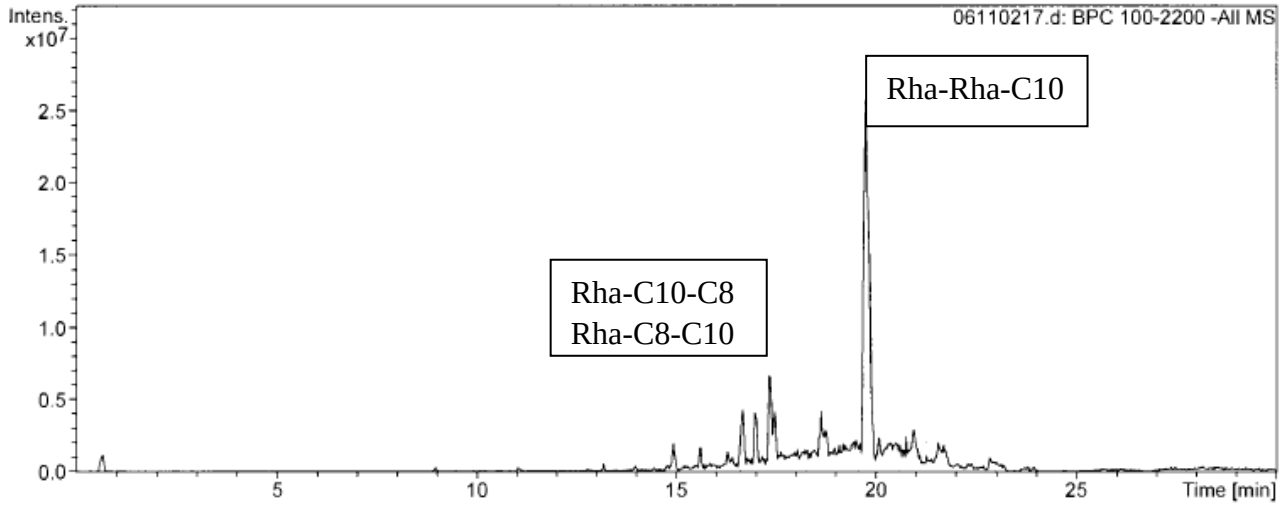
With this screening method, those mutants with the greatest inhibition for *Bacillus subtilis* cell growth were selected. Then, mutant rhamnolipids were further screened through IFT measurement and structure analysis by HPLC/MS. Finally, one mutant which has mutation in RhlB (L168P, Figure 7-5-7) was confirmed to have different properties in IFT behavior and structure components (Figure 7-5-8 and Table 10)

```
RHLB-WT      MHAILIAIGSAGDVFPFIGLARTLKLRGHRVSLCTIPVFRDAVEQHGIAF   50
RHLB-MUTANT  MHAILIAIGSAGDVFPFIGLARTLKLRGHRVSLCTIPVFRDAVEQHGIAF   50
Consensus    mhailiaigsagdvfpfiglartlklrghrvslctipvfrdaveqhgiaf
RHLB-WT      VPLSDELTYRRTMGDPRLWDPKTSFGVLWQAIAGMIEPVYEYVSAQRHDD  100
RHLB-MUTANT  VPLSDELTYRRTMGDPRLWDPKTSFGVLWQAIAGMIEPVYEYVSAQRHDD  100
Consensus    vplsdeltyrrtmgdprlwdpktsfgvlwqaiagmiepvyeyvsaqrhdd
RHLB-WT      IVVVGSLWALGARIAHEKYGIPYLSAQVSPSTLLSAHLPPVHPKFNVPEQ  150
RHLB-MUTANT  IVVVGSLWALGARIAHEKYGIPYLSAQVSPSTLLSAHLPPVHPKFNVPEQ  150
Consensus    ivvvgslwalgariahekygipylsaqvspstllsahlppvhpkfnvpeq
RHLB-WT      MPLAMRKLLWRCIERFKLDRTCAPEINAVRRKVGLETPVKRIFTQWMHSP  200
RHLB-MUTANT  MPLAMRKLLWRCIERFKPDRTCAPEINAVRRKVGLETPVKRIFTQWMHSP  200
Consensus    mplamrkllwrcierfk drtcapeinavrrkvgletpvkriftqwmhsp
RHLB-WT      QGVVCLFPAWFAPPQQDWPQPLHMTGFPLFDGSIPGTPLDDELQRFLDQG  250
RHLB-MUTANT  QGVVCLFPAWFAPPQQDWPQPLHMTGFPLFDGSIPGTPLDDELQRFLDQG  250
Consensus    qgvvclfpawfappqqdwpqplhmtgfplfdgsipgtplddelqrfldqg
RHLB-WT      SRPLVFTQGSTEHLQGDFYAMALRALERLGARGIFLTGAGQEPLRGLPNH  300
RHLB-MUTANT  SRPLVFTQGSTEHLQGDFYAMALRALERLGARGIFLTGAGQEPLRGLPNH  300
Consensus    srplvftqgstehlqgdfyamalralerlgargifltgagqeplrglpnh
RHLB-WT      VLQRAYAPLGALLPSCAGLVHPGGIGAMSLALAAGVPQVLLPCAHDQFDN  350
RHLB-MUTANT  VLQRAYAPLGALLPSCAGLVHPGGIGAMSLALAAGVPQVLLPCAHDQFDN  350
Consensus    vlqrayaplgallpscaglvhpggigamslalaagvpqvllpcahdqfdn
RHLB-WT      AERLVRLGCGMRLGVPLREQELRGALWRLLEDPAMAAACRRFMELSQPHS  400
RHLB-MUTANT  AERLVRLGCGMRLGVPLREQELRGALWRLLEDPAMAAACRRFMELSQPHS  400
Consensus    aerlvrlgcgmrlgvplreqelrgalwrlledpamaaacrrfmelsqphs
RHLB-WT      IACGKAAQVVERCHREGDARWLKAAS                         426
RHLB-MUTANT  IACGKAAQVVERCHREGDARWLKAAS                         426
Consensus    iacgkaaqvverchregdarwlkaas
```

Figure 7-5-7. Alignment of amino acid sequence of RhlB WT and Mutant H. Mutant H has a single mutant at position L168P.



(a)



(b)

Figure 7-5-8. Base peak ion intensity chromatograms of rhamnolipids from engineered *E. coli* ETRAB-SacI WT and Mutant strains. (a) WT; (b) Mutant H. The main compositions are labeled in each chromatogram. Note for practical purposes, the ETRAB-SacI WT microbe is the same as ETRAB.

Table 10. IFT, structure and relative abundance of the rhamnolipids from *E. coli* ETRAB-SacI WT and Mutant strains

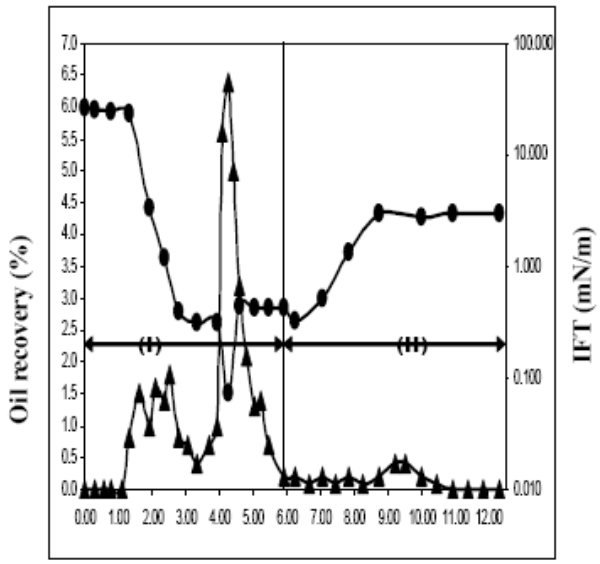
Strains	IFT (mN/m)*		Rha-C10-C10**	Rha-C10-C8	Rha-C8-C10
	pH7	pH8	m/z 503	m/z 475	m/z 475
WT	0.019	0.23	100	8.0	16.5
Mutant H	0.22	0.018	100	11.5	3.7

* rhamnolipids concentration is 100mg/L, ** Given the relative amount of the Rha-C10-C10 100

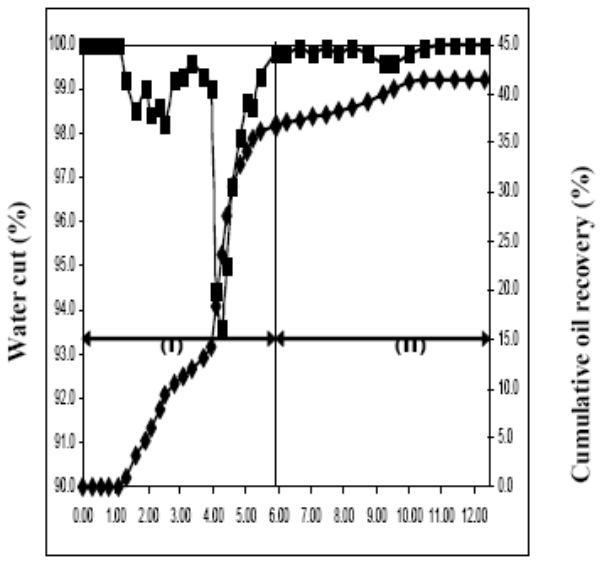
7.6 Core flooding test of rhamnolipid for enhanced oil recovery

7.6.1 Core flooding test of PEER02 rhamnolipid for enhanced oil recovery

After an oil well loses its self flow (primary recovery by natural forces), and secondary flow (secondary recovery by pressurization using water flood and gas), approximately 2/3 of the original oil remains in the reservoir. Much of the remaining oil can be recovered by enhanced oil recovery techniques such as polymer flooding or surfactant flooding. Effective emulsifying surfactants and a better understanding of the formation of their emulsions with the crude oil are of paramount importance to economically recover the abundant residual oil in reservoirs. Entrapment of petroleum hydrocarbon by capillary forces is a major factor that limits oil recovery. Hydrocarbon displacement can occur if interfacial tension between the hydrocarbon and aqueous phases is reduced by several orders of magnitude. Microbial-produced biosurfactants may be an economical method to recover residual hydrocarbons since they are effective at low concentration. The IFT analysis of our engineered rhamnolipids showed that they can reduce remarkably interfacial tension of oil (n-octane)/water at the certain combination of higher salinity and acidic pH. Based on these data from IFT analysis, sand-packed core flooding tests of rhamnolipids from *P. aeruginosa* with soybean oil as carbon source were performed. .



(a)



(b)

Figure 7-6-1. Oil recovery test of sand-packed core by rhamnolipids flooding with 240 ppm of PEER02. (a) Profile of oil recovery and IFT during flooding; (b) Water cut and cumulative oil recovery. (I) rhamnolipid flooding; (II) Brine flooding. Water cut: is the percent of water phase in the effluent.

Playground Sand purchased from Home Depot was packed into a 1" diameter by 2-foot long stainless steel tube to make a sand packed core. The sand pack was evacuated and saturated with brine. The brine composition and make-up brine for the rhamnolipid surfactant solution were the same as the pH5 buffer-2% NaCl brine – the conditions for minimum IFT. The pore volume and porosity of this core were calculated, and also brine permeability of this core (approximately 50 Darcies) was measured at different flow rates. Then, the brine was displaced with oil (n-Octane) in the core until no water (brine) comes out. The oil-saturated core was aged for 1 day at room temperature. After 1day aging, brine was injected to displace oil, and the oil production was recorded. Water injection continues until no oil comes out. Next, 3 PV of 250mg/L rhamnolipids solution was injected and then the core with rhamnolipids was shut in overnight at room temperature. On the next day, an additional 3 PV of 250mg/L rhamnolipids solution was injected, followed then by injection of 6 PV of the brine. The recovered oil was measured and oil recovery change with injected pore volume of brine/rhamnolipids solution was calculated (Figure 7-6-1a and 7-6-1b).

Two-stage rhamnolipids flooding gave two peaks of oil recovery (Figure 7-6-1a). The first 3 PV of rhamnolipids injection only gave a small amount of oil recovery, with a cumulative oil recovery of about 12% (Figure 7-6-1b). Then, another 3 PV of rhamnolipids was flooded following shut-in overnight. Then, a larger peak of oil recovery was noted (Figure 7-6-1a), and by the end of this stage, the cumulative oil recovery reached 37% (Figure 7-6-1b). After rhamnolipids injection and displacement by a 6 PV brine flush, the final cumulative oil recovery reached 42% (Figure 7-6-1b). The profile of IFT of the effluent shows IFT decreased with oil recovery. At the point of maximum oil cut, the lowest IFT was observed. Thereafter, with lower oil cut, IFT increased, and then maintained a relatively stable level. The subsequent brine flooding made IFT of the effluent increase yet further. This is because the concentration of rhamnolipids decreased with brine

flooding. Our flooding technology showed that overnight incubation of core with rhamnolipids would increase oil recovery effectively and reduce the water cut.

After an oil well loses its self flow (primary recovery by natural forces), and secondary flow (secondary recovery by pressurization using water flood and gas), approximately 2/3 of the original oil remains in the reservoir. Much of the remaining oil can be recovered by enhanced oil recovery techniques such as polymer flooding or surfactant flooding. Effective emulsifying surfactants and a better understanding of the formation of their emulsions with the crude oil are of paramount importance to economically recover the abundant residual oil in reservoirs. Entrapment of petroleum hydrocarbon by capillary forces is a major factor that limits oil recovery. Hydrocarbon displacement can occur if interfacial tension between the hydrocarbon and aqueous phases is reduced by several orders of magnitude. Microbial-produced biosurfactants may be an economical method to recover residual hydrocarbons since they are effective at low concentration. The IFT analysis of our engineered rhamnolipids showed that they can reduce remarkably interfacial tension of oil (n-octane)/water at the certain combination of higher salinity and acidic pH. Based on these data from IFT analysis, sand-packed core flooding tests of rhamnolipids from *P. aeruginosa* with soybean oil as carbon source were performed. .

7.6.2 Sand-packed Core Flooding Tests Showed that only Strains Produced Rhamnolipids that were Detected by LC-MS can Recover Oil Effectively

The fermentation products of the each engineered E.coli strains were adjusted to 250ppm based on oil spreading assay and their optimal pH which gives the lowest IFT value. Each of those solutions was then tested for its oil recovery capability by the Sand-packed Core Flooding experiments and the results were summarized in Figure 7-6-2 to Figure 7-6-3. The strains ETRhl, ETRABC and ETRhl-RC, which can produce rhamnolipids according to table 2 in Section 3.4, could effectively recover 24-45% of the remaining oil after water flooding. While the products of the strains ETRA and ETRAC, which did not produce LC-MS detectable rhamnolipids, could not recover oil effectively. Thus, although those two strains produced some surface-active products that gave low IFT values, they could not recover oil nearly as well as the strains that produced either mono or di-rhamnolipid biosurfactants. Apparently, the oil recovery efficiency is closely correlated to the IFT

values (Figure 7-6-3). Interestingly, strain ETRAB could produce rhamnolipids in good quantity, but it recovered least amount of oil among the six E.coli strains. It seemed that the high adsorption of the rhamnolipids produced by ETRAB could be the contributing factor of its low oil recovery (Figure 7-6-4). Summary of the surfactant adsorption of the fermentation products of the six E.coli strains.). Further experiments need to be conducted to investigate the difference between ETRAB and other stains that contain rhamnosyltransferase-B gene.

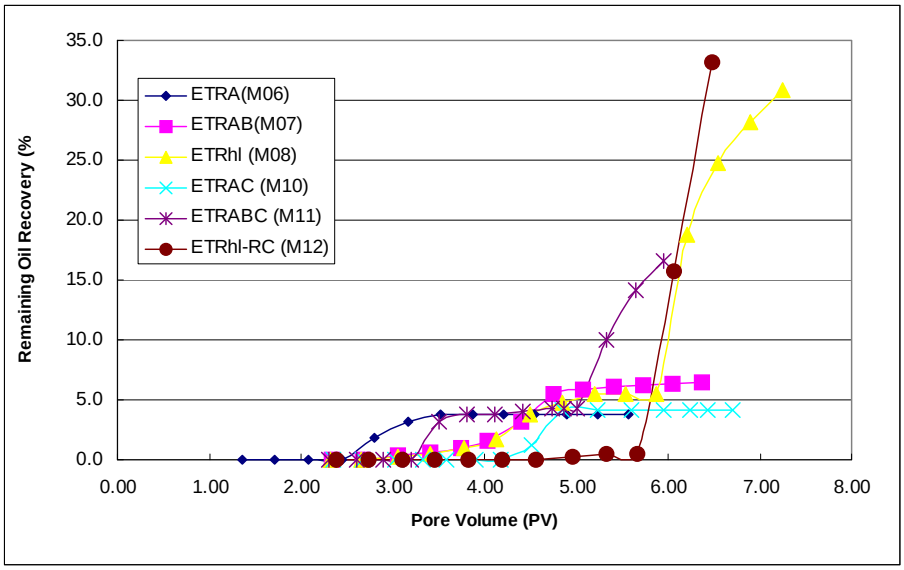


Figure 7-6-2. Sand-packed core flooding tests of the rhamnolipids produced by six engineered E.coli strains. Rhamnolipids were adjusted to 250ppm and optimal pH values based on the IFT tests. Blue diamonds – ETRA; Pink Squares- ETRAB; Yellow triangles – ETRhl; Light blue crosses – ETRAC; Brown stars – ETRABC and Brown circles – ETRhl-RC.

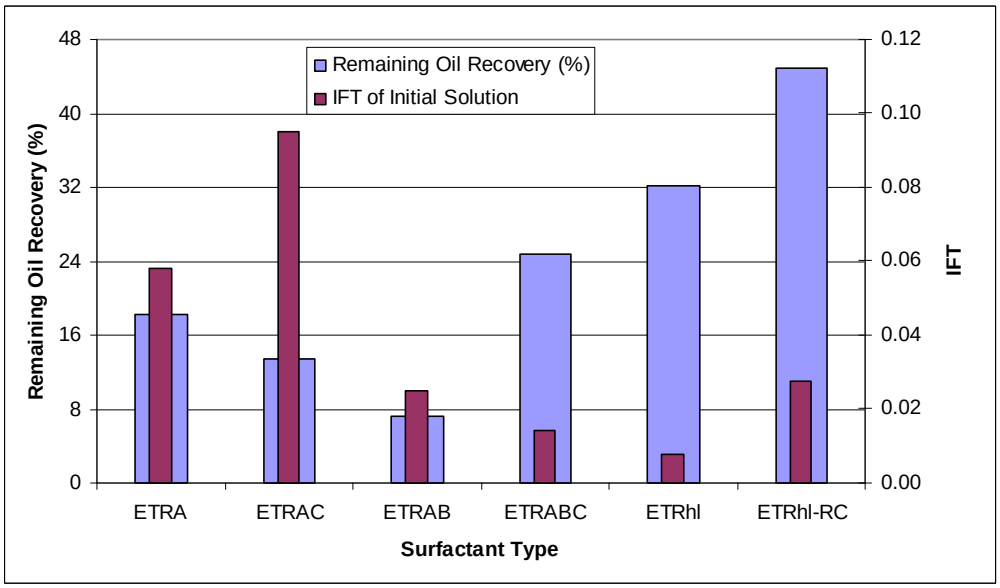


Figure 7-6-3. Summary of the remaining oil recovery and IFT of the initial rhamnolipids injected of the fermentation products of the six E.coli strains.

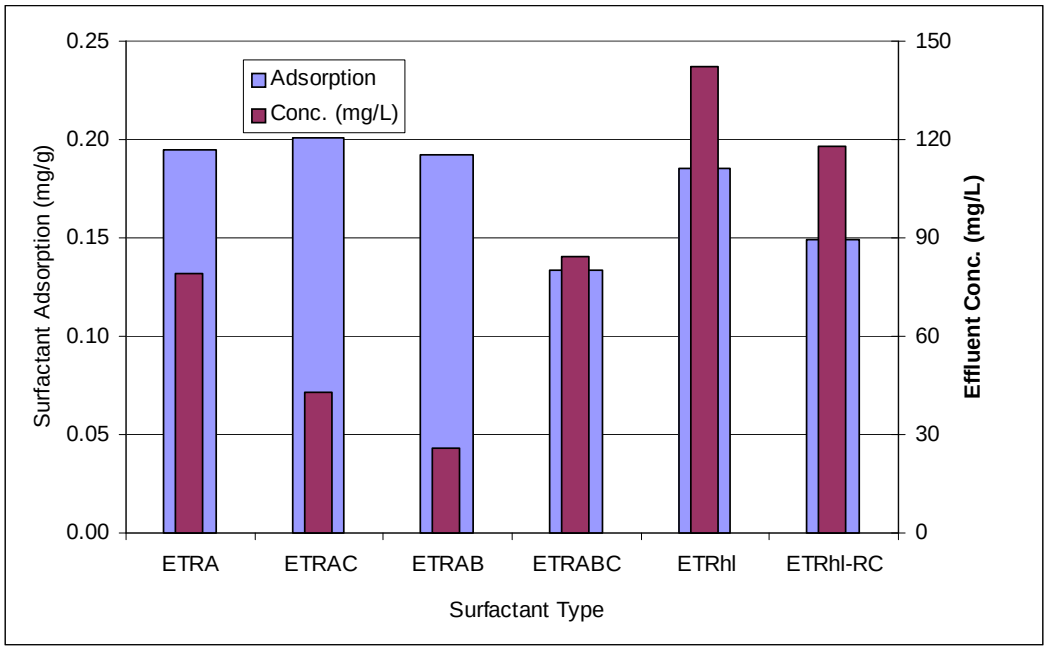


Figure 7-6-4. Summary of the surfactant adsorption of the fermentation products of the six E.coli strains.

8. Research work during the no-cost extension period (Year 3)

Rhamnolipids, as the extensively studied biosurfactants, are a subclass of glycolipids and mainly produced from *Pseudomonas aerugina.* Besides the comprehensive application in environmental engineering, rhamnolipids also have potential application in medicine, such as from their antimicrobial activity and anti-adhesive activity. Rhamnolipids production in Pseudomonads is involved in the process of quorum sensing with complex regulation and quite difficult for optimization. In addition, many *P. aeruginosa* strains are pathogenic strains that are limited or not suitable for industrial development. Here, through the methodology of synthetic biology and metabolic engineering, *E. coli* was designed to produce mono-rhamnolipids by introducing the key genes of rhamnolipid biosynthesis, *RhlAB.*

Usually, the type of rhamnolipids produced depends on the bacterial strain, the carbon source used, and the process strategy (Mulligan et al., 1993). Due to the structure diversity of rhamnolipid, here, we used directed evolution technology to improve rhamnolipid production in engineered *E. coli*.

Using high-throughput screening method based on bioactive assay a mutant with different product selectivity was found. Oil recovery test was carried with rhamnolipid samples from wild type strain and mutant at different condition to evaluate the performance the different rhamnolipid samples.

8.1 Plasmid-based engineered bacteria were constructed for rhamnolipid production

Rhamnolipids production in Pseudomonads is a part of quorom sensing process, which is complicately regulated. Thus, optimizing production of rhamnolipids in Pseudomonads is quite difficult. In addition, many *P. aeruginosa* strains are pathogenic strains that are limited or not suitable for industrial applicatiion. *E. coli*, as generally recoginzed as safe (GRAS) microorgainsm, was comprehensively applied as engineered host strains for many bioproducts production, including proteins, enzymes, metabolites and other pharmaceuticals. According to the metabolic engineering methodology for engineering rhamnolipid-producing *E. coli*, RhlAB genes were constructed under T7 promoter (Figure 8-1-1) and transformed into *E. coli* BL21(DE3) to obtain the corresponding plasmid-based engineered strain *E. coli* ETRAB which can produce either mono-rhamnolipids (Table 11). Preliminary fermentation of rhamnolids by engineered *E. coli* in minimal (MS) media plus 0.4% glucose was carried out (Table 11). The *E. coli* ET30 containing pET30a(+) was a control strain. For the subsequent experiment of directed evolution, we also introduce a site of SacI between RhlA and RhlB. Thus, we obtained another rhamnolipid-producing strain *E. coli* ETRAB-SacI. The rhamnolipids from this strain is same as that from *E. coli* ETRAB (Table 11)

Table 11 Production, structure and relative abundance of the rhamnolipids from various plasmid-based engineered *E. coli*

Strains	Rhamnolipid (mg/L)	Type of Rhamnolipid	Rha-Rha-C10-C10 m/z 649	Rha-C10-C10 m/z 503	Rha-Rha-C10-C12 (or C12-C10) m/z 677	Rha-C8-C10 (or C10-C8) m/z 475
E. coli ET30	ND	-	-	-	-	-
E. coli ETRAB	115.0±14.2	Mono-	-	73.1	-	26.9
E. coli ETRAB-SacI	120.0±15.1	Mono-	-	69.7	-	30.3

ND: can't detect. The media for rhamnolipid production is minimal salt plus 0.4% glucose. The fermentation condition is 30°C and 24h with shaking. LB media was the media for seed culture.

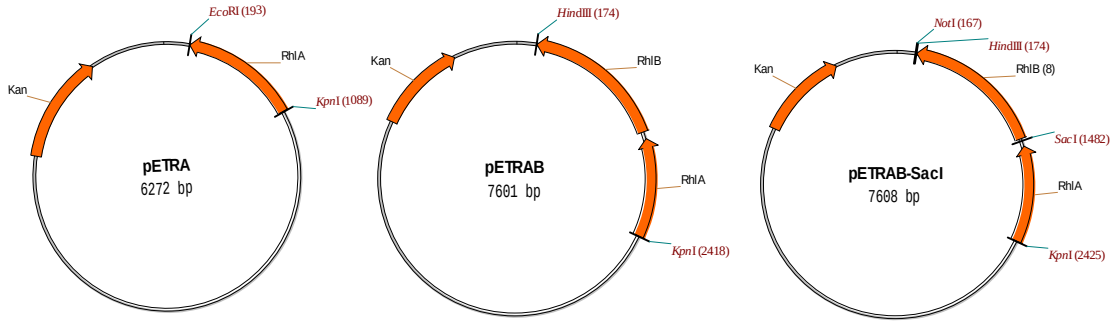


Figure 8-1-1. Plasmid map for engineering rhamnolipid-producing *E. coli.* Parental plasmid is pET30a(+). *RhlA*: Rhamnosyltransferase Gene A; *RhlB*:Rhamnosyltransferase Gene B.

8.2 Oil recovery test of rhamnolid from engineered strain with Limestone core in Amott cell

From the result of directed evolution for improved rhamnolipid production, we have found the rhamnolipid from a mutant which showed different IFT performance at different pH compared with that from wild-type strain (Table 12). Our final goal to engineering strains for improving rhamnolipid is to apply them in EOR. To evaluate the performance of our rhamnolipid samples, we designed a simple oil recovery test with limestone core at Amott cell (Figure 8-2-1). Briefly, the 1×2 inch cores (a slab of limestone obtained by New Mexico Travertine) were at 120°C for hours to remove adsorbed moisture, vacuumed for 4 hours to remove the air inside of core, and saturated with n-octane containing 0.05% red ink dye overnight. The cores were weighed before and after oil saturation, and the amount of adsorbed oil in the cores were recorded. Then, the n-octane-saturated cores were placed in Amott cell containing 200ppm rhamnolipid solution in 50 mM Citrate-Na_2HPO_4 buffer, pH7 or pH8, and the oil was measured that came out the volumetric burette of Amott cell (As the aqueous phase imbibes into the core, oil expelled and captured in the volumetric burette. The Amott cell was maintained at room temperature and the oil recovery was monitored versus time (Figure 8-2-1).

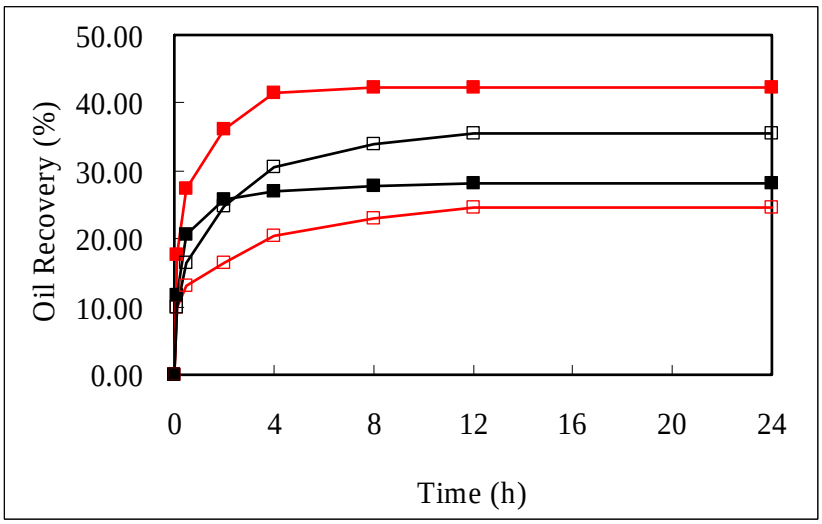


Figure 8-2-1. Oil recovery test with Limestone core at Amott cell. Open dark square: wild-type sample at pH7; Open red square; mutant sample at pH7; Filled dark square: wild-type sample at pH8; Filled red square: mutant sample at pH8.

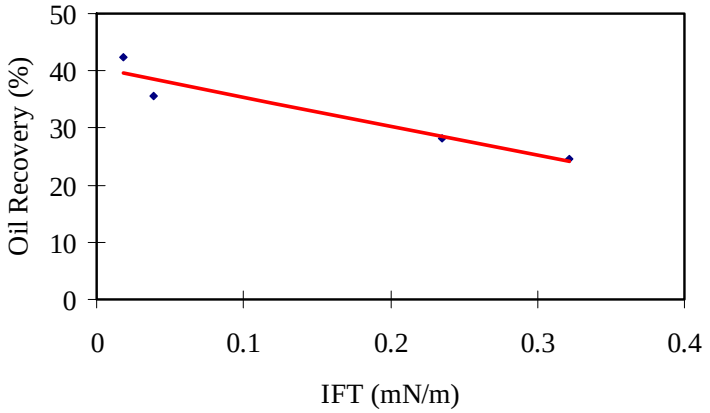


Figure 8-2-2. The relationship of IFT and Oil recovery from Limestone core at Amott cell.

From the results of oil recovery test with limestone core at Amott cell, we found that if the rhamnolipid showed lower IFT at the test condition, higher oil recovery was reached. For example, at pH7, the rhamnolipid sample from wild-type strain *E. coli* ETRAB-SacI WT had the lower IFT of 0.039mM/m, and the oil recovery in this condition at Amott cell was 35.6% after 24h. At pH8, the sample from mutant strain H had the lower IFT of 0.019mN/m, and oil recovery 42.2%. From these four oil recovery tests, we made a plot of the relationship of IFT and oil recovery (Figure 8-2-2). It seems that a linear trend was presented. The lower IFT is, the more oil is recovered by imbibition.

8.3 Global transcriptional machinery engineering for improving rhamnolipid production in *Escherichia coli*

Engineering transposon-based E. coli for rhamnolipid production

From the biosynthetic pathway (Figure 8-3-1), the synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyltransferase with TDP-rhamnose acting as a rhamnosyl donor and 3-hydroxyalkanoyl-3-hydroxyalkanoate acting as acceptor. L-Rhamnosyl-L-rhamnosyl- 3-hydroxyalkanoyl 3-hydroxyalkanoate and L-rhamnosyl-3-hydroxyalkanoyl-3- hydroxyalkanoate, referred to as rhamnolipids 1 (mono-rhamnolipids) and 2 (di-rhamnolipds), respectively, are the principal glycolipids produced in liquid cultures. Rhamnosyltransferase 1 is encoded by the *rhlAB* genes, which are organized in an operon and responsible for biosynthesis mono-rhamnolipds. The active enzyme complex is located in the cytoplasmic membrane, with the RhlA protein being localized in the periplasm and the catalytically active RhlB component crossing the membrane (Ochsner et al., 1994).

RhlAB was the key enzyme of rhamnolipids biosynthesis, but this biosynthesis was modulated by the complex transcriptional regulatory network in *P. aeruginosa* (Soberon-Chavez and Aguirre-Ramirez, 2005). However, to achieve the rhamnolipids production in the strains which can not produce rhamnolipids, integrating RhlAB is indispensable. In this circumstance, convenient molecular biotechniques would give good success. Here, we show an improvement via the state-of-the-art transposome-mediated chromosomal integration to modify metabolic pathway of rhamnolipid biosynthesis.

Briefly, RhlAB coding region which was fused with T7 promoter and selection maker gene Cm (chloramphenicol resistance gene) were cloned into Tn5 derived transposon plamid pMOD-2 (Epcentre Biotechnologies, Madison, WI) to produce the recombinant plasmid pMOD-2CRABb (Wang et al., 2007). pMOD-2CRABb was digested with PshAI to obtain chimeric transposon TnRABb (Figure 8-3-1a) which can bind transposase to produce transposome TnRABbsome (Figure 8-3-1b). This new transposon TnRAB was inserted into the chromosome of *E. coli* BL21(DE3) by transposome-mediated integration to engineer strain *E. col*i TnERAB which was also confirmed by PCR (data not shown). With IPTG induction, *E. col*i TnERAB can produce

rhamnolipid both in rich (LB) and minimal (MS) media and glucose as substrate. Because *E. coli* can grow better in LB media than minimal media, rhamnolipid yield is higher in LB media (Table 12).

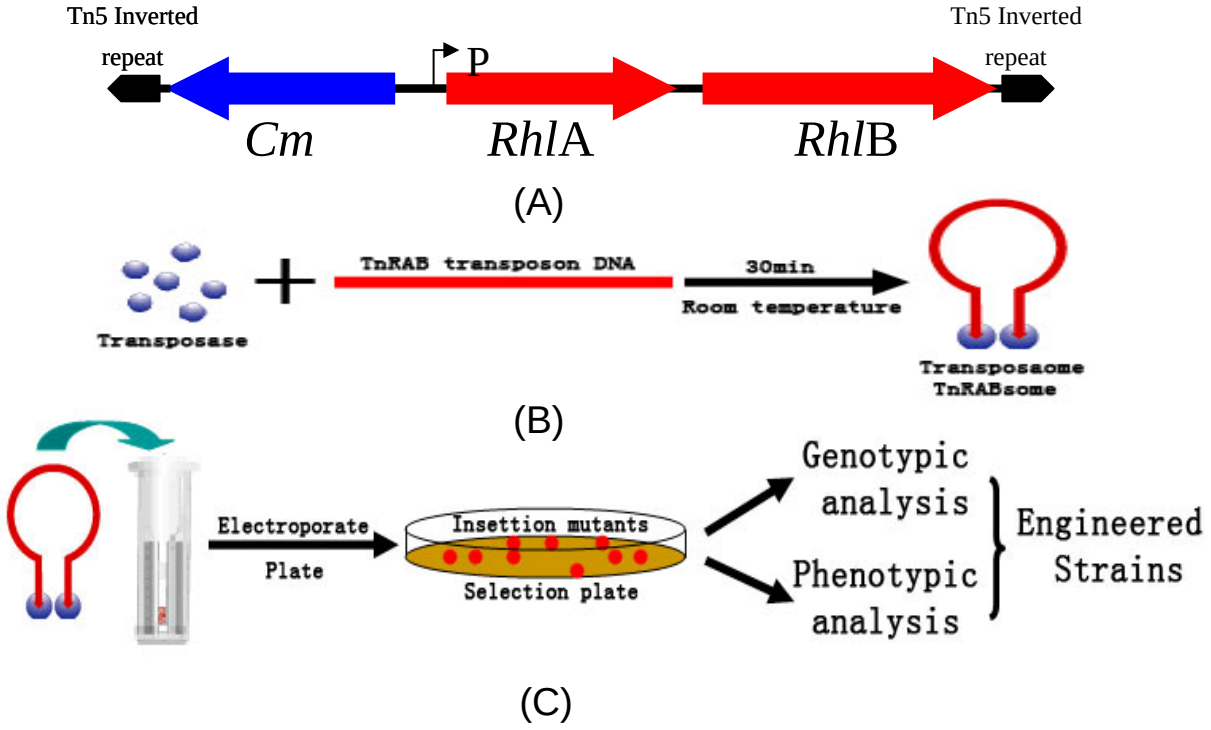


Figure 8-3-1. Diagram of Engineered Rhamnolipids-producing *E. coli* Construction (a) Structure of rhamnolipid biosynthetic gene RhlAB-containing Transposon TnRAB); Cm: chloramphenicol resistance gene cassette; P: T7 promoter; (b) Transposome TnRABsome construction; (c) Transposome eletroevaporation and insertion mutants selection.

Table 12 Rhamnolipid fermentation by engineered *E. coli* in rich or minimal media

Stains	Rhamnolipid (mg/L)	
	LB plus 0.4% glucose	MS plus 0.4% glucose
E. coli BL21(DE3)	ND	ND
*E. col*i TnERAB	175.3±13.2	75.6±11.2

ND: can't detect

Global transcriptional machinery engineering for improving rhamnolipid production

Prior efforts in metabolic engineering have attempted to improve strain properties through the modification of components of localized pathways and certainly using gene-by-gene strategies. To this end, many successful examples have been reported that were based on re-designing regulatory networks, metabolite balancing systems, and rational or combinatorial gene deletion and amplification approaches. However, these approaches lack a cell-wide perspective and mostly fail in eliciting desired phenotypes dependent on *simultaneous multiple gene* modifications. A central reason is the vast size of the space of possible combinations of multiple-gene modifications combined with limited transformation capacity needed to probe this space. As a result, a huge fraction of the phenotype space that depends on multiple gene interactions has been largely unexplored.

Here, we present a method that departs from the traditional gene-phenotype mapping approach and regards the phenotype as the manifestation of a particular transcriptional profile. Hence, the method attempts to elicit new phenotypes by manipulating directly the transcriptome of a cell through engineering of specially selected global regulators. This approach allows modulation of simultaneous multiple gene expression at the highest level with profound implications for phenotype improvement of prokaryotic and eukaryotic cells alike. Specifically, we modify the sigma factors σ^{70} (RpoD) of *Escherichia coli* to elicit a possible multigenic reprogramming of the transcriptome using an approach termed global Transciption Machinery Engineering (gTME). RpoD with native promoter was amplified by error-prone PCR, digested with NheI/HindIII, and cloned into low-copy-number plasmid pACYC177. The recombinant plasmids were transformed rhamnolipid-producing strain *E. col*i TnERAB to create a mutant library. The mutants were applied for screening after plating in the blood agar (Figure 8-3-2). The mutant colonies which formed the largest clearing zone on blood agar were picked out and followed to further analysis of rhamnolipid production by fermentation. After two rounds of screening with blood agar and fermentation, a mutant with enhanced yield was found. This mutant (*E. col*i TnERAB-45II harboring pArpoD-45II) can produce 263.9mg/L rhamnolipid in LB plus 0.4% glucose at 30°C for 24 hours. Compared with the rhanmolipid yield of wild type strain *E. col*i TnERAB-WT harboring pArpoD-WT, the yield of mutant strain has 1.5-fold higher in the same fermentation condition.

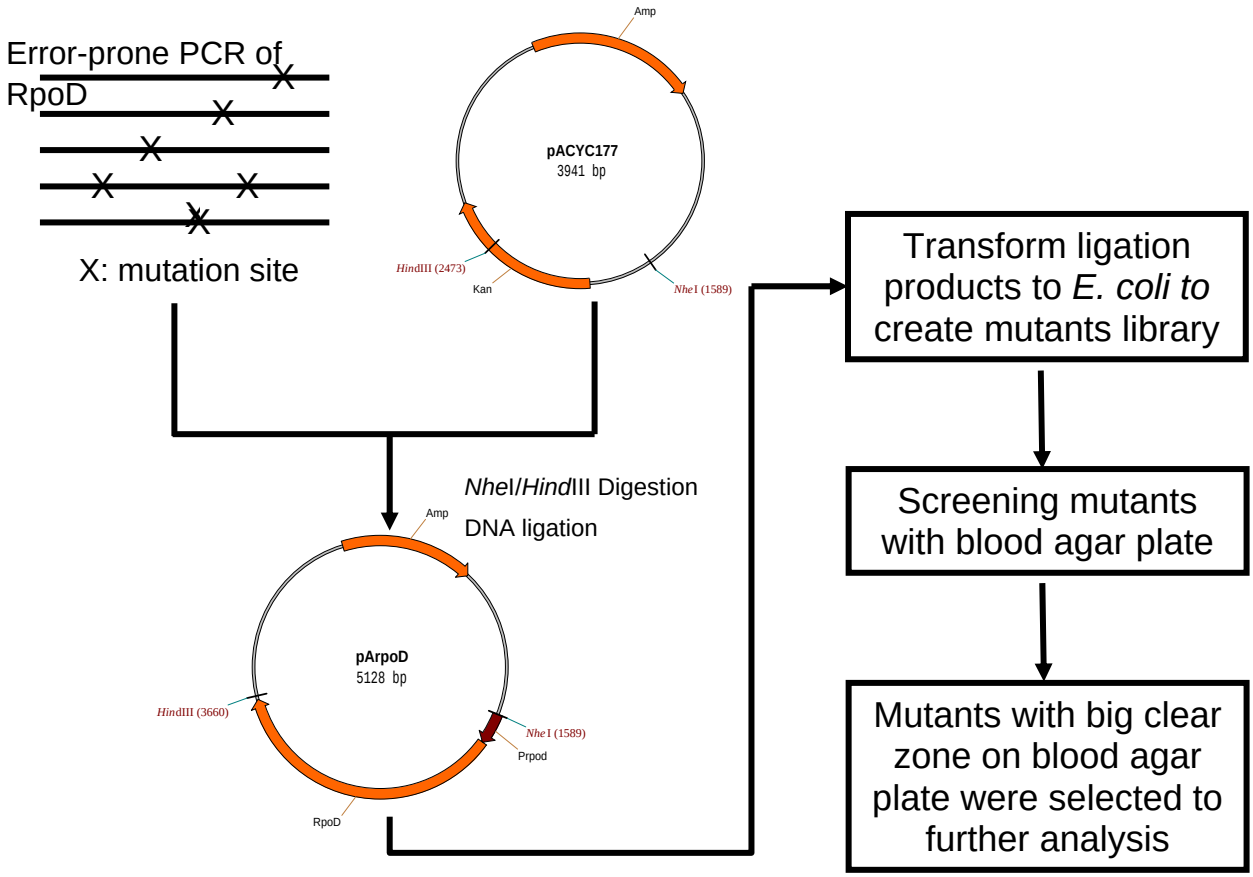


Figure 8-3-2. Construction of mutant library of RpoD by error-prone PCR.

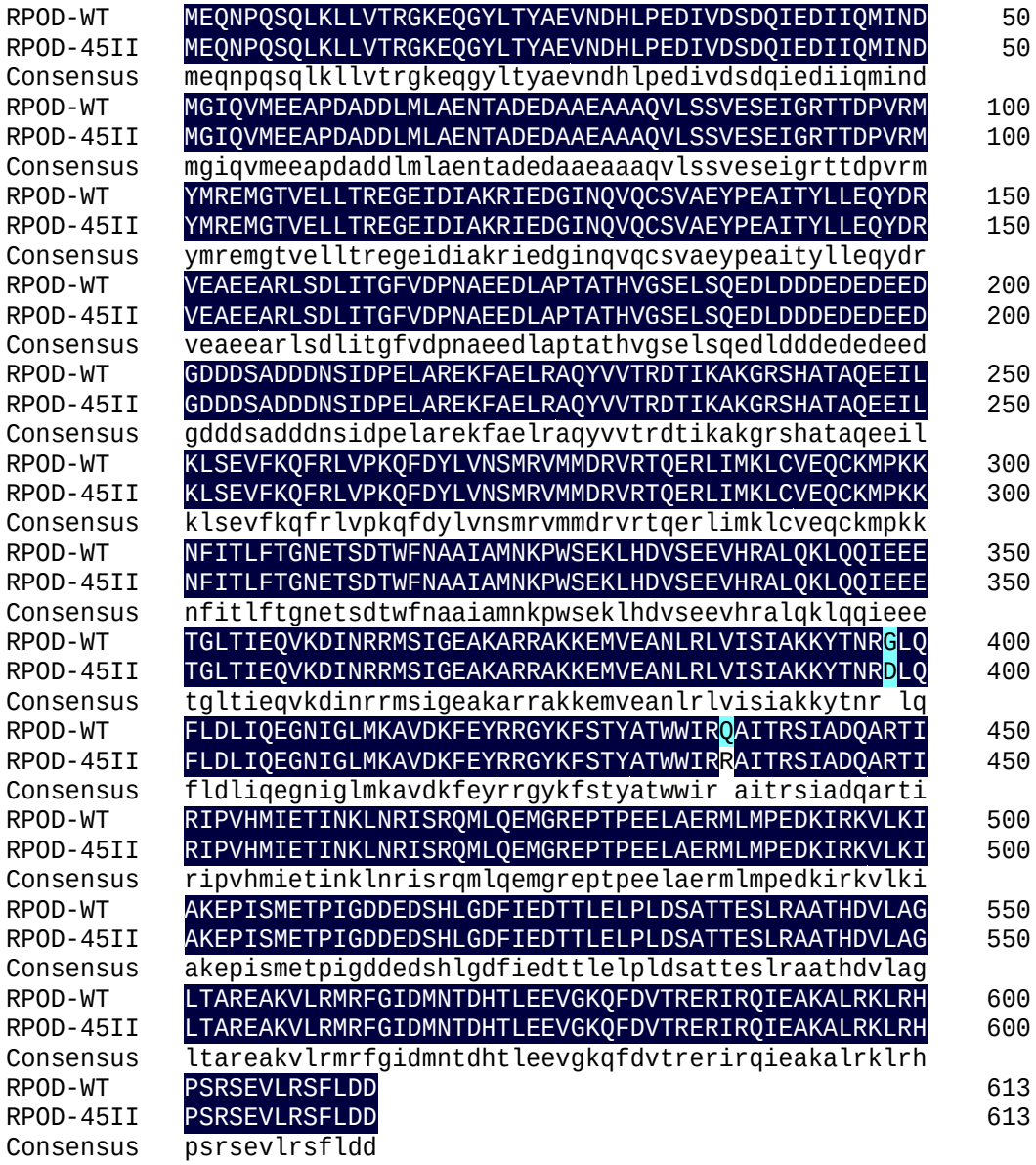


Figure 8-3-3. Alignment of amno acid sequence of RpoD WT and Mutant 45II. Mutant H have two-point mutation, G398D and Q437R.

Aligning the protein sequence of wild-type and mutant sigma factors σ^{70} (RpoD), we found that there are two point mutation in mutant protein, G398D and Q437R (Figure 8-3-3). These mutations will lay a foundation for our further engineering of strains to improve rhamnolipid production in *E. coli* and elucidate the mechanism of yield enhancement.

9. **Milestones Status**

During this three-year of study, we have met the following milestones for the project:

1. The genes involved in the rhamnolipid bio-synthesis were successfully cloned.

2. By using the Transposon containing Rhamnosyltransferase gene rhlAB, we have successfully produced rhamnolipds in both *P. aeroginosa* PAO1-RhlA$^-$ strain and *P. fluorescens* ATCC15453 strain, with the increase of 55 to 175 fold in rhamnolipid production comparing with wild type bacteria strain.

3. We have completed several rounds of direct evolution studies and have constructed the library of RhlAB-containing Transposon to express mutant gene in heterologous hosts.

4. The comprehensive evaluation studies of rhamnolipid as a surfactant product for conventional chemical EOR applications were completed.

5. We are continuing to improve lab methods to clone a bio-surfactant mutant into candidate thermophilic oil field microbes.

6. We have successfully engineered *P. aeruginosa* PEER02 strain and several E. Coli strains and mutants to produce rhamnolipid biosurfactants effectively.

10. Summary of Significant Accomplishments

1. We successfully engineered the new mutant strains *P. aeruginosa* PEER02 and *E. coli* TnERAB that can produce rhamnolipids.

2. Rhamnolipid biosurfactants produced by our engineered bacteria have been evaluated by core flooding tests and are proven to be effective EOR agent

3. We have successfully produced rhamnolipids from many different plant oils and other agriculture products and waste products.

4. A Bioactive assay based on a high throughput screening method for screening our mutants and improving rhamnolipids production in *E coli* was successfully developed.

5. A complete LC-MS analytical methods have been documented for structural analysis of the rhamnolipids.

11. Future Work / Potential Problems

If funds are available, we will continue our research into following directions,

1. Further improve the methods to evaluate rhamnolipid and surfactin for their EOR characteristics.

2. Improve our mutant strains and optimize fermentation process to increase the yield of rhamnolipid production using agriculture products and waste streams as substrate.

3. Continue our directed evolution studies, screen and improve our mutant E.Coli strains to produce rhamnolipids with different structural verities.
4. Continue to search for better oilfield microbe strains that could be a carrier of our improved mutant genes for potential in-situ oilfield EOR applications.

12. Technology Transfer Activities

We have discussed our research work and potential technology value with several major oil companies, such as Chevron, Exxon-Mobil and Shell. We also presented some of our research results as a poster at the 28th Symposium on Biotechnology for fuels and Chemicals in Nashville, TN in May 2006, the SPE Oilfield Chemistry Symposium in Feb, 2007, and the AIChE 2007 Annual Meeting.

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