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Enhanced Rhamnolipid Production of *Pseudomonas aeruginosa* DN1 by Metabolic Engineering under Diverse Nutritional Factors

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Abstract

Rhamnolipids are a very promising class of biosurfactants exhibiting properties of great interest in several industrial applications, but they are not widely used because of the low yield and the high cost of production. In this study, a metabolic engineering strategy was used to construct the engineered strains DNAB and DNC through introducing *rhl*AB and *rhl*C genes respectively to *Pseudomonas aeruginosa* wild-type strain DN1, as well as optimization of nutritional parameters such as carbon and nitrogen sources were assessed simultaneously, with the purpose of promoting the productivity of rhamnolipids. Both engineered strain DNAB and DNC had higher yield of rhamnolipids than the DN1 under the same conditions by means of increasing the copy number of *rhl*AB and *rhl*C genes, respectively. Of particular importance was olive oil and sodium nitrate as the optimal sole carbon and nitrogen source separately, engineered strain DNAB had the highest rhamnolipid yields 1.28-fold and 1.25-fold of the DN1, and engineered strain DNC had the highest rhamnolipid yields 1.36-fold and 1.43-fold of the DN1. The ideal C/N ratio was found to be 20 that increased specific rhamnolipid productivity to 19.5 g/L and 22.5 g/L of the engineered strains, a certain amount to 1.39-fold and 1.61-fold of DN1 strain respectively. Meanwhile, there was a difference of the identified rhamnolipids between wild-type and engineered strains by ESI-MS analysis, and Rha-Rha-C10 and Rha-Rha-C10-C10 were the most dominant structure of rhamnolipids produced by the engineered strains through altering the expression levels of *Rhl*AB and *Rhl*C.

Keywords: Rhamnolipids; *P. aeruginosa* DN1; Engineered strains DNAB and DNC; Diverse nutritional factors; Structural characterization

Introduction

Rhamnolipids are amphipathic molecules with surface activities to reduce surface tensions, which are able to reduce surface tension of water from 72 mN/m to values below 30 mN/m at room temperature under atmospheric pressure, and their critical micellar concentrations are between 50 and 200 mg/L [1,2]. Correspondingly, they have a tremendous potential in improving residual oil recovery, IFT reduction and wettability alteration which make them one of the most important topics of research in microbial enhanced oil recovery (MEOR) field [3]. Despite the advantages of rhamnolipids, such as the high biodegradability, structural diversity, low toxicity apart from the wide-type range of industrial applications, they are not widely used in industry due to their high financial costs after 65 years of studies and process optimization attempts since the first rhamnolipid was described [3,4].

The type of rhamnolipids produced depends on the culture conditions, carbon source used and the microbial strains [5]. Rhamnolipids are generally thought to be produced by hydrocarbon-degrading microorganisms as amphipathic characteristics of rhamnolipids are essential for bacteria to uptake and utilize the hydrophobic hydrocarbons as the carbon source [6]. Quite of a few rhamnolipid-producing strains have been isolated and characterized in the last decades [7,8]. Among the available strains, *Pseudomonas aeruginosa* is still the most competent producer of rhamnolipids [9,10]. However, the complex gene regulation in *P. aeruginosa* represents a challenge to industrial production, which has been the object of a growing number of studies [11,12]. To aid in the development of rhamnolipid synthetic processes in *P. aeruginosa*, conceivable strategies based on the metabolic engineering for rhamnolipid production improvement were studied, such as strain modifications by overexpression of the key genes and increase the copy numbers of the key genes [7,10,13,14]. Moreover, extensive studies on factors affecting rhamnolipid production have been carried out to explore safe and economical methods aiming at their large-scale production based on renewable resources, and numerous reports are available showing that production and structural characterization of this secondary metabolite is dependent on microbial growth medium composition, e.g. carbon and nitrogen sources, as well as the expression levels of the key enzymes for rhamnolipid biosynthesis [4,15-18]. While metabolic pathway engineering and fermentation strategies are widely acknowledged, it has not been shown that rhamnolipids could be simultaneously improved the yield in low cost by using modification of the strains under optimized nutritional parameters.

In this study, two main strategies including metabolic engineering and optimization nutritional parameters were performed synchronously to enhance rhamnolipid yields of *P. aeruginosa* DN1. The key genes *rhl*AB and *rhl*C were genetically manipulated with plasmid pAK1900 to be recombinant plasmids, and then transformed to wild-type strain DN1 to construct the engineered strains named as DNAB and DNC, respectively. Rhamnolipid production by the DN1 strain and engineered strains was comparatively investigated grown on diverse carbon and nitrogen sources as well as different C/N ratio. The structural characterization of produced rhamnolipids was also carried out on the comparative analysis.

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Material and Methods

Bacterial strains, plasmids and primers

P. aeruginosa DN1 strain (GeneBank Accession No. CP017099) used in this study was originally isolated from petroleum-contaminated soil in Shaanxi, China, and was known to produce rhamnolipids [19,20]. The bacterial strains, plasmids, and oligonucleotide primers used in this study were presented in Table 1.

Media and culture condition

Bacterial strains were grown in Lysogeny broth (LB) containing 10 g tryptone, 5 g yeast extract and 5 g NaCl per liter of deionized water, or Biosurfactant Production Liquid Medium (BPLM, pH 7.4) supplemented with various carbon and nitrogen sources respectively, where the pH value was kept constant by means of adding either sodium hydroxide solution or dilute hydrochloric acid [20,21]. In order to prepare seed culture, a loop of cells taken from LB agar plate was added to a 250 ml flask containing 50 ml LB medium.

Construction of recombinant plasmids and engineered strains

For constructing engineered strains, the key genes *rhl*AB and *rhl*C with the native operon promoter was amplified from DN1 genomic DNA with the primers, respectively (Table 1). The PCR product of the *rhl*AB and *rhl*C operons were digested with *Eco*RI and *Hind*III and cloned into pAK1900 to produce the recombinant plasmid pAK-AB and pAK-C, and then they were transferred into *P. aeruginosa* wild-type strain DN1 by electroporation respectively, giving rise to the engineered strains named as DNAB and DNC [22]. The recombinant plasmids, such as pAK-AB and pAK-C isolated in the LB medium supplemented with 250 mg/L carbenicillin, were screened by PCR with the primers used for *rhl*AB and *rhl*C amplification, and then confirmed by double digested analysis of recombinant plasmids pAK-AB and pAK-C, respectively. After genotypic and phenotypic analysis, the engineered strains DNAB and DNC were selected for further study of rhamnolipid production.

Rhamnolipid production by wild-type and engineered strains

Rhamnolipid production studies were carried out in 500 ml flasks containing 200 ml sterilized BPLM medium supplemented with diverse carbon or nitrogen sources, respectively. The flasks were inoculated with 1% (v/v) seed culture (optical density 0.8 at Abs_{600}) taken from LB and incubated for 7 days at 37°C and 180 rpm under aerobic batch conditions. The parameters studied for the production included various carbon source such as palm oil, glycerol, corn oil, rapeseed oil, soybean oil, and olive oil (2%, w/v) with fixed nitrogen source, or nitrogen source addition such as urea, ammonium sulfate, potassium nitrate, ammonium nitrate, ammonium chloride, sodium nitrate (0.5%, w/v) with fixed carbon source, and different C/N ratio like 5, 10, 15, 20, and 25 with the fixed optimum carbon and nitrogen source. All experiments were performed in triplicate, and cultures were sampled every day during the whole incubation process. The amount of rhamnolipids in samples was quantified by the colorimetric determination of sugars with orcinol [23].

Rhamnolipid extraction and purification

These experiments were performed in three sets for 7 days utilizing the resultant optimum parameters, and then fermentation broth was centrifuged at 8,000 g for 10 min at 4°C. The supernatant was acidified to a pH 2-3 with concentrated HCl and extracted three times with ice-cold chloroform/methanol (2:1 v/v). The organic phases were collected and pooled, and the solvent was evaporated in a rotary vacuum evaporator. The residue was further purified twice with dichloromethane and dried with a rotary evaporator to obtain the pure rhamnolipids. These products were stored at -20°C for further analysis.

Determination of surface tension

The surface tension and critical micelle concentration (CMC) were measured by a JYW-200A automatic interfacial tension meter (Chengde Jinhe Equipment Manufacture Co. Ltd) using the ring methods. Based on the surface tension measurement, the CMC was then obtained by plotting the surface tension as a function of the serial concentration of rhamnolipids, the surface tension at this point was designated as the CMC value [24].

Strain and plasmid and primer	Characteristics	Source
	Strains	
E. coli DH5α	*F- mcrA Δ (mrr-hsdRMS-mcrBC) 80dlacZ Δ M15 Δ lacX74 deoR recA1 endA1 araD139 Δ (ara leu)7697 galU galKλ- rpsL nupG*	Takara, Japan
P. aeruginosa DN1	Rhamnolipid-producing strain; wild-type strain	Laboratory collection
E. coli pAK*rhl*AB	Cb^r; Plasmid pAK*rhl*AB was transformed into *E. coli* DH5α	This study
E. coli pAK*rhl*C	Cb^r; Plasmid pAK*rhl*C was transformed into *E. coli* DH5α	This study
P. aeruginosa DNAB	Cb^r; Plasmid pAK*rhl*AB was transformed into *P. aeruginosa* DN1	This study
P. aeruginosa DNC	Cb^r; Plasmid pAK*rhl*C was transformed into *P. aeruginosa* DN1	This study
	Plasmids	
pAK1900	Broad host range vector; 4750 bp	Laboratory collection
pAK*rhl*AB	Cb^r; pAK1900 containing 2.2 kb *rhl*AB genes with native promoter	This study
pAK*rhl*C	Cb^r; pAK1900 containing 0.9 kb *rhl*C genes with native promoter	This study
Primers		
*rhl*AB-F	GAATCTCTAGAATGCGGCCGAAAGTCTGT	29 bp,*Xba*I
*rhl*AB-R	CGGTAAGCTTTCAGGACGCAGCCTTCAGCC	30 bp,*Hind*III
*rhl*C-F	ATGAAGCTTGGATAGACATGGGCGTGCTG	30bp,*Hind*III
*rhl*C-R	ATCGAATTCTTGGCCTTGCCGGAAGCT	27bp,*Eco*RI
	Cb^r: Carbenicillin resistance.	

Table 1: Bacterial strains, plasmids and oligonucleotide primers used in this study.

Structural characterization of rhamnolipids

Rhamnolipids were dissolved in methanol and subjected to mass spectrometry characterization using LCQTM quadrupole ion-trap mass spectrometer (LCMS-2010 EV, Shimadzu, Japan) utilizing ES). Injection volume of the sample was 30 µl. ESI-MS analysis was performed in the positive ion mode. Ion spray voltage was set at 30 V and the scan range of the instrument was set at m/z 0–1000. Nitrogen was used as a nebulizer gas at flow rate 1.5 L/min. Flow injection analysis (FIA) was used for sample introduction into the heated ESI (H-ESI) ion source. 0.05% ammonium acetate + 0.1% formic acid: 100% methanol (80:20, v/v) was used as mobile phase at flow rate 0.3 ml/min. The H-ESI temperature was set to 200°C at 2 kV. LC solution software was used for integration of results [25-27].

Results and Discussion

Rhamnolipid production by wild-type and engineered strains

Strains were grown in BPLM supplemented with olive oil (2% w/v) and sodium nitrate (5 g/L) as the carbon and nitrogen source respectively and rhamnolipid production by the wild-type and engineered strains was comparatively investigated under aerobic conditions. As shown in Figure 1, there was similar growth kinetics of rhamnolipid production with respect to the DN1 and engineered strains, and the yields of rhamnolipids were fast increase during the stationary growth phase and reached maximum after the stationary growth phase. Nevertheless, the maximum yields of rhamnolipids produced by three strains were significantly different. For the engineered strains, the final maximum yield of rhamnolipids was 22.71 g/L and 24.25 g/L respectively, while 17.83 g/L rhamnolipids was determined in culture of the DN1. Through genetic manipulation, both engineered strains enhanced the rhamnolipid production by means of increasing the copy number of *rhl*AB and *rhl*C genes separately. Compared to the DN1 strain of which the surface tension of water decreased from 61.2 mN/m to 30.5 mN/m when rhamnolipid concentration was 100 mg/L, the engineered strains possessed higher surface activity, which was consistent with the data of rhamnolipid production capacity.

Metabolic engineering has been widely used to generate a broad number of modified strains, and some conceivable strategies for rhamnolipid production improvement have been achieved [28]. Herein, several constructs were made including the overexpression of the *rhl*AB and *rhl*C, leading to the increase of rhamnolipid production from the yield of 17.83 g/L to 22.71 g/L and 24.25 g/L separately under the same conditions. Comparative analysis of numerous studies during the last few years, in which metabolism remodeling was performed either in Pseudomonads or other related bacterial species mostly using heterologous expression of *Pseudomonas* genes, the molecular manipulation is an efficient strategy for improvement of rhamnolipid productivity [7,13,14]. In particular, increasing the copy number of key genes with the native promoter by indigenous expression can make much more efficiently the involved genes overexpression to enhance the production of desirable product by strains, when compared with several engineered strains that have proven to produce rhamnolipids in bacteria such as *E. coli* and *P. putida* [14,19,29,30].

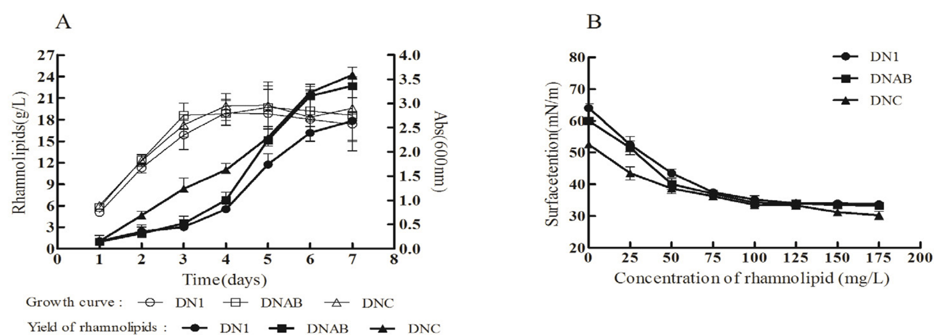

Figure 1: Time course profiles of cell growth and rhamnolipid production as well as critical micelle concentration (CMC) of rhamnolipids produced by wild-type and engineered strains. (**A**) rhamnolipid production and cell growth; (**B**) critical micelle concentration (CMC) of rhamnolipids.

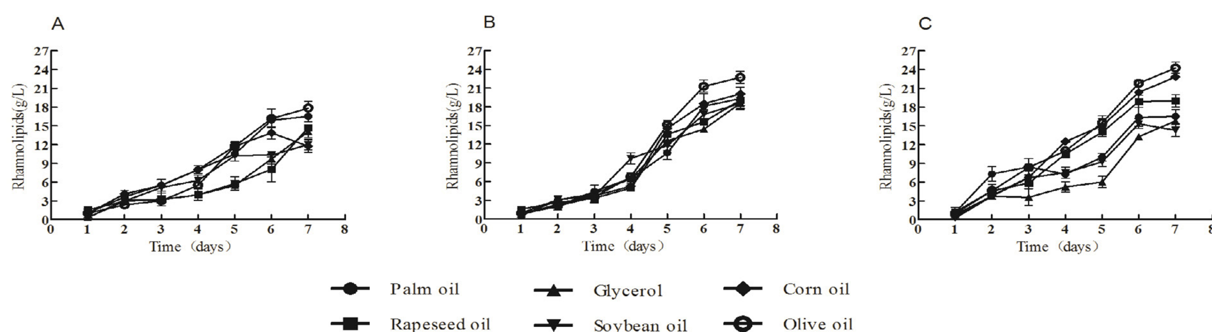

Figure 2: Effect of different carbon sources on rhamnolipid production of *P. aeruginosa* wild-type and engineered strains. (**A**) DN1 strain; (**B**) engineered strain DNAB; (**C**) engineered strain DNC.

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Optimization of nutritional parameters for rhamnolipid production

Rhamnolipid production using different carbon and nitrogen sources was carried out by three strains under shake flake fermentation conditions. As shown in Figure 2, it could be clearly seen that the optimum carbon source was olive oil, regardless of whether the strain was modified or not. At this point, engineered strain DNAB and DNC had the highest rhamnolipid yields 1.28-fold and 1.36-fold of wild-type strain DN1, respectively.

The optimal nitrogen source for rhamnolipid production in this study was sodium nitrate, at that time the engineered strains had the highest rhamnolipid yields 1.25-fold and 1.43-fold of the DN1 strain separately (Figure 3). This finding agrees with other reports in which nitrate nitrogen was more effective than ammo nitrogen for rhamnolipid production [31]. Moreover, it also had price advantage over organic nitrogen, though studies verified that different kinds of nitrogen sources had a significant effect on the biosurfactant production [32]. From Figure 4, it was found that the ideal C/N ratio for rhamnolipid production was 20:1 whether in the wild-type strain or engineered strains, which gave preferable rhamnolipid productivity of 19.5 g/L and 22.5 g/L by the engineered strains and 14.2 g/L by the DN1. C/N ratio was certified to be one of the most vital factors affecting the performance of microorganisms in biosurfactant production, and a low C/N culture condition might result in bacterial lysis in advance while excess C/N may result in bacteria metabolic disturbance [33,34]. This study indicated that both the type and relative concentrations of carbon and nitrogen substrates can significantly impact on the rhamnolipid production, and an appropriate C/N ratio could enhance the bacterial metabolism as well as promote the yield of rhamnolipids [35].

Numbers of reports are available showing that rhamnolipid production is dependent on medium components, and carbon source in culture medium plays important role in production of rhamnolipids by microbes [18]. Based on the accumulated studies, the yield of rhamnolipids utilizing the hydrophobic hydrocarbons is higher than that using the hydrophilic substrates as the carbon source. For instance, a study from Wei et al., has reported that olive oil was the best carbon source among seven different C-sources (glucose, glycerol, olive oil, sunflower oil, grape seed oil, diesel and kerosene) by rhamnolipid producing *P. aeruginosa* J4 isolated from petrochemical factory wastewater in southern Taiwan [36]. Another study using soybean oil by *P. aeruginosa* LB1 gives 11.72 g/L of rhamnolipids, whereas *P. aeruginosa* EM1 producing biosurfactant from glucose shows a higher production biosurfactant about 7.50 g/L compare to olive oil and soybean oil about 3.70 and 2.63 g/L [17,37]. The variation of the result suggests that both types of *P. aeruginosa* strain and substrate play an important role in growth and concentration of biosurfactant production [2]. Our result was in agreement with the fact that carbon source preference for rhamnolipid production by microorganisms is strain-dependent, whenever the strain was modified or not. Furthermore, in the economical production strategy over production was important as well as high activity of the product.

Rhamnolipid characteristics produced by wild-type and engineered strains

The molecular mass of the purified rhamnolipids was measured using ESI-MS analysis, which revealed the presence of different amounts of major and minor congeners with respect to the wild-type strain DN1 and engineered strains, although majority portion of rhamnolipids was

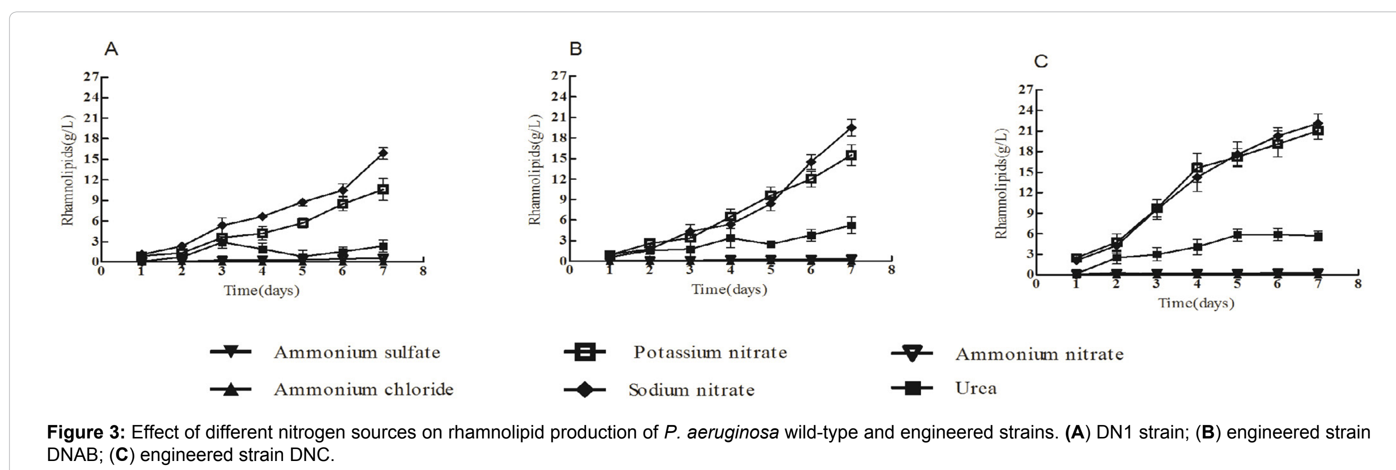

Figure 3: Effect of different nitrogen sources on rhamnolipid production of *P. aeruginosa* wild-type and engineered strains. (**A**) DN1 strain; (**B**) engineered strain DNAB; (**C**) engineered strain DNC.

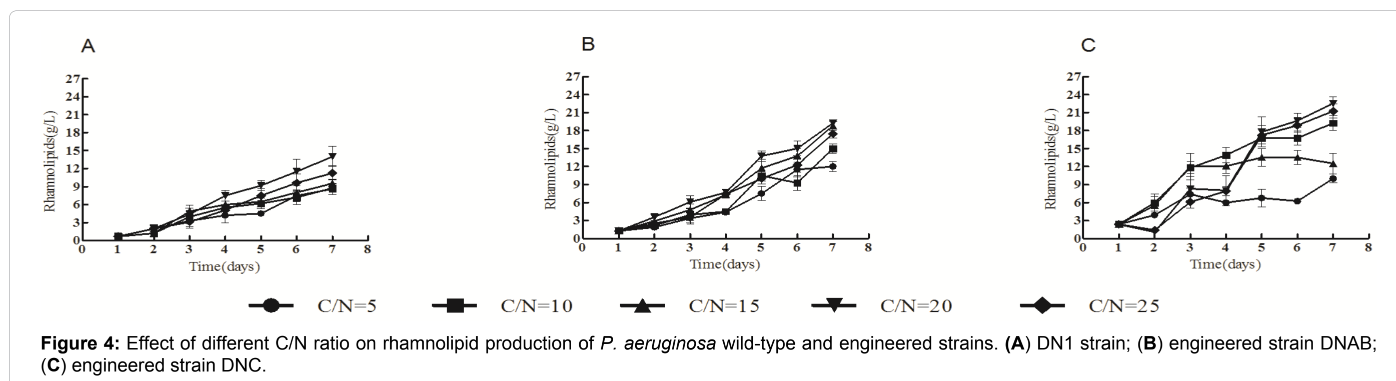

Figure 4: Effect of different C/N ratio on rhamnolipid production of *P. aeruginosa* wild-type and engineered strains. (**A**) DN1 strain; (**B**) engineered strain DNAB; (**C**) engineered strain DNC.

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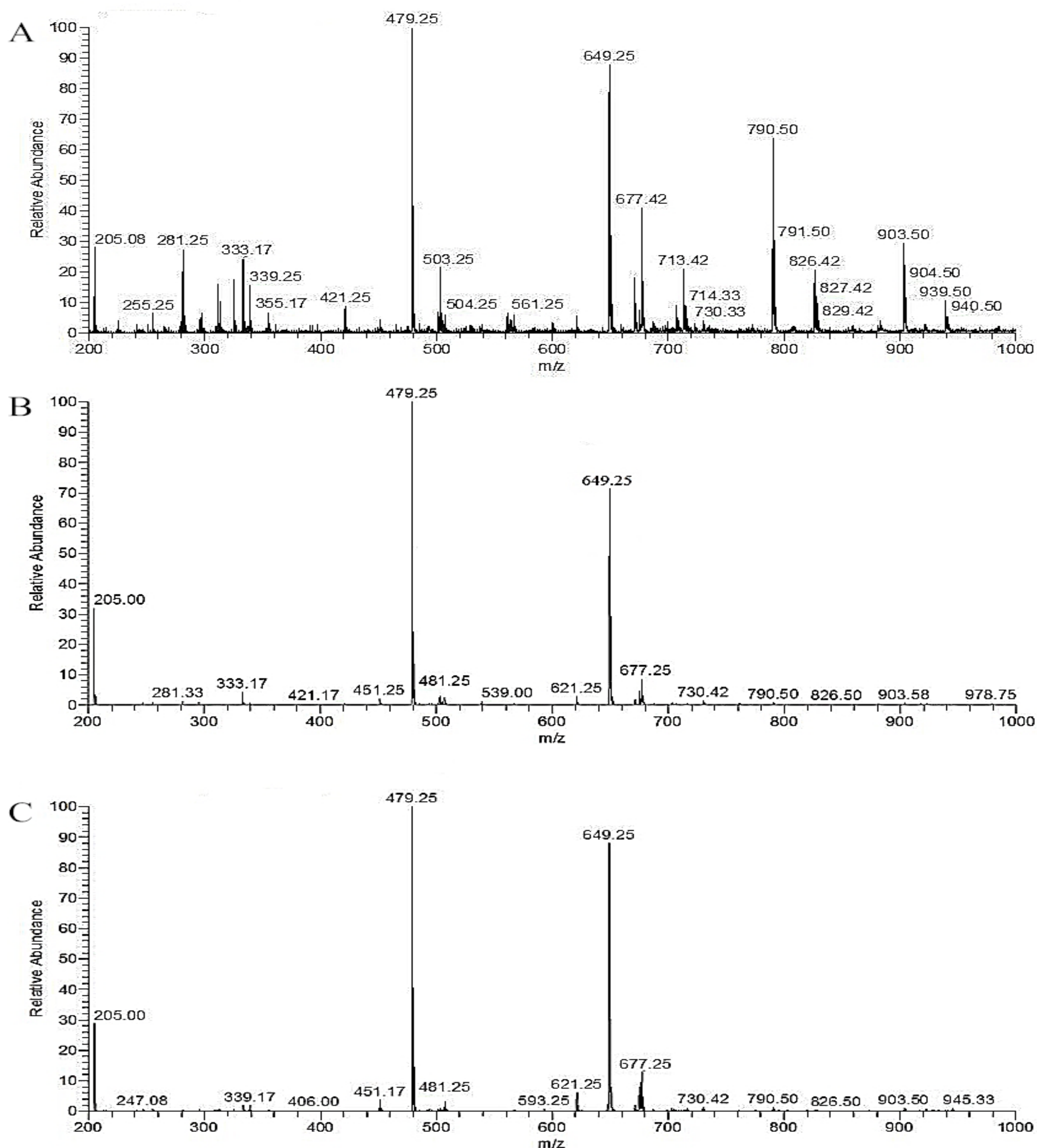

Figure 5: ESI-MS spectra of the identified rhamnolipid congeners produced by *P. aeruginosa* wild-type and engineered strains. (**A**) DN1 strain; (**B**) engineered strain DNAB; (**C**) engineered strain DNC.

occupied by dirhamnolipids (Figure 5). The proportions of various rhamnolipids listed in Table 2 were obtained from relative intensities of their corresponding parent ions, including monorhamnolipid like Rha-C_{10}-C_{10} and various dirhamnolipid such as Rha-Rha-C_{10}-C_{12}, Rha-Rha-C_{10}-C_{16}, Rha-Rha-C_{8}, Rha-Rha-C_{12}, and so on. Among those, two [M-H]- pseudomolecular ions with m/z 479.25 and 679.25 were observed as the dominant ions by those strains, and was consistent with the structure Rha-Rha-C_{10} and Rha-Rha-C_{10}-C_{10}, respectively. Of particular importance were engineered strains as the foremost ions, which could be because of the overexpression of the *rhl*AB and *rhl*C. The occurrence of high percentage of dirhamnolipids compared to monorhamnolipids acquiesces with the previous reports in the

Rhamnolipids	DN1 strain	DNAB strain	DNC strain	$[M-H]^{-}$ Obsd
Monorhamnolipids	Rha- C_{10}-C_{10}	-	-	503.25
Dirhamnolipids	-	Rha-Rha-C_8	Rha-Rha-C_8	451.25
Dirhamnolipids	Rha-Rha-C_{10}	Rha-Rha-C_{10}	Rha-Rha-C_{10}	479.25
Dirhamnolipids	Rha-Rha-C_{12}	-	-	504.25
Dirhamnolipids	-	-	Rha-Rha-C_8-C_8	593.25
Dirhamnolipids	-	Rha-Rha-C_8-C_{10}	Rha-Rha-C_8-C_{10}	621.25
Dirhamnolipids	Rha-Rha-C_{10}-C_{10}	Rha-Rha-C_{10}-C_{10}	Rha-Rha-C_{10}-C_{10}	649.25
Dirhamnolipids	Rha-Rha-C_{10}-C_{12}	Rha-Rha-C_{10}-C_{12}	Rha-Rha-C_{10}-C_{12}	677.25
Dirhamnolipids	Rha-Rha-C_{10}-C_{16}	Rha-Rha-C_{10}-C_{16}	Rha-Rha-C_{10}-C_{16}	730.42
Dirhamnolipids	Rha-Rha-C_{14}-C_{16}	Rha-Rha-C_{14}-C_{16}	Rha-Rha-C_{14}-C_{16}	790.5

Table 2: Identified rhamnolipids produced by *P. aeruginosa* wild-type strain DN1 and engineered strains DNAB and DNC using the analysis of ESI-MS.

literature [38]. Gong et al., studied cultivation of *P. aeruginosa* TIB-R02 in coconut oil, palm oil, olive oil, grapeseed oil and soybean oil and reported various rhamnolipid congeners, where the dirhamnolipids (Rha-Rha-C_{10}-C_{10}) were abundant, while same strain produced higher proportion of monorhamnolipids (Rha-C_{10}-C_{10}) when cultivated in corn oil and frying oil. In contrast, *P. aeruginosa* LB1 produced higher percentage of monorhamnolipids (44%) than dirhamnolipids (29%) when cultivated on soybean oil [39,40]. Thus the variation in rhamnolipid congeners and the predominance of a particular type depends on various factors like type of carbon substrate, culture conditions, and analytical method used [41]. The results shown here supported that the chemical composition of rhamnolipid congeners diverges by the presence of unsaturated bonds, the length of the carbon chain and the size of the hydrophilic head-group owing to multiple factor, like individual strain, carbon source, culture conditions and isolation procedures, and emphasized the important specific strain and carbon source [21,39].

Conclusion

The results showed that simultaneously increasing the copy number of key genes and optimization of nutritional parameters was more efficiently to enhance the production of rhamnolipids by *P. aeruginosa* DN1 under aerobic conditions, and the optimum carbon source was olive oil, as well as the ideal C/N ratio for rhamnolipid production was 20:1 regardless of whether the strain was modified or not. The produced rhamnolipids exhibited excellent surface activity, and difference was observed in case of congeners of rhamnolipids though dirhamnolipids were predominant with irrespective of the strain used.

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