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RESEARCH

Statistical Screening of Medium Components for Recombinant Production of *Pseudomonas aeruginosa* ATCC 9027 Rhamnolipids by Nonpathogenic Cell Factory *Pseudomonas putida* KT2440

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Abstract Rhamnolipids (RLs) produced by the opportunistic human pathogen *Pseudomonas aeruginosa* are considered as potential candidates for the next generation of surfactants. Large-scale production of RLs depends on progress in strain engineering, medium design, operating strategies, and purification procedures. In this work, the *rhlAB* genes extracted from a mono_RLs_producing strain of *P. aeruginosa* (ATCC 9027) were introduced to an appropriate safety host *Pseudomonas putida* KT2440. The capability of the recombinant strain was evaluated in various media. As a prerequisite for optimal medium design, a set of 32 experiments was performed in two steps for screening a number of macro-nutritional compounds. In the experiments, a two-level fractional factorial design resolution IV was followed by a two-level full factorial one. By means of this approach, it was observed that glycerol, yeast extract, and peptone have significant positive influence on recombinant RLs production while the yeast extract/peptone two-factor and glycerol/yeast extract/peptone three-factor interactions have considerable negative effects. A wide range of variation from 0 to 570 mg/l was obtained for RLs production during the screening experiments indicating the importance of medium optimization. The results point out the opportunity for possible higher yields of RLs through further screening, mixture/combined mixture designs, and high-cell-density cultivations.

Keywords Heterologous rhamnolipids production · *Pseudomonas aeruginosa* ATCC 9027 · *Pseudomonas putida* KT2440 · Transformation · Experimental screening design · Two-level factorial design

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Introduction

Rhamnolipids (RLs), an interesting group of useful microbial products, have been extensively studied as the next generation of surfactants in recent years. They are one of the most useful biosurfactants with a wide range of applications from industry, agriculture, oil recovery, and soil/water remediation to personal care and medicine [1–3]. RLs are surface active anionic glycolipids containing L-(+)-rhamnose (hydrophilic part) and β-hydroxyalkanoic acids (hydrophobic part) moieties [4]. Based on the number of rhamnose molecules (one or two), RLs are categorized into two main groups: mono-RLs and di-RLs. They are exoproducts mainly produced by a number of gram-negative bacteria, such as *Pseudomonas* sp., *Burkholderia* sp., *Thermus thermophilus*, *Acinetobacter calcoaceticus*, *Enterobacter* sp. [4–7]. Relatively high RLs productivities (i.e., 16.67 mg/l/h from sunflower oil) have been mainly reported using well-studied opportunistic human pathogen *Pseudomonas aeruginosa* [8].

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RLs have received considerable attention from two points of view. First, RLs as well as pyocyanin, cyanide, and lipase are virulence factors resulting in pathogenesis of *P. aeruginosa*. Their production is regulated by complex quorum sensing systems (cell population density-dependent regulatory systems) at both transcriptional and translational levels [9–11]. Second, RLs are known as natural biosurfactants of great commercial interest with various applications and high industrial potential. They are low toxic and biodegradable organic molecules with potent surface tension-reducing and emulsifying activities as well as admirable detergency along with wonderful foaming properties [1]. They have bioactive functionalities and antimicrobial and antiviral effects [12]. Due to these properties, RLs are target of investigation for a wide range of potential markets [2, 3].

Biosynthesis of RLs includes the following two principle steps:

(1) Synthesis of the relevant precursors, which are dTDP-L-rhamnose (stemmed from D-glucose-6-phosphate), and 3-(3-hydroxyalkanoyloxy) alkanoate (HAA) resulted from Acetyl-CoA through fatty acid de novo synthesis. The enzyme responsible for condensation of two activated β-hydroxy fatty acids and formation of HAA is *RhlA* (rhamnosyltransferase A).
(2) Assembling of the two precursors (dTDP-L-rhamnose and HAA) by *RhlB* (rhamnosyltransferase B) gene and formation of mono-RL molecules.

The mentioned genes are both organized in a single operon named *rhlAB* and co-expressed from the same promoter [1, 4, 13, 14].

Another rhamnosyltransferase called *RhlC*, which is present in genome of the most *P. aeruginosa* sp., is responsible for di-RL biosynthesis via additional rhamnosyl transfer with dTDP-L-rhamnose.

Since most of high RLs producers are opportunistic human pathogens, for large-scale RLs production and commercialization, an appropriate alternative would be the application of nonpathogenic microorganisms; particularly the ones potentially able to produce the main precursors of RLs due to their metabolic versatility.

Based on their significant characteristics, *Pseudomonas putida* and its related subspecies have drawn the researchers' attention as proficient cell factories for production of various valuable bioproducts [15]. Moreover, it was demonstrated that *P. putida* KT2440 can tolerate high concentrations of RLs (>90 g/l) [14]. This fact makes *P. putida* KT2440 an appropriate host for potential high-RLs-yield bioprocesses.

Considerable studies have been conducted on heterologous RLs production in *P. putida* species. Ochsner et al. [16] studied the expression of cloned *rhlAB* rhamnosyltransferase genes in a number of heterologous hosts using two different constructs (pUO101 and pUO98). They achieved the highest yield of 0.6 g/l RLs in a recombinant *P. putida* strain KT2442 carrying the plasmid pUO98 cultivated in Luria–Bertani medium supplemented with 1 % glucose [16]. Using molecular cloning of *P. aeruginosa* EMS1 *rhlAB* genes with the quorum-sensing system-relevant genes (*rhlRI*) via easy vector pGEM-T, Cha et al. [17] investigated the heterologous RLs production in a strain of *P. putida*. They could produce up to 7.3 g/l RLs utilizing soybean oil as carbon source. Employing *P. putida* KT42C1 pVLT31_*rhlAB* (an engineered strain of KT2440 in which polyhydroxyalkanoate formation as competing pathway has been removed), Wittgens et al. [14] could increase the yield of RLs from 0.22 to 1.5 g/l in Luria–Bertani medium supplemented with 1 % glucose. They achieved the highest yet reported conversion rate (0.15 g/g) of recombinant RLs production with water-soluble substrates [2, 14]. Heterologous production of RLs entails other considerable advantages; for instance, disconnecting the RLs biosynthesis from the complex quorum-sensing regulation [14, 16]. This property leads to more controllable RLs bioprocesses as well as less difficult and costly product purification.

To produce RLs in large-scale, the same as other valuable secondary metabolites, developments in various fields such as strain engineering, medium design, optimal operating strategies, and purification procedures are required [2, 3]. Optimization of the culture medium is an important part for fermentative bioprocess development [18–22]. The preliminary step to achieve optimal process performance is to screen and evaluate the nutritional compounds influencing the biosynthesis of the desired product. Statistical approaches for multivariate design of experiments (DoEs) provide an ingenious style for designing of the most appropriate cultivation medium [18]. Plackett–Burman design and two-level fractional factorial design are two of the most widely applied approaches to screen the critical medium components with the significant effects on the production of biochemicals of interest. The first one is a suitable approach for detection of the significant factors without examination of the interactions. The second approach can check up the main effects along with the two-factor interactions (resolution V). The design is orthogonal and therefore can gives pure effect of every single factor not confounded with the interactions [18, 21, 23]. Despite lowering the number of the required experiments, using lower resolution IV, the two-factor interactions may aliased with each other. However, since the resolution IV clearly examines the main effects, it is a proper choice for initial screening design for systems with high numbers of variables. Insight gained from the initial screening helps us to reduce the number of factors that must

be taken into account. Hence, it enables us to utilize higher resolutions or even full factorial screening sets of experiments as the complementary steps to identify the most effective compounds and to study the effects of their interactions on the objective of interest.

In this work, in order to study the feasibility of recombinant production of RLs, the genes responsible for mono-RLs production (promoterless *rhlAB* genes) in *P. aeruginosa* ATCC 9027 (PTCC 1074) were cloned in pVLT33 broad-host-range expression vector and the construct (pVLT33–*rhlAB*) was transferred to *P. putida* KT2440. The heterologous production of RLs by the engineered strain in a number of cultivation media was examined. Afterwards, as the preliminary step for optimal medium design, a set of experiments was performed in two steps for screening compounds such as different sources of carbon, nitrogen, and phosphate. As mentioned before, in the experiments, a two-level fractional factorial design resolution IV was followed by a two-level full factorial one. Employing the two-step procedure, the compounds with significant positive influences on RLs production as well as the effects of the two- and three-factor interactions were revealed.

Materials and Methods

Strains and Plasmids

Pseudomonas aeruginosa ATCC 9027 was utilized for extraction of *rhlAB* genes. This strain as a mono-RLs-producing member of *Pseudomonas* sp. has been widely studied [24–26] and its produced congeners of mono-RLs have been systematically surveyed [27]. According to Lebrón-Paler's [27] studies, *P. aeruginosa* ATCC 9027 produces a significantly higher number of mono-RL homologs in comparison to other studied species. Using HPLC, mass spectrometry, and NMR spectroscopy, the structural characterization of up to 30 identified mono-RL congeners produced by this strain was determined. Also, it was shown that the species with molecular weight (MW) 504 constitute about 82 % of the produced RL compounds [27, 28]. Figure 1 illustrates the chemical structures of these types of identified mono-RL congeners [27].

Escherichia coli BL21DE3 was used as the host strain for maintenance of the plasmids. *P. putida* KT2440 (DSM 6125) was employed as the host for the heterologous production of *P. aeruginosa* RLs. All strains were grown in Lennox Broth (LB: 10 g/l peptone, 5 g/l yeast extract, and 5 g/l NaCl) liquid medium or on LB agar plates.

pVLT33 plasmid was employed for cloning of *rhlAB* genes. It is a *lacI*^{q}/*Ptac*-based broad-host-range plasmid encoding kanamycin resistance gene. The vector is derived from the basic plasmid pMMB207. pVLT33 plasmid permits the construction of gram-negative strains with conditional phenotypes depending upon IPTG (isopropyl-β-D-1-thiogalactopyranoside) addition to the medium [29]. It was provided by Professor Victor de Lorenzo, Centro Nacional de Biotecnología, CSIC Madrid 28049, Spain. The construction scheme of pVLT33 plasmid is shown in Fig. 2.

Rhamnolipids Identification and Quantification

A large number of analytical techniques have been developed for identification and quantification of RLs [30]. Two simple colorimetric methods: cetyltrimethylammonium bromide (CTAB) agar test [31, 32] and orcinol assay [33] were employed to detect production of RLs by *P. aeruginosa* ATCC 9027 and the recombinant *P. putida*, respectively. Moreover, thin layer chromatography (TLC) was used to confirm the production of heterologous RLs by the recombinant *P. putida*.

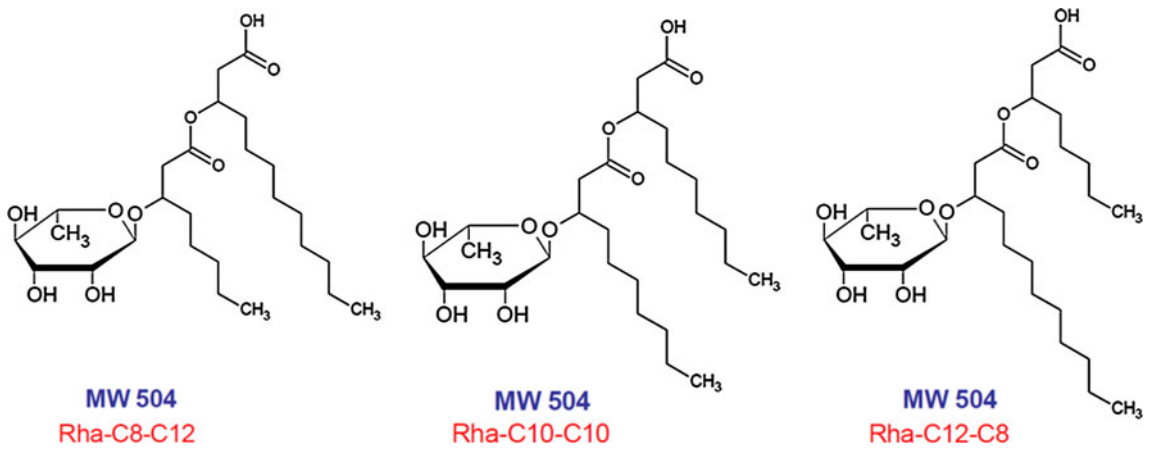

Fig. 1 Chemical structures of the dominant mono-RL congeners produced by *P. aeruginosa* ATCC 9027 [27]

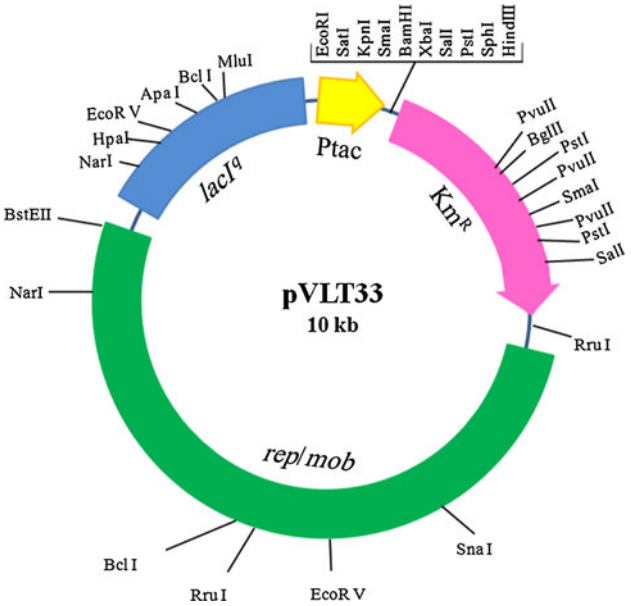

Fig. 2 Construction scheme of pVLT33 plasmid

CTAB Agar Test

CTAB agar test is a semiquantitative agar plate technique proposed by Siegmund and Wagner [31] for detection of RLs as a group of anionic extracellular biosurfactants. This method is stemmed from formation of an insoluble ion pair of anionic tenside with the cationic surfactant CTAB and the basic dye methylene blue included in mineral agar plates (MB). In this method, dark blue halos are formed around the colonies of RLs productive microorganisms and the spot diameters depend on RLs concentrations [30, 31].

Orcinol Assay

The orcinol assay is based on the reaction of the rhamnose moiety of RL molecules with colored chemical compound (orcinol) in the presence of a strong acid (sulfuric acid) at high temperatures (above 80 °C) leading to formation of a dye which can be quantified by absorbance measurements at 421 nm [33–35] or 404 nm (this study). This method has been introduced in [33]. The detailed procedure for extraction of the samples and subsequently, the orcinol analysis is also available in [34, 35]. The procedure can be briefly explained as follows: RLs molecules of 400 µl of supernatant were extracted twice with diethyl ether (750 µl for each time) and transferred to a microtube. The ether fractions were pooled and left to evaporate in air for a while up to dryness. 400 µl of pH 8 phosphate buffer was utilized to redissolve the participant left in the microtube. 900 µl of a solution containing 0.19 % orcinol (in 53 % H_2SO_4) was added to 100 µl of each sample. The samples were heated at high temperature (for instance, in a floating rack in boiling water) for 20 min and left to be cooled in a dark cupboard for 35 min at room temperature. The optical density as a representative of L-(+)-rhamnose concentration in the sample was measured at 421 nm [34, 35] or 404 nm (this study). This method as an effective and simple analysis approach has been widely utilized by various research groups using different protocols for quantification of RLs [30].

For quantification of RLs, firstly, a standard curve should be prepared to correlate the L-(+)-rhamnose concentrations in defined rhamnose solutions with the relevant values for optical density. The standard curve was prepared for 404 nm using defined L-(+)-rhamnose solutions in pH 8 phosphate buffer according to the method mentioned in [35]. Direct measurement of rhamnose concentrations in broth samples was conducted employing the method of extraction presented in [35]. It should be mentioned that in this study, the orcinol assay was also used for detection of heterologous RLs production and the capabilities of the recombinant *P. putida* for RLs production in a number of cultivation media.

Thin Layer Chromatography

Since the orcinol assay confirms only the excretion of the reducing saccharide (rhamnose) into the medium, TLC was also used to demonstrate the production and secretion of the heterologous glycolipids. The method described in [14] for extraction by diethyl ether was used to extract samples of dried RLs. They were dissolved in 10 µl ethanol and 5 µl of the solutions were spotted on a silica gel TLC plate (Merck, Germany). Also, a sample of an aqueous solution of concentrated crude RLs (containing mostly mono-RLs and a little di-RLs) produced by *P. aeruginosa* sp. (provided by Professor Ibrahim Banat, School of Biomedical Sciences, University of Ulster, County Londonderry, Northern Ireland, to Research Institute of Petroleum Industry, Tehran, Iran) was prepared. The extracted dried RLs from the mentioned aqueous solution were also dissolved in 10 µl ethanol and 5 µl of the solution was spotted on the plate. The running buffer used for this experiment was a solution of chloroform, methanol, and acetic acid with 65:15:2 ratios. After running, an orcinol detection agent (containing 0.15 g orcinol, 8.2 ml sulfuric acid (60 %), and 42 ml deionized water) was used to visualize the RLs. The dried plate was incubated at 90 °C for 20 min in order to observe the red-brown spots of RLs.

Observation of RLs Production by *P. aeruginosa* ATCC 9027

In order to determine the RLs production by *P. aeruginosa* ATCC 9027, CTAB agar test was used. The medium for

preparation of the RLs-detection agar plates contained (per liter): 20 g glycerol, 0.7 g KH_2PO4, 0.9 g Na_2HPO4, 2 g $NaNO_3$, 0.4 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.2 g CTAB, 0.005 g MB, 12 g microbial agar, and 2 ml of a trace element solution with the following composition (per liter): 2 g $FeSO_4{\cdot}7H_2O$, 1.5 g $MnSO_4{\cdot}_2O$, and 0.6 g $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ [31, 32]. Five wells in each plate were prepared for different volumes of inoculums and an additional one for prior medium as negative control. In this part of study, firstly, four different volumes (25, 50, 75, and 100 µl) of the solution of the mentioned concentrated crude RLs were used as positive controls for testing the method and observation of the appeared dark blue halos. The aqueous solutions of the crude RLs were added to the plates and the plates were incubated at 37 °C for 48 h. After that, the plates were stored in the refrigerator for at least 24 h [31, 32]. This procedure was repeated for the *P. aeruginosa* ATCC 9027 culture as follows: different volumes (25, 50, 75, and 100 µl) of overnight-grown precultures of *P. aeruginosa* ATCC 9027 in LB medium were added to the wells of the agar plates, and a 100 µl of the fresh LB was also added to the central well as negative control. The similarity of the dark blue halos resulted from the culture and those from the crude RLs solution would confirm RLs production by *P. aeruginosa* ATCC 9027 and the function of this strain's *rhlAB*.

Cloning of *rhlAB* Genes

Total DNA of *P. aeruginosa* ATCC 9027 was extracted by CTAB method [36] and served as template for the polymerase chain reaction (PCR) amplification of *rhlAB* with forward primer 5′-TTGAATTCATGCGGCGCGAAAGTC TGTT-3′ and reverse primer 5′-TTTTAAGCTTTCAGGA CGCAGCCTTCAGCC-3′. PCR of *rhlAB* was performed with Pfu-Taq polymerase (Fermentas). The PCR product was digested by *Eco*RI and *Hin*dIII, and subsequently ligated into the *Eco*RI/*Hin*dIII sites of pVLT33 in order to generate the pVLT33–*rhlAB* construct. This construct was then used to transform *E. coli* BL21DE3. Transformation was done by electroporation with a Gene Pulser apparatus (Bio-Rad) according to the manufacturer's specifications. Cells were plated on LB agar containing kanamycin (50 mg/l). Plates were incubated at 37 °C overnight. Individual colonies were transferred to 5 ml LB medium containing kanamycin (50 mg/l) for 24 h. To confirm plasmid existence, colony PCR and digestion by the same restriction enzymes (*Eco*RI and *Hin*dIII) was done and the band of interest (2.23 kb) was observed on 1 % agarose gel. Transformation of *P. putida* was done using electroporation method. Also, plasmid insertion was verified by DNA sequencing (Macrogen company, Korea).

Identification of RLs Production by *P. putida* KT2440

The first step after confirmation of the transformation was to prove the heterologous RLs production in a typical medium such as LB. An experiment was set up to study RLs production by the recombinant *P. putida* strain induced by two IPTG concentrations (0.4 and 1.2 mM). The following two cultivation media were chosen for this aim:

- Complex medium 1: LB (5_day cultivation).
- Complex medium 2: LB supplemented with 20 g/l glucose (5_day cultivation).

Cultures of 100 ml complex media in 250 ml Erlenmeyer flasks were inoculated with 500 µl of overnight-grown precultures and incubated in a shaker incubator at 28 °C and 170 rpm for 5 days. Kanamycin (50 mg/L) and IPTG were added to the media before incubation. The wild-type strain as well as non-induced recombinant one was also cultivated as negative controls for comparison. After 120 h, cells were harvested (by centrifugation at $5{,}000\times g$ for 20 min) and supernatants were utilized for extraction and identification of RLs via TLC and orcinol assay. Using an oven, the harvested biomass pellets were dried at 50 °C in order to measure the dry total biomass.

After confirmation, as the second step, a number of cultivation media (mostly the mineral ones) were checked regarding the capability of the recombinant *P. putida* for RLs production. Since one of the common and practical protocols for heterologous production of valuable bioproducts such as recombinant proteins, is to grow cultures to intermediate or high densities followed by induction of product formation [37], the media were chosen concerning the feasibility of achievement of high-cell-density cultivations (HCDC) of *P. putida* [38, 39]. It should be noted that in this step the main objective was just to investigate the possibility of product formation in each considered medium. HCDC and optimization of the medium composition as well as the operating policies were not regarded for the current study. With regards to each medium, cultures of 100 ml media in 250 ml Erlenmeyer flasks were inoculated with 500 µl from a starter culture and incubated at 28 °C and 170 rpm for 5 days. Kanamycin (50 mg/L) and IPTG were added to the media before incubation. The associated cultures of non-induced recombinant strain were also cultivated as negative controls for comparison. The following media were examined to check in which medium RLs production is possible in a detectable manner:

- Mineral solution 1 contained (per liter): 10 g glucose, 3.0 g $(NH_4)_2SO_4$, 3.32 g $Na_2HPO_4{\cdot}12H_2O$, 0.83 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 20 mg $FeSO_4{\cdot}7H_2O$, 10 mg $CaCl_2$, and 1 ml of trace element 1. The trace

element 1 composition was: 0.3 H_3BO_3, 0.2 g $CoCl_2{\cdot}6H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 30 mg $MnCl_2{\cdot}4H_2O$, 30 mg $Na_2MoO_4{\cdot}2H_2O$, 20 mg $NiCl_2{\cdot}6H_2O$, 10 mg $CuSO_4{\cdot}5H_2O$ per liter of 0.1 N HCl [38] (5_day cultivation).

– Dual cultivation media: In addition to the above media, inspired by the protocols for high-cell-density expression methods presented in [40], another case study was considered. The proposed method potentially enables us to achieve HCDC and high product yields with normal laboratory settings using shaking flasks and does not require fermenters. The method begins cell cultivation in rich (complex) media to enhance initial cell density before IPTG-induction. After the cells reach a significant OD_{600} value, while they are still in their exponential growing phase, they are switched into the same volume of mineral/minimal media. 1.0–1.5 h later, they are induced with IPTG for production of the desired biomolecules. Based on this method, LB was chosen as the preliminary rich medium for initial cell growth, and the mineral solution 1 was chosen as the producing medium. It should be noted that for this case, cells were grown in 100 ml LB for 1 day and then harvested and transferred to 100 ml of mineral solution 1 and incubated for 5 days. After 1.5 h of medium switching, IPTG was added to the mineral medium for induction. Both the rich and the mineral media contain 50 mg/l kanamycin.

Regarding the importance of glycerol as a renewable water-soluble feedstock and an appropriate carbon source for RLs biosynthesis [3], it is utilized in the following media:

– Mixture medium (120 ml): Inspired by the dual cultivation media, a new medium was designed which is a blend of LB, the mineral solution 1 and glycerol. The medium contained: 47.5 volume % LB, 47.5 volume % mineral solution 1, and 5 volume % aqueous solution of 150 g/l glycerol (the final glycerol concentration was 7.5 g/l in the medium).

After 5 days, cells were harvested (by centrifugation at 5,000×*g* for 20 min) and supernatants were used for extraction and identification of RLs.

Experimental Design and Evaluation

As a prerequisite to achieve optimal RLs biosynthesis performance, a screening based on a two-step factorial design (a two-level fractional factorial design resolution IV followed by a full factorial one) was set up. The initial two-level fractional factorial design resolution IV was applied for eight different factors including carbon (glycerol and sunflower oil), nitrogen (NH_4NO_3, $NaNO_3$, peptone, and yeast extract), and phosphate (Na_2HPO_4 and KH_2PO_4) macro-nutritional compounds while the full factorial one was applied for the most considerable factors resulted from the initial step. Glycerol as a water-soluble carbon source and sunflower oil as a hydrophobic one were selected due to their extent utilization in RLs production [3]. As the initial screening test, resolution IV was chosen to detect the significant factors affecting the RLs production. Also, it would be possible to gain suitable insights for further screening and mixture designs from two-factor interactions in spite of probable confounds. Based on the initial tests, the most significant factors were determined. The second screening step was applied for these factors in order to thoroughly study the effects of each individual factor in addition to two- and three-factor interactions. The software package of Design Expert version 7.1.5 (State Ease Inc.) was employed to perform the experimental design and statistical analysis. According to the first step of the experimental design, 16 different medium compositions were prepared. The macro-nutritional factors were supplemented to the media in two levels of concentration (high or low). Table 1 represents the two concentration levels considered for each variable factor. These quantities are chosen based on the magnitude of each component concentration in LB medium or M9 minimal medium. The layout of the two-level fractional factorial design is illustrated in Table 2. It should be noted that to all the 16 media, the same micro-nutritional compounds with the same concentrations (0.2 g $MgSO_4{\cdot}7H_2O$, 10 mg $CaCl_2$, and 1 ml of trace element 1 per liter of the medium) were added. Cultures of 100 ml media in 250 ml Erlenmeyer flasks were inoculated with 500 µl of overnight-grown precultures and incubated in a shaker incubator at 28 °C and 170 rpm for 7 days. Kanamycin (50 mg/l) and IPTG (0.4 mM) were added to the media before incubation. After 7 days, cells were harvested (by centrifugation at 5,000×*g* for 20 min) and supernatants were used for extraction and measurement of RLs. At least four samples for each case were analyzed and the mean values of the

Table 1 Concentrations of macro-nutritional factors in the media according to the screening design

Factor no.	Design variable factors	Low level (%w/v)	High level (%w/v)
1	Peptone	0.0	1.0
2	Yeast extract	0.0	0.5
3	NH_4NO_3	0.0	0.5
4	$NaNO_3$	0.0	0.5
5	$Na_2HPO_4{\cdot}12H_2O$	0.0	1.2
6	KH_2PO_4	0.0	0.3
7	Glycerol	0.0	1.0
8	Sunflower oil	0.0	1.0

Table 2 Two-level fractional factorial design (resolution IV) for the 8 factors (16 testing media)

Media no.	Peptone (%w/v)	Yeast extract (%w/v)	NH_4NO_3 (%w/v)	$NaNO_3$ (%w/v)	$Na_2HPO_4{\cdot}12H_2O$ (%w/v)	KH_2PO_4 (%w/v)	Glycerol (%w/v)	Sunflower oil (%w/v)
1	0.0	0.5	0.5	0.0	0.0	0.0	1.0	1.0
2	0.0	0.5	0.0	0.5	1.2	0.0	1.0	0.0
3	0.0	0.5	0.0	0.0	1.2	0.3	0.0	1.0
4	1.0	0.5	0.5	0.0	1.2	0.0	0.0	0.0
5	1.0	0.5	0.5	0.5	1.2	0.3	1.0	1.0
6	1.0	0.0	0.5	0.0	0.0	0.3	0.0	1.0
7	0.0	0.0	0.5	0.0	1.2	0.3	1.0	0.0
8	0.0	0.0	0.5	0.5	1.2	0.0	0.0	1.0
9	1.0	0.0	0.0	0.0	1.2	0.0	1.0	1.0
10	1.0	0.5	0.0	0.5	0.0	0.0	0.0	1.0
11	1.0	0.0	0.0	0.5	1.2	0.3	0.0	0.0
12	1.0	0.5	0.0	0.0	0.0	0.3	1.0	0.0
13	0.0	0.5	0.5	0.5	0.0	0.3	0.0	0.0
14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	1.0	0.0	0.5	0.5	0.0	0.0	1.0	0.0
16	0.0	0.0	0.0	0.5	0.0	0.3	1.0	1.0

responses were applied for statistical analysis. Using an oven, the harvested biomass pellets were dried at 50 °C in order to measure the dry total biomass.

Based on the results obtained from the first screening, the most considerable compounds were selected for the second screening set of experiments. The conditions of the second set were similar to those of the first one, except for the initial IPTG concentration. In order to examine whether or not the increase of IPTG concentration from 0.4 to 1.2 mM had significant effect on the results, it was considered to be 1.2 mM in the second set of experiments.

Results

Observation of RLs Production by *P. aeruginosa* ATCC 9027

The normal and UV-transilluminated pictures of agar plates supplemented with concentrated crude RLs are shown in Fig. 3a, b. It is observable that the area of the halos depends on the amount of RLs [31, 32]. Also, Fig. 4a, b illustrates the normal and UV-transilluminated pictures of agar plates inoculated with *P. aeruginosa* ATCC 9027. The dark halos appeared around the wells indicate the function of the *rhlAB* genes and the ability of this strain for RLs production.

Cloning of *rhlAB* Genes

rhlA and *rhlB* are arranged as an operon and are clustered with *rhlR* and *rhlI*. Since *rhlAB* operon promoter is under the control of the *rhlR* and *rhlI* gene products, we attempted to achieve heterologous RL production in the host *P. putida* KT2440 by molecular cloning of just *rhlAB* rhamnosyltransferase genes. Using pVLT33 vector enables their expression by IPTG-induction. This fact helps us to avoid the complexity of quorum sensing and to get close to a more controllable biosynthesis processes. The expected bands of *rhlAB* by PCR of extracted *P. aeruginosa* DNA were detected on agarose gel 1 % (two samples represented in Fig. 5). The pVLT33–*rhlAB* construct is schematically shown in Fig. 6. The expected bands of *rhlAB* by colony PCR of *E. coli* BL21DE3 (Fig. 7) were also detected on agarose gel 1 %.

In order to confirm pVLT33–*rhlAB* sequence, the recombinant plasmid was extracted from *P. putida* KT2440 and sent to Macrogen company, Korea for sequencing. The sequence was aligned with BLAST database of NBCI. It has a complete identity with rhamnosyltransferase (*rhlAB*) genes sequence.

Identification of RLs Production by *P. putida* KT2440

First of all, using standard aqueous solutions of L-(+)-rhamnose (10, 20, 40, 80, 100, 120, 160, and 200 mg/l), a survey has been conducted for selection of the best wave length regarding orcinol assay. Figure 8 depicts the results of survey for two samples (100 and 200 mg/l rhamnose). Due to the more significant differences between the optical densities of the samples at 404 nm, this wave length was chosen and a calibration curve (linear correlation) was prepared (Fig. 9). The data points shown are the average of

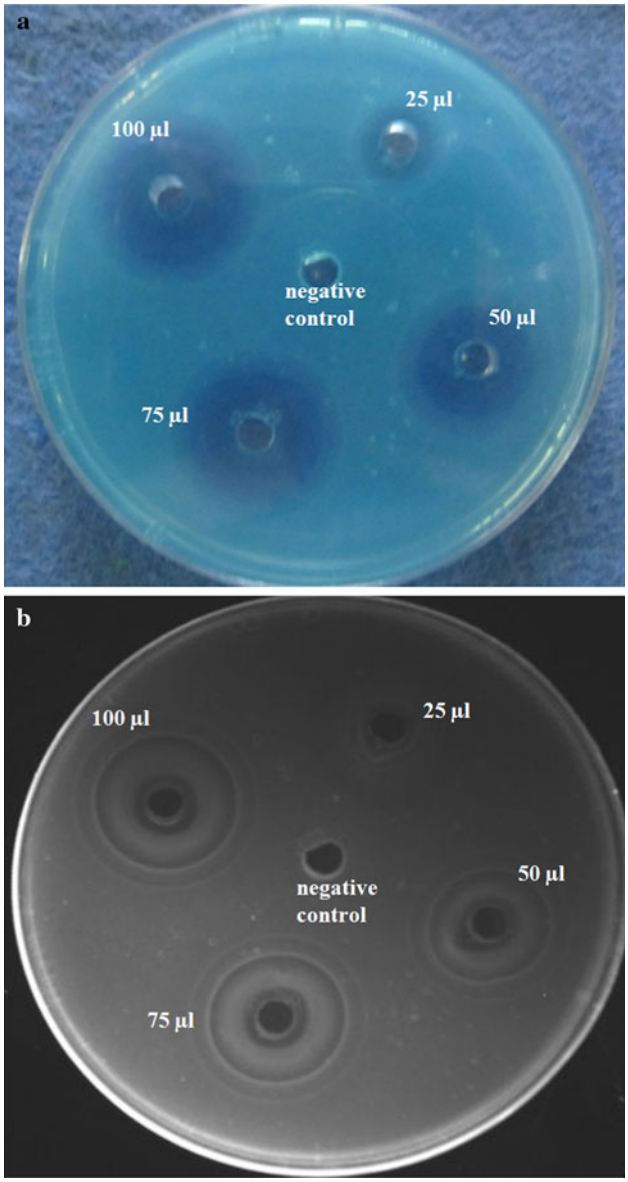

Fig. 3 Normal (**a**) and UV-transilluminated (**b**) pictures of agar plates supplemented with concentrated crude RLs solutions

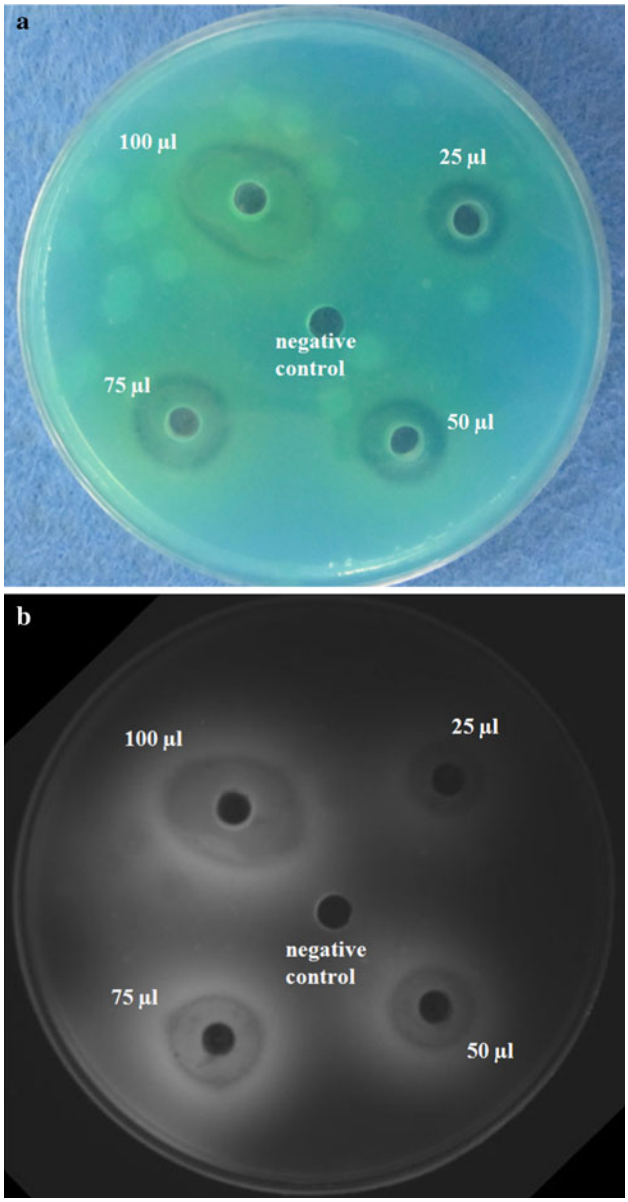

Fig. 4 Normal (**a**) and UV-transilluminated (**b**) pictures of agar plates inoculated with *P. aeruginosa* ATCC 9027

two samples. Based on the prepared calibration (standard) curve, the concentrations of the produced RLs in the mentioned media were determined. Table 3 presents the amounts of dry total biomass as well as RLs produced in the first two complex media.

According to Table 3, increase of IPTG concentration from 0.4 to 1.2 mM led to approximately 10 % increase of RLs formation. This indicates the effectiveness of the construct in the host *P. putida* KT2440 as well as the successful expression and the function of *rhlAB* genes. Also, the positive effect of glucose addition on increase of biomass yield and subsequently, rise of RLs production is obviously comprehensible. Adding 20 g/l glucose to the LB medium resulted in about 90 % increase of biomass and RLs production. This fact points out the importance of utilization of carbon sources in RLs biosynthesis even when rich media are utilized.

The detection of RLs by TLC was conducted as previously described. For this purpose, four samples were prepared from the complex media cultures of the non-induced recombinant (negative control) as well as the induced recombinant *P. putida* KT2440 and the aqueous solution of concentrated crude RLs (positive control). Figure 10 depicts the results of TLC for the four samples. These results confirmed that the recombinant strain *P. putida* KT2440 pVLT33_*rhlAB* is able to produce mono-RLs. After the confirmation, it was time to examine the abilities

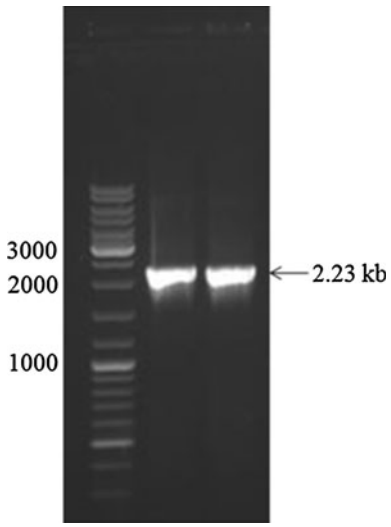

Fig. 5 The expected bands of *rhlAB* by PCR of extracted *P. aeruginosa* (ATCC 9027) DNA

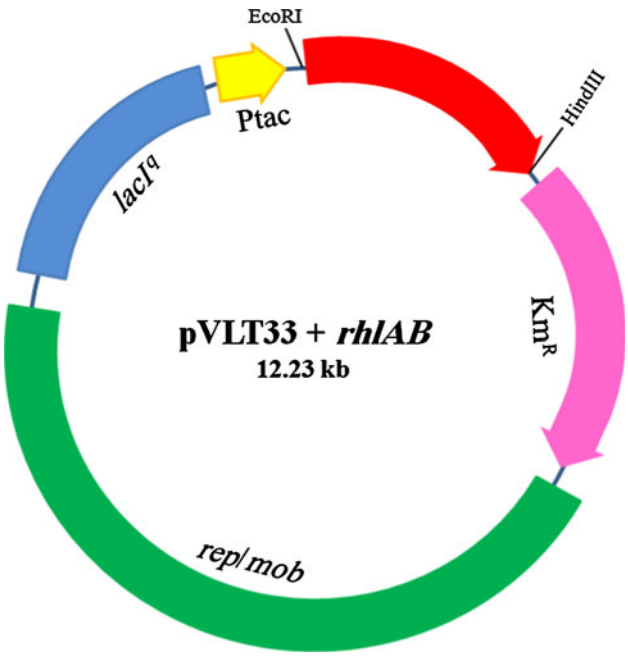

Fig. 6 Construction scheme of the construct pVLT33-*rhlAB*

of the recombinant strain for production of RLs in other cultivation media.

The amounts of RLs secreted in the second set of cultivation media are listed in Table 4.

According to the results shown in Table 4, no detectable RLs production occurred using the mineral solution 1. Despite the fact that this medium is potentially considerable for achievement of *P. putida* HCDC and high polyhydroxyalkanoates (PHAs) production, it is not individually a good choice for heterologous RLs production. The reason is that $(NH_4)_2SO_4$ might not be an appropriate

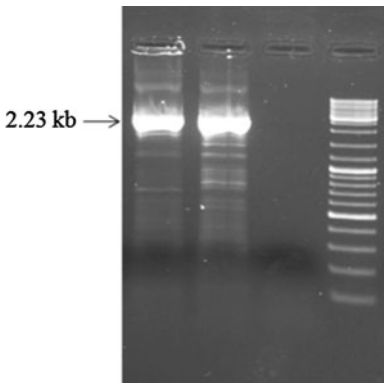

Fig. 7 The expected bands of *rhlAB* by colony PCR of transformed *E. coli* BL21DE3 and *P. aeruginosa* (ATCC 9027)

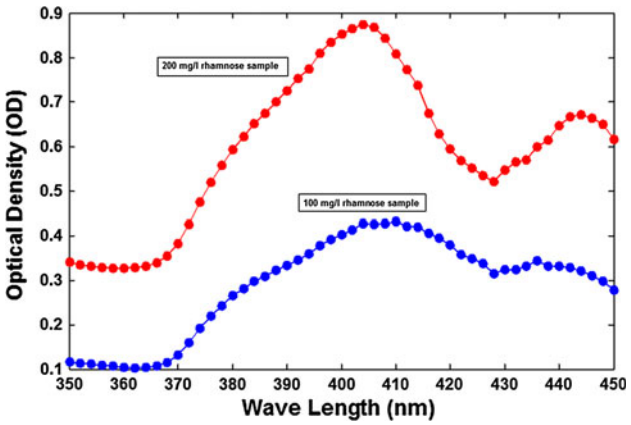

Fig. 8 Optical density survey for two samples of 100 and 200 mg/l rhamnose solutions

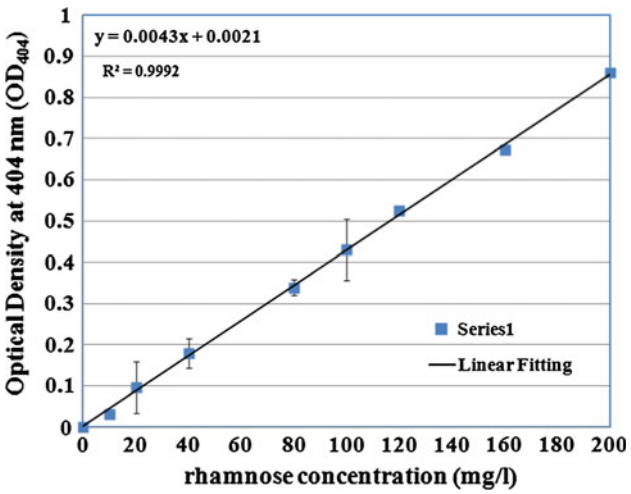

Fig. 9 Standard curve (linear correlation) for correlating OD_{404} and rhamnose concentration using orcinol assay

nitrogen source for simultaneous biomass and RLs formation. Using a complex medium at the first stage of dual cultivation method, might provide the opportunity for

Table 3 The amounts of dry total biomass, OD_{404} values, and the concentrations of produced RLs in complex media 1 and 2

Strain	Media	IPTG concentration (mM)	Dry total biomass (g/l)	OD_{404}[a]	Rhamnose concentration (mg/l)	RLs concentration (mg/l)[b]
Wild-type *P. putida* KT2440	LB + 20 g/l glucose	0.0	1.3	–	No detectable	No detectable
Recombinant *P. putida* KT2440	LB	0.0	1.1	–	No detectable	No detectable
Recombinant *P. putida* KT2440	LB	0.4	1.04	0.311	72	222
Recombinant *P. putida* KT2440	LB	1.2	1.0	0.346	80	247
Recombinant *P. putida* KT2440	LB + 20 g/l glucose	0.0	1.87	–	No detectable	No detectable
Recombinant *P. putida* KT2440	LB + 20 g/l glucose	0.4	2.38	0.593	138	425
Recombinant *P. putida* KT2440	LB + 20 g/l glucose	1.2	2.28	0.653	152	468

[a] The reported values are the average of at least two samples

[b] The values were estimated based on the ratio of the molecular weights of the predominant mono-RLs (504) to rhamnose (164.16)

formation of requisite precursors and help the cells to be prepared for production of such secondary metabolites. The result stemmed from the mixture medium indicates that it is possible to simultaneously utilize mixtures of complex and mineral media instead of dual cultivation systems. Both the dual cultivation system and mixture medium prepare considerable opportunities for further developments in heterologous biosynthesis of valuable secondary metabolites.

Inspired by the obtained results, the screening design was conducted using the components of LB and M9 mineral media.

Experimental Design and Analysis

The First Screening Step: Two-Level Fractional Factorial Design Resolution IV

After demonstration of the capability of the recombinant *P. putida* strain for RLs production, the screening set of experiments was implemented. The estimated amounts of RLs produced (mean ± standard deviation) as well as the dry total biomass formed in the media is illustrated in Table 5.

The responses listed in Table 5 showed the discrepancy from 0 (medium no. 8) to 0.788 (medium no. 12) of measured OD_{404} and 0 to 565 mg/l of estimated RLs production. The discrepancy indicates the critical role of medium composition in RLs biosynthetic performance. Also, the variation makes the statistical analysis easy and reliable. The highest levels of produced RLs were related to medium no. 5 and medium no. 12. Medium no. 5 contained the high levels of all the components (leading to the highest biomass yield) while medium no. 12 contained only four of them (leading to the highest RLs yield). A primary comparison results in identification of the effectiveness of some macro-nutritional compounds such as peptone, yeast extract, and glycerol. For factor analysis, the responses (OD_{404} and dry total biomass) were introduced to Design Expert software package. According to the responses,

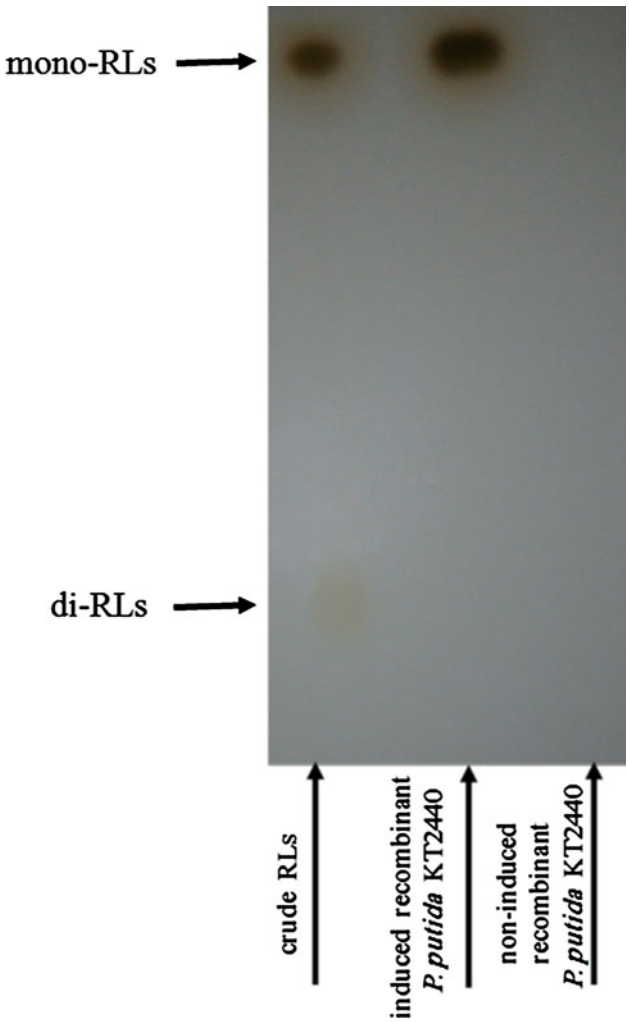

Fig. 10 Thin layer chromatography of RLs

Table 4 OD_{404} values and the produced RLs concentrations in the second set of cultivation media

Media	IPTG concentration (mM)	OD_{404}[a]	Rhamnose concentration (mg/l)	RLs concentration (mg/l)[b]
Mineral solution 1	1.0	–	No detectable	No detectable
Dual cultivation	1.0	0.221	51	157
Mixture medium	1.0	0.327	76	234

[a] The reported values are the average of at least two samples

[b] The values were estimated based on the ratio of the molecular weights of the predominant mono-RLs (504) to rhamnose (164.16)

Table 5 The amounts of dry total biomass, OD_{404} values, and the concentrations of produced RLs in the initial 16 screening-designed media

Media no.	Dry total biomass (g/l)	OD_{404}[a]	Rhamnose concentration (mg/l)	RLs concentration (mg/l)[b]
1	2.01	0.585 ± 0.078	136 ± 18	419 ± 56
2	2.63	0.687 ± 0.036	160 ± 8	492 ± 26
3	1.20	0.206 ± 0.018	48 ± 4	147 ± 13
4	0.78	0.215 ± 0.027	50 ± 6	153 ± 20
5	3.50	0.750 ± 0.048	175 ± 11	537 ± 34
6	1.37	0.105 ± 0.009	24 ± 2	74 ± 6
7	1.73	0.284 ± 0.030	66 ± 7	203 ± 22
8	Not measurable	No detectable	0	0
9	3.04	0.528 ± 0.028	123 ± 7	378 ± 20
10	2.08	0.219 ± 0.030	51 ± 7	156 ± 22
11	0.77	0.167 ± 0.012	39 ± 3	118 ± 9
12	3.14	0.788 ± 0.049	184 ± 11	565 ± 35
13	0.64	0.225 ± 0.027	52 ± 6	160 ± 20
14	0.00	0.00	0.00	0.00
15	2.56	0.530 ± 0.043	124 ± 10	380 ± 31
16	0.84	0.223 ± 0.018	52 ± 4	159 ± 13

[a] The reported values are the average of at least four samples

[b] The values were estimated based on the ratio of the molecular weights of the predominant mono-RLs (504) to rhamnose (164.16)

Table 6 Ranking list of the individual factors based on their impacts on RLs production

Design variable factors	Sum of squares	Effect	Contribution (%)
Peptone	0.074	+	7.33
Yeast extract	0.212	+	20.85
NH_4NO_3	0.001	−	0.094
$NaNO_3$	0.0005	+	0.050
$Na_2HPO_4{\cdot}12H_2O$	0.0016	+	0.16
KH_2PO_4	1.655E−005	−	0.0016
Glycerol	0.656	+	64.55
Sunflower oil	0.005	−	0.488

analysis of variance (ANOVA) was done and the sum of squares for each factor was calculated. Based on the sum of squares values, ranking lists of the factors were made (Table 6) and their impacts on the response were determined.

All the factors except NH_4NO_3, KH_2PO_4, and sunflower oil (hydrophobic carbon source) had positive effects on the response. However, the values of sum of squares clearly point out that glycerol, yeast extract, and peptone had significant positive impacts on the response with contributions of 64.55, 20.85, and 7.33 %, respectively. Although the two-level fractional factorial design (resolution IV) used might not clearly examine the two-factor interactions and these interactions might confound with each other, it had two main advantages. The first one is the main effects of individual factors are clear of the two-factor interactions. Thus, the influences of glycerol, yeast extract, and peptone are reliable. The second one is some insights can be achieved for further experimental designs considering the two-factor interactions in the current step. Therefore, the half-normal plot was drawn depicting the impacts by each factor or two-factor interactions on RLs yield (Fig. 11a). Based on the half-normal plot, peptone/glycerol, peptone/yeast extract, and peptone/KH_2PO_4 two-factor interactions should be considered in the analysis. Taking peptone/KH_2PO_4 interaction into account caused KH_2PO_4 to be required to support hierarchy. Thus, four factors (glycerol, yeast extract, peptone, and KH_2PO_4) with three two-factor interactions were regarded in the mathematical model developed by the software package. The

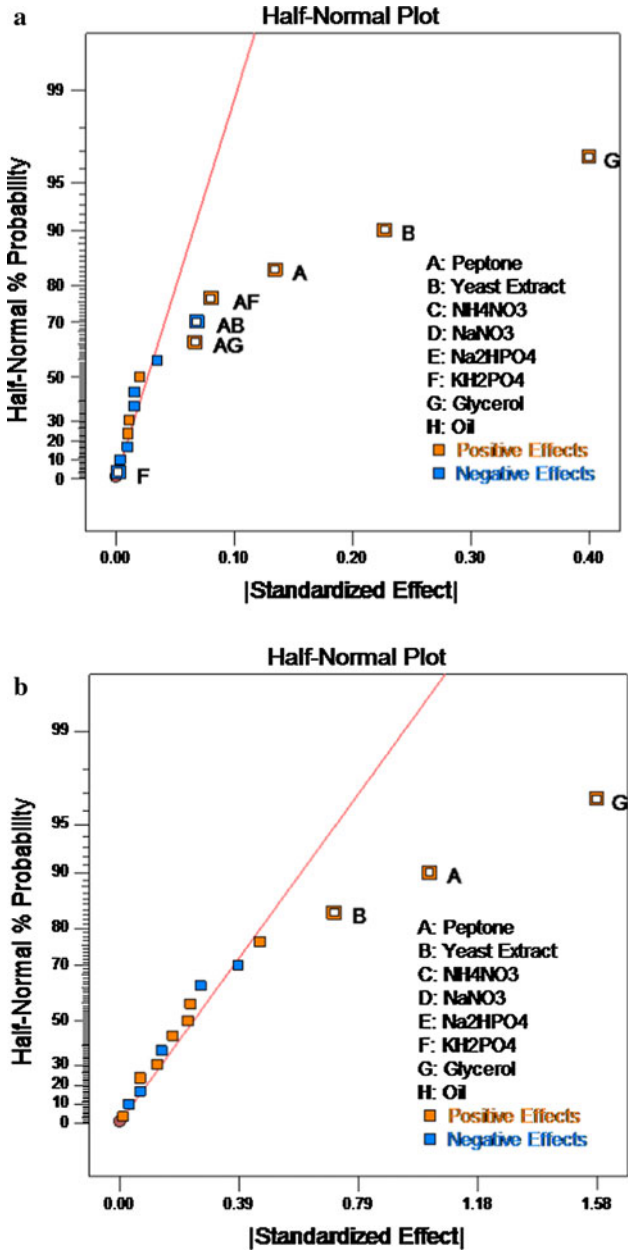

Fig. 11 The half-normal plot depicting the impacts by individual factors or two-factor interactions on RLs yield (**a**) and dry total biomass (**b**) in the two-level fractional factorial screening design

ranking list of the considered elements is illustrated in Table 7.

The second-order polynomial function fitted to the experimental results of OD_{404} in terms of actual factors is as follows:

$$y_{OD_{404}} = 0.056x_1 + 0.6x_2 - 0.278x_3 + 0.337x_4 - 0.276x_1x_2 + 0.542x_1x_3 + 0.136x_1x_4, \quad (1)$$

where x_1, x_2, x_3, and x_4 are, respectively, the concentrations (%w/v) of peptone, yeast extract, KH_2PO_4, and glycerol.

Table 7 Ranking list of the considerable single factors and significant two-factor interactions based on the impacts on RLs production

Design variable factors	Sum of squares	Effect	Contribution (%)	*p* value
Peptone	0.074	+	7.33	<0.0001*
Yeast extract	0.212	+	20.85	<0.0001*
KH_2PO_4	1.655E−005	−	0.0016	0.9110
Glycerol	0.656	+	64.55	<0.0001*
Peptone/yeast extract	0.019	−	1.88	0.0045*
Peptone/KH_2PO_4	0.026	+	2.60	0.0017*
Peptone/glycerol	0.0184	+	1.81	0.0049*

* The *p* values less than 0.05 indicate model term is significant

The value of the determination coefficient R^2 is equal to 0.9902 for the mathematical model. Equation (1) would be suitable for further investigation and decision making for future designs.

As resulted from Table 7, peptone/yeast extract and KH_2PO_4 had negative influences on the response. The important object which should be pointed out is that despite the important individual roles of peptone and yeast extract in RLs formation, the presence of both the terms may lead to a slight reduction in production. Moreover, although KH_2PO_4 had a negligible negative effect, it would increase the product formation when used with peptone. It might be stemmed from the impact of KH_2PO_4 on pH of the cultivation medium as an element of phosphate buffer.

Figure 11b illustrates the half-normal plot, which shows the impacts by each factor or two-factor interactions on dry total biomass. Regarding this plot and the relevant analysis on dry total biomass (data not shown) it is revealed that glycerol, peptone, and yeast extract had the most significant positive impacts (53.36, 22.52, and 10.79 % of contribution, respectively) on total biomass formation. This fact demonstrated that the contribution of peptone was much more than that of yeast extract. However, peptone/yeast extract two-factor interaction had also a slight negative effect (1.54 % of contribution) on the response.

The Second Screening Step: Two-Level Full Factorial Design

Regarding the results obtained in the first screening step, glycerol, yeast extract, peptone, and KH_2PO_4 were selected as considerable factors for further investigation. A two-level full factorial set of experiments was designed in which the low and high values for each factor were regarded similar to the previous step (listed in Table 1). For this aim, 16 different medium compositions were prepared. The layout of the full factorial design is illustrated in

Table 8. The estimated amounts of produced RLs as well as the dry total biomass are presented in Table 9.

The responses listed in Table 9 showed the discrepancy from 0 (media no. 5, 9, 10 and 13) to 0.794 (medium no. 8) of measured OD_{404} and 0 to approximately 570 mg/l of estimated RLs production. The maximum production was related to medium no. 8. Also, the media no. 6, 1, 12, and 15 had significant amounts of RLs comparing to the others. Medium no. 8 contained the high levels of all the components (leading to the highest biomass yield). Comparison of the medium no. 8 in the second series of experiments and the medium no. 12 in the initial series indicated that the increase of IPTG induction did not affect the responses in a straightforward manner. This means that 0.4 mM IPTG concentration would be enough and appropriate for the factor screening design through which only the effects of medium components were evaluated. For factor analysis, the responses (OD_{404} and dry total biomass) were given to the Design Expert software package. Then, ANOVA was performed and the sum of squares for each factor was calculated. Based on the sum-of-square values, ranking lists of the factors were made (Table 10) and their impacts on the response were determined.

Table 8 Two-level full factorial design for the 4 factors (16 testing media)

Media no.	Glycerol (%w/v)	Yeast extract (%w/v)	Peptone (%w/v)	KH_2PO_4 (%w/v)
1	1.0	0.0	1.0	0.0
2	0.0	0.5	0.0	0.0
3	0.0	0.5	0.0	0.3
4	0.0	0.5	1.0	0.0
5	0.0	0.0	0.0	0.0
6	1.0	0.5	0.0	0.3
7	1.0	0.0	1.0	0.3
8	1.0	0.5	1.0	0.3
9	1.0	0.0	0.0	0.3
10	1.0	0.0	0.0	0.0
11	0.0	0.0	1.0	0.0
12	1.0	0.5	0.0	0.0
13	0.0	0.0	0.0	0.3
14	0.0	0.5	1.0	0.3
15	1.0	0.5	1.0	0.0
16	0.0	0.0	1.0	0.3

Table 10 Ranking list of the considerable single factors and significant two/three-factor interactions based on the impacts on RLs production

Design variable factors	Sum of squares	Effect	Contribution (%)	*p* value
Glycerol	0.39	+	32.78	<0.0001*
Yeast extract	0.27	+	22.09	<0.0001*
Peptone	0.25	+	21.02	<0.0001*
Glycerol/yeast extract	0.056	+	4.62	0.0004*
Glycerol/peptone	0.034	+	2.86	0.0017*
Yeast extract/peptone	0.14	−	12.0	<0.0001*
Glycerol/yeast extract/peptone	0.043	−	3.56	0.0009*

* The *p* values less than 0.05 indicate model term is significant

Table 9 The amounts of dry total biomass, OD_{404} values and the concentrations of produced RLs in the second 16 screening-designed media

Media no.	Dry total biomass (g/l)	OD_{404}[a]	Rhamnose concentration (mg/l)	RLs concentration (mg/l)[b]
1	2.60	0.664 ± 0.028	155 ± 7	476 ± 20
2	0.32	0.225 ± 0.022	52 ± 5	160 ± 16
3	0.38	0.227 ± 0.005	53 ± 1	162 ± 4
4	0.64	0.289 ± 0.037	67 ± 9	206 ± 27
5	Not measurable	No detectable	0.0	0.0
6	2.74	0.674 ± 0.038	157 ± 9	483 ± 27
7	2.88	0.610 ± 0.038	142 ± 9	437 ± 27
8	3.20	0.794 ± 0.059	185 ± 14	569 ± 43
9	Not measurable	No detectable	0.0	0.0
10	Not measurable	No detectable	0.0	0.0
11	0.39	0.239 ± 0.023	55 ± 5	170 ± 17
12	1.24	0.663 ± 0.060	155 ± 14	475 ± 43
13	Not measurable	No detectable	0.0	0.0
14	0.62	0.308 ± 0.030	72 ± 7	220 ± 22
15	2.36	0.645 ± 0.052	150 ± 12	462 ± 38
16	0.60	0.251 ± 0.008	58 ± 2	179 ± 6

[a] The reported values are the average of at least four samples

[b] The values were estimated based on the ratio of the molecular weights of the predominant mono-RLs (504) to rhamnose (164.16)

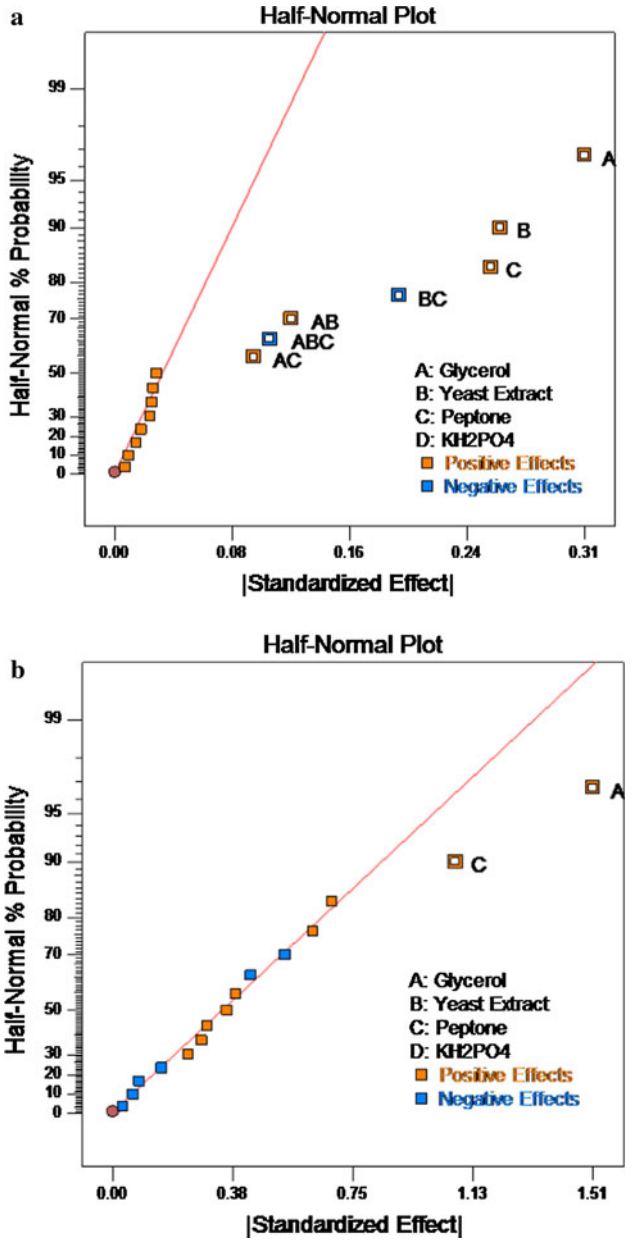

Fig. 12 The half-normal plot depicting the impacts by individual factors or two/three-factor interactions on RLs yield (**a**) and dry total biomass (**b**) in the full factorial screening design

The third-order polynomial function fitted to the experimental results of OD_{404} in terms of actual factors is as follows:

$$v_{OD_{404}} = 0.453u_2 + 0.245u_3 + 0.885u_1u_2 + 0.392u_1u_3 - 0.346u_2u_3 - 0.827u_1u_2u_3, \quad (2)$$

where u_1, u_2, and u_3 are, respectively, the concentrations (%w/v) of glycerol, yeast extract, and peptone.

The value of the determination coefficient R^2 is equal to 0.9893 for the mathematical model. The half-normal plot was also drawn in order to depict the impacts by individual factors as well as two- and three-factor interactions on RLs yield (Fig. 12a).

Regarding the results shown in Table 10 and Fig. 12a, the significant positive effects of glycerol, yeast extract, and peptone on heterologous RLs production were confirmed. However, it should be noted that based on the terms of the mathematical model (Eq. 2), the influence of glycerol in the presence of yeast extract or peptone was significant. Comparing its contribution in the first (64.55 %) and the second (32.78 %) sets of experiments highlights this fact even more. Also, the negative impact of yeast extract/peptone two-factor interaction was demonstrated. This contributed to the negative impact of glycerol/yeast extract/peptone three-factor interactions while glycerol/yeast extract and glycerol/peptone two-factor interactions had positive impacts on RLs production. It should be noted that the presence of KH_2PO_4 in the culture medium affected the RLs production in a positive manner although not significantly.

The half-normal plot, which depicts the impacts of each factor or two/three-factor interactions on dry total biomass is illustrated in Fig. 12b. According to this plot and the relevant analysis on dry total biomass (data not shown), it is revealed that glycerol and peptone had, respectively, the most significant effects on biomass formation. The positive impact of peptone was more than three times in comparison to that of yeast extract. Also, yeast extract/peptone two-factor and glycerol/yeast extract/peptone three-factor interactions had negative influences on biomass formation while glycerol/peptone and glycerol/yeast extract two-factor interactions affects biomass formation in a positive manner.

Discussion

Heterologous Production of *P. aeruginosa* ATCC 9027 Rhamnolipids in *P. putida* KT2440

The cloned genes (*rhlAB*) were extracted for the first time from the well-studied mono-RLs-producing strain of *P. aeruginosa* (ATCC 9027). Due to the rich pieces of information available about ATCC 9027 and the wide range of different mono-RLs produced by this strain [27], the current study will be the preliminary step for investigation of the capabilities of the recombinant *P. putida* KT2440 in comparison to *P. aeruginosa* ATCC 9027 for production of a vast variation of mono-RLs/or specific ones with limited range of molecular weights.

It should be noted that the current work was mainly a conceptual study which investigated the feasibility of heterologous RLs production in different types of culture

systems such as dual cultivation and mixture media. The main aim was to provide appropriate insights for further studies to improve the RLs biosynthesis processes. According to the results obtained from the media utilized in this study, the recombinant *P. putida* KT2440 pVLT33_*rhlAB* could able to produce more than 0.2 and 0.4 g/l RLs in LB medium and LB supplemented with glucose, respectively. The results had the same order of magnitude as those of Ochsner et al. [16] (*P. putida* KT2442 pUO98) and Wittgens et al. [14] (*P. putida* KT2440 pVLT33_*rhlAB*). However, these values are rather low compared to the RLs concentrations produced by the parental strain *P. aeruginosa* ATCC 9027 (i.e., 4.261 g/l [41]) and the genetically modified strain of *P. putida* studied in [17]. The reason is that the production of RLs by the parental strain and other *P. aeruginosa* species are highly regulated by the complex quorum-sensing systems (las and rhl) at the transcriptional and translational levels. Through autoinducer signal molecules (*N*-(3-oxododecanoyl) homoserin lactone and *N*-butyryl homoserin lactone) these complex systems contribute to coordination of population activities such as highly upregulation of *rhlAB* genes [10, 42] especially under nitrogen or phosphate limitation [14, 41]. These well-studied systems are very important in *P. aeruginosa* species pathogenesis [10]. As mentioned before, the *rhlAB* operon promoter is under the control of the *rhlR* and *rhlI* gene products [17]. Since *N*-acyl homoserine lactone (AHL) autoinducers are produced by some *P. putida* strains [43], cloning of the whole *rhlABRI* operon might contribute to high yields of heterologous RLs as reported by [17]. According to [37], using synthetic quorum sensing systems enables us to engineer microorganisms to sense their cell density and coordinate/activate the desired genes expression at the suitable time. Due to the complexity of quorum sensing systems and lack of studies on these systems in *P. putida* species, an alternative method can be chosen in which the cultures are grown to an intermediate or high density before inducing product formation at the appropriate time [37]. This method helps us to avoid the complexity of quorum sensing and achieve a more controllable biosynthetic process. However, as a result of the fact that both precursors of RLs are stemmed from the central metabolism, the overproduction of heterologous RLs is somehow difficult [2, 13, 16]. It seems that in addition to metabolic engineering, optimal medium design can be an appropriate solution for the problem through which the suitable sources with proper ratios are supplied to the recombinant microbes. Thus, the main goal of the current work was to investigate the feasibility of heterologous production of *P. aeruginosa* ATCC 9027 RLs in a number of considerable media as well as the application of the screening design as the preliminary step for optimal medium design that paves the way for further investigations.

With the aim of utilization of renewable resources, due to the advantages of glycerol (the substrate of natural origin with various main sources) it is utilized in the main part of the experiments [3]. The results pointed out that unlike sunflower oil (the hydrophobic carbon source) glycerol has a significant impact on heterologous RLs biosynthesis. This fact leads us to further studies for examination of the other hydrophilic and hydrophobic carbon sources.

Experimental Design

While the previous studies in [14] confirmed the importance of metabolic engineering to improve the recombinant RLs formation yields, the current study mainly focused on the importance of medium composition and identification of the most critical and effective compounds. The wide range of variation between the results of screening tests demonstrated the role of medium composition on RLs biosynthesis performance. Applying the proposed two-step screening approach helped us to gain maximum insight via conducting a minimum number of experiments. The initial two-level fractional factorial design provided useful pieces of information for the complementary steps and further studies. Although we could have confined ourselves to the two-factor interactions when resolution IV was employed, considering the interactions would provide suitable suggestions as initial screening designs. Thus, it may be a better choice compared to Plackett–Burman method. Based on the individual factor analysis, glycerol, yeast extract, and peptone were determined as the best compounds. However, taking the two-factor interactions into account, negative effect of peptone/yeast extract and positive effect of peptone/KH_2PO_4 on RLs formation were revealed. The combination of the initial fractional factorial screening with the two-level full factorial design for the four mentioned components allowed for a thorough investigation of the two- and three-factor interactions on RLs production in addition to confirming the impacts of individual compounds. Investigating the two-factor interactions besides the individual main effects would be the first step of two- or three-step procedures including mixture experiment or combined mixture designs in which the process factors such as IPTG-induction time, the times of induction, the process time, agitation, and aeration as well as feeding strategies and HCDC-relevant techniques can be studied. It should be noted that through stepwise screening procedures such as initial Plackett–Burman method followed by fractional factorial (resolutions IV or V) and full factorial designs, it would be possible to screen a large number of components and identify the critical ones via small numbers of experimental series.

To sum up, it is determined that glycerol, yeast extract, and peptone have positive influences on heterologous RLs

production. Peptone is more suitable for biomass formation, which implicitly influences the RLs production, while yeast extract more significantly affects the RLs formation. However, their interaction influences the biomass and RLs formation in a negative manner. Thus, there exists a trade-off between biomass formation and RLs production using both peptone and yeast extract in the medium composition. This fact contributes to the definition of the most efficient ratios of the compounds glycerol, yeast extract, and peptone in the cultivation media via further experimental designs such as mixture/combined mixture experiments.

Conclusion

In this work, the heterologous production of *P. aeruginosa* ATCC 9027 RLs in the safety host *P. putida* KT2440 was investigated. The heterologous production of RLs was studied and demonstrated in a number of cultivation media. Thanks to the techniques of experimental design, as a prerequisite of the optimal medium design, a two-step approach for screening of eight macro-nutritional components (containing carbon, nitrogen, and phosphate sources) was proposed through which the most effective ones were identified and the two/three-factor interactions besides their individual impacts were studied conducting only two 16-experimental series. Glycerol, yeast extract, and peptone were chosen for the complementary experimental designs such as mixture experiments or combined mixture ones. Due to the wide range of advantages of DoE methodologies and the proposed screening procedure, optimal medium designs and optimal operating policies can be determined through small numbers of experimental series. Moreover, stepwise screening procedures can enhance the screening of large numbers of media components, environmental conditions, and process factors, as well as determining the multi-factor interactions.

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References

1. Soberón-Chávez, G., Lepine, F., & Déziel, E. (2005). Production of rhamnolipids by *Pseudomonas aeruginosa*. *Applied Microbiology and Biotechnology, 68*, 718–725.
2. Müller, M. M., Kügler, J. H., Henkel, M., Gerlitzki, M., Hörmann, B., Pöhnlein, M., et al. (2012). Rhamnolipids—next generation surfactants. *Journal of Biotechnology, 162*, 366–380.
3. Henkel, M., Müller, M. M., Kügler, J. H., Lovaglio, R. B., Conttiero, J., Syldatk, Ch., et al. (2012). Rhamnolipids as biosurfactants from renewable resources: Concepts for next-generation rhamnolipid production. *Process Biochemistry, 47*, 1207–1219.
4. Lietermann, F., Walter, V., Syldatk, C., & Hausmann, R. (2010). Rhamnolipids. In K. N. Timmis (Ed.), *Handbook of hydrocarbon and lipid microbiology* (pp. 3037–3051). Berlin: Springer.
5. Abdel-Mawgoud, A. M., Lépine, F., & Déziel, E. (2010). Rhamnolipids: Diversity of structures, microbial origins and roles. *Applied Microbiology and Biotechnology, 86*, 1323–1336.
6. Pantazaki, A. A., Dimopoulou, M. I., Simou, O. M., & Pritsa, A. A. (2010). Sunflower seed oil and oleic acid utilization for the production of rhamnolipids by *Thermus thermophilus* HB8. *Applied Microbiology and Biotechnology, 88*, 939–951.
7. Řezanka, T., Siristova, L., & Sigler, K. (2011). Rhamnolipid-producing thermophilic bacteria of species *Thermus* and *Meiothermus*. *Extremophiles, 15*, 697–709.
8. Müller, M. M., Hörmann, B., Syldatk, Ch., & Hausmann, R. (2010). *Pseudomonas aeruginosa* PAO1 as a model for rhamnolipid production in bioreactor systems. *Applied Microbiology and Biotechnology, 87*, 167–174.
9. Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., et al. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Molecular Microbiology, 24*, 309–319.
10. Wagner, V. E., Frelinger, J. G., Barth, R. K., & Iglewski, B. H. (2006). Quorum sensing: dynamic response of *Pseudomonas aeruginosa* to external signals. *Trends in Microbiology, 14*, 55–58.
11. Schuster, M., & Greenberg, E. P. (2007). Early activation of quorum sensing in *Pseudomonas aeruginosa* reveals the architecture of a complex regulon. *BMC Genomics, 8*, 287.
12. Vatsa, P., Sanchez, L., Clement, Ch., Baillieul, F., & Dorey, S. (2010). Rhamnolipid biosurfactants as new players in animal and plant defense against microbes. *International Journal of Molecular Sciences, 11*, 5095–5108.
13. Ochsner, U. A., Hembach, Th., & Fiechter, A. (1995). Production of rhamnolipid biosurfactants. *Advances in Biochemical Engineering/Biotechnology, 53*, 89–118.
14. Wittgens, A., Tiso, T., Arndt, T. T., Wemk, P., Hemmerich, J., Müller, C., et al. (2011). Growth independent rhamnolipid production from glucose using the non-pathogenic *Pseudomonas putida* KT2440. *Microbial Cell Factories, 10*, 80–97.
15. Poblete-Castro, I., Becker, J., Dohnt, K., dos Santos, V. M., & Wittmann, Ch. (2012). Industrial biotechnology of *Pseudomonas putida* and related species. *Applied Microbiology and Biotechnology, 93*, 2279–2290.
16. Ochsner, U. A., Reiser, J., Fiechter, A., & Witholt, B. (1995). Production of *Pseudomonas aeruginosa* rhamnolipid biosurfactants in heterologous hosts. *Applied and Environment Microbiology, 61*, 3503–3506.
17. Cha, M., Lee, N., Kim, M., Kim, M., & Lee, S. (2008). Heterologous production of *Pseudomonas aeruginosa* EMS1 biosurfactant in *Pseudomonas putida*. *Bioresource Technology, 99*, 2192–2199.
18. Kennedy, M., & Krouse, D. (1999). Strategies for improving fermentation medium performance: A review. *Journal of Microbiology and Biotechnology, 23*, 456–475.
19. Parekh, S., Vinci, V. A., & Strobel, R. J. (2000). Improvement of microbial strains and fermentation processes. *Applied Microbiology and Biotechnology, 54*, 287–301.
20. Abdel-Fattah, Y. R., & Olama, Z. A. (2002). L-Asparginase production by *Pseudomonas aeruginosa* in solid-state culture:

Evaluation and optimization of culture conditions using factorial designs. *Process Biochemistry, 38*, 115–122.
21. Rispoli, F., & Shah, V. (2007). Mixture design as a first step for optimization of fermentation medium for cutinase production from *Colletotrichum lindemuthianum*. *Journal of Industrial Microbiology and Biotechnology, 34*, 349–355.
22. Mandenius, C. F., & Brundin, A. (2008). Bioprocess optimization using design-of-experiments methodology. *Biotechnology Progress, 24*, 1191–1203.
23. Broedel, S. E., Jr., Papciak, S. M., & Jones, W. R. (2001). The selection of optimum media formulations for improved expression of recombinant proteins in *E. coli*. *Technical Bulletin, 2*, 1–6.
24. Zhang, Y., & Miller, R. M. (1992). Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). *Applied and Environment Microbiology, 58*, 3276–3282.
25. Zhang, Y., & Miller, R. M. (1994). Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. *Applied and Environment Microbiology, 60*, 2101–2106.
26. Al-Tahhan, R. A., Sandrin, T. R., Bodour, A. A., & Maier, R. M. (2000). Rhamnolipid-induced removal of lipopolysaccharide from *Pseudomonas aeruginosa*: Effect on cell surface properties and interaction with hydrophobic substrates. *Applied and Environment Microbiology, 66*, 3262–3268.
27. Lebrón-Paler, A. (2008) Solution and interfacial characterization of rhamnolipid biosurfactant from *P. aeruginosa* ATCC 9027. PhD Thesis, University of Arizona, Arizona.
28. Bai, G. Y., Brusseau, M. L., & Miller, R. M. (1997). Biosurfactant-enhanced removal of residual hydrocarbon from soil. *Journal of Contaminant Hydrology, 25*, 157–170.
29. de Lorenzo, V., Eltis, L., Kessler, B., & Timmis, K. N. (1993). Analysis of *Pseudomonas* gene products using *lacI*^{q}/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. *Gene, 123*, 17–24.
30. Heyd, M., Kohnert, A., Tan, T. H., Nusser, M., Kirschhofer, F., Brenner-Weiss, G., et al. (2008). Development and trends of biosurfactant analysis and purification using rhamnolipids as an example. *Analytical and Bioanalytical Chemistry, 391*, 1579–1590.
31. Siegmund, I., & Wagner, F. (1991). New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnology Techniques, 5*, 265–268.
32. Pinzon, N., & Ju, L. K. (2009). Improved detection of rhamnolipid production using agar plates containing methylene blue and cetyl trimethylammonium bromide. *Biotechnology Letters, 31*, 1583–1588.
33. Chandrasekaran, E. V., & Bemiller, J. N. (1980). Constituent analyses of glycosaminoglycans. In R. L. Whistler (Ed.), *Methods in carbohydrate chemistry* (pp. 89–96). New York: Academic Press.
34. Koch, A. K., Käppeli, O., Fiechter, A., & Reiser, J. (1991). Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *Journal of Bacteriology, 173*, 4212–4219.
35. Ballot, F. (2009). Bacterial production of antimicrobial biosurfactants. MSc Thesis, University of Stellenbosch, Stellenbosch, South Africa.
36. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., et al. (1987). *Current protocols in molecular biology*. New York: Greene Publishing Associates/ Wiley Interscience.
37. Holtz, W. J., & Keasling, J. D. (2010). Engineering static and dynamic control of synthetic pathways. *Cell, 140*, 19–23.
38. Kim, G. J., Lee, I. Y., Choi, D. K., Yoon, S. C., & Park, Y. H. (1996). High cell density cultivation of *Pseudomonas putida* BM01 using glucose. *Journal of Microbiology and Biotechnology, 6*, 221–224.
39. Sun, Z., Ramsay, J. A., Guay, M., & Ramsay, B. A. (2006). Automated feeding strategies for high-cell-density fed-batch cultivation of *Pseudomonas putida* KT2440. *Applied Microbiology and Biotechnology, 71*, 423–431.
40. Sivashanmugam, A., Murray, V., Cui, Ch., Zhang, Y., Wang, J., & Li, Q. (2009). Practical protocols for production of very high yields of recombinant proteins using *Escherichia coli*. *Protein Science, 18*, 936–948.
41. Clarke, K. G., Ballot, F., & Reid, S. J. (2010). Enhanced rhamnolipid production by *Pseudomonas aeruginosa* under phosphate limitation. *World Journal of Microbiology & Biotechnology, 26*, 2179–2184.
42. Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I., & Iglewski, B. H. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. *Journal of Biotechnology, 185*, 2080–2095.
43. Bertani, I., & Venturi, V. (2004). Regulation of the *N*-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase *RpoS* sigma factor and the global regulator *GacA*. *Applied and Environment Microbiology, 70*, 5493–5502.