Optimization and characterization of microbial rhamnolipid production from renewable resources

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von

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Preamble

The thesis at hand is based on a compendium of peer-reviewed works that were independently published during the last three years. These publications compose of one general introducing book chapter, two original papers which reflect a part of the laboratory work, and a review article that is concluding the last years of rhamnolipid research and gives an outlook of the possible and necessary works for the future. Chapter 1 is a general introduction and was published from PanStanford Publishing Pte. Ltd. (New Jersey, London, Singapore) in 2011 as “Chapter 16: Microbial rhamnolipids” in “Carbohydrate-Modifying Biocatalysts” which was edited by Prof. Dr. P. Grunwald of the Hamburg-Harburg University.

The Chapters 2 and 3 reflect a principal part of the laboratory work performed during the last three years. The main problems encountered with *Pseudomonas aeruginosa* during rhamnolipid bioreactor production are presented and discussed. Chapter 2 was published as “*Pseudomonas aeruginosa* PAO1 as a model for rhamnolipid production in bioreactor systems” in 2010 and Chapter 3 as “Evaluation of rhamnolipid production capacity of *Pseudomonas aeruginosa* PAO1 in comparison to the rhamnolipid over-producer strains DSM 7108 and DSM 2874” in 2011, both in the Applied Microbiology and Biotechnology Journal from Springer (Heidelberg).

Finally, in Chapter 4 the main conclusions and encountered problems of the last 60 years of rhamnolipid research were put into this Mini-Review which will be published as “Regulatory and metabolic network of rhamnolipid biosynthesis: Traditional and advanced engineering towards biotechnological production” in 2011 in the Applied Microbiology and Biotechnology Journal from Springer (Heidelberg).

The presented publications are identical in content but not in layout with the published versions. If necessary, urgent changes were made and highlighted by footmarks.

Publications and presentations

Original papers and reviews

2011 **Müller MM** and Hausmann R.
Regulatory and metabolic network of rhamnolipid biosynthesis: Traditional and advanced engineering towards biotechnological production.
Applied Microbiology and Biotechnology;
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2011 **Müller MM**, Hörmann B, Kugel M, Syldatk C and Hausmann R.
Evaluation of rhamnolipid production capacity of *Pseudomonas aeruginosa* PAO1 in comparison to the rhamnolipid over-producer strains DSM 7108 and DSM 2874.
Applied Microbiology and Biotechnology; 89; (3): 585-592

2010 Hörmann B, **Müller MM**, Syldatk C and Hausmann R.
Rhamnolipid production by *Burkholderia plantarii* DSM 9509^{T}.
European Journal of Lipid Science and Technology; 112; 674-680.

2010 **Müller MM**, Hörmann B, Syldatk C and Hausmann R.
Pseudomonas aeruginosa PAO1 as a model for rhamnolipid production in bioreactor systems.
Applied Microbiology and Biotechnology. 87; (1): 167-174.

2009 Martinez JC, **Müller MM**, Turley H, Steer G, Choteau L, Li J-L. Saison R, Harris AL, Pezzella F and Gatter KC.
Nuclear and membrane expression of the angiogenesis regulator delta-like 4 (DLL4) in normal and malignant human tissues.
Histopathology; 54; 598-606.

Book chapters

2011 **Müller MM**, Hörmann B, Syldatk C and Hausmann R.
Microbial Rhamnolipids IN *Carbohydrate Modifying Biocatalysts* Prof. Peter Grunwald (ed.), Pan Stanford Publishing Pte Ltd.
ISBN-10: 981-4-241-679 *in press*

2011 Abdel-Mawgoud AM, Hausmann R, Lépine F, **Müller MM** and Déziel E.
Biosurfactants: From genes to applications IN *Microbiology Monographs series Vol. 20* Gloria Soberón-Chávez (ed.), Springer.
ISBN: 978-3-642-14489-9

Talks and presentations at conferences

Sept. 2011	**Talk** "Rhamnolipids: Next Generation Biosurfactants" 1st European Congress of Applied Biotechnology in Berlin (announced)
April 2011	**Talk** "Rhamnolipids: Green surfactants based on renewables" VAAM Annual Meeting in Karlsruhe
Sept. 2010	**Poster presentation** 28. Annual Meeting of DECHEMA in Aachen
May 2010	**Poster presentation** DECHEMA Symposium: "Bioprozessorientiertes Anlagendesign" in Nürnberg
Sept. 2009	**Poster presentation** 27. Annual Meeting of DECHEMA in Mannheim

Zusammenfassung

In den letzten zehn Jahren hat das Interesse der chemischen Industrie an wirtschaftlichen und umweltverträglichen Bioprozessen zur Herstellung von Bioprodukten, die in der Lage sind etablierte petrochemisch hergestellte Produkte zu ersetzen, deutlich zugenommen. Rhamnoselipide sind Glykolipide und werden auf Basis nachwachsender Rohstoffe mikrobiell hergestellt, weshalb sie als Biotenside bezeichnet werden. Diese Glykolipide wurden vor 60 Jahren erstmalig erwähnt. Heutzutage werden alle Glykolipide, welche aus ein bis zwei L-Rhamnoseeinheiten bestehen und mit meist zwei β-Hydroxyalkansäuren verbunden sind, als Rhamnoselipide bzw. Rhamnolipide zusammengefasst. Die L-Rhamnose stellt hierbei den hydrophilen Teil des Biotensids dar, während das hydrophobe Ende durch die β-Hydroxyalkansäuren gebildet wird. Mittlerweile sind mehr als 60 Strukturhomologe der Rhamnoselipide bekannt. Sie gehören zu der relativ kleinen Gruppe von bereits kommerziell erhältlichen Biotensiden und werden hauptsächlich von *Pseudomonas* und *Burkholderia* Stämmen synthetisiert. Aufgrund der sehr guten tensidischen Eigenschaften, der Umweltverträglichkeit und der Möglichkeit sie nachhaltig herzustellen, könnten Rhamnoselipide eine wichtige Rolle als vielseitig einsetzbare Biotenside einnehmen. Trotzdem gibt es verschiedene Faktoren, wie zum Beispiel die bisher sehr niedrige Produktivität, eine relativ komplexe und kostenintensive Produktaufarbeitung, sowie die vergleichsweise hohen Rohstoffkosten, welche den wirtschaftlichen Einsatz als Grundchemikalie bisher verhindert haben.

Kapitel 1 „Microbial rhamnolipids“ gibt eine allgemeine Einführung zu von *P. aeruginosa* produzierten Rhamnoselipiden und “alternativen“ nicht-pathogenen Mikroorganismen. Hier wird unter anderem auf die Struktur, die tensidischen Eigenschaften, die Biosynthese, die genetische Regulation, die biotechnologische Produktion, die Produktaufarbeitung, einige Anwendungsbeispiele sowie auf die aktuellen und zukünftigen Erfordernisse in der Prozessentwicklung zur Herstellung von Rhamnoselipiden eingegangen.

Bisherige Versuche einen Fed-Batch-Prozess zu etablieren waren unbefriedigend. Das liegt vor allem daran, dass die auf molekularer Ebene stattfindenden regulatorischen Vorgänge bisher noch nicht quantitativ beschrieben sind. Dies gilt im Speziellen für die Produktion von Rhamnoselipiden im Bioreaktor mit *P. aeruginosa*.

Ein systembiotechnologischer Ansatz bietet hier eine vielversprechende Möglichkeit zur weiteren gentechnischen und prozesstechnischen Optimierung. Eine Grundvoraussetzung für die erfolgreiche Anwendung eines solchen Ansatzes ist ein sequenzierter Modellstamm.

In Kapitel 2 „*Pseudomonas aeruginosa* PAO1 as a model for rhamnolipid production in bioreactor systems" werden Arbeiten mit dem sequenzierten *P. aeruginosa* Typstamm PAO1 vorgestellt. Hier ist es durch ein Batch-Verfahren im 30 L Maßstab gelungen bis zu 39 g/L Rhamnoselipide zu produzieren. Im Gegensatz zu den bereits etablierten Produktionsstämmen, erlaubt PAO1 in Verbindung mit der bisher verwendeten, zumeist heuristischen, bioverfahrenstechnischen Vorgehensweise einen wissensbasierten, systembiotechnologischen Ansatz. Die spezifische Rhamnoselipidproduktivität war zwischen der 40. und 70. Prozessstunde am höchsten und betrug 0,088 $g_{RL}/(g_{BDM} \cdot h)$. In diesem Zeitintervall verschob sich das Verhältnis von di- zu mono-Rhamnoselipid von 1:1 zu 2:1. Während des Prozesses wurden der Kohlenstoffdioxidausstoß (CER) und die Sauerstoffaufnahmerate (OUR) aufgezeichnet. OUR und CER zeigten jeweils einen lokalen Höchstwert zwischen der 40. und 60. Prozessstunde. Dies entspricht dem Zeitintervall mit maximaler spezifischer Produktivität. PAO1 scheint nicht nur ein geeigneter Modellstamm zu sein, sondern zeigte überraschenderweise auch das Potential als Grundlage für die weitere Entwicklung eines zukünftigen Produktionsstammes zu dienen.

Die Bedeutung dieses Stammes wurde in einer vergleichenden Studie mit den bereits etablierten Produktionsstämmen DSM 7108 und DSM 2874 weiter untersucht. In Kapitel 3: „Evaluation of rhamnolipid production capacity of *Pseudomonas aeruginosa* PAO1 in comparison to the rhamnolipid over-producer strains DSM 7108 and DSM 2874" werden die Ergebnisse aus Kultivierungen bei 30 und 37 °C im 30 L Bioreaktor mit Sonnenblumenöl als einziger Kohlenstoffquelle und unter stickstofflimitierenden Bedingungen diskutiert. Die maximale Produktkonzentration variierte hierbei zwischen 7 und 38 g/L, die volumetrische Produktivität zwischen 0,16 und 0,43 g/(L·h), wobei PAO1 jeweils die besten Ergebnisse erzielte. Es wird explizit gezeigt, dass die spezifische Rhamnoselipidsynthese pro Zelle einen für Sekundärmetaboliten typischen Verlauf aufweist. Dieser zeigt sich in einer zum Wachstum verschobenen spezifischen Produktivitätskurve, deren Maximum mit dem Übergang des logistischen Wachstums in eine stationäre Phase zusammenfällt. In der Literatur wird häufig die Aussage getätigt, dass stickstofflimitierende Bedingungen nicht per se die Rhamnoselipidsynthese induzieren, diese jedoch nach

dem Verbrauch des vorhandenen Stickstoffs einsetzt. In dieser Arbeit wird für drei verschiede Stämme gezeigt, dass im Bioreaktor der Stickstoff zwar das Wachstum limitierte, die Produktsynthese jedoch schon zu einem Zeitpunkt eingesetzt hatte, zu dem die Stickstoffquelle nicht limitierend vorlag. Die spezifische Produktivität war stark von der verwendeten Temperatur und dem untersuchten Stamm abhängig. Die quantitative Darstellung der spezifischen Wachtsums- und Produktionsraten war durch eine mathematische Datenschätzung (Fitting) der Biomasse- und Rhamnoselipidverläufe möglich. Erstmalig konnte dadurch der zeitliche Verlauf der spezifischen Rhamnoselipidbildungsrate dargestellt werden. Die für Sekundärmetabolite typische Zu- und Abnahme der spezifischen Produktivität trat bei allen untersuchten Stämmen auf. Einzig *P. aeruginosa* DSM 7108 zeigte bei 30 °C eine geringfügige Abweichung hiervon.

In Kapitel 4: „Regulatory and metabolic network of rhamnolipid biosynthesis: Traditional and advanced engineering towards biotechnological production" wird detailliert auf das aktuelle Wissen über das metabolische und regulatorische Netzwerk der Rhamnoselipidbiosynthese eingegangen. Zudem werden in diesem Kapitel die in den letzten 60 Jahren angewendeten bioverfahrenstechnischen Strategien zur Prozessentwicklung für die Herstellung von Rhamnoselipiden verglichen. Gleichzeitig wird zwischen „klassischen" und „modernen" Methoden unterschieden. Der Fokus liegt dabei auf *P. aeruginosa* als Modellorganismus. Schließlich wird ein holistischer, systembiotechnologischer Ansatz diskutiert. Dieser sollte sowohl auf der Ebene der gesamten Zelle als Biokatalysator, als auch auf der Bioprozessebene durchgeführt werden. Als sequenzierter Modellstamm kann *P. aeruginosa* PAO1 eine zentrale Rolle in einem solchen Ansatz einnehmen.

Abstract

During the last decade the demand for economical and sustainable bioprocesses replacing petrochemical derived products has significantly increased. Rhamnolipids are biosurfactants which can be produced by microorganisms based on sustainable substrates. Over 60 years have passed since these glycolipids constituted of a hydrophilic head group with two sugar moieties, namely, L-rhamnose, and two β-hydroxydecanoic acid residues, forming the hydrophobic tail of the molecule, were described for the first time. Today, these glycolipids and over 60 known structural congeners are generally termed rhamnolipids. They belong to the limited group of commercially available biosurfactants and are mainly produced by *Pseudomonas* and *Burkholderia* species. Due to their surfactants properties, environmental friendly nature, and sustainable character rhamnolipids will, eventually, play a prominent role as versatile surfactants in the future. However, economic obstacles for rhamnolipid production, like low productivity, comparatively expensive raw material, and relative high costs for downstream processing, prevented them from being applied in bulk applications.

Chapter 1: “Microbial rhamnolipids” gives an introduction on rhamnolipids produced by strains of *Pseudomonas aeruginosa* and “alternative” non-pathogenic strains, their structure, properties, biosynthesis, genetic regulation, biotechnological production, purification, areas of application, as well as current needs and future trends in rhamnolipid biotechnology. A lack of understanding the rhamnolipid production regulation in bioreactor systems is a main reason for unsatisfying fed-batch production with *P. aeruginosa* till now.

However, one strategy with good chances of positive outcome in the near future is a successfully adopted systems biotechnological approach for strain and process development. For such an approach a sequenced model strain is indispensable.

Chapter 2: “*Pseudomonas aeruginosa* PAO1 as a model for rhamnolipid production in bioreactor systems” shows that the sequenced *P. aeruginosa* strain PAO1 is able to produce about 39 g/L of rhamnolipid during a 30 L batch process. In contrast to well-established production strains, PAO1 allows knowledge-based systems biotechnological process development combined with the frequently used heuristic bioengineering approach. The specific rhamnolipid productivity showed a maximum

between 40 and 70 h of process time of 0.088 $g_{RL}/(g_{BDM} \cdot h)$. At the same time interval, a shift of the molar di- to mono-rhamnolipid ratio from 1:1 to about 2:1 was observed. Additionally, by exhaust gas analysis the oxygen uptake rate (OUR) and the carbon-dioxide emission rate (CER) could directly be associated with the specific rhamnolipid productivity, both showing peaks between 40 and 60 h of process time. PAO1 not only seems to be an appropriate model, but also surprisingly has the potential as a strain of choice for actual biotechnological rhamnolipid production.

The "Evaluation of rhamnolipid production capacity of *P. aeruginosa* PAO1 in comparison to the rhamnolipid over-producer strains DSM 7108 and DSM 2874", as presented in Chapter 3, shows the cultivation in a 30 L bioreactor with a medium containing nitrate and sunflower oil as sole carbon source at 30 and 37 °C for these three strains. The maximum rhamnolipid concentrations varied from 7 to 38 g/L and the volumetric productivities from 0.16 to 0.43 g/(L·h), with PAO1 showing the best results. This study explicitly shows that the specific rhamnolipid synthesis rate per cell follows secondary metabolite-like courses coinciding with the transition to the stationary phase of typical logistic growth behaviour. An often stated general presumption is that N-limiting conditions do not favor rhamnolipid production per se, but production starts with the exhaustion of nitrogen. However, in this study the rhamnolipid synthesis was already induced before N-limitation occurred. The lapse of the specific rhamnolipid productivity was strongly dependent on the temperature and the applied strain. By logistic fitting of the biomass and rhamnolipid courses a quantitative description of the specific growth and rhamnolipid production rate was possible. A typical secondary metabolite-like increase and decrease of the specific productivity was, apart from *Pseudomonas aeruginosa* DSM 7108 (30 °C), apparent in all cultivations.

In Chapter 4 "Regulatory and metabolic network of rhamnolipid biosynthesis: Traditional and advanced engineering towards biotechnological production" the current knowledge about the regulatory and metabolic network of rhamnolipid synthesis is shown in detail. Additionally, traditional and advanced engineering strategies performed during the last 60 years for rhamnolipid production improvement, focusing on *P. aeruginosa*, are compared. Finally, the opportunities of applying the systems biotechnology toolbox on the whole-cell biocatalyst and bioprocess level for further rhamnolipid production optimization are discussed. As a model *P. aeruginosa* PAO1 will play a key role in the discussed approaches.

Table of contents

1 Microbial rhamnolipids

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1.1 Introduction

In 1949 Jarvis and Johnson reported an anionic glycolipid with a molecular weight of 650 g/mol produced by three different strains of *Pseudomonas aeruginosa.* They showed that this amphiphilic glycolipid was constituted of a hydrophilic head group with two sugar moieties, namely, L-rhamnose, and two β-hydroxydecanoic acid residues, forming the hydrophobic tail of the molecule. Today, glycolipids composed of L-rhamnose and β-hydroxyalkanoic acid residues are generally termed rhamnolipids. Rhamnolipids belong to the limited group of biosurfactants commercially available. Although over 60 years have passed since Jarvis and Johnson described the first rhamnolipid, economic obstacles for rhamnolipid production, like low productivity, comparatively expensive raw material, and relative high costs for downstream processing, prevented them from being applied in bulk applications. Nevertheless, they are non-polluting, sustainable, and show excellent physicochemical properties, which make them interesting for cosmetic, pharmaceutical, and detergent manufacturers. This chapter focuses on rhamnolipids produced by strains of *P. aeruginosa*, their structure, properties, biosynthesis, genetic regulation, biotechnological production, purification, areas of application, as well as current needs and future trends in rhamnolipid biotechnology.

1.2 Chemical structure and properties of microbial rhamnolipids

Rhamnolipids are mainly synthesized by the genus *Pseudomonas*, especially *P. aeruginosa* strains; under growth-limiting conditions with water-immiscible carbon sources (cf. Chapter 1.3.3). *P. aeruginosa* is an opportunistic pathogen. It is a Gram-negative, monotrichous, and ubiquitous present soil bacterium. *P. aeruginosa* synthesizes different cell associated and extracellular virulence factors, one of them being rhamnolipids.

Rhamnolipid structure

The di-rhamnolipid (α-L-rhamnopyranosyl-(1-2)-α-L-rhamnopyranosyl-3-hydroxy-decanoyl-3-hydroxydecanoate also called rhamnolipid 3 or Rha-Rha-C_{10}-C_{10}) characterized by Jarvis and Johnson (1949) is only one of over 28 rhamnolipid congeners described for *P. aeruginosa*, varying mostly in fatty acid chain length and

degree of saturation (Déziel et al. 1999). Chain lengths vary from octanoic (-C_8) to dodecanoic (-C_{12}) acids. Single and double bonds are observed for all chain lengths (e.g., -$C_{10:1}$, -$C_{12:1}$). The major part of the rhamnolipids contains two fatty acid residues, generally of the same length. Most rhamnolipid congeners are produced in traces. Apart from di-rhamnolipid[1], a mono-rhamnolipid[2] (α-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate also called rhamnolipid 1 or Rha-C_{10}-C_{10}) is predominant in *P. aeruginosa* cultivations. The so-called rhamnolipids 2 and 4, are a di- and a mono-rhamnolipid (Rha-Rha-C_{10} and Rha-C_{10}, respectively), with only one β-hydroxydecanoic acid residue, respectively. They were reported for resting cells of *Pseudomonas* sp. DSM 2874 cultivated at 37 °C (Syldatk et al. 1985a,b). The generalized chemical structure and nomenclature for mono- and di-rhamnolipids is presented in Figure 1-1.

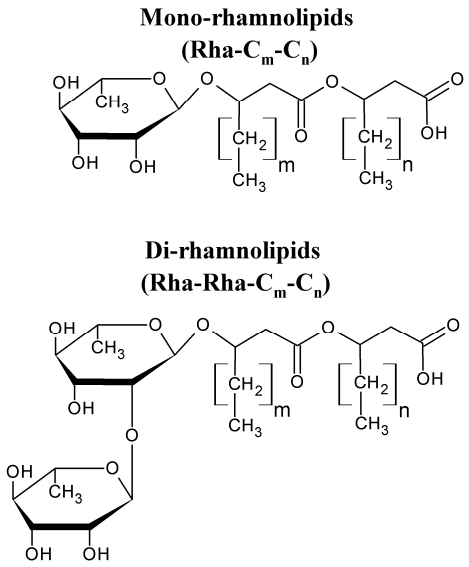

Figure 1-1. Generalized structures and nomenclatures for mono-rhamnolipids and di-rhamnolipids, *m*, *n* = 4–8 (Jarvis and Johnson 1949; Edwards and Hayashi 1965; Itoh et al. 1971; Syldatk et al. 1985a,b; Gruber et al. 1993).

The rhamnolipids 1–4 are termed after their order of appearance on a thin-layer chromatography of an extract from *Pseudomonas* sp. DSM 2874 (Syldatk et al. 1985a,b). Although the terms rhamnolipid 1–4 are obsolete, they are still often used. However, in the following sections the generalized nomenclature will be used (cf. Figure 1-1). Two further rhamnolipids are α$C_{10:1}$-Rha-C_{10}-C_{10} (rhamnolipid A) and

[1] dirhamnolipid was changed to di-rhamnolipid throughout this chapter
[2] monorhamnolipid was changed to mono-rhamnolipid throughout this chapter

$\alpha C_{10:1}$-Rha-Rha-C_{10}-C_{10} (rhamnolipid B), which were reported by Yamaguchi and Sato (1976) and represent α-decenoic acid ($\alpha C_{10:1}$) acylated derivatives of Rha-C_{10}-C_{10} (rhamnolipid 1) and Rha-Rha-C_{10}-C_{10} (rhamnolipid 3), respectively. However, this is the only report about acylated rhamnolipid congeners.

Surfactant properties of rhamnolipids

Rhamnolipids are weak acids. Rha-C_{10}-C_{10} has a pK_a value of between 4.1 and 5.6, below and above the critical micelle concentration (CMC), respectively (Lébron-Paler et al. 2006). Rhamnolipids display excellent surface active features. Table 1-1 shows the values for CMC, interfacial and surface tension properties for the most important rhamnolipids of *P. aeruginosa*.

Table 1-1. Surfactant properties of the main reported rhamnolipid congeners (Syldatk et al. 1985a; Yamaguchi and Sato 1976; Dubeau et al. 2009).

Structure	σ_{min} (mN/m)	γ_{min} (mN/m)	CMC (mg/L)	MW (g/mol)
Rha-C_{10}-C_{10}	26 – 40	4	20 – 600	505
Rha-C_{10}	25 – 40	<1	200 – 600	334
Rha-Rha-C_{10}-C_{10}	27 – 40	<1	10 – 600	651
Rha-Rha-C_{10}	30 – 40	<1	200 – 600	481
Rha-Rha-C_{14}-C_{14}*	42	n.r.	225 – 250	763

Note: σ_{min}, surface tension; γ_{min}, interfacial tension against n-C_{16}; CMC, critical micelle concentration; MW, molecular weight; n.r., nothing reported; *rhamnolipid of *Burkholderia* sp.

From Table 1-1 results, the surfactant properties of *P. aeruginosa* rhamnolipids are reported to be able to lead to a reduction of the surface tension of water from 72 to below 30 mN/m and a decrease of the interfacial tension of oil/water systems from 43 mN/m to <1 mN/m. Additionally, rhamnolipids stabilize oil-in-water emulsions (Patel and Desai 1997a,b).

Physiological role of rhamnolipids

According to Sobéron-Chávez et al. (2005), of physiological relevance may be the solubilization and uptake of hydrophobic substrates, virulence mediation by interfacial and haemolytic activity and as protective agents due to their extensive antimicrobial effects. Rhamnolipids are extracellular virulence factors like exotoxin A, pyocyanin, and diverse proteases. Rhamnolipids show haemolytic activity and may influence biofilm formation of *P. aeruginosa* (Davey et al. 2003). Recent results support that

rhamnolipids are key protective agents of *P. aeruginosa* against several cellular components of the human immune system (Van Gennip et al. 2009).

1.3 Rhamnolipid production by *Pseudomonas aeruginosa*

1.3.1 Rhamnolipid biosynthesis pathway

A putative rhamnolipid biosynthesis pathway is recapitulated in Figure 1-2 (Burger et al. 1963; Ochsner et al. 1996; Kanehisa and Goto 2000; Rahim et al. 2001; Soberón-Chávez et al. 2005; Winsor et al. 2009). An up-to-date review of the putative rhamnolipid biosynthesis pathway is given by Sobéron-Chávez et al. (2005). Altogether the biosynthesis can be separated into three major parts. In the first two steps the precursor for the hydrophilic part, namely, dTDP-L-rhamnose, and of the hydrophobic part, namely, activated 3-(3-hydroxyalkanoyloxy) alkanoate (HAA), are synthesized *de novo* (Hauser and Karnovsky 1957, 1958; Burger et al. 1963). The final step is characterized by the reaction of two special rhamnosyltransferases catalyzing the sequential rhamnosyl transfer reactions from the precursors over mono- toward di-rhamnolipids. The *de novo* synthesis of the sugar moiety occurs by the subsequent conversion of D-glucose-6-phosphate, deriving from the glycolysis respectively the gluconeogenisis, toward dTDP-glucose. After further dehydration, epimerization, and reduction, dTDP-L-rhamnose is formed. These steps are performed by the gene products of the *rmlBDAC* operon (Rahim et al. 2000). Both, dTDP-glucose and dTDP-L-rhamnose can also be incorporated into the bacterial cell membrane as part of the lipopolysaccharide (LPS) side chains. When cultivated on vegetable oil as sole carbon source the triglycerides are cleaved by exolipase activity in *P. aeruginosa* (Gilbert et al. 1991, 1993; Jaeger et al. 1996). While the resulting glycerol diffuses passively through the microbial membrane the fatty acids need active transport into the cell. Recently Marsudi et al. (2008) reported that the glycerol part is used for rhamnolipid formation via gluconeogenisis and fatty acid *de novo* synthesis, while the fatty acids are used for cell growth and polyhydroxyalkanoate (PHA) production via β-oxidation. The *de novo* synthesis of the fatty acid moiety for rhamnolipid catabolism is described in detail by Rehm et al. (2001). The rhamnolipid biosynthesis pathway is closely connected to alginate and PHA production (Pham et al. 2004); both of them are unwanted byproducts when considering the biotechnological rhamnolipid production.

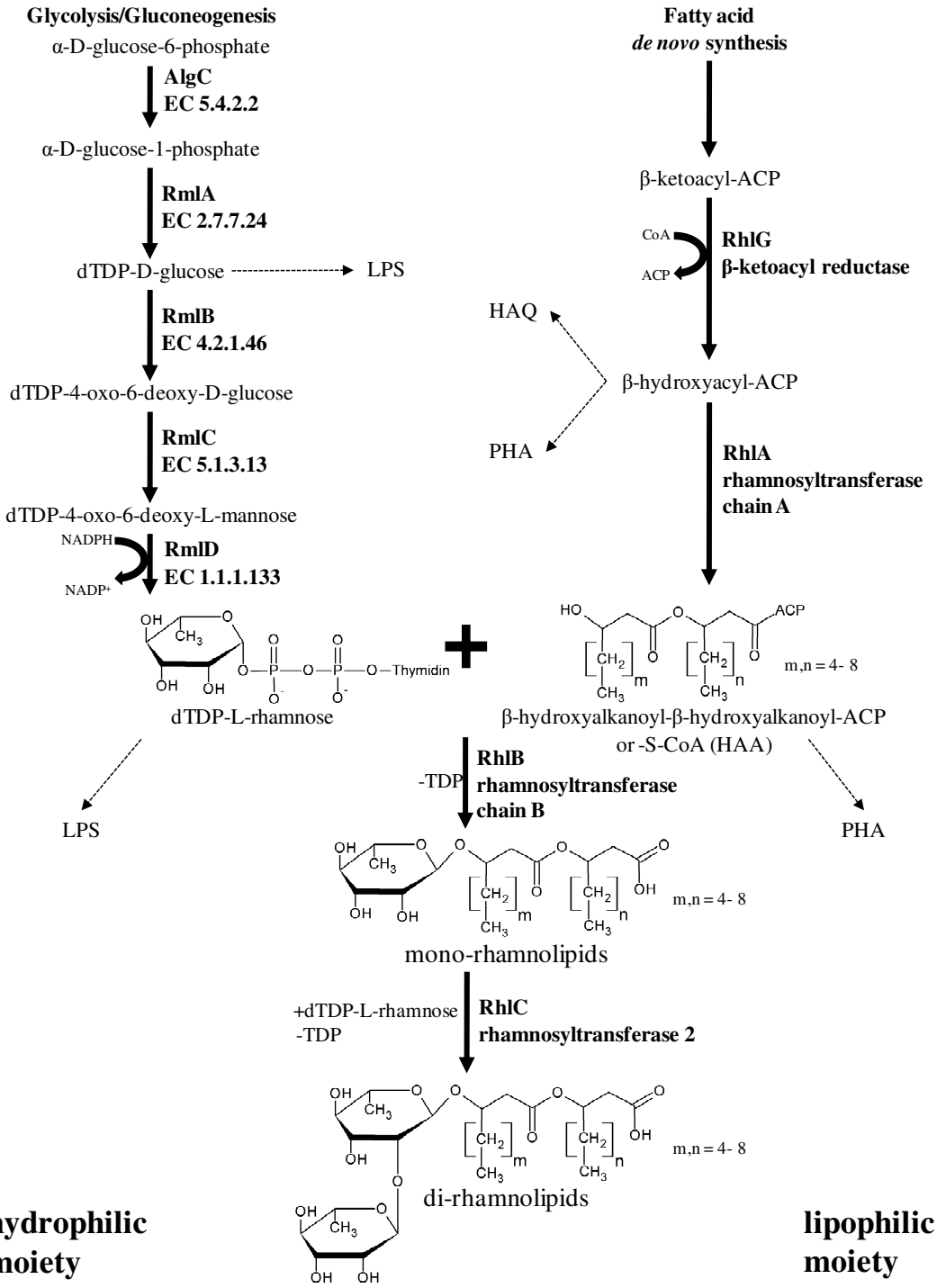

Figure 1-2. Putative rhamnolipid biosynthesis pathway according to Soberón-Chávez (2005), with minor changes. LPS, lipopolysaccharides; PHA, polyhydroxyalkanoates; HAQ, 4-hydroxy-2-alkylquinolines; ACP, acyl carrier protein; CoA, coenzyme A; dTDP, deoxythymidine 5'-diphosphate; NADPH/NADP$^+$, nicotinamide adenine dinucleotide phosphate; HAA, 3-(3-hydroxyalkanoyloxy)alkanoate; EC, enzyme commission number; *m*, *n* = 4–8.

RhlG performs the NADPH-dependent reduction of β-ketoacyl-ACP to β-D-hydroxyacyl-ACP (Campos-García et al. 1998; Miller et al. 2006). However, Miller et al. (2006) reported that RhlG was 2000-fold less active than FabG in carrying out the

same reaction. Additionally, structural and biochemical studies further suggest that the ACP of fatty acid synthesis does not carry the substrates for RhlG. The following steps are catalyzed by RhlAB, termed rhamnosyltransferase 1. The formation of the activated HAAs, whether it is HAA-ACP or HAA–S-CoA (Maier and Soberón-Chavez 2000) is still not clear, is presumably done by rhamnosyltransferase chain A (RhlA) of the rhamnosyltransferase 1, while the rhamnosyltransferase chain B (RhlB) catalyses the formation of mono-rhamnolipids from dTDP-L-rhamnose and activated HAAs. Both chains are encoded and co-expressed within the *rhlABRI* regulon, which is strongly cross-linked within the cell density N-acylhomoserine lactone (AHL)-dependent quorum sensing (QS) system (Sullivan 1998) described in Chapter 1.3.2. Subsequently a fraction of the mono-rhamnolipids is converted to di-rhamnolipids by rhamnosyltransferase 2 (RhlC) through transfer of a second rhamnosyl group. The biosynthesis mechanism for mono- and di-rhamnolipids with only one β-hydroxy-alkanoic acid moiety (e.g., Rha-Rha-C_{10} and Rha-C_{10}) is still unravelled (Soberón-Chávez et al. 2005). The fact, that both precursors for rhamnolipid synthesis are derived from central metabolic pathways makes it difficult to overproduce rhamnolipids in heterologous hosts (Ochnser et al. 1994; Ochsner and Reiser 1995).

1.3.2 Quorum sensing regulation of rhamnolipid biosynthesis

The rhamnolipid biosynthesis in *P. aeruginosa* is regulated by cell-to-cell communication termed QS (Sullivan 1998). QS signals are the determinant of cell population density and integrate into a global regulatory network of signal molecules (Williams and Camara 2009). The superior QS system responsible for the rhamnolipid regulation is the AHL-dependent or *las/rhl* system (Ochsner and Reiser 1995; Pearson et al. 1997), which is connected to the minor 2-alkylquinolone (AQ)-dependent or *Pseudomonas* quinolone signal (PQS) system (Williams and Camara 2009). The main regulatory network of AHL-dependent QS important for rhamnolipid biosynthesis is outlined in Figure 1-3 (Ochsner and Reiser 1995; Sullivan 1998; Soberón-Chávez et al. 2005; Williams and Camara 2009).

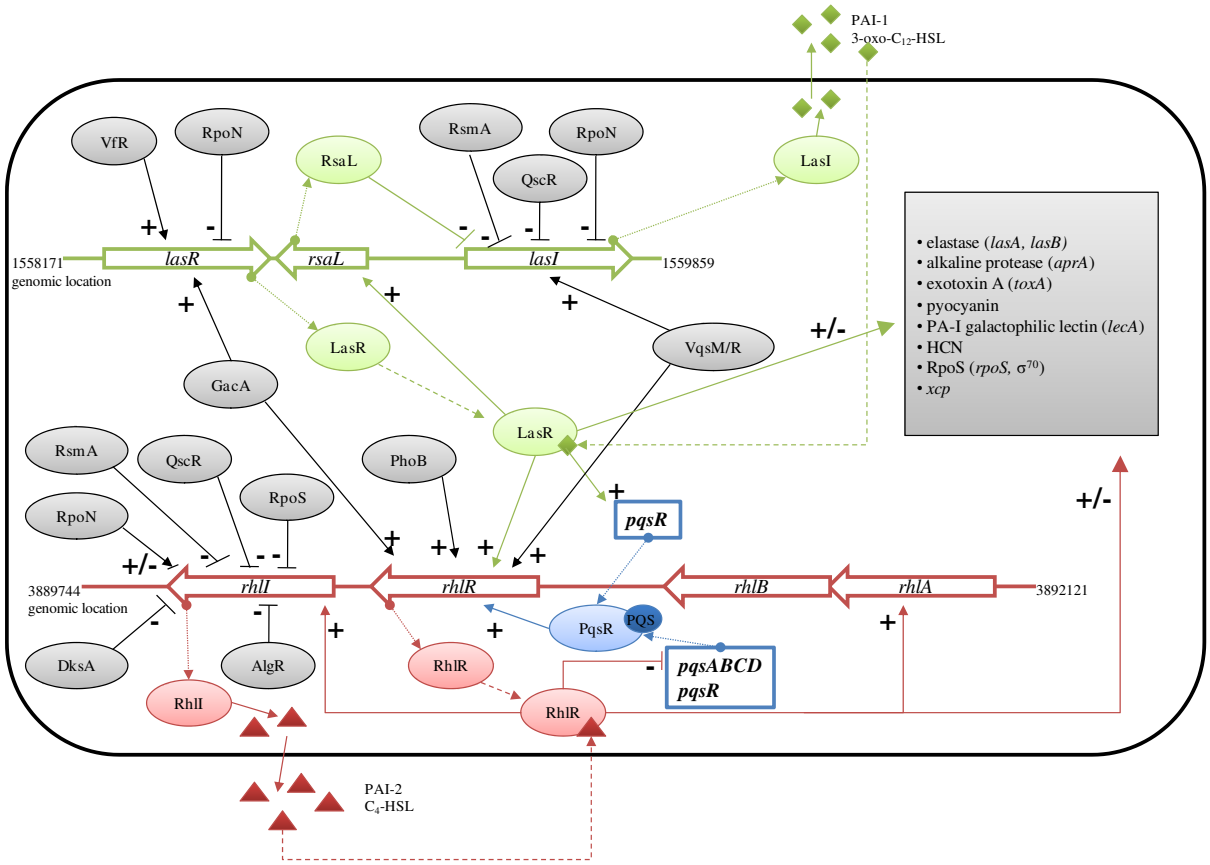

Figure 1-3. Overview of the AHL-dependent *las/rhl* quorum sensing (QS) network and interconnection with the AQ-dependent QS. Detailed functions and abbreviations are described in Table 1-2 or within the text. The *las* system is marked in green, the *rhl* system is marked in red and the *Pseudomonas* quinolene signal (PQS) is marked in blue, respectively. Gray symbols do not directly belong to the *las/rhl* or PQS regulon. Arrows (⟶) or T-bars (⟞) with solid lines indicate positive (+) or negative (-) regulatory effects. Dotted arrows with circles (●⋯▶) show expression of genes. Genomic locations are related to the PAO1 genome from the *Pseudomonas* Genome Database (Windsor et al. 2009).

Two *N*-AHLs are responsible for the cellular answer toward environmental factors. The transcriptional regulator VfR and the global activator GacA initiate the expression of *lasR*. Within the *las* system, LasI is responsible for the synthesis of *N*-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}-HSL), also termed *P. aeruginosa* autoinducer 1 (PAI-1). LasR binds 3-oxo-C_{12}-HSL and positively regulates the expression of the *rhlABRI* regulon. RhlI synthesizes *P. aeruginosa* autoinducer 2 (PAI-2), chemically characterized as *N*-butanyol-homoserine lactone (C_4-HSL). Bound to RhlR, C_4-HSL induces the expression of the rhamnosyltransferase genes *rhlAB* and *rhlC* (Ochsner et al. 1994; Pearson et al. 1997; Rahim et al. 2001). Complex positive and negative feedback loops lead to increase and decrease of autoinducer signals between surrounding cells by regulation on transcriptional and posttranscriptional level (Schuster and Greenberg et al. 2007).

Table 1-2. Main regulators and effectors of the AHL-dependent *las/rhl* quorum sensing (QS) system and rhamnolipid biosynthesis of *P. aeruginosa* (in particular PAO1).

Gene	Description	Role
aprA	Alkanline protease	Positive regulated by *las/rhl* system
lasA	Protease precursor	Positive regulated by *las/rhl* system
lasB	Elastase	Positive regulated by *las/rhl* system
lecA	Galactophilic lectin	Positive regulated by *las/rhl* system
rmlBDAC	*rml* locus	dTDP-L-rhamnose synthesis pathway
toxA	Endotoxin A	Positive regulated by *las/rhl* system
xcp	General secretion of proteins	Positive regulated by *las/rhl* system
Effector	**Description**	**Role**
AlgC	Phosphomannomutase	Converts G-6-P to G-1-P
AlgR	Transcriptional regulator	Repression of *rhlI* in biofilms
DksA	Transcriptional regulator	Negative control of *rhlI*
GacA	Global activator	Positive regulator of *lasR* and *rhlR*
LasI	PAI-1 synthesis protein	Synthesis of 3-oxo-C_{12}-HSL
LasR	Transcriptional regulator	Key regulator of *las/rhl* system
PhoB	Response regulator	Stimulator of *rhlR* under P-limitation
PQS	*Pseudomonas* quinolone signal	Positive regulator of *rhl* QS
QscR	QS control repressor	Repression of *lasI* and *rhlI*
RhlA	Rhamnosyltransferase chain A	HAA-ACP/S-CoA synthesis
RhlB	Rhamnosyltransferase chain B	Mono-rhamnolipid synthesis
RhlC	Rhamnosyltransferase 2	Di-rhamnolipid synthesis
RhlG	β- ketoacyl reductase	HAA precursor synthesis
RhlI	PAI-2 synthesis protein	Synthesis of C_4-HSL
RhlR	Transcriptional regulator	Key regulator of *las/rhl* system
RpoN	Alternative sigma factor, σ^{54}	Negative regulator of *lasRI* and *rhlI*; can also be a positive activator of *rhlI*
RpoS	Stat. phase sigma factor, σ^{70}	Negative regulator of *rhlI*
RsaL	Regulatory protein; repressor of QS	Repression of *lasI*
RsmA	Regulator of secondary metabolites; posttranscriptional regulator	Repression of *lasI* and *rhlI*
VfR	Transcriptional regulator; cyclic AMP receptor protein	Induction of *lasR*
VqsM/R	Virulence and QS regulator protein	Positive regulation of *lasI* and *rhlR*

Table 1-2 summarizes the main regulators and effectors in rhamnolipid production and their annotation according to current knowledge of rhamnolipid biosynthesis (Soberón-Chávez et al. 2005), QS regulation (Williams and Camara 2009), and genomics of *P. aeruginosa* PAO1 (*Pseudomonas* Genome Database, Windsor et al. 2009).

1.3.3 Characteristic rhamnolipid production curves

Rhamnolipid production under growth-limiting conditions

Cultures of *P. aeruginosa* synthesize mixtures of various rhamnolipid congeners (Déziel et al. 2003). The composition depends on the applied process conditions (temperature, medium composition, pH, etc.) and is strongly influenced by the strain used for cultivation (Lang and Trowitzsch-Kienast 2002; Leitermann 2008).

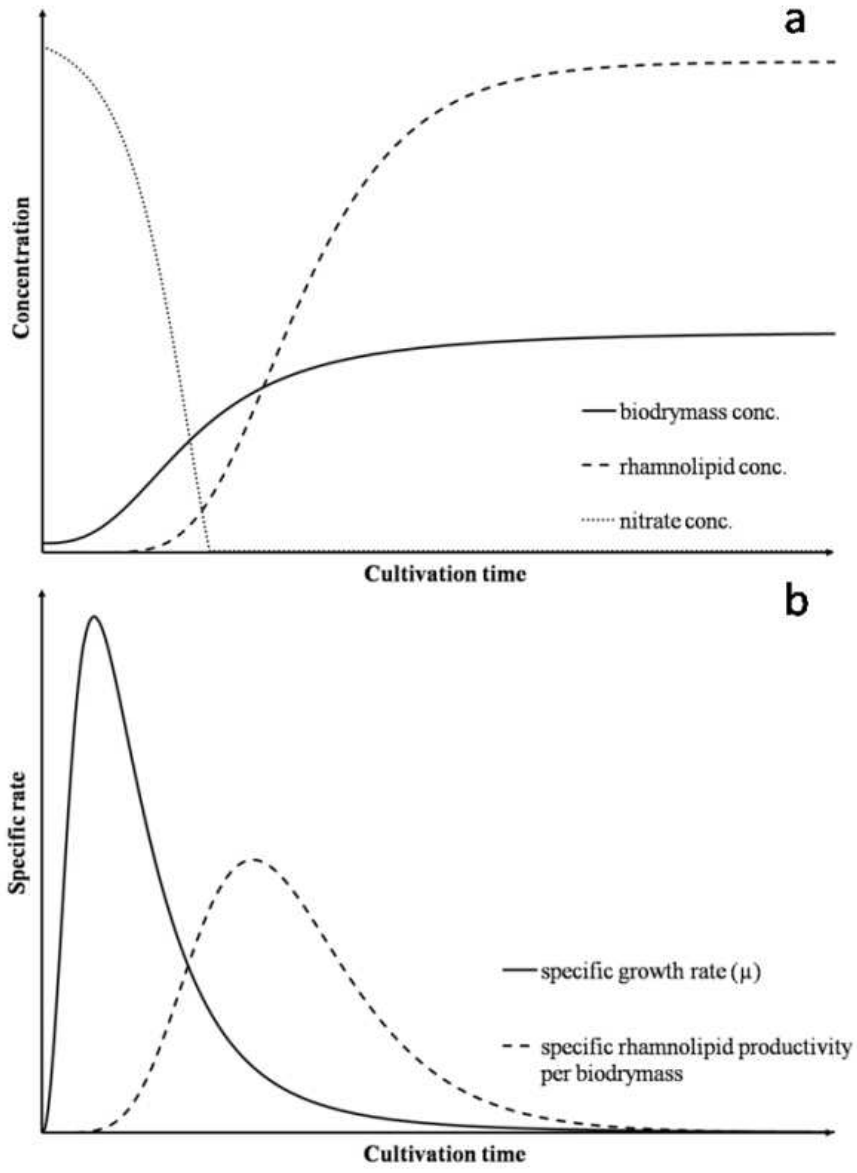

Figure 1-4. (a) Characteristic time course of *P. aeruginosa* grown on hydrophobic C-sources, present in excess under nitrogen-limiting conditions, of biodrymass, N-source and rhamnolipid concentrations. **(b)** Specific growth and specific rhamnolipid productivity per biodrymass.

As previously described (cf. Chapter 1.2), the rhamnolipids Rha-C_{10}-C_{10} and Rha-Rha-C_{10}-C_{10}, are the predominant mono- and di-rhamnolipids, respectively. However, in most cases rhamnolipid concentrations are quantified indirectly by the orcinol method for carbohydrate analysis (Chandrasekaran and BeMiller 1980) and converted into rhamnolipid concentrations by extrapolation of di- to mono-rhamnolipid ratios. Müller et al. (2010) and Leitermann (2008) showed by HPLC analysis, that during cultivations of *P. aeruginosa* PAO1, DSM 7108, and DSM 2874, different strain-specific ratios of di- to mono-rhamnolipids occur with the progress of the production process. Thus indirect rhamnolipid analysis and the use of different *P. aeruginosa* strains make it difficult to properly compare reported data for rhamnolipid production.

In 1997 Giani et al. claimed to produce 74 – 112 g/L of rhamnolipids by batch and fed-batch cultivations of DSM 7107 and DSM 7108. However, insufficient details on the experimental data are given and no verifications of these claims on laboratory scale have been reported since then. High concentrations of rhamnolipids in bioreactor scale were mainly reported under nitrogen-limiting conditions and with water-immiscible carbon sources. Figure 1-4a shows a generalized time course of biodrymass, rhamnolipid, and nitrogen concentration of cultivations with nitrate as nitrogen source compiled from Hauser and Karnovsky (1954), Mulligan et al. (1989), Robert et al. (1989), Manresa et al. (1991), and Ochsner et al. (1996). Figure 1-4 is based on original data from bioreactor cultivations with *P. aeruginosa* PAO1 by Müller et al. (2010).

Specific rhamnolipid production and growth rate

The typical specific growth and specific productivity time course was recently reported for *P. aeruginosa* PAO1 by Müller et al. (2010) and is shown in Figure 1-4b. A similar behavior was reported by Mulligan et al. (1989) under phosphate-limiting conditions for a biosurfactant, most likely rhamnolipid, produced by *P. aeruginosa* ATCC 9027. Even though the precursors for rhamnolipid biosynthesis (cf. Figure 1-2) are compounds produced by central metabolic pathways, the production curve shows the characteristic behavior of a secondary metabolite produced under growth-limiting conditions. The cell growth under rhamnolipid production conditions follows a typical

logistic behavior (Ramana et al. 1991). The specific productivity per biodrymass (Figure 1-4b) shows a distinct maximum after the limiting substrate is depleted. The maximum specific productivity coincides with the transition to the stationary phase. Shortly after, the specific productivity decreases.

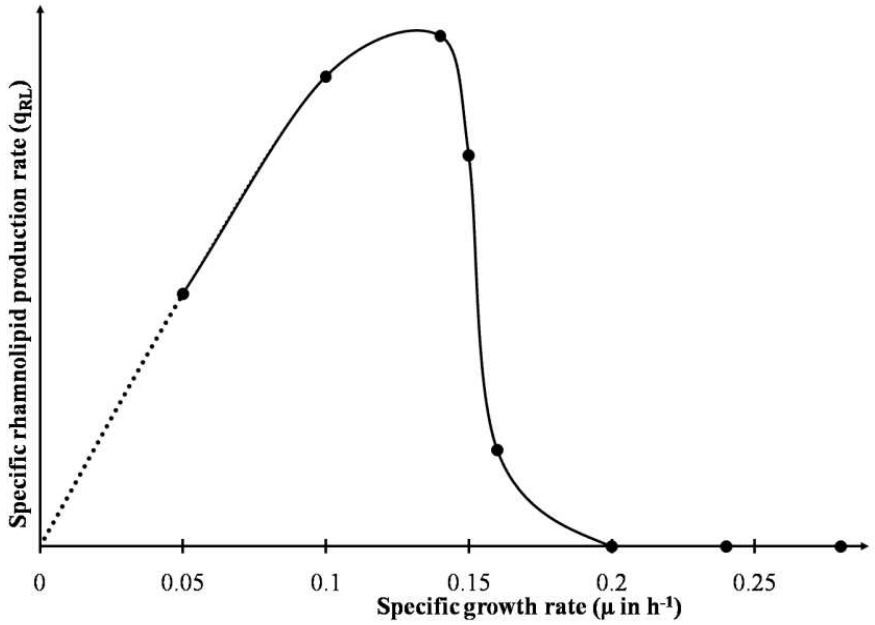

Figure 1-5. Specific rhamnolipid productivity vs. specific growth rate extracted from a continuous culture of *P. aeruginosa* DSM 2659 (Guerra-Santos et al. 1986). *Dotted line* indicates the putative course of the specific rhamnolipid productivity at specific growth rates lower than 0.05 h^{-1}.

Guerra-Santos et al. (1986) showed that the specific rhamnolipid productivity is dependent of the specific growth rate. Under continuous culture biosurfactant production (33 °C, pH 6.25, 20 g/L glucose) *P. aeruginosa* DSM 2659 showed maximum specific rhamnolipid productivity (q_{RL}) when growing at specific growth rates of μ = 0.1 – 0.15 h^{-1}, with rapidly decreasing productivity at higher and lower specific growth rates (Figure 1-5). The maximum specific rhamnolipid productivity under the given conditions was q_{RL} = 0.029 g/(g·h) at a specific growth rate of μ = 0.135 h^{-1}.

Genetic regulation under growth-limiting rhamnolipid production

On genetic level RpoN, PhoB, and the regulator of secondary metabolites (RsmA) are transcriptional regulators in cell density mediated rhamnolipid synthesis under substrate-limiting conditions (cf. Chapter 1.3.2). Biosurfactant production of

P. aeruginosa is enhanced under limitation of media components like nitrogen, phosphate, and trace elements (Guerra-Santos et al. 1984; Mulligan et al. 1989; Manresa et al. 1991). The alternative sigma factor RpoN (σ^{54}) is associated to nitrogen metabolism and respective binding sites are reported for *rhlR* (Medina et al. 2003), *rhlAB* operon, and the *PA1131-rhlC* operon (Rahim et al. 2001). PhoB is a response regulator and stimulates *rhlR* expression under phosphate-limiting conditions via the AQ-dependent QS (Jensen et al. 2006).

1.3.4 Cultivation strategies for rhamnolipid production

Cultivation strategies applied for rhamnolipid production implicate shake flask, batch, fed-batch, continuous, and integrated microbial/enzymatic processes. Mainly glucose, glycerol, *n*-alkanes, and triglycerides were used as carbon sources. Nitrogen sources reported are nitrate, ammonium, urea, and complex amino acids containing supplements (Syldatk and Wagner 1987; Zhang and Miller 1992; Lee et al. 2004). Table 1-3 shows different published processes for rhamnolipid production. The most important cultivation conditions applied for rhamnolipid overproduction are according to Lang and Wullbrand (1998):

- (Fed-)batch cultivations under growth-limiting conditions
- Batch cultivations under resting cells condition
- Semicontinuous production with immobilized cells (excluding any N-source)
- Continuous cultivation and production with free cells

Batch and fed-batch strategies

Batch and fed-batch processes with *P. aeruginosa* DSM 7107 and DSM 7108 under growth-limiting conditions (30 °C, pH 6.3, soybean oil, *N*-limited) are reported to result in >100 g/L rhamnolipid (Giani et al. 1997). Most of the process strategies aim to limit at least one of the media components, mainly the nitrogen or phosphate sources, however, not the carbon source (Guerra-Santos et al. 1984; Soberón-Chávez et al. 2005). Fed-batch cultivations of *P. aeruginosa* BYK-2 KCTC 18012P with 25 g/L fish oil in the feed medium resulted in $Y_{RL/S}$ of 0.75 g/g in contrast to batch cultivations with $Y_{RL/S}$ of 0.68 g/g (Lee et al. 2004). Chen et al. (2007b) used a pH-stat fed-batch strategy to improve rhamnolipid production with 6% glucose in their

feed medium. In general fed-batch cultivation is the most popular process strategy for high productivities. However, for rhamnolipid production this strategy was not effectively adopted yet. Even though considerable final yields have been reported, the total rhamnolipid concentrations are generally lower than reported in batch cultivations (cf. Table 1-3). The difficulties for fed-batch cultivations are mainly due to the complex regulation of rhamnolipid production and excessive foam formation during cultivation. Additionally, most of the reported fed-batch strategies just rely on heuristic approaches.

Resting cells cultivations

Trummler et al. (2003) report an integrated microbial/enzymatic process with resting cells of *Pseudomonas* sp. DSM 2874. By a two-step reaction, first biomass is produced and harvested. The resting cells are then cultured in a buffer solution (30 °C, pH 6.5, glucose) and the rhamnolipid production is induced by addition of the carbon source (rapeseed oil). A volumetric productivity (P_V) of about 0.14 g/(L·h) was achieved by this method. Syldatk et al. (1985a,b) showed for the same strain an improvement of rhamnolipid yield coefficient $Y_{RL/S}$ from 0.16 to 0.23 g/g and $Y_{RL/X}$ from 0.61 to 3.30 g/g, respectively, when cultivated under resting cell conditions compared to growth-limiting conditions with nitrogen limitation.

Semicontinuous cultivations

A profound problem encountered during rhamnolipid production under aerobic conditions is the excessive foaming. Chemical antifoaming agents were shown to negatively influence the product quality, mechanical foam control being an alternative to be considered (Leitermann 2008). Because of foaming problems semicontinuous strategies were developed with integrated continuous product removal by flotation. Screenings with *Pseudomonas* sp. DSM 2874 revealed, that a cultivation with calcium alginate immobilized cells and glycerol as carbon source are the best conditions for semicontinuous rhamnolipid production (Syldatk et al. 1984). It was shown that it is possible to reuse the immobilized biocatalyst several times (Siemann and Wagner 1993) after appropriate regeneration of the cells.

Continuous cultures

Most of the continuous cultivations for rhamnolipid production were performed with *P. aeruginosa* DSM 2659 and glucose as C-source (cf. Table 1-3). The main characteristics of the performed experiments involve carbon and phosphate excess in addition to nitrogen and iron limitation. As outlined in Figure 1-5, the peak of specific productivity occurs at relatively low growth rates (Guerra-Santos et al. 1984a,b; 1986) when *P. aeruginosa* DSM 2659 is cultivated in continuous cultivation (33 °C, pH 6.25, 20 g/L glucose). Ochsner et al. (1996) could reach volumetric productivities (P_V) of 2 g/(L·h) and a product yield ($Y_{RL/S}$) of 0.48 g/g when using corn oil as carbon source for continuous cultivation of *P. aeruginosa* DSM 2659.

Table 1-3. Reported rhamnolipid production processes with different *P. aeruginosa* strains.

P. aeruginosa strain	C-source (g/L)	Type	t (h) resp. D (h^{-1})	$c_{RL,\,max}$ (g/L)	BDM (g/L)	$Y_{RL/S}$	$Y_{RL/X}$	P_V (g/(L·h))	Reference
44T1	olive oil (20)	S	120	7.65	6	0.38	1.28	0.11	Robert et al. (1989)
BYK-2 KCTC 18012P	fish oil (25)	B	216	17	5.3	0.68	3.2	0.08	Lee et al. (2004)
	fish oil (30)	FB	264	22.7	6.1	0.75	3.72	0.09	
	fish oil (10)	B^{IC}	192	6	-	0.6	-	0.03	Jeong et al. (2004)
	fish oil (10)	C^{CR}	0.018	4.3 – 5.8	10(w)	0.43–0.58	0.43–0.58(w)	0.08	
DS 10-129	soybean oil (6)		288	4.31	8	0.72	0.53	0.01	Rahman et al. (2002)
DSM 2659	corn oil (40)	B	42	8.94	7	0.22	1.28	0.21	Hembach, (1994)
	corn oil (84)	FB	107	33.78	5	0.40	6.76	0.52	
	corn oil (40)	C	0.02 - 0.1	4 – 18	7 - 7.5	0.18–0.46	0.53–2.57	0.45 - 0.69	
	glucose (30)	C	0.065	2.25	2.5	0.08	0.9	0.15	Reiling et al. (1986)
	glucose (20)	C	0.135	1	2.4	0.05	0.42	0.13	Guerra-Santos (1985)
	glucose (20)	C	0.14	0.12	2.4	0.05	0.06	0.13	Gruber et al. (1993)
	glucose (20)	C	0.05	0.2	2.5	0.08	0.03	0.01	
	glucose (20)	C^{CR}	0.18	2	13.3	0.15	0.04	0.55	
	glucose (20)	C	0.10	-	7.7	0.15	0.04	0.29	
	corn oil (40)	C^{CR}	0.1	19.36	-	0.48	-	2	Ochsner et al. (1996)

Table 1-3 (continued).

P. aeruginosa strain	C-source (g/L)	Type	*t* (h) resp. D (h^{-1})	$c_{RL,\,max}$ (g/L)	BDM (g/L)	$Y_{RL/S}$	$Y_{RL/X}$	P_V (g/L·h)	Reference
DSM 2874	*n*-$C_{14}/_{15}$ (40)	B^{IC}	168	10	5	0.25	2	0.06	Syldatk et al. (1985a,b)
	soybean oil (40)	B^{IC}	168	7.5	5	0.18	1.5	0.04	
	glycerol (40)	B^{IC}	168	8.5	5	0.21	1.7	0.05	
	glucose (20)	B^{IC}	168	4.5	5	0.11	0.9	0.03	
	n-C_{14} (100)	B^{IC}	210	13.2	3.6 – 4.1	0.13	3.22– 3.66	0.06	
	n-C_{14} (80)	B^{IC}	280	18.5	5	0.23	3.3	0.07	Syldatk et al. (1984)
	rapeseed oil (198)	FB^{RC}	321	45	48 (w)	0.2	0.83(w)	0.14	Trummler *et al.* (2003)
DSM 7107 DSM 7108	soybean oil (125)	B	167	78	-	0.62	-	0.47	Giani et al. (1997)
	soybean oil (163)	B	264	112	-	0.69	-	0.42	
	soybean oil (163)	FB	264	95	-	0.74– 0.69	-	0.44	
IFO 3924	ethanol (55)	S	168	32	3.4	0.58	9.41	0.19	Matsufuji et al. (1997)
	ethanol (65)	S	192	32	-	0.49	-	0.17	Nakata et al. (1998)
J4	olive oil (100)	B	139	3.6	-	0.04	-	0.03	Wei et al. (2005)
KY 4025	*n*-paraffin (50)	B	144	8.5	-	0.09	-	0.06	Itoh et al. (1971)
LBI	soybean oil (10)	S	144	11.72	1.48	1.17	7.91	0.08	Nitschke *et al.* (2005)
MUB	*n*-$C_{14}/_{15}$ (20)	B	48	2.9	3.4	0.15	0.85	0.06	Wagner et al. (1983)
PAO1	sunflower oil (250)	B	90	39	16	0.23	2.43	0.43	Müller et al. (2010)
PTCC 1637	corn oil (250)	S	120	12.5	-	0.05	-	0.1	Tahzibi et al. (2004)
S2	glucose (40)	B	144	5.31	2.4	0.13	0.22	0.04	Chen et al. (2007a)
	glucose (40)	FB	195	6.06	2.62	0.15	2.31	0.03	Chen et al. (2007b)
UI 29791	corn oil (75)	B	192	46	12	0.61	3.83	0.24	Linhardt et al. (1989)

Note: S/B/FB/C, shake flask/batch/fed-batch/continuous cultivation; RC, resting cells; IC, immobilized cells; CR, cell retention; *w,* wet; $c_{RL,max}$, maximum rhamnolipid concentration; BDM, final biodrymass; D, dilution rate; $Y_{RL/S}$, rhamnolipid yielded per used C-source; $Y_{RL/X}$, rhamnolipid yielded per produced biodrymass; P_V, volumetric productivity per working volume.

Several factors are known to influence the rhamnolipid productivity and yield, especially the type of carbon source, the limiting compound, temperature, pH, and others discussed below.

Type of carbon source

Generally, biosurfactant production is induced by applying hydrocarbon or water immiscible substrates (Syldatk and Wagner 1987). However, also hydrophilic carbon sources like ethanol, mannitol, glycerol, and glucose are described as substrates for rhamnolipid production (Robert et al. 1989; Sim et al. 1997). In comparison *n*-alkanes and vegetable oil lead to higher concentrations of rhamnolipids (Syldatk et al. 1985b; Robert et al. 1989). For example cultivations of *P. aeruginosa* UG2 on hydrophobic substrates like corn oil, lard, and long-chain alcohols lead to $Y_{RL/S}$ = 100 – 165 mg/g while growth on glucose and succinic acid only resulted in $Y_{RL/S}$ = 12 – 36 mg/g (Mata-Sandoval et al. 2001). When *n*-alkanes were added to cultivations of *P. aeruginosa* SB30 biosurfactant production was induced (Chakrabarty 1985). On the contrary catabolic repression of rhamnolipid production was observed when applying glucose, acetate and tricarboxylic acids (Hauser and Karnovsky 1954; 1957).

Influence of the growth-limiting component

For the induction of rhamnolipid formation in a biotechnological setup an appropriate limitation must be achieved. For this purpose the limitation of nitrogen, phosphor, or multivalent ions in combination with carbon excess have been employed (cf. Chapter 1.3.3). Interestingly, nitrate as nitrogen source showed to promote rhamnolipid production, however, ammonium did not (Guerra-Santos et al. 1986; Manresa et al. 1991; Arino et al. 1996). Under anaerobic denitrifying conditions with *P. aeruginosa* ATCC 10145 (28 °C, pH 6.8, 2% (*v/v*) hexadecane) with P-limitation good rhamnolipid productivities were observed (Chayabutra et al. 2001). Chayabutra et al. (2001) showed that a P-limitation under denitrifying conditions resulted in a four- to five-fold higher rhamnolipid productivity as compared to an N-limitation, using hexadecane as C-source in excess. For rhamnolipid production under batch and fed-batch conditions at 25 °C and pH 7 with *P. aeruginosa* BYK-2 KCTC 18012P urea turned out to be the best nitrogen source in combination with fish oil as carbon source (Lee et al. 2004). Not only the type of carbon and nitrogen source, but also the respective C/N ratios showed strong influences on the total rhamnolipid productivity (Guerra-Santos et al. 1984; Santa Anna et al. 2002; Wu et al. 2008).

Guerra-Santos et al. (1984; 1986) showed that for *P. aeruginosa* DSM 2569 (37 °C, pH 6.5, glucose, nitrate) between C/N (*w/w*)[3] ratios of 16/1 to 18/1 the rhamnolipid productivity was highest and no rhamnolipid production could be observed at C/N (*w/w*) ratios lower than 11/1. Apart from P- and N-limitation, the limitation of multivalent ions like Mg, Ca, K, Na, and trace element salts also cause increasing of the rhamnolipid yield (Guerra-Santos et al. 1984, 1986). Highest final rhamnolipid concentrations were observed for *P. aeruginosa* DSM 7107 and DSM 7108 (30 °C, pH 6.3, sunflower oil) in Ca-free media (Giani et al. 1996; Müller et al. 2010).

Other factors influencing rhamnolipid production

The dependence of the rhamnolipid production on temperature and pH value has been extensively studied. Guerra-Santos et al. (1984) reported highest productivities at pH of 6 – 6.5 for *P. aeruginosa* DSM 2659. Robert et al. (1989) found that a temperature of 37 °C was optimum for the rhamnolipid production with *P. aeruginosa* 44T1. However, the highest reported yield was gained at 30 °C (Giani et al. 1997) and a starting pH of 6.3. Temperature influenced the composition of rhamnolipids when resting cells of *Pseudomonas* sp. DSM 2874 were cultivated on rapeseed oil using a fed-batch or continuous cultivation process (Syldatk et al. 1985b; Trummler et al. 2003). Syldatk et al. (1985a) showed that resting cells of *Pseudomonas* sp. DSM 2874 produced up to 15 g/L of different rhamnolipids in simple phosphate buffer or a sodium chloride solution with optimal pH value in the range 6 – 7.2. While rhamnolipid formation with glycerol as the sole C-source showed a wide optimum ranging from 27 °C up to 37 °C, production of rhamnolipids from *n*-alkanes had a sharp optimum at 37 °C.

Typical production media compositions

Typical production media compositions for the different process strategies are summarized in Table 1-4. In semicontinuous culture the production media is only composed of a buffer system and the added C-source. For continuous cultivation Guerra-Santos et al. (1984a,b; 1986) developed different media, mainly based on glycerol as C-source. The best rhamnolipid yields in batch and fed-batch conditions

[3] was changed from *m/m* to *w/w* throughout this chapter

have been achieved with the Ca-free medium from Giani et al. (1996) and modifications thereof (Leitermann 2008; Müller et al. 2010). The P-limited proteose peptone glucose ammonia salt (PPGAS) medium is often used for the investigation of rhamnolipid regulation on molecular level (Zhang and Miller 1992; Medina et al. 2003).

Table 1-4. Representative rhamnolipid production media compositions for resting cell, continuous, N-limited, or P-limited batch/fed-batch process strategies.

Component	Resting Cells	Continuous	N-Limited	P-Limited
Main salts	**(g/L)**	**(g/L)**	**(g/L)**	**(g/L)**
$NaNO_3$	-	2.75	15.00	-
PO_4^{3-} source	-	1.90	0 – 0.30	-
Buffer (M)	0.1 Na-phosphate	-	0 – 0.1 Na-phosphate	0.12 Tris-HCl
$MgSO_4 \times 7H_2O$	-	0.44	0.5	0.394
KCl	-	0.60	1	1.49
NaCl	-	0.60	-	-
$CaCl_2 \times H_2O$	-	0.03	Ca-free	-
NH_4Cl	-	-	-	1.07
Trace elements	**(mg/L)**	**(mg/L)**	**(mg/L)**	**(mg/L)**
$ZnSO_4 \times 7H_2O$	-	1.65	1.40	-
$MnSO_4 \times H_2O$	-	1.65	0.80	-
H_3BO_3	-	0.33	-	-
$CoCl_2 \times 6H_2O$	-	0.17	1.20	-
$CuSO_4 \times 5H_2O$	-	0.17	1.20	-
$NaMoO_4 \times H_2O$	-	0.11	-	-
Na-citrate x H_2O	-	-	2.00	-
$FeCl_3 \times 6H_20$	-	-	0.28	-
$FeSO_4 \times 7H_2O$	-	0.55	-	-
Others				
pH	6.5	6.2 – 6.4	6.3 – 7.0	7.2
temperature (°C)	30 – 37	32 – 34	30 – 40	37
C-source (complex component)	Rapeseed oil	2% glycerol	Sunflower oil, soybean oil	0.5% glucose (proteose peptone)
(g/L)	198	20	125 – 250	5 (10)
References	Syldatk et al. (1985a,b), Trummler et al. (2003)	Guerra-Santos et al. (1986)	Giani et al. (1997), Müller et al. (2010)	Zhang and Miller, (1992), Medina et al. (2003)

1.3.5 Downstream processing of rhamnolipids

Apart from the expenses for raw materials, and invest costs, purification is the highest cost factor for biosurfactant production (Mulligan and Gibbs 1993). Fine chemicals imply very high purities. Thus, on one side, the versatility of rhamnolipids is a valuable aspect with regard to product portfolio. On the other hand it becomes difficult to separate complex mixtures of up to 28 different rhamnolipid congeners

(Déziel et al. 1999). Purification costs are also dependent on the employed strain and the rhamnolipid mixtures produced thereof. However, for some applications this purity grades will not be necessary and thus purification costs should be more moderate.

Production of rhamnolipid crude extracts

After the production process is finished, the rhamnolipids have to be recovered and separated from the cell-containing medium. Different methodologies are generally used for the acquisition of rhamnolipids. The first step mostly includes acid precipitation. At a pH of 2 – 3 rhamnolipids are protonated and become insoluble in water, thus precipitate. Yields of up to 98% were reported for acid precipitation after cell removal and subsequent heat treatment (Mixich et al. 1997). This aims to obtain aqueous culture medium-free crude extract. The precipitate is collected by filtration or centrifugation, and further processed. However, at this pH other molecules also precipitate. Therefore precipitation is generally followed by organic solvent extraction methods. The used organic solvents are mostly ethyl acetate (Syldatk et al. 1985a; Schenk et al. 2005) and chloroform (Mata-Sandoval et al. 1999). Sometimes, remains of vegetable oil have to be removed before acidification by *n*-hexane extraction. Solvent extraction methods are also applied for preparation of rhamnolipid quantification during bioreactor cultivation. Although various methods are available the most commonly used is organic solvent extraction. Subsequent evaporation of the organic solvent leads to a crude extract, which contains all synthesized rhamnolipid congeners in a highly concentrated manner. Further steps are needed to separate the single rhamnolipid species from each other if requested.

Further purification steps

The crude extract can be further concentrated by filtration and chromatographic methods (Andrä et al. 2006). The rhamnolipid production is closely related to LPS and alginate production (cf. Chapters 1.3.1 and 1.3.2). Thus such impurities will affect the purification process. Reiling et al. (1986) has reported about clogging by polysaccharides, when applying column chromatography for purification. Rhamnolipids are, by default, purified with the help of normal phase chromatography

columns with embedded silica gels (Itoh et al. 1971; Schenk et al. 1995; Monteiro et al. 2007). Alternatively reverse-phase C_{18} columns (Mata-Sandoval et al. 1999; Heyd et al. 2008), ion-exchange chromatography (Reiling et al. 1986; Schenk et al. 1995), and hydrophobic interaction chromatography (Reiling et al. 1986; Matulovic 1987; Abalos et al. 2001; Haba et al. 2003) have been applied. Reiling et al. (1986) used a purification process composed of three subsequent chromatographic steps. The three steps comprised hydrophobic interaction, ion exchange, and again hydrophobic interaction chromatography. Over 60% rhamnolipid recovery was achieved using this order of separation methods. Small samples of single rhamnolipid species can be purified by preparative thick-layer chromatography based on silica-gel basis (Syldatk et al. 1985a; Déziel et al. 2000; Monteiro et al. 2007). Appropriate running solutions are generally mixtures of chloroform, methanol, acetic acid, and water. The rhamnolipids may be eluted from the thick-layer chromatography plate. However, this is not a method to purify large amounts of rhamnolipids but it is reliable for small quantities like needed for HPLC as references. From crude extracts Rha-Rha-C_{10}-C_{10} can be recovered by recrystallization with *n*-hexane (Pajarron et al. 1993).

In situ product removal

Alternatively, *in situ* product removal (ISPR) can be used instead of a batch purification. By the means of ISPR a continuous product flow can be provided. Additionally ISPR allows reducing side effects like product inhibition, product degradation or the accumulation of toxic byproducts. For rhamnolipid production diverse filtration and foam fractionation methods have been extensively studied. Membrane filtration methods applied for continuous removal of produced rhamnolipid often failed due to rapid membrane fouling, which is mainly caused by exopolysaccharides and bacteria. Nevertheless, rhamnolipid gain of 50% (Gruber 1991) and 92% (Mulligan and Gibbs 1990) were reported for micro- and ultrafiltration, respectively. During cultivation excessive foam formation can be observed. Thus foam fractionation could be an interesting way to remove the produced rhamnolipid, which is highly concentrated in the foam. The foam is stabilized due to the interfacial properties of the rhamnolipids. The method of foam fractionation is well established for biosurfactant production like with *B. subtilis* (Chen

et al. 2006). Foam fractionation columns have been tested for rhamnolipid recovery (Matulovic 1987; Gruber 1991). Unfortunately the removal of foam during rhamnolipid processes also leads to a washout of biomass, which is even more enriched in the foam than rhamnolipids. Thus working with immobilized or retained cells should be preferred when using foam fractionation. Recently, a biosurfactant recovery of 97% and an enrichment ratio of 4 was reported when applying foam fractionation technique for the continuous recovery of rhamnolipids (Sarachat et al. 2010).

1.4 Rhamnolipid production by other microorganisms

Other organisms apart from *P. aeruginosa* have been identified and characterized as rhamnolipid producers. Table 1-5 gives a summary of other reported microorganisms synthesizing rhamnolipids. Reports about Bacilli- and Actinobacteria-producing rhamnolipids have to be handled with care, unless further reports confer these findings. Especially *Burkholderia* sp. like *B. glumae* (Pajarron et al. 1993), *B. pseudomallei* (Häußler et al. 1998), *B. plantarii* (Azegami et al. 1988; Andrä et al. 2006; Hörmann et al. 2010), and recently *B. thailandensis* (Dubeau et al. 2009) have been reported to produce rhamnolipids. The investigated *Burkholderia* sp. mainly synthesizes di-rhamnolipids like Rha-Rha-C_{10}-C_{10} up to Rha-Rha-C_{16}-C_{16}. However, the most predominant di-rhamnolipid is Rha-Rha-C_{14}-C_{14}. Because some of these strains are nonpathogenic and therefore need lower security measures than *P. aeruginosa,* they are sometimes summarized as “alternative” production strains. But more interesting than potential lower costs for security precautions during production is the fact that longer fatty acid side chains lead to higher hydrophobicity than decanoic acid (-C_{10}) containing rhamnolipids. However, the crude rhamnolipid mixture produced by *B. thailandensis* reduces the surface tension of water from 72 to 42 mN/m while displaying a CMC value of 225 mg/L (Dubeau et al. 2009). Thus the reported surfactant properties seem to be not as good as for the purified *P. aeruginosa* rhamnolipids (cf. Table 1-1). The purified Rha-Rha-C_{14}-C_{14} rhamnolipid of *B. plantarii* reduced the surface tension of water from 72 mN/m to <29 mN/m and showed CMC of 15 mg/L (Hörmann et al. 2010). These features are quiet similar to the properties reported for Rha-Rha-C_{10}-C_{10}. However, the low product concentrations of the current production processes described for these strains are not competitive with *P. aeruginosa* rhamnolipid yields. Dubeau et al. (2009) achieved

rhamnolipid concentrations of 0.4 – 1.5 g/L, after 13 days of culture with *B. thailandensis* in shake flask experiments (34 °C, nutrient broth with 4% glycerol or canola oil). *B. plantarii* produced 46 mg/L in 25 h under non-optimized conditions (30 °C, pH 7, nutrient broth with 10 g/L glucose) in a 0.5 L parallel bioreactor system (Hörmann et al. 2010). *P. aeruginosa* concentrations can be 50 – 280 fold higher (Giani et al. 1997) than the maximum reported *Burkholderia* sp. concentrations. Apart from the investigation of natural rhamnolipid producers also the construction of heterologous rhamnolipid hosts were reported (Ochsner et al. 1995; Wang et al. 2007). Here mainly *P. putida* and *E. coli* strains were used. Ochsner et al. (1995) report 0.6 g/L rhamnolipid when using *P. putida* strain KT2442 as heterologous host for rhamnolipid biosurfactant production. While for *P. aeruginosa* the molecular regulation of rhamnolipid production is already known in large part (cf. Chapter 1.3), the rhamnolipid production by *Burkholderia* sp. is a younger field with many uncertainties. Recent studies propose a different regulation of rhamnolipid production for *Burkholderia* sp. like *B. thailandensis* (Dubeau et al. 2009). For *B. thailandensis* an *rhlABC* operon was identified. This proposes an explanation for the occurrence of mostly di-rhamnolipids, due to the possibly same stoichiometric ratio of the homologous rhamnosyltransferases. In *P. aeruginosa rhlAB* and *PA1131-rhlC* constitute two independently transcribed operons cross-linked by complex QS signalling.

Table 1-5. Reported rhamnolipid-producing bacteria besides *P. aeruginosa* and the main identified rhamnolipid species thereof.

Strain	Structure Determination	Main Structures	Phylum and Safety Level	Reference
B. cereus	Not determined	Not reported	Bacilli L2	Tuleva et al. (2005)
B. glumae	FAB-MS	Rha-Rha-C_{10}-C_{10} Rha-Rha-C_{14}-C_{14}	Proteobacteria L1	Pajarron et al. (1993)
B. plantarii	NMR	Rha-Rha-C_{14}-C_{14} Rha-Rha-C_{12}-C_{14} Rha-Rha-C_{14}-C_{12}	Proteobacteria L1	Azegami et al. (1988), Andrä et al. (2006), Hörmann et al. (2010)
B. pseudomallei	GC-MS, NMR	Rha-Rha-C_{14}-C_{14}	Proteobacteria L2	Häußler et al. (1998)
B. thailandensis	LC-MS-MS	Rha-Rha-C_{14}-C_{14} Rha-Rha-C_{12}-C_{14} Rha-Rha-C_{14}-C_{16}	Proteobacteria L1	Dubeau et al. (2009)
P. boreopolis	Not determined	not reported	Proteobacteria L1	Itoh and Suzuki (1972), Käppeli and Guerra-Santos (1986)
P. chlororaphis	HPLC-MS	Rha-C_{10}-$C_{12:1}$ Rha-C_{10}-C_{12} Rha-C_{12}-C_{12} Rha-C_{10}-C_{10}	Proteobacteria L1	Gunther et al. (2005)
P. cruciviae	Not determined	not reported	Proteobacteria L1	Itoh and Suzuki (1972), Käppeli and Guerra-Santos (1986)
P. fluorescens	Not determined	not reported	Proteobacteria L1	Itoh and Suzuki (1972), Käppeli and Guerra-Santos (1986)
P. oleovorans	Not determined	not reported	Proteobacteria L1	Itoh and Suzuki (1972), Käppeli and Guerra-Santos (1986)
P. putida	GC of lipophilic part	-$C_{18:2}$, -C_{20}, -C_{22}	Proteobacteria L1	Tuleva et al. (2002), Martínez-Toledo (2006)
P. stutzeri	Not determined	not reported	Proteobacteria L1	Janiyani et al. (1992)
R. salmoninarum	IR comp. with *P.aeruginosa* RLs	Rha-C_{10}-C_{10} Rha-Rha-C_{10}-C_{10}	Actinobacteria L2	Christova et al. (2004)
T. koreensis	not determined	Not reported	Bacilli L1	Lee et al. (2005)

1.5 Commercial aspects of rhamnolipids

The estimated manufacturing cost per ton of produced rhamnolipid in a 20 – 100 m³ scale bioreactor was estimated to be around 5,000 – 20,000 USD (Lang and Wullbrand 1999). This reflects 2 – 20 times higher expenses as being described for chemical bulk surfactants (e.g., ethoxylates and alkyl polyglycosides). Therefore, only a substantial reduction of the costs (cf. Chapter 1.6) combined with an expected increase of crude oil prices and a shortage of crude oil availability will lead to economic competitive rhamnolipid production for bulk applications. However, the excellent surfactants properties, biodegradability, environmental friendliness and the fact, that rhamnolipids can be produced by the means of renewable resources, lead

to an increasing interest in rhamnolipid applications. Especially, if there is a need for “greener” products and environmental concerns. At the beginning of 2010 the web-based research at the European Patent Office (http://ep.espacenet.com) resulted into a total of 152 patents worldwide related to “biosurfactant” and 63 directly related to “rhamnolipid.” There is a huge heterogeneity among the claimed applications for rhamnolipids. Rhamnolipids may be used in agriculture, for bioremediation and enhanced oil recovery (EOR), in cosmetic industry, in food process engineering, as detergents, for medical applications or as emulsifiers in general. Some of these applications are sketched in this chapter.

Agriculture and environment

Zonix™, a commercially available rhamnolipid biosurfactant mixture (Jeneil Biosurfactant Co., Saukville, USA), is a liquid contact biofungicide used in agricultural, horticultural, and turf settings to prevent and control plant pathogens such as downy mildews, Pythium and Phytophthora and is officially approved by the U.S. Environmental Protection Agency (USEPA). Rhamnolipids can potentially be used in bioremediation (Nguyen et al. 2008), biodegradation (Zhang et al. 2005) and microbial enhanced oil recovery (MEOR) as described by Yang and Li (1999) and Wang et al. (2007). In 1989, the Exxon *Valdez* oil spills bioremediation by applying *P. aeruginosa* biosurfactants was effectively tested. The rhamnolipids of *P. aeruginosa* enhanced the bioavailability of the crude oil and thus improved degradation thereof by MEOR (Harvey et al. 1990). Rhamnolipids increase the mobility of phenantren and hexadecane in contaminated soils better than chemical surfactants like SDS or Tween 80 (Rahman et al. 2003). Such properties emphasize the efficient use in MEOR (Kowalewski et al. 2006).

Cosmetics and medicine

Cosmetics and wound healing applications have recently been claimed by Paradigm Biomedical, Inc. (Piljac and Piljac 2007). The considerable antimicrobial effects of rhamnolipids (Abalos et al. 2001; Rodrigues et al. 2006) emphasize the application in the pharmaceutical sector. Aurora Advanced Beautylabs (St. Petersburg FL, USA) announced in 2008 to launch cosmoceutical products based on rhamnolipids.

However, no such product has reached the marked yet. Hwang et al. (2001) and Gandhi et al. (2007) reported that the antimycotic and antifungal activity of rhamnolipids is well worth to be used in pharmaceutical applications. Rhodrigues et al. (2006) for example reported an antiadhesive and antimicrobial effect of rhamnolipids on *Mycobacterium tuberculosis*. Yet, there are no approvals by the U.S. Food and Drug Administration (USFDA) for the use of rhamnolipids in or as pharmaceuticals.

Food technology

The Suedzucker AG (Mannheim, Germany) claimed in 1990 a process for the production of rhamnose from rhamnolipids for the purpose of food additive (Mixich and Rapp 1990). Rhamnose can serve as a precursor for high-quality flavor products like sweeteners. Rhamnose is produced by hydrolysis of rhamnolipids (Linhardt et al. 1989). However, microbial produced rhamnose is in competition with rhamnose from plant origin. The use as emulsifiers in food engineering is another possible application for rhamnolipids (Van Hasendonck and Vanzeveren 2006). Rhamnolipids enhance the storage life and quality of bakery products. Rhamnolipids shall be used as feed additives for avian or mammal farm animal to provide a means for preventing or treating an inflammatory disease by a Gram-negative bacterium (Motoshi et al. 2009). Yet, there are no approvals by the USFDA for the use of rhamnolipids in nutrition.

Cleaning detergents

Surfactants are major ingredients of detergents and household cleaning agents. In this area there is a constant trend toward environmentally sound products. Biosurfactants are currently entering this market and there is a good chance of biosurfactants in general representing the next generation of detergents for household products. Ecover (Malle, Belgium) produces a glass and surface cleaner containing rhamnolipid. This cleansing agent was classified as biodegradable (OECD-test 301F) and shows minimal impact on aquatic life (OECD-tests 201 and 202). Thus rhamnolipids perfectly match the requirements of eco-friendly surfactants and could find extensive use as detergent additives.

1.6 Needs and future trends in rhamnolipid production

Although it seems like rhamnolipids are already established in the market, this is not the case. The low productivities of the current production processes and the lack of companies producing rhamnolipids still make them not applicable in bulk applications. Additionally, approvals of the USFDA and USEPA for most of the possible applications are still pending. However, their biodegradability, environmental friendliness, low aquatic toxicity, and the fact that they can be produced by the means of renewable resources make them interesting surfactants for a biosustainable future.

The research about rhamnolipid production in the recent decades mainly focused on heuristic process engineering approaches. Future steps toward economical and ecological rhamnolipid production should include the following:

- Characterization of QS during bioreactor cultivations in pilot plant scale
- Use of cheap substrates from waste (e.g., glycerol from biodiesel production) or renewable (e.g., nonfood vegetable oils) resources
- Improvement of process strategies with the help of model-based process understanding
- Improvement of downstream processing and product recovery avoiding organic solvents
- Stabilization of rhamnolipid production by nonchemical foam control
- Use of “alternative” and heterologous host strains for rhamnolipid production to avoid costly safety measures
- Pathway engineering to decrease formation of unwanted byproducts (e.g., polysaccharides, alginate, PHA, and LPS) and increase precursor pools

Systems biotechnology for the improvement of rhamnolipid production

According to Lee et al. (2005b), up-to-date production strain improvement must include “systems biotechnology” approaches. For this purpose X-omics technologies, namely transcriptomics, proteomics, and metabolomics are available. However, these techniques need the sequences and annotation of the respective microbial genome. Thus the sequenced *P. aeruginosa* PAO1 (Stover et al. 2000) should be one strain of choice. Recently it was shown that this strain is capable to produce high-enough quantities to be used as a model for rhamnolipid production in bioreactor systems (Müller et al. 2010). Additionally, this strain is an important model for QS studies. Thus eased cooperation of bioengineers and basic research groups

should be possible by application of this strain. The knowledge, which will be accumulated by “systems biotechnology,” could be integrated into advanced process control of rhamnolipid production. The use of a knowledge-based approach should lead to economic rhamnolipid processes in the not-so-distant future. Therefore it will be necessary to reduce process times and increase productivities (cf. Table 1-3) for batch and fed-batch fabrication of rhamnolipids. For the development of new fed-batch strategies it will be necessary to implicate the knowledge of nitrogen source lapse and growth rate dependence of the specific rhamnolipid production rate (Figure 1-5). None of the reported fed-batch strategies focused on nitrogen control during cultivation. However, it was shown that the phase of nitrogen depletion is most important for rhamnolipid overproduction. Thus future fed-batch strategy will integrate knowledge about QS regulation and has to focus on the depleting substrate. Nevertheless, such approaches should be accompanied with online process control systems. For this purpose, modern online probes based on NMR and FTIR technology (Leitermann 2008), as well as exhaust gas analysis (Müller et al. 2010) should lead to advanced controlled fed-batch processes.

Future trends and needs for downstream processing

The expenses for raw materials can make up 10 – 50% of the total costs (Mulligan and Gibbs 1993). Downstream techniques like crystallization should lead to higher yields and would allow highly pure rhamnolipids. The relatively high costs for current extraction methods are an important economic obstacle toward bulk application. The reduction of the use of organic solvents is also an ecologic issue. Depending on the application the need of purity can be very high. If used as fine chemicals for pharmaceutical or food products an approximate purity of >99% will be requested. As an antifungal agent like Zonix™ or as washing detergent, the rhamnolipid purity should be less important. For MEOR, even the whole culture suspension could be applied after deactivation of the cells. Nevertheless, it seems to be inevitable for economic rhamnolipid production to reduce the produced mixtures of rhamnolipid congeners.

Strain improvement and development

Future strains preferentially producing only one type of rhamnolipid (mono- or di-rhamnolipids) would be an ideal solution for the problems encountered during downstream process. The mixtures not only complicate purification but also influence the yield of the wanted final rhamnolipid product. Undesirable byproducts like PHA, LPS, alginate, and polysaccharide formation should be excluded by pathway engineering. The use of “alternative” bacteria, especially *Burkholderia* sp., and heterologous hosts, should not only allow reducing the cost intensive safety measures for production but also broadens the available rhamnolipid portfolio. For some of the *Burkholderia* sp. the genomes are already sequenced, thus allowing “systems biotechnology” approaches in the future. However, an increase in productivity is even more required as for established *P. aeruginosa* rhamnolipid manufacturing.

1.7 Outlook

Rhamnolipids are versatile biosurfactants with a broad spectrum of possible applications ranging from cleansing agents to high-value pharmaceuticals. However, even though over 60 years have passed since the first rhamnolipid was described, there is still a long way before achieving an economic bioproduction for bulk applications. Amongst other things the main reason therefore is the lack of understanding the rhamnolipid production regulation. However, there are good chances of success in the near future, if a systems biotechnological approach is effectually adopted for strain and process development. The use of new heterologous rhamnolipid producing hosts and “alternative” strains will help to broaden the product spectrum and make it possible to produce single rhamnolipid congeners.

Due to their surfactants properties, environmental friendly nature, and sustainable character rhamnolipids will, eventually, play a prominent role as versatile surfactants in the future.

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2 *Pseudomonas aeruginosa* PAO1 as a model for rhamnolipid production in bioreactor systems

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2.1 Abstract

Rhamnolipids are biosurfactants with interesting physico-chemical properties. However, the main obstacles towards an economic production are low productivity, high raw-material costs, relatively expensive downstream processing, and a lack of understanding the rhamnolipid production regulation in bioreactor systems. This study shows that the sequenced *Pseudomonas aeruginosa* strain PAO1 is able to produce high quantities of rhamnolipid during 30 L batch bioreactor cultivations with sunflower oil as sole carbon source and nitrogen limiting conditions. Thus PAO1 could be an appropriate model for rhamnolipid production in pilot plant bioreactor systems. In contrast to well-established production strains, PAO1 allows knowledge-based systems biotechnological process development combined with the frequently used heuristic bioengineering approach. The maximum rhamnolipid concentration obtained was 39 g/L after 90 h of cultivation. The volumetric productivity of 0.43 g/(L·h) was comparable with previous described production strains. The specific rhamnolipid productivity showed a maximum between 40 and 70 h of process time of 0.088 $g_{RL}/g_{BDM}h$. At the same time interval, a shift of the molar di- to mono-rhamnolipid ratio from 1:1 to about 2:1 was observed. PAO1 not only seems to be an appropriate model, but surprisingly has the potential as a strain of choice for actual biotechnological rhamnolipid production.

2.2 Introduction

Glycolipids, consisting of one or two L-rhamnose moieties and one or two β-hydroxy-alkanoic acid residues, are termed rhamnolipids (RL). Various microorganisms, mainly *Pseudomonas aeruginosa,* are able to produce rhamnolipids (Jarvis and Johnson 1949; Manso Pajarron et al. 1993; Häußler et al. 1998; Andrä et al. 2006; Gunther 2007). Rhamnolipids are biosurfactants of special interest for cosmetic, pharmaceutical and detergent manufacturers, and already proved their potential in enhanced oil recovery (Wang et al. 2007), bioremediation (Nguyen et al. 2008), and biodegradation (Zhang et al. 2005). They show excellent biodegradability, low toxicity, thus environmental friendliness, and can be produced by using renewable or waste resources (Giani et al. 1997; Rahman et al. 2002; Wei et al. 2005; Marsudi et al. 2008), while showing comparable physico-chemical properties to synthetic surfactants (Syldatk et al. 1985b). Rhamnolipids were first described by Jarvis and

Johnson (1949). The main rhamnolipid producing bacteria applied are Pseudomonads, especially strains of the species *P. aeruginosa*. Rhamnolipid production in *P. aeruginosa* is quorum sensing (QS) regulated (Ochsner and Reiser 1995). In 2000, the complete genome of *P. aeruginosa* PAO1 (Stover et al. 2000), a QS model organism, was published and thus PAO1 became the best studied *P. aeruginosa* strain (Venturi 2006; Potvin et al. 2008; Williams and Camara 2009). However, today there is still a lack of understanding the rhamnolipid regulation in pilot plant bioreactor systems on genetic, protein, and metabolic level. Two main types of rhamnolipids (Figure 2-1) are typically secreted when *P. aeruginosa* is growing on water immiscible substrates (Lang and Trowitzsch-Kienast 2002).

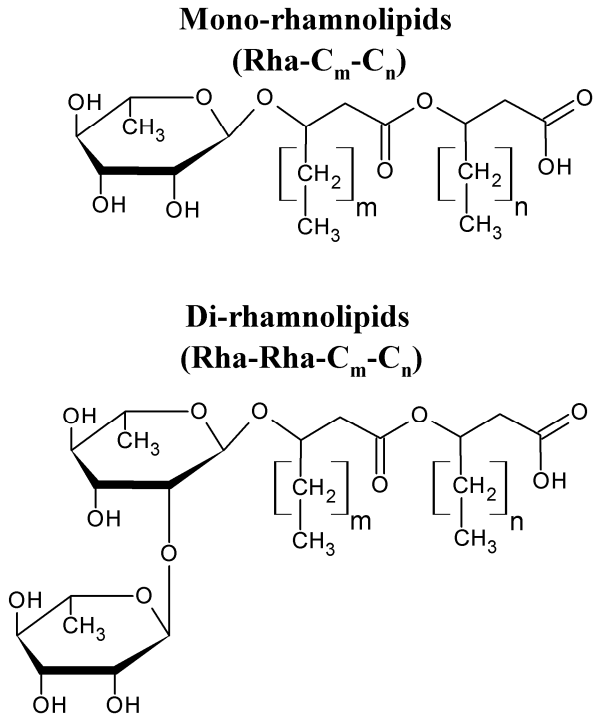

Figure 2-1. Generalized structures of mono-rhamnolipids and di-rhamnolipids, m,n = 4 – 8.

The most frequently mono- and di-rhamnolipids consist of β-hydroxydecanoic acid residues, namely α-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-C_{10}-C_{10}) and α-L-rhamnopyranosyl-(1-2)-α-L-rhamnopyranosyl-3-hydroxy-decanoyl-3-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}), respectively. In a patent Giani et al. (1997) claimed to achieve 74 - 112 g/L of rhamnolipids. No verification of these claims on laboratory scale was reported since then. Typical obstacles encountered during rhamnolipid production include, excessive foaming, insufficient rhamnolipid

yields, expensive raw materials and relatively high costs for downstream processing (Banat et al. 2000). The fact that many different strains have been used and the quantification of rhamnolipids is mostly done by indirect carbohydrate analysis (Chandrasekaran and BeMiller 1980), leads to confusing comparisons in literature. Mainly heuristic approaches of process development were used in the last decades but according to Lee et al. (2005) up-to-date process development should include systems biotechnological approaches (e.g., prote-, metabol-, transcriptomics). As many of the tools are established for PAO1, this strain should be an appropriate model for rhamnolipid processes. The intention of this study was to investigate whether *P. aeruginosa* PAO1 produces high enough quantities of rhamnolipids to be used as a model for pilot plant scale bioreactor process development. Additionally, the introduction of a platform batch process for further knowledge-based systems biotechnological and bioengineering approaches, rather than heuristics, was pursued.

2.3 Materials and methods

2.3.1 Chemicals

Sunflower oil (Bellasan®, *Aldi Süd,* Rastatt, Germany) was of food grade. The di-rhamnolipid (Rha-Rha-C_{10}-C_{10}) standard was a gift from former *Hoechst AG* (Hoechst, Germany) and the mono-rhamnolipid (Rha-C_{10}-C_{10}) standard for high performance liquid chromatography (HPLC) analysis was prepared as described before by Trummler et al. (2003). For rhamnolipid derivatization triethylamine and 4-bromophenacylbromide were used (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) like described by Schenk et al. (1995). Other chemicals were from Carl Roth GmbH (Karlsruhe, Germany). All chemicals were of analytical grade.

2.3.2 Microorganism

The bacterial strain *P. aeruginosa* PAO1 (Holloway et al. 1979; Stover et al. 2000) was used for all experiments described below. The strain *P. aeruginosa* PAO1 was kindly provided by Frank Rosenau, Forschungszentrum Jülich (Jülich, Germany). Glycerol stocks were prepared of a culture in Lysogeny Broth (Bertani 1951) from the exponential growth phase and stored at -80 °C until needed.

2.3.3 Culture methods

Media

Lysogeny broth (LB) was used for pre-cultivation step one. For the seed culture, a nitrogen-limited medium consisting of 125 g/L sunflower oil and a Ca-free mineral salt solution with 1.5 g/L $NaNO_3$, 0.05 g/L $MgSO_4$ x 7 H_2O, 0.1 g/L KCl, containing a 0.1 M sodium phosphate buffer at pH 6.5, was used. A total of 1 mL/L of trace element solution was added. The trace element solution contained 2.0 g/L sodium citrate x 2 H_2O, 0.28 g/L $FeCl_3$ x 6 H_2O, 1.4 g/L $ZnSO_4$ x 7 H_2O, 1.2 g/L $CoCl_2$ x 6 H_2O, 1.2 g/L $CuSO_4$ x 5 H_2O and 0.8 g/L $MnSO_4$ x H_2O.

Nitrogen-limited production medium consisted of a Ca-free mineral salt solution with 15.0 g/L $NaNO_3$, 0.5 g/L $MgSO_4$ x 7 H_2O, 1.0 g/L KCl and as a phosphate source 0.3 g/L K_2HPO_4. As sole carbon source sunflower oil with a starting concentration of 250 g/L was used and 1 mL/L of the above mentioned trace element solution was added. Mineral salt solutions, phosphate sources, and sunflower oil were autoclaved separately for all experiments. Trace elements were filter-sterilized through a 0.22 µm membrane filter (Carl Roth GmbH, Karlsruhe, Germany). Production medium was adjusted to pH 6.5 and controlled during cultivation using 4M NaOH or 4M H_3PO_4, respectively.

Preparation of seed culture

All shake flask cultures were incubated in a shake incubator chamber (Multitron II, HT Infors, Bottmingen, Switzerland). First 25 mL of LB in a 100-mL baffled shake flask were inoculated with a total volume of 100 µL from the glycerol stock solution of *P. aeruginosa* PAO1 and incubated for 24 h at 37 °C, 120 rpm. Seed cultures containing 200 mL medium were cultivated in a 1-L baffled shake flask and inoculated using a total of 5 mL from the 24-h LB culture. This culture was incubated for at least 24 h at 37 °C, 120 rpm. The seed culture was used to inoculate with a resulting OD_{580} of 0.06 in the aqueous phase at the start of the bioreactor cultivation.

Production of rhamnolipids using *P. aeruginosa* PAO1 in 30 L bioreactor scale

A 42-L stirred tank, with a nominal capacity of 30 L, was used for all cultivations (Biostat® Cplus, Sartorius Stedim Biotech, Göttingen, Germany). The bioreactor was equipped with an integrated process control system for temperature, pH, pO_2, airflow and foam level regulation (MFCS/win 2.1, Sartorius BBI, Göttingen, Germany). The foaming was controlled by a modified foam breaker in the headspace of the bioreactor, which was automatically activated when the foam level reached the 35 L scale of the bioreactor (approximately 15 L of foam). Stirrer speed was set fix at 400 rpm, temperature at 37 °C and dissolved oxygen was set at minimum 5% and controlled by variation of the aeration rates between 0.1 L/(L·min) and 3.3 L/(L·min), related to the aqueous phase of the production medium (15 L). For the cultivation, 19 L of the production medium was used. The trace element solution was added at cultivation times of 0, 20, 40, 70, and 120 h, as described before (Giani et al. 1997). Exhaust gas was continuously analyzed (EGAS-L gas analyzer, Sartorius BBI, Göttingen, Germany). The cultivation was performed as a triplicate. Online parameters (exhaust gas, pH control, etc.) were monitored for 165 h.

2.3.4 Analytical methods

Sampling and processing

Samples were taken for offline analysis of biodrymass (BDM), sunflower oil and RL concentrations. Therefore, the culture suspension was mixed vigorously with n-hexane 1:1 (*v/v*) and centrifuged (4,600 g, 4 °C, 30 min) for separation of cells, aqueous and n-hexane phase. The n-hexane phase was used for gravimetric determination of sunflower oil concentrations, after evaporation of n-hexane. It was confirmed by HPLC (cf. below), that no rhamnolipids were extracted by the n-hexane extraction step. The cell pellet was washed once in 0.9% NaCl solution (4,600 g, 4 °C, 30 min), dried till constant weight and BDM was quantified gravimetrically. An aliquot of the aqueous phase was acidified with 85% phosphoric acid 1:100 (*v/v*) to adjust a pH of about 2 - 3, leading to precipitation of the rhamnolipids. Rhamnolipids were extracted twice with ethyl acetate 1:1.25 (*v/v*). Appropriate amounts of ethyl acetate extracts were evaporated and used for rhamnolipid quantification by HPLC

as described below. Offline analysis was continued till 100 h of process time for all triplicates and till 165 h for one of the cultivations.

Data analysis

Bacterial growth was fitted using a logistic equation (BDM_{fit}) with four parameters in a scientific data analysis and graphing software (Sigma Plot 9.0, Systat, San Jose, USA). The used equation was:

$$y\,(x) = y_0 + \frac{a}{1+\left(\frac{x}{x_0}\right)^b} \quad \text{(Equation 2–1)}$$

The four parameters are the following: y_0 indicates the BDM_{inoc} after inoculation; a indicates the maximum additional BDM_{add} produced in addition to BDM_{inoc} ($BDM_{max} = a + y_0$); x_0 indicates the process time when ½BDM_{add} is reached. The fourth parameter b in the four-parameter equation is a shape parameter and difficult to explain biologically (Erkmen and Alben 2002). Derivation of the fitting was used for determination of, specific (μ_{fit}) and maximum ($\mu_{fit,max}$), growth rates.

HPLC-UV/vis quantification of rhamnolipids

Rhamnolipid standard solutions of 1 g/L in 0.1 M sodium buffer solution pH 6.5 were used for calibration. Phenacyl esters of rhamnolipids for HPLC analysis were obtained as described before by Schenk et al. (1995) with minor changes. The analysis was performed with a standard HPLC device (Agilent 1100 Series, Agilent, Waldbronn, Germany) equipped with a 15 cm reverse phase column (Supelcosil® LC-18, Supelco, Deisenhofen, Germany) at 30 °C. Mobile phase solutions were solution A with 5% methanol and solution B with 95% methanol in ultrapure water, respectively. To achieve separation, a gradient of solution B from 80% to 100% was used according to the following protocol: from t = 0 to t = 17 min increase of solution B from 80% to 100%. Holding 100% solution B up to t = 25 min and decrease to 80% solution B until t = 30 min. Holding 80% solution B for 5 min more to equilibrate. The flow rate was 0.4 mL/min and rhamnolipids were monitored at 254 nm. Retention times were (21.5 ± 0.1) min for Rha-Rha-C_{10}-C_{10} and (22.2 ± 0.1) min for Rha-C_{10}-C_{10}.

2.4 Results

2.4.1 Triplicate production of rhamnolipids in a 30-L bioreactor system

The cultivation of *P. aeruginosa* PAO1 in a 30 L bioreactor system was performed three times. Logistic growth of the bacterial culture was determined (Figure 2-2). A logistic equation with four parameters was used to fit the bacterial growth (BDM_{fit}, Equation 2–1). The maintained parameters were the following: BDM_{inoc} was 0.65 g/L; BDM_{add} was 15.63 g/L; time (½BDM_{add}) was 31.41 h and the shape factor *b* was -2.68. Thus, BDM_{max} was 16.28 g/L. After 24 h of cultivation rhamnolipid concentrations of 0.65 g/L were quantified. Rhamnolipid production continued up to 90 h of cultivation and reached a maximum of approximately 39 g/L. No further increase of the total rhamnolipid concentration (c_{RL}) was observed from 90 h of process time. After 40 - 45 h of cultivation, the mechanical foam control was activated automatically due to excessive foaming. Visually, the foam was uncongested at the beginning while getting more and more compact with progress of the cultivation, leading to a heterogeneously composed broth of a 35 L dense airy emulsion at the end of the process. The sunflower oil was not degraded completely by the microorganisms resulting in a resting concentration of about 50 g/L. Although BDM and rhamnolipid concentration were stable after 90 h, still a small decrease of sunflower oil was observed. From the BDM_{fit} results, the specific growth rate (μ_{fit}) lapse was determined. Figure 2-3 shows values for specific growth (μ_{calc}), based on the measured data, together with μ_{fit} and the specific rhamnolipid productivity (P_{RL}). The relatively high standard deviations derive from the error propagation of the BDM, *t*, and c_{RL} data of Figure 2-2. The maximum specific growth rate ($\mu_{fit,max}$) determined by the deviation was 0.133 h^{-1} at 11 h of cultivation. This reflects a minimum cell division (t_d) time of 5.21 h. The maximum rhamnolipid production was not growth related, what is in accordance to literature data and a typical behavior of secondary metabolites (Sobéron-Chávez et al. 2005).

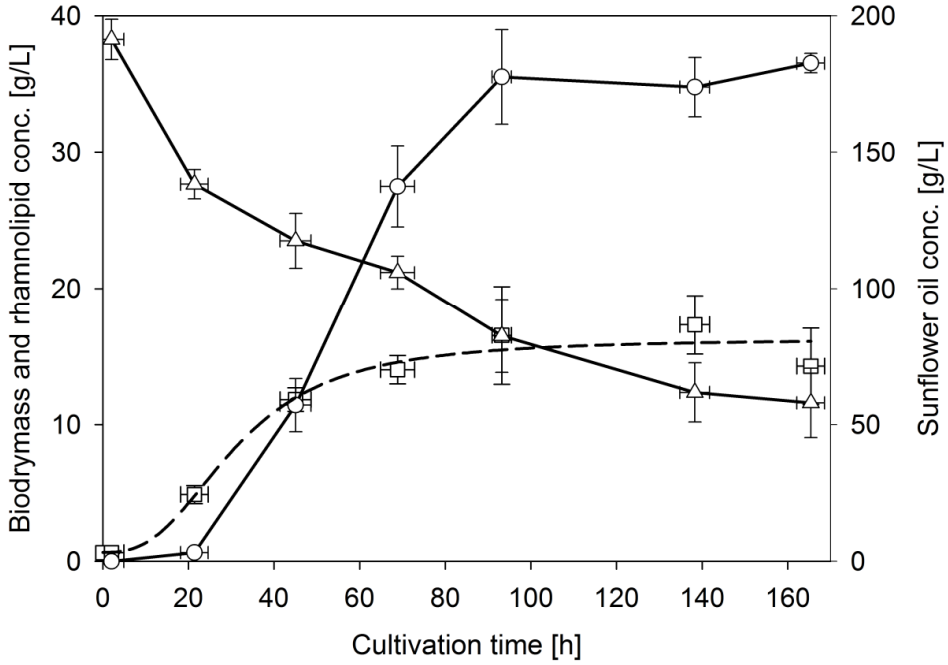

Figure 2-2. Production of rhamnolipids with *P. aeruginosa* PAO1 in a 30 L pilot plant bioreactor with sunflower oil as sole carbon source. Measured biodrymass (BDM, *square*), rhamnolipid (c_{RL}, *circle*) and sunflower oil (*triangle*) concentrations are given as averages of cultivation triplicates. The *dashed line* represents a logistic fitting of the biodrymass concentration (BDM_{fit}) based on Equation 2–1.

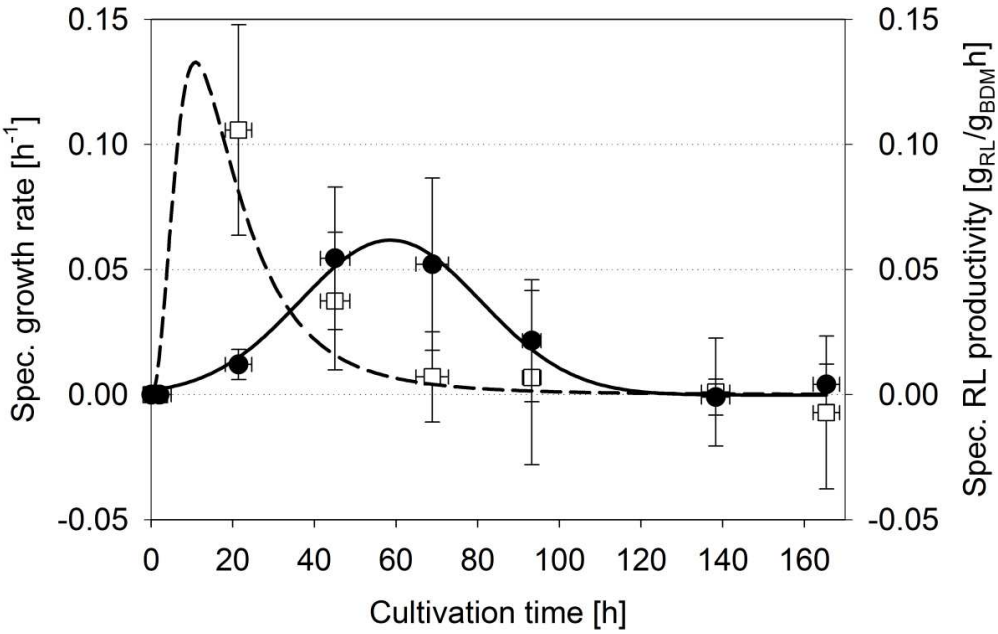

Figure 2-3. Calculated averaged specific growth rate (μ_{calc}, *blank square*) and rhamnolipid productivities (P_{RL}, *filled circle*) of a triplicate cultivation of *P. aeruginosa* PAO1 in a 30 L pilot plant bioreactor using sunflower oil as sole carbon source. *Dashed line* represents the μ_{fit} course derived from the logistic fitting in (Figure 2-2).

The maximum of the P_{RL} was calculated for between 40 - 70 h of cultivation (max. P_{RL}= 0.088 $g_{RL}/g_{BDM}h$) in the transition towards stationary phase. However, small quantities of rhamnolipid were already produced in the early growth phase. The production process was finished after 90 h and all below calculated rates are therefore related to this process length. A volumetric productivity (P_V) of 0.43 g/(L·h), which is comparable with the best-reported productivities (Table 2-1), was achieved. The overall specific productivity per final BDM was 2.4 g_{RL}/g_{BDM}[5], the product yield per consumed sunflower oil ($Y_{P/S}$) was 0.23 g_{RL}/g_{oil} and in total 8.17 g_{RL}/h were produced.

Table 2-1. Representative literature values for batch and fed-batch bioreactor cultivations of *P. aeruginosa* strains with vegetable oil as sole carbon source.

P. aeruginosa strain	Vegetable oil (g/L)	Process type (scale)	RL_{max}[a] (g/L)	Process time (h)	P_V[b] (g/(L·h))	Reference
DSM 2659	Corn oil (84)	Fed-batch (7.5 L)	34	107	0.52	Hembach 1994
DSM 2874	Rapeseed (198)	Fed-batch (2 L)	45	321	0.14	Trummler et al. 2003
DSM 7107	Soybean (125)	Batch (30 m^3)	78	166	0.47	Giani et al. 1997
DSM 7108	Soybean (163)	Batch (300 L)	112	267	0.42	Giani et al. 1997
DSM 7108	Soybean (163)	Fed-batch (18.5 m^3)	95	216	0.44	Giani et al. 1997
PAO1	**Sunflower (250)**	Batch (30 L)	**39**	**90**	**0.43**	**This study**
UI 29791	Corn oil (75)	Batch (14 L)	46	192	0.24	Linhardt et al. 1989

[a]Maximum rhamnolipid concentration
[b]Volumetric productivity

[5] the unit was changed from g/gh to g/g, due to incorrectness

2.4.2 Molar ratio of di-rhamnolipid (RL3) to mono-rhamnolipid (RL1)

Figure 2-4a shows the molar di-rhamnolipid (Rha-Rha-C_{10}-C_{10})/mono-rhamnolipid (Rha-C_{10}-C_{10}) ratio over the time course of the cultivations. While starting at about 1:1 the molar di- to mono-rhamnolipid ratio shifts towards 1.5:1 during the phase of maximum rhamnolipid productivity between 40 and 70 h of cultivation. In later stages, even a ratio close to 2:1 was observed. After 90 h of cultivation, the total rhamnolipid concentration stayed on a level of about 60 mM. Rha-C_{10}-C_{10} decreased about 3 – 4 mM, while Rha-Rha-C_{10}-C_{10} showed an increase of a similar amount (Figure 2-4b), respectively.

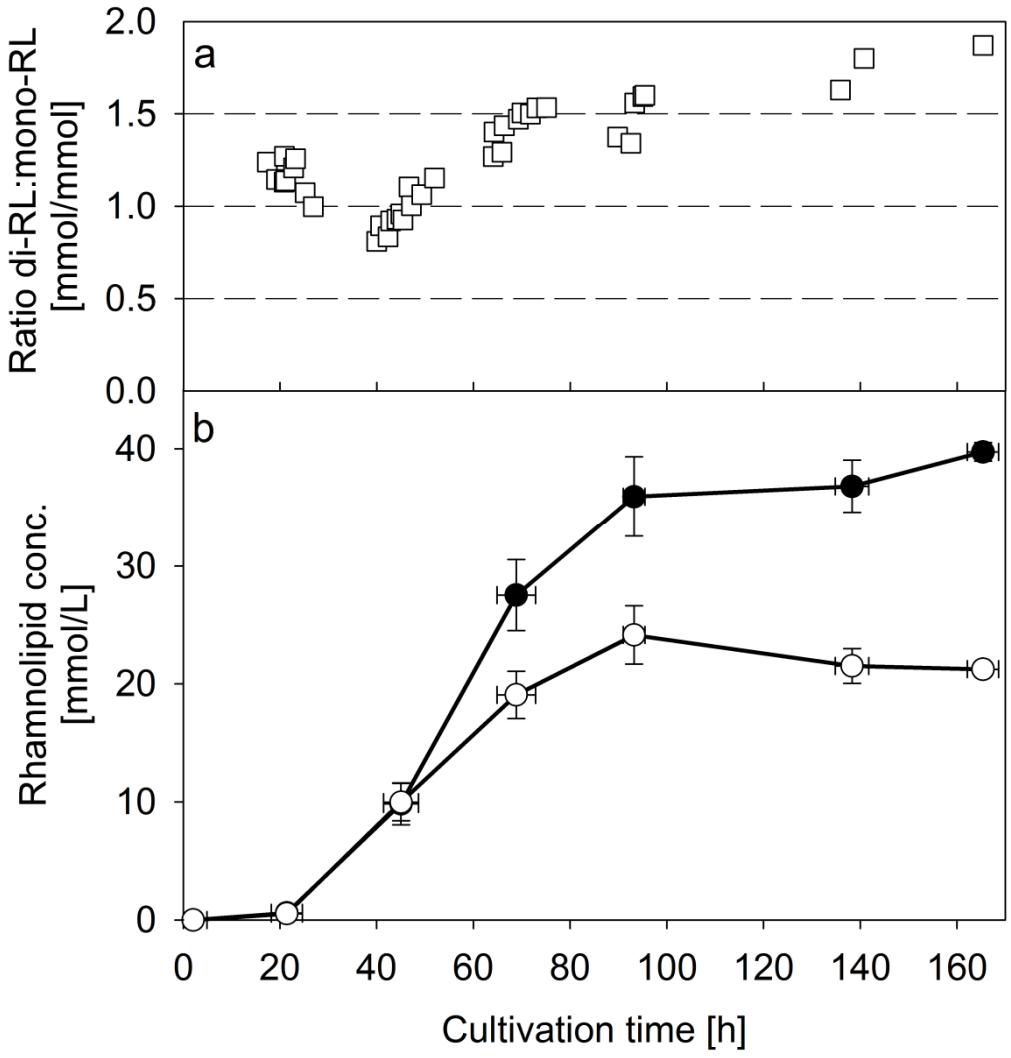

Figure 2-4. Investigation of the molar variation of mono-rhamnolipid (Rha-C_{10}-C_{10}) and di-rhamnolipid (Rha-Rha-C_{10}-C_{10}) derived from three 30 L bioreactor cultivations of *P. aeruginosa* PAO1 with sunflower oil as sole carbon source. **a** Lapse of molar ratios Rha-Rha-C_{10}-C_{10}: Rha-C_{10}-C_{10} (di-RL:mono-RL (*blank square*)). **b** Course of averaged rhamnolipid concentrations for Rha-C_{10}-C_{10} (*blank circle*) and Rha-Rha-C_{10}-C_{10} (*filled circle*).

2.4.3 Exhaust gas analysis

During the cultivations, oxygen and carbon dioxide partial pressures were analyzed in the exhaust gas and recorded by the process control system. Figure 2-5a shows the calculated oxygen uptake rate (OUR) and carbon-dioxide[6] evolution rate (CER) which increased during the logistic growth phase. Both showed a distinct peak within the time interval of maximum rhamnolipid production. The specific OUR and CER (Figure 2-5b) per BDM were calculated with the BDM_{fit} results from the logistic fitting described above (Figure 2-2) by dividing OUR and CER through BDM_{fit}, respectively. The specific rates showed a maximum in the growth phase but also allowed to distinguish a small peak during the max. P_{RL}. The respective CER/OUR ratios (respiratory quotient or RQ) allowed that four different phases of microbial metabolism could be distinguished (Figure 2-5c). The relatively high standard deviations are due to error propagation of averaged exhaust gas data. Higher RQs of about 0.66 - 0.76 were observed at the growth phase (I), the second phase (II) was characterized by lower RQs of 0.6. The third phase (III) showed RQs of between 0.63 and 0.66 decreasing to a phase (IV) with average RQs of 0.62.

[6] „carbon“ was changed to „carbon-dioxide“

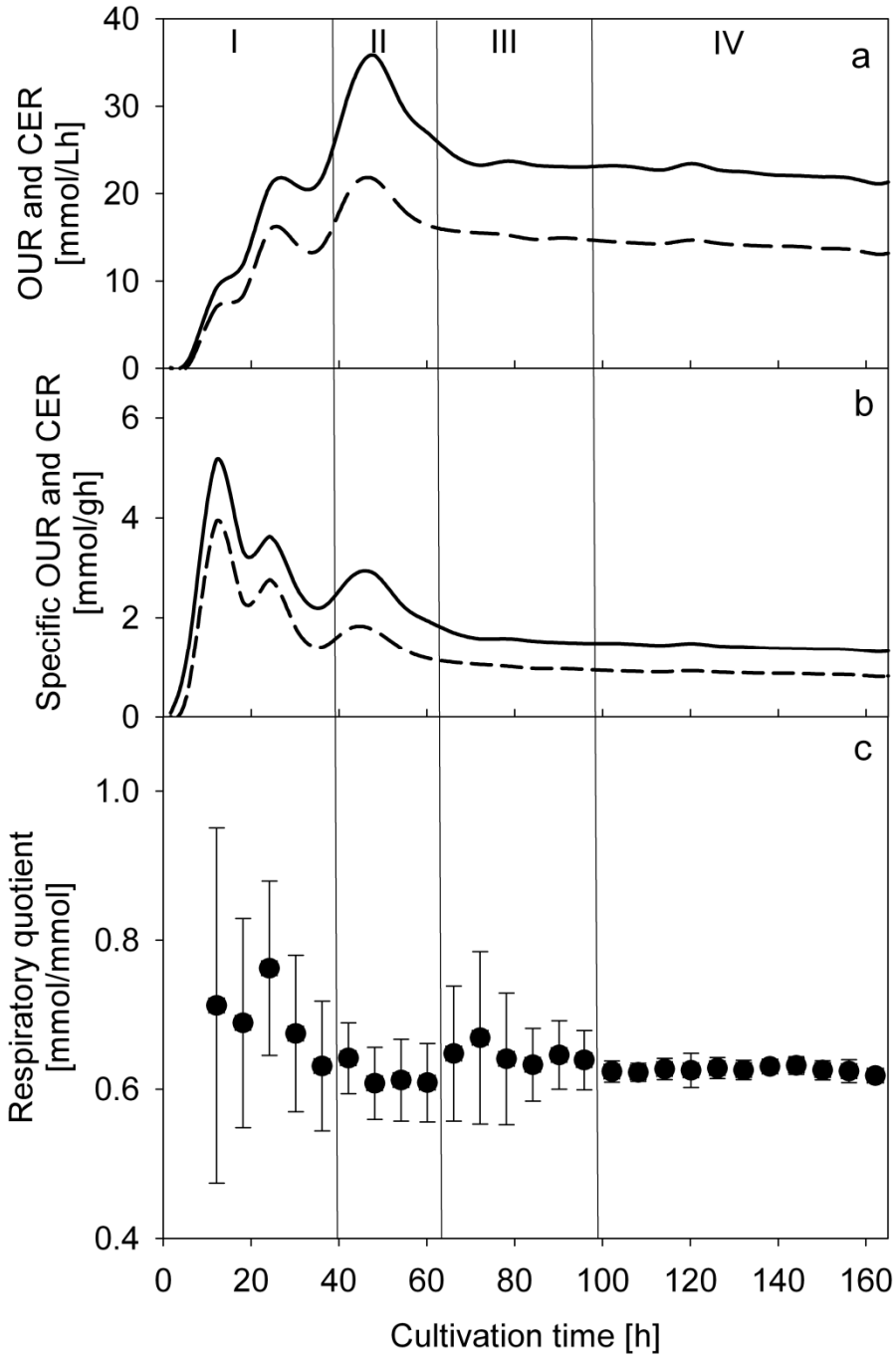

Figure 2-5. Results of exhaust gas analysis for the cultivation of *P. aeruginosa* PAO1 with sunflower oil as the sole carbon source. **a** OUR (*straight*) and CER (*dashed*) courses; **b** BDM related spec. OUR (*straight*) and spec. CER (*dashed*) courses; **c** respective respiratory quotients (RQ= CER/OUR). Indicated phases are: I- maximum growth, II- maximum P_{RL}, III- end of growth and decrease of rhamnolipid production, IV- stationary phase.

2.5 Discussion

It was shown that PAO1 has the potential to produce rhamnolipids in high enough quantities to match with well-established production strains like DSM 2874 (Syldatk et al. 1985a+b; Matulovic 1987; Trummler et al. 2003), DSM 7107 and DSM 7108 (Giani et al. 1997). Inquiries with PAO1 aiming to overproduce rhamnolipids were mostly restricted to shake flask (Wang et al. 2007) or small bioreactor scale, and did not surpass 2.2 g/L (Ochsner et al. 1994; Ochsner et al. 1995; Ochsner and Reiser 1995; Rahim et al. 2001). Although intensive research was done on QS regulation (Dockery and Leener 2001; Chen et al. 2004; Wagner et al. 2006; Williams and Camara 2009), the rhamnolipid production was studied and optimized with mostly heuristic approaches during the last decades. In comparison to shake flask experiments, the rhamnolipid concentrations in bioreactor experiments are up to 100-fold higher. A main finding under the investigated experimental set-up was that *P. aeruginosa* PAO1 produces high quantities of rhamnolipids in batch culture of up to 39 g/L with a P_V of 0.43 g/(L·h). Additionally, the OUR and the CER could directly be associated with P_{RL}, both showing peaks between 40 and 60 h of process time. Thus exhaust gas analysis will allow online observation and could support future investigations and development of rhamnolipid production. The complete sequenced and annotated genome of PAO1 (Stover et al. 2000) gives the possibility to use the up-to-date systems biotechnological tools like microarray, mass spectrometric, and *in-silico* modeling techniques. Basic research groups working in the field of QS regulation have already established many of these technologies for PAO1 (Wagner et al. 2003; Nouwens et al. 2003; Wagner et al. 2004). During PAO1 experiments, it was possible to distinguish different process stages (Figure 2-5) by exhaust gas analysis, which now could be investigated by the help of process analytical technologies like transcriptome, proteome, and metabolome analysis. Comparative experiments of PAO1 wild-type and already available mutant strains should lead to new insights about rhamnolipid production in bioreactor cultivations. Among the main issues must be the regulation of rhamnolipid production, oil degradation, lipase activity, assimilation of substrate, and rhamnolipid feed-back, which all leads to a characteristic, nearly Gaussian distribution, of the specific productivity in the pilot plant bioreactor system (Figure 2-3). The analysis of the single rhamnolipids by HPLC instead of the commonly used total or indirect quantification (Chandrasekaran

and BeMiller 1980; Pinzon and Ju 2009) lead to important results like the transition from mono- to di-rhamnolipids (Figure 2-4a), the difference in final mono- and di-rhamnolipid concentrations and the potential extracellular conversion of Rha-C_{10}-C_{10} to Rha-Rha-C_{10}-C_{10} (Figure 2-4b). These findings indicate a more complex rhamnolipid mechanism of regulation then thought before (Sullivan 1998). The importance of sigma and other transcriptional factors has already been discussed (Rahim et al. 2001; Medina et al. 2003; Potvin et al. 2008) for rhamnolipid regulation. However, their characteristic devolution during batch cultivation should reveal important information about the regulation on genetic level.

After 40 h of cultivation mostly di-rhamnolipids were synthesized, leading to nearly four times more L-rhamnose incorporated in di-rhamnolipids than in mono-rhamnolipids (1 mol di-rhamnolipid equals 2 mol of L-rhamnose). There was still enough L-rhamnose for this step of synthesis catalyzed by RhlC (Rahim et al. 2001), or rhamnosyltransferase II, while the production pathway towards Rha-C_{10}-C_{10} must have been limited or suppressed somewhere. Additional metabolome analyzes could show whether there is a limitation in the respective precursor pools for rhamnolipid production. Such information on genomic and metabolomic level should give the possibility to integrate the findings in a systems bio(techno)logic network (Wagner et al. 2004; Wang et al. 2006).

The present results suggest PAO1 being used as a model organism also for the biotechnological production of rhamnolipids. Advantages would be an enhancement of the comparability of experimental results throughout different laboratories and the possibility of closer cooperation between engineering and basic research groups. New insights will be applicable to the established high production strains like DSM 7107, DSM 7108 and DSM 2874. After all, it has to be mentioned that the established batch cultivation was not optimized, thus leaving space for higher productivities and yields with PAO1 in future studies. PAO1 not only seems to be an appropriate model, but surprisingly has the potential as a strain of choice for actual biotechnological rhamnolipid production. With further research and application of culture and process development strategies, and additional incorporation of exhaust gas analysis into an advanced process control, economic rhamnolipid production should be in not-so-distant future.

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3 Evaluation of rhamnolipid production capacity of *Pseudomonas aeruginosa* PAO1 in comparison to the rhamnolipid over-producer strains DSM 7108 and DSM 2874

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3.1 Abstract

A lack of understanding of the quantitative rhamnolipid production regulation in bioreactor cultivations of *Pseudomonas aeruginosa* and the absence of respective comparative studies are important reasons for achieving insufficient productivities for an economic production of these biosurfactants. The *Pseudomonas* strains DSM 7108 and DSM 2874 are described to be good rhamnolipid over-producers. The strain PAO1 on the other hand is the best analyzed type strain for genetic regulation mechanisms in the species *P. aeruginosa*. These three strains were cultivated in a 30-L bioreactor with a medium containing nitrate and sunflower oil as sole C-source at 30 and 37 °C. The achieved maximum rhamnolipid concentrations varied from 7 to 38 g/L, the volumetric productivities from 0.16 to 0.43 g/(L·h) and the cellular yield from 0.67 – 3.15 g/g, with PAO1 showing the highest results for all of these variables. The molar di- to mono-rhamnolipid ratio changed during the cultivations; was strain dependent but not significantly influenced by the temperature. This study explicitly shows that the specific rhamnolipid synthesis rate per cell follows secondary metabolite-like courses coinciding with the transition to the stationary phase of typical logistic growth behaviour. However, the rhamnolipid synthesis was already induced before N-limitation occurred.

3.2 Introduction

Jarvis and Johnson (1949) observed that *Pseudomonas* strains can produce anionic glycolipids containing two units each of L-rhamnose and β-hydroxyalkanoic acid. Today, glycolipids of this composition are generally termed rhamnolipids. Depending on the number of L-rhamnose moieties, a distinction is drawn between mono- and di-rhamnolipids. Mainly *Pseudomonas* and *Burkholderia* strains were described to produce rhamnolipids (Jarvis and Johnson 1949; Pajarron et al. 1993; Häußler et al. 1998; Dubeau et al. 2009; Hörmann et al. 2010), but high quantities for production purposes were only described for *Pseudomonas* spp. (Linhardt et al. 1989; Hembach 1994; Giani et al. 1997; Trummler et al. 2003; Nitschke et al. 2005; Müller et al. 2010), two of the most outstanding strains being DSM 7108 and DSM 2874. About 60 rhamnolipid congeners were described (Déziel et al. 2000; Abdel-Mawgoud et al. 2010). The most frequently produced mono- and di-rhamnolipid congeners are α-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-C_{10}-C_{10}) and

α-L-rhamnopyranosyl-(1-2)-α-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxy-decanoate (Rha_2-C_{10}-C_{10}), respectively.

Rhamnolipid biosurfactants are alternatives to the commonly used synthetic surfactants. However, obstacles for economic rhamnolipid production, like relatively low productivity, comparatively expensive raw materials, and relative high costs for downstream processing, prevented them from being applied in bulk applications. Nevertheless, they are non-polluting while showing comparable physico-chemical properties to synthetic surfactants (Syldatk et al. 1985b) and can be produced by using renewable or waste substrates (Giani et al. 1997; Rahman et al. 2002; Wei et al. 2005; Marsudi et al. 2008; Müller et al. 2010). Rhamnolipid production of *Pseudomonas aeruginosa* is *de novo* (Hauser and Karnovsky 1957; 1958; Burger et al. 1963), regulated by the *las/rhl*-quorum-sensing (QS) network (Ochsner and Reiser 1995; Williams and Camara 2009) and enhanced under limitation of media components like nitrogen, phosphate, and trace elements (Guerra-Santos et al. 1984; Mulligan et al. 1989; Manresa et al. 1991). However, high concentrations were mainly reported under N-limiting conditions and when NO_3^- is applied as the N-source in combination with water-immiscible C-sources (Giani et al. 1997; Trummler et al. 2003; Leitermann 2008; Müller et al. 2010). The comparability of such reports is confusing due to the use of different *Pseudomonas* strains, cultivation conditions, (pH, T, scale, etc.) and media compositions. The intention of this study was to fill this gap with a comparative study of the rhamnolipid over-producing *Pseudomonas* strains DSM 7108 and DSM 2874, with PAO1 in a 30-L bioreactor system under N-limiting conditions with sunflower oil as sole C-source and two different cultivation temperatures. The former Hoechst AG rhamnolipid production strain *P. aeruginosa* DSM 7108 (Giani et al. 1997) was claimed to produce over 100 g/L under similar conditions as tested. *Pseudomonas* sp. DSM 2874 was reported to produce over 45 g/L of rhamnolipids under resting cell conditions (Syldatk et al. 1985a; Syldatk et al. 1985b; Trummler et al. 2003), and *P. aeruginosa* PAO1 (DSM 22644) is a sequenced type strain (Hancock and Carey 1979; Stover et al. 2000) for QS research and a promising rhamnolipid production model (Müller et al. 2010). A matter of particular interest was the specific productivity per cell ($q_{RL,spec}$) and the di- to mono-rhamnolipid ratio. Former studies mainly focused to investigate

volumetric or overall yields, but with respect to future fed-batch process development, $q_{RL,spec}$ is the more appropriate parameter.

3.3 Materials and methods

3.3.1 Chemicals

The di-rhamnolipid standard (Rha_2-C_{10}-C_{10}) for HPLC analysis was a gift from former Hoechst AG (Hoechst, Germany). Mono-rhamnolipid standard (Rha-C_{10}-C_{10}) was prepared as described before (Trummler et al. 2003). Triethylamine and 4-bromophenacylbromide were used (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) for derivatization at the carboxylic acid group of the rhamnolipids (Schenk et al. 1995). Sunflower oil (Bellasan®, Aldi Süd, Rastatt, Germany) was of food grade. Spectroquant® nitrate test was from Merck KGaA (Darmstadt, Germany). Other chemicals were from Carl Roth GmbH (Karlsruhe, Germany). All chemicals were of analytical grade.

3.3.2 Microorganism

The *Pseudomonas* strains *P. aeruginosa* DSM 7108, *Pseudomonas* sp. DSM 2874, and *P. aeruginosa* PAO1 (DSM 22644) were used for the experiments described below. Glycerol stocks of each strain were prepared of cultures in lysogeny broth (Bertani 1951) from the exponential growth phase and stored at -80 °C until needed. All strains are available at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

3.3.3 Culture methods

Media

For the first pre-cultivation step LB with 10 g/L NaCl was used. Consecutively, seed cultures were prepared in an N-limited medium consisting of 125 g/L sunflower oil and a Ca-free mineral salt solution with 1.5 g/L $NaNO_3$, 0.05 g/L $MgSO_4$ x $7H_2O$, 0.1 g/L KCl, containing a 0.1 M sodium phosphate buffer at pH 6.5. A total of 1 mL/L of trace element solution was added. The trace element solution contained 2.0 g/L sodium citrate x $2H_2O$, 0.28 g/L $FeCl_3$ x $6H_2O$, 1.4 g/L $ZnSO_4$ x $7H_2O$, 1.2 g/L $CoCl_2$ x $6H_2O$, 1.2 g/L $CuSO_4$ x $5H_2O$ and 0.8 g/L $MnSO_4$ x H_2O and was filter-sterilized with a 0.22-µm membrane filter (Carl Roth GmbH, Karlsruhe, Germany) before usage. The medium utilized for the production experiments was N-limited and,

apart from the phosphate buffer, adopted from Giani et al. (1997). This production medium consisted of a Ca-free mineral salt solution with 15.0 g/L $NaNO_3$, 0.5 g/L $MgSO_4$ x $7H_2O$, 1.0 g/L KCl, and 0.3 g/L K_2HPO_4 as a phosphate source. Sunflower oil was used as sole C-source at 250 g/L. The pH value was adjusted to 6.5 and controlled during cultivation using 4 M NaOH or 4 M H_3PO_4, respectively.

Preparation of seed culture

Incubation of all shake flasks was performed in a shake incubator chamber (Multitron II, HT Infors, Bottmingen, Switzerland). First, a total volume of 100 µL glycerol stock solution of each strain was used to inoculate 25 mL of LB in 100-mL baffled shake flasks, respectively. These cultures were incubated at 37 °C, 120 rpm. After 24 h a total volume of 5 mL was transferred to 1-L baffled shake flasks containing 200 mL seed medium and incubated for at least 24 h, at 37 °C, 120 rpm. For inoculation with a theoretical OD_{580} of 0.06, appropriate volumes of the seed cultures were used.

Production of rhamnolipids in 30-L bioreactor scale at 30 and 37 °C

For all cultivations, a 42-L stirred tank bioreactor, with a nominal capacity of 30 L, was used (Biostat® Cplus, Sartorius Stedim Biotech, Göttingen, Germany). The bioreactor was equipped with an integrated process control system for temperature, pH, pO_2, airflow, and foam level regulation (MFCS/win 2.1, Sartorius BBI, Göttingen, Germany). A foam separator was installed in the headspace of the bioreactor to control the foaming. The cultivations were performed with constant stirrer speed (400 rpm), temperature (30 or 37 °C, respectively), and minimal dissolved oxygen (pO_2) of 5%, which was controlled by variation of the aeration rate between 0.1 L/(L·min) and 3.3 L/(L·min). For the cultivation, 15 L of the aqueous production medium was used. After 0, 20, 40, 70, and 120 h of cultivation time, 1 mL/L of the abovementioned trace element solution was added. In total, 14 batch cultivations were performed. Each strain was cultivated at least twice at 30 and at 37 °C, respectively. *P. aeruginosa* PAO1 (37 °C) was cultivated once and combined with previously published data from a triplicate cultivation (Müller et al. 2010).

3.3.4 Analytical methods

Sampling and processing

Samples were taken routinely for offline analysis of biodrymass (BDM), sunflower oil, mono-/di-rhamnolipid, and nitrate concentrations. Therefore, the culture suspension was mixed vigorously with *n*-hexane 1:1 (*v/v*) and centrifuged (4,618xg, 4 °C, 30 min) for separation of biomass, aqueous, and organic solvent phase. The organic solvent phase was used for gravimetric estimation of sunflower oil concentrations, after evaporation of *n*-hexane. It was confirmed by HPLC (cf. below) that no rhamnolipids were extracted by the *n*-hexane extraction step. The cell pellet was washed once in 0.9% NaCl (*w/v*) solution (4,618xg, 4 °C, 30 min), dried till constant weight, and BDM was quantified gravimetrically. Nitrate analysis was performed in the aqueous phase according to assay instructions analogous to ISO 7890/1. Rhamnolipids were extracted twice with ethyl acetate 1:1.25 (*v/v*) from the aqueous phase after adjusting the pH to about 2 – 3 with 85% phosphoric acid 1:100 (*v/v*). Rhamnolipid quantification by HPLC was done as described before (Trummler et al. 2003; Hörmann et al. 2010; Müller et al. 2010).

3.3.5 Data analysis

A logistic equation with four parameters was used to fit the data for the bacterial growth under limiting conditions (Zwietering et al. 1990). A logistic model for biomass growth was shown to be satisfactory under rhamnolipid producing circumstances (Ramana et al. 1991; Babu et al. 1996). For this purpose, a scientific data analysis and graphing software (Sigma Plot 9.0, Systat, San Jose, USA) was used. The applied equation was

$$BDM\ (\mathrm{t}) = BDM_0 + \frac{BDM_{max,add}}{1+\left(\frac{t}{t_0}\right)^b} \quad \text{(Equation 3–1)}$$

where BDM_0 indicates the BDM concentration after inoculation, $BDM_{max,add}$ indicates the maximum additional achieved BDM concentration (BDM_{max} = $BDM_{max,add}$ + BDM_0), t_0 indicates the process time when one half of $BDM_{max,add}$ is reached. The shape parameter *b* is difficult to explain biologically (Erkmen and Alben 2002). The fitting quality was improved by including the theoretical produced BDM, which was calculated by the means of $Y_{N/BDM}$ (cf. Table 3-1) and the consumed nitrogen

concentrations, into the data points for the gravimetrically determined BDM. Derivation of the fitting was used for determination of specific (μ) and maximum (μ_{max}) growth rates. Rhamnolipid concentration lapse and specific productivity per BDM ($q_{RL,spec}$) were derived with an analogous procedure applying a logistic equation with three parameters (cf. Equation 3–1 , but excluding a value for RL_0), respectively.

3.4 Results

3.4.1 Rhamnolipid production at 30 and 37 °C in a 30-L bioreactor

The following results represent the average data for the respective replicates. All cultures showed characteristic logistic growth like described under rhamnolipid production conditions (Ramana et al. 1991; Babu et al. 1996) and were fitted according to Equation 3–1. The root squares for the growth curve fittings were ≥0.98, which was sufficient to calculate the lapse of μ (see below). The comparison of the *Pseudomonas* strains, but also of the two different temperatures, revealed a high variability of the rhamnolipid production (cf. Table 3-1). In the cultivations with *P. aeruginosa* DSM 7108 (30 °C), *Pseudomonas* sp. DSM 2874 (30 and 37 °C), and *P. aeruginosa* PAO1 (37 °C), the N-source was completely consumed during the time course of the cultivation and BDM_{max} was about 17 g/L. However, with *P. aeruginosa* DSM 7108 (37 °C) and *P. aeruginosa* PAO1 (30 °C), the N-source was not entirely consumed and the biomass reached values of about 10 g/L. Figure 3-1 compares two exemplary cultivations for each of these two outcomes. However, the biomass yields with regards to the consumed nitrogen ($Y_{N/BDM}$) showed similar results for all cultivations (cf. Table 3-1) varying from 0.14 to 0.18 g/g. Analogously, the rhamnolipid concentration lapse (mono-/di-rhamnolipid) was fitted with a three-parameter version of Equation 3–1. The root squares for the total rhamnolipid concentration lapse were ≥0.96 for all cultivations and sufficient for the calculation of the lapse of $q_{RL,spec}$ (see below). Maximum rhamnolipid concentrations (RL_{max}) ranged from 7 g/L for *P. aeruginosa* DSM 7108 (37 °C) up to 38 g/L for *P. aeruginosa* PAO1 (37 °C) and showed temperature and strain dependency (cf. Table 3-1). The highest volumetric productivity (P_V) was achieved by *P. aeruginosa* PAO1 (37 °C) with 0.43 g/(L·h) and was also reported before (Müller et al. 2010). The lowest P_V was observed for *P. aeruginosa* DSM 7108 (37 °C) and *Pseudomonas* sp. DSM 2874 (30 °C) with 0.16 g/(L·h), respectively. The cultivations with *P. aeruginosa* DSM 7108

(37 °C) and *Pseudomonas* sp. DSM 2874 (30 °C) also resulted in the lowest rhamnolipid yields per BDM ($Y_{RL/BDM}$) of 0.75 and 0.86 g/g, respectively. The highest $Y_{RL/BDM}$ were reached by *P. aeruginosa* PAO1 (30 and 37 °C) with up to about 3 g/g. The rhamnolipid production period was finished between 45 h for *P. aeruginosa* DSM 7108 (37 °C) and 140 h for *P. aeruginosa* DSM 7108 (30 °C), respectively.

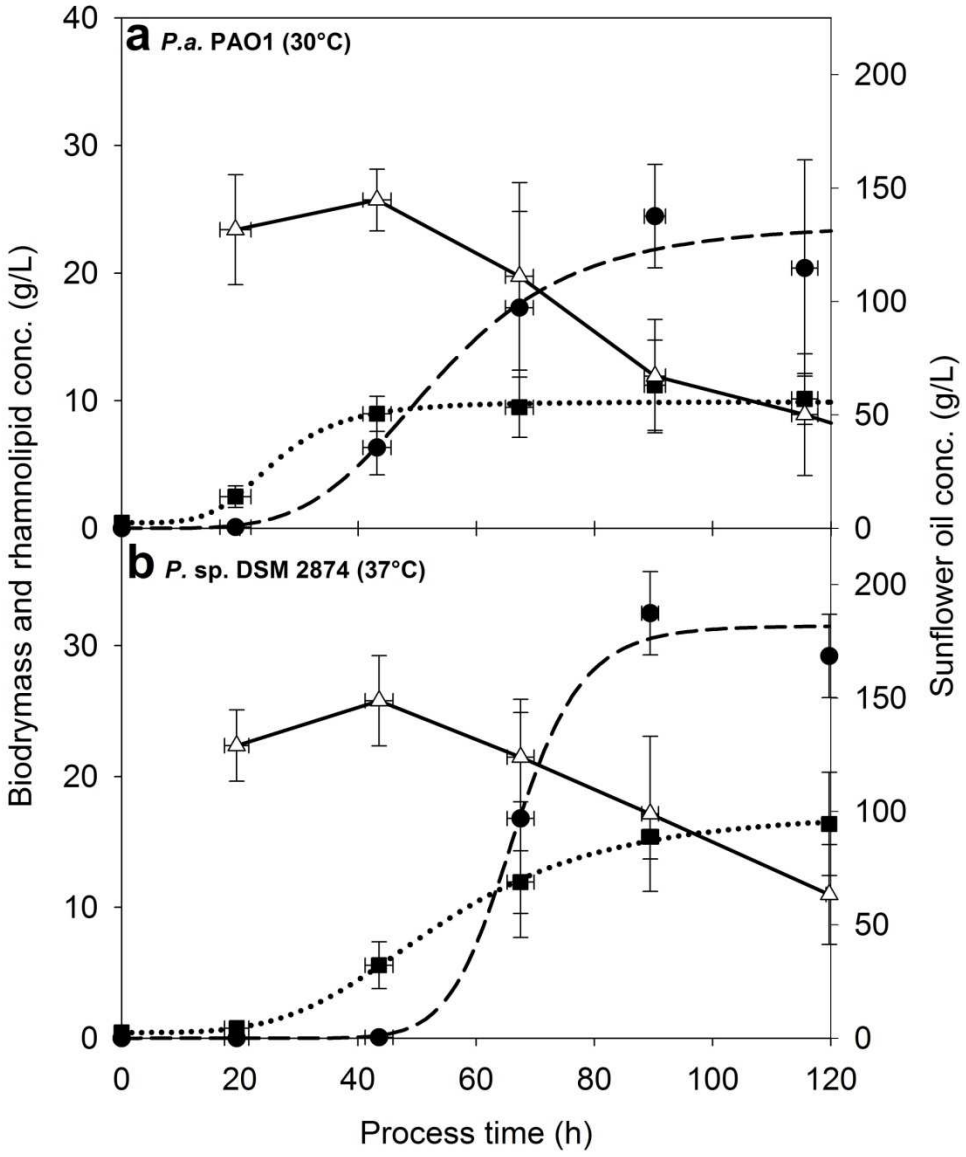

Figure 3-1. a, b Exemplary production of rhamnolipids in a 30-L pilot plant bioreactor with sunflower oil as sole C-source and N-limiting conditions. BDM (*filled square*) and rhamnolipid (*filled circle*) concentrations, and the concentration of the hexane extractable portion of sunflower oil (*blank triangle*) are given as averages of cultivation replicates. *Dotted line* represents the logistic fitting of the BDM, and *dashed line* represents the rhamnolipid concentration based on the four- and three-parameter version of Equation 3–1, respectively.

Table 3-1. Summarized results for rhamnolipid production of the *Pseudomonas* strains in a 30-L pilot plant bioreactor with sunflower oil as sole C-source and N-limiting conditions

Strain	T (°C)	BDM_{max} (g/L)[a]	μ_{max} (h^{-1})[a]	RL_{max} (g/L)[a]	Max $q_{RL,spec}$ (g/g·h)[a]	Avrg. $q_{RL,spec}$ (g/g·h)[a]	$t_{RL,max}$ (h)	P_V (g/L·h)[b]	$Y_{RL/BDM}$ (g/g)	$Y_{N/BDM}$ (g/g)
P. a. PAO1	30	10.1 ± 1.6	0.17	25.8 ± 2.0	0.06	0.03	110	0.23	2.55 ± 0.60	0.17 ± 0.02
	37[c]	16.5 ± 2.6	0.13	36.7 ± 2.2	0.08	0.04	85	0.43	2.22 ± 0.42	0.16 ± 0.03
P. a. DSM 7108	30	16.0 ± 1.1	0.11	35.7 ± 2.0	0.07	0.03	140	0.26	2.23 ± 0.28	0.16 ± 0.01
	37	9.7 ± 0.3	0.18	7.3 ± 0.6	0.19	0.02	45	0.16	0.75 ± 0.08	0.18 ± 0.02
P. sp. DSM 2874	30	16.6 ± 2.4	0.10	14.2 ± 0.2	0.06	0.01	90	0.16	0.86 ± 0.14	0.16 ± 0.02
	37	17.5 ± 0.8	0.09	30.8 ± 1.5	0.12	0.03	100	0.35	1.76 ± 0.17	0.14 ± 0.01

μ_{max} maximum specific growth rate, RL_{max} maximum rhamnolipid concentration, $q_{RL,spec}$ specific productivity per cell, $t_{RL,max}$ process time when RL_{max} was reached, P_V volumetric productivity, $Y_{RL/BDM}$ rhamnolipid produced per BDM, $Y_{N/BDM}$ nitrogen used per BDM
[a]Values derived from logistic fitting
[b]Volumetric productivity related to end of production ($t_{RL,max}$)
[c]Combined with Müller et al. 2010

3.4.2 Specific growth and specific rhamnolipid productivity

The courses of μ and $q_{RL,spec}$ were derived from the fittings according to Equation 3–1 of the BDM and rhamnolipid concentration lapses (cf. Figure 3-1). Figure 3-2a-f shows the resulting courses of μ and $q_{RL,spec}$ together with the respective nitrogen concentration lapse for each of the cultivation set-ups. The cultivations of *P. aeruginosa* DSM 7108 (37 °C) and *P. aeruginosa* PAO1 (30 °C) resulted into the highest μ_{max} of 0.17 h^{-1} and 0.18 h^{-1}, respectively, and did not entirely consume the N-source. For the other cultivations, μ_{max} varied from 0.09 to 0.13 h^{-1} and the N-source was totally consumed. The *P. aeruginosa* strains PAO1 and DSM 7108 showed growth variation with the different temperatures, while for *Pseudomonas* sp. DSM 2874, no significant difference was observed. The maximum $q_{RL,spec}$ was between 0.06 $g_{RL}/(g_{BDM}·h)$ for *P. aeruginosa* PAO1 (30 °C) as well as for *Pseudomonas* sp. DSM 2874 (30 °C) and 0.19 $g_{RL}/(g_{BDM}·h)$ for *P. aeruginosa* DSM 7108 (37 °C). The average $q_{RL,spec}$ varied between 0.01 $g_{RL}/(g_{BDM}·h)$ and 0.04 $g_{RL}/(g_{BDM}·h)$ for the respective rhamnolipid production periods.

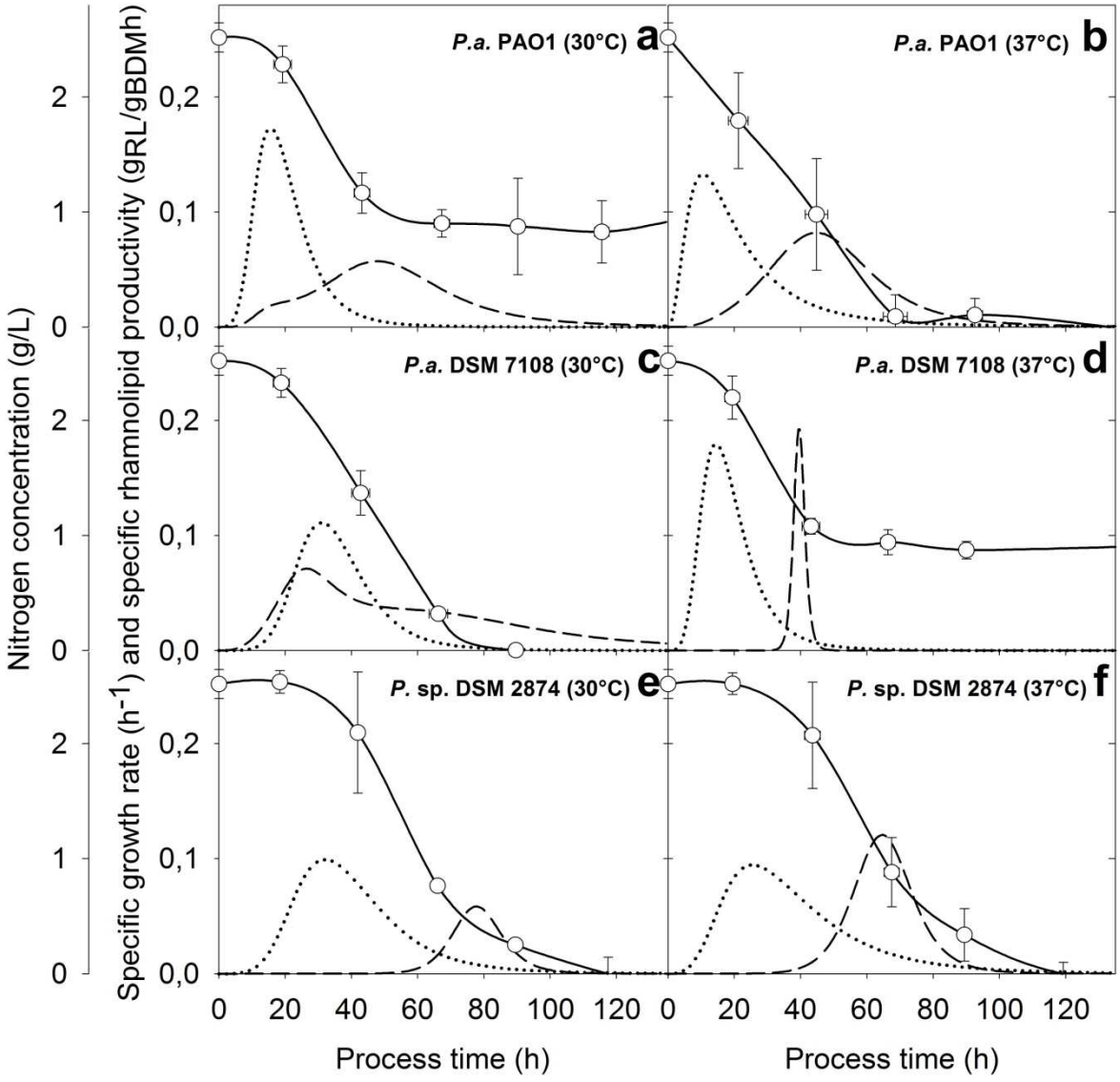

Figure 3-2. a–f Course of nitrogen concentration (*blank circle*) during rhamnolipid production of the *Pseudomonas* strains at two different temperatures in a 30-L pilot plant bioreactor with sunflower oil as sole C-source and N-limiting conditions. *Dotted line* represents the specific growth rate (μ) and *dashed line* represents the specific rhamnolipid productivity ($q_{RL,spec}$) deviated from the logistic fitting according to Equation 3–1 (cf. Figure 3-1).

Altogether, the lapse of $q_{RL,spec}$ can be roughly divided into two types, one showing a relatively long production phase from 75 to 100 h (cf Figure 3-2a–c) like the example of *P. aeruginosa* PAO1 (30 °C) in Figure 3-1a. The second type is characterized by a shorter, more stringent production period (cf. Figure 3-2d–f) of about 20 to 60 h as shown for *Pseudomonas* sp. DSM 2874 (37 °C) in Figure 3-1 b. Apart from *P. aeruginosa* DSM 7108 (30 °C), the cultivations showed their max $q_{RL,spec}$ in the transition from growth to the stationary phase. The $q_{RL,spec}$ typically increased and

decreased in a secondary metabolite-like course manner. This was also characterized by the transition from nitrogen usage to depletion or stationary concentrations. In all cultivations, rhamnolipids were already synthesized before nitrogen was depleted and still present in excess (>1 g/L). In contrast to the cultivations represented in Figure 3-2a, b, d-f, *P. aeruginosa* DSM 7108 (30 °C) showed max $q_{RL,spec}$ shortly before μ_{max} was reached (cf. Figure 3-2 c), which does not reflect typical secondary metabolite-like behavior. Additionally, *P. aeruginosa* DSM 7108 (30 °C) showed the longest overall production period of >140 h.

3.4.3 Molar ratio of di- to mono-rhamnolipid

Figure 3-3 shows the molar ratio of di-rhamnolipid (Rha_2-C_{10}-C_{10}) to mono-rhamnolipid (Rha-C_{10}-C_{10}) over the devolution of the cultivations. The *P. aeruginosa* strains PAO1 and DSM 7108 showed similar time courses of the molar ratio resulting in twice as much di-rhamnolipid than mono-rhamnolipid after a cultivation time of 150 h. Although the temperature showed influence on the total rhamnolipid concentrations (cf. Table 3-1), the lapse of the molar ratio was quasi superimposed for 30 and 37 °C. For *Pseudomonas* sp. DSM 2874, some temperature-dependent variance was especially observed for the relatively low concentrations at the beginning of the rhamnolipid production periods. Nevertheless, while starting with a molar ratio of di-rhamnolipid (Rha_2-C_{10}-C_{10}) to mono-rhamnolipid (Rha-C_{10}-C_{10}) of 1:2, finally a ratio of 1:1 was reached.

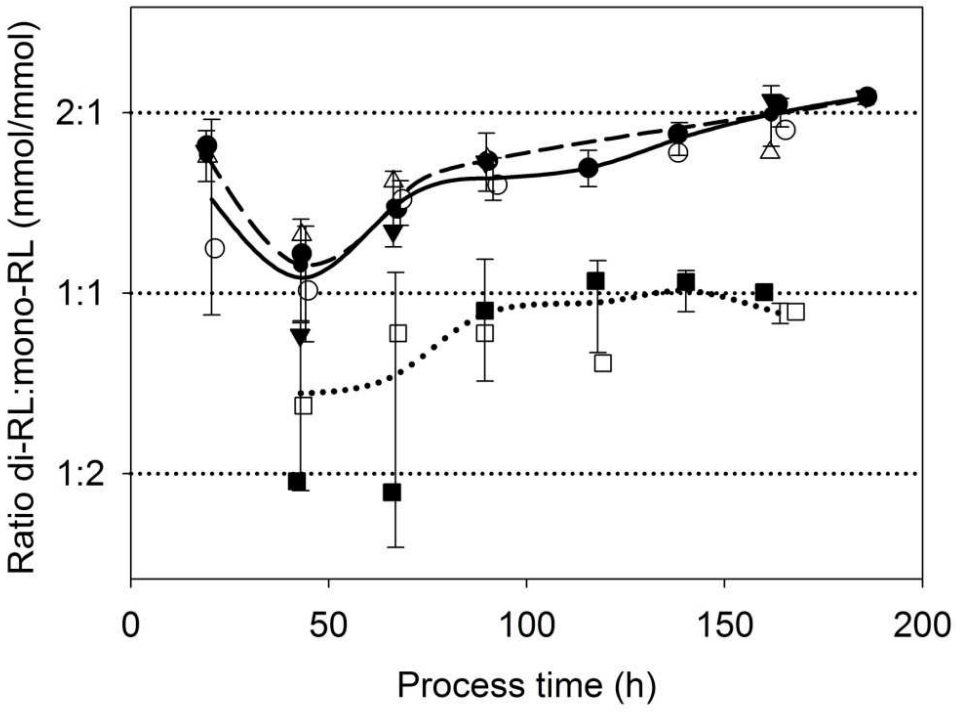

Figure 3-3. Investigation of the molar devolution of di-rhamnolipid (Rha_2-C_{10}-C_{10}) to mono-rhamnolipid (Rha-C_{10}-C_{10}) during rhamnolipid production of the *Pseudomonas* strains at two different temperatures in a 30-L pilot plant bioreactor with sunflower oil as sole C-source and N-limiting conditions. Lapse of molar ratio of Rha_2-C_{10}-C_{10} to Rha-C_{10}-C_{10} (di-RL to mono-RL) from *P. aeruginosa* PAO1 (*circle*), *P. aeruginosa* DSM 7108 (*triangle*), and *Pseudomonas* sp. DSM 2874 (*square*) at temperatures of 30 °C (*blank*) and 37 °C (*filled*), respectively. *Straight line* represents the temperature-independent average lapse (mean values for cultivations at 30 and 37 °C together) of the di-RL to mono-RL ratio for *P. aeruginosa* PAO1, *dashed line* for *P. aeruginosa* DSM 7108, and *dotted line* for *Pseudomonas* sp. DSM 2874.

3.5 Discussion

It was shown, that under the applied experimental set-up in a 30-L bioreactor, with sunflower oil as sole C-source and N-limiting medium conditions, *P. aeruginosa* PAO1 resulted in higher yields than the over-producer strains DSM 7108 and DSM 2874 (cf. Table 3-1). Thus, *P. aeruginosa* PAO1 has the potential to produce rhamnolipids in high enough quantities to match with well-established production strains. However, although a medium similar to Giani et al. (1997) was used, *P. aeruginosa* DSM 7108 did not reach the claimed rhamnolipid concentrations of >100 g/L (Giani et al. 1997). When cultivated at 30 °C, *P. aeruginosa* DSM 7108 showed a growth associated $q_{RL,spec}$. This can be related to the fact that this strain was actually a result of mutant screenings with the purpose of high production rates at 30 °C (Giani et al. 1997). *Pseudomonas* sp. DSM 2874 was reported to produce

45 g/L (Trummler et al. 2003) when an integrated microbial/enzymatic process was used (resting cells, 30 °C, pH 6.5, rapeseed oil). However, Trummler et al. (2003) report of $Y_{RL/BDM}$ = 0.83 g/g and P_V = 0.14 g/(L·h) which is comparable with the achieved $Y_{RL/BDM}$ = 0.86 g/g and P_V = 0.16 g/(L·h) at 30 °C (cf. Table 3-1). All three strains showed different response towards the applied temperature, and the synthesis rate of rhamnolipids appears to be a strain-specific feature. Although the temperature showed influence on the total rhamnolipid concentrations (cf. Table 3-1), the lapse of the molar ratio was quasi superimposed for 30 and 37 °C. For *Pseudomonas* sp. DSM 2874, some temperature-dependent variance was especially observed for the relatively low concentrations at the beginning of the rhamnolipid periods. The di- to mono-rhamnolipid ratio is an important parameter with respect to final product composition and could further influence the effort needed for purification of the different rhamnolipid species if necessary. Additionally, the lapse of the molar ratio could hinder towards the regulation of rhamnolipid production on enzymatic and molecular level.

The lapse of the $q_{RL,spec}$ was strongly dependent on the temperature and the applied strain. The fitted results were sufficient for the purpose of lapse comparison and deviation. A typical secondary metabolite-like increase and decrease of the specific productivity was, apart from the abovementioned *P. aeruginosa* DSM 7108 (30 °C), apparent in all cultivations. Previous reports mainly focused on overall and volumetric productivities, but with regard to process optimization by bioengineering approaches, it is necessary to have detailed information about the specific productivity per cell. The discrepancy between the maximum $q_{RL,spec}$ and the average $q_{RL,spec}$ reflects a potential space of optimization. It is reasonable to assume that the increase of $q_{RL,spec}$ is a matter of genetic regulation. However, the subsequent decrease is still a point of question. Either there is a genetic down-regulation, a metabolic lack in precursor pools, or enzyme degradation. Also, the depletion or inhibitory accumulation of metabolites within the medium could indirectly lead to the decrease of $q_{RL,spec.}$ In future, suitable fed-batch strategies aiming to avoid the decrease of the specific rhamnolipid productivity per cell have the potential to substantially increase the overall rhamnolipid yield.

It has been demonstrated repeatedly that nitrate is the best N-source for the induction of rhamnolipid production (Guerra-Santos et al. 1984; Manresa et al. 1991; Arino et al. 1996). Soberón-Chávez et al. (2005) stated that N-limiting conditions do not favor rhamnolipid production per se, but production starts with the exhaustion of nitrogen (Ramana and Karanth 1989; Robert et al. 1989; Manresa et al. 1991). However, in all cited works shake flask experiments were performed. Nevertheless, in this study the highest productivities were observed during the transition phase of nitrate usage towards depletion or stationary nitrogen concentrations. Rhamnolipid was produced, even though nitrate was not depleted and still present in excess (cf. Figure 3-3). The growth curves and rhamnolipid concentration lapse showed a typical logistic behavior (Ramana et al. 1991; Babu et al. 1996), thus implicating growth limiting conditions and secondary metabolite-like character (Desai and Banat 1997). It is reasonable that under the applied conditions, multiple limitations occur which have positive influence on rhamnolipid formation, thus leading to an increase of the $q_{RL,spec}$. The alternative sigma factor RpoN (σ^{54}) is associated with nitrogen metabolism, and respective binding sites are reported for *rhlR* (Medina et al. 2003), *rhlAB* operon, and the *PA1131-rhlC* operon (Rahim et al. 2001). The *las*-QS system of *P. aeruginosa* controls the expression of rhamnolipids. However, parallel nitrogen and carbon starvation can force a mutant producing these rhamnolipids in spite of a *las*-QS mutation (Van Delden et al. 1998) by regulation with the alternative sigma factor RpoS (σ^{S}).

In view of the complex QS-regulated induction of rhamnolipid synthesis in *P. aeruginosa*, the further optimization of the large-scale rhamnolipid production will almost certainly be dependent on a more precise understanding of the up-regulation and decrease of the rhamnolipid productivity. The type-strain *P. aeruginosa* PAO1 showed higher yields than the long-known production strains DSM 7108 and DSM 2874. Therefore, this strain has the potential as a strain of choice for biotechnological rhamnolipid process development. The comparison of the three strains revealed that the specific rhamnolipid productivity per cell explicitly shows a typical secondary metabolite-like production course. However, the rhamnolipid synthesis was already induced before N-limitation occurred.

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4 Regulatory and metabolic network of rhamnolipid biosynthesis: Traditional and advanced engineering towards biotechnological production

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4.1 Abstract

During the last decade the demand for economical and sustainable bioprocesses replacing petrochemical derived products has significantly increased. Rhamnolipids are interesting biosurfactants that might possess a broad industrial application range. However, despite of 60 years of research in the area of rhamnolipid production, the economic feasibility of these glycolipids is pending. Although the biosynthesis and regulatory network are in a big part known, the actual incidents on the cellular and process level during bioreactor cultivation are not mastered. Traditional engineering by random and targeted genetic alteration, process design and recombinant strategies did not succeed by now. For enhanced process development there is an urgent need of in-depth information about the rhamnolipid production regulation during bioreactor cultivation to design knowledge-based genetic and process engineering strategies. Rhamnolipids are structurally comparable, simple secondary metabolites and thus have the potential to become instrumental in future secondary metabolite engineering by systems biotechnology. This review summarises current knowledge about the regulatory and metabolic network of rhamnolipid synthesis and discusses traditional and advanced engineering strategies performed for rhamnolipid production improvement, focusing on *Pseudomonas aeruginosa*. Finally, the opportunities of applying the systems biotechnology toolbox on the whole-cell biocatalyst and bioprocess level for further rhamnolipid production optimization are discussed.

4.2 Introduction

Glycolipid biosurfactants which are comprised of L-rhamnose and β-hydroxyalkanoic acid residues are termed rhamnolipids (RL). Hereof about 60 congeners and homologues are produced in mixtures at various quantities by different *Pseudomonas* species and by bacteria belonging to other families, classes, or even phyla (Abdel-Mawgoud et al. 2010b). *P. aeruginosa* is the best studied producer mainly synthesizing the mono-RL congener Rha-C_{10}-C_{10} and the di-RL congener Rha_2-C_{10}-C_{10} (Syldatk et al. 1985a). RLs show good physico-chemical properties compared to conventional petrochemical derived surfactants (Abalos et al. 2001; Haba et al. 2003). Their application in bioremediation (Nguyen et al. 2008), enhanced oil recovery (Wang et al. 2007), and biodegradation (Zhang et al. 2005) has been

reported for several times. Apart from this, a high interest exists in the cosmetic, pharmaceutical and detergent industry (Banat et al. 2010), especially because of their anti-microbial activity (Vatsa et al. 2010). They can be produced using waste or renewable substrates and show excellent biodegradability as well as low aquatic toxicity (Giani et al. 1997; Rahman et al. 2002; Wei et al. 2005; Marsudi et al. 2008). Over 60 years have passed since the first RL was described (Jarvis and Johnson 1949), however, industrial bulk application is still restricted by the insufficient productivity, relatively high raw-material costs and expensive downstream processing. Basically, three key strategies are generally conceivable for RL production improvement (cf. Figure 4-1).

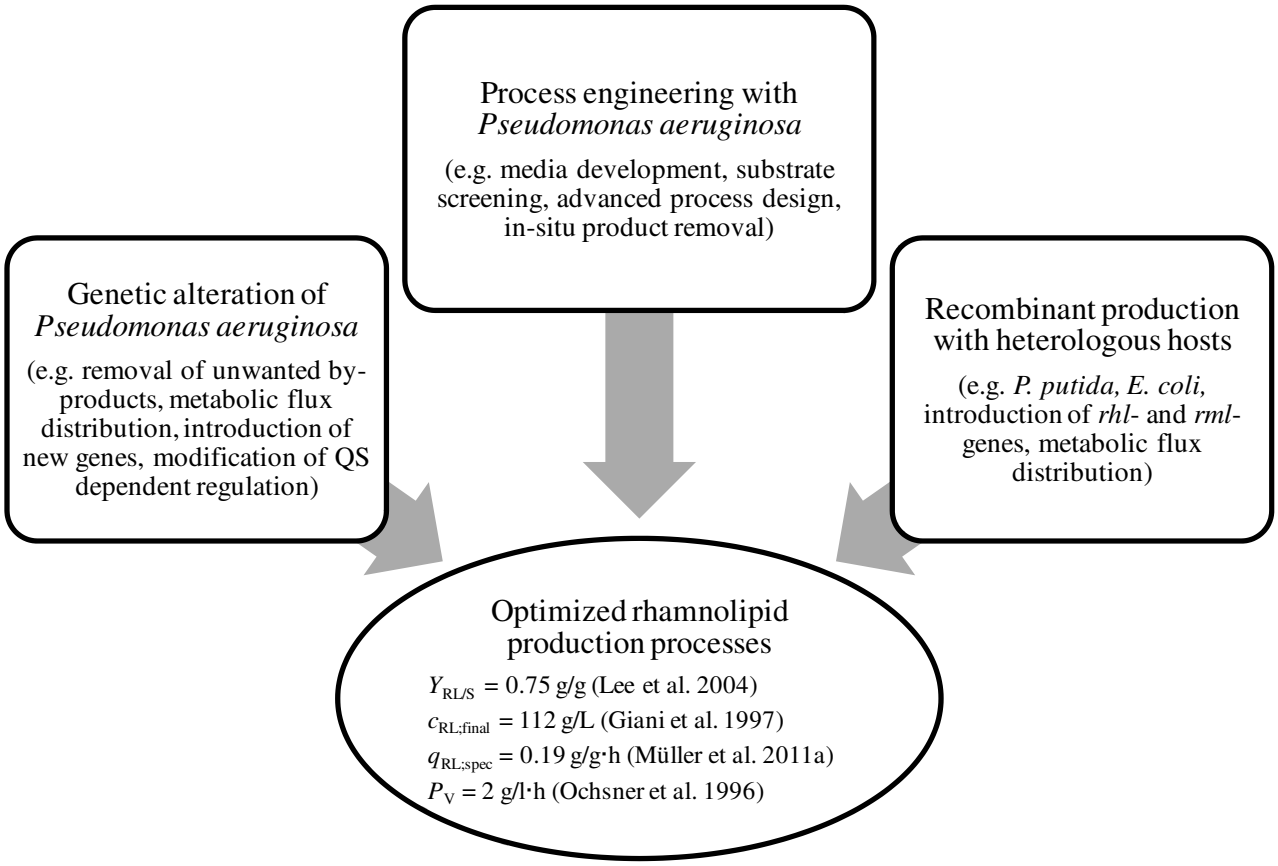

Figure 4-1. Presentation of three generally conceivable strategies for rhamnolipid production optimization based on *Pseudomonas aeruginosa* and the best yet with these strategies achieved results for substrate conversion ($Y_{RL/S}$), final rhamnolipid concentration ($c_{RL;final}$), specific rhamnolipid production rate per biodrymass ($q_{RL;spec}$) and volumetric productivity (P_V).

Random or targeted genetic alteration is used to enhance the production with already available or newly isolated *P. aeruginosa* strains. These manipulations may implicate the removal of unwanted by-products, the modification of RL production and growth regulation, introduction of recombinant proteins and metabolic flux distribution changes. Second, RL production, by established *P. aeruginosa* strains, optimized using engineering strategies like design of experiment (DOE) or heuristic process design. Thirdly, to circumvent the complex quorum sensing (QS) dependent regulatory network and pathogeneses issues of *P. aeruginosa,* recombinant production of rhamnolipids by heterologous hosts like *P. putida* and *E. coli* has been investigated. However, a limited understanding of the physiology of the microbial regulation and the lack of predictive models for whole-cell biocatalyst operations are the main reason for low productivities of microbial products in general (Woodley 2008) and specifically for rhamnolipids. Recently, integrative concepts for the implementation of systems biology tools in bioprocess development were discussed (Wang et al. 2009; Kuhn et al. 2010; Otero and Nielsen 2010). These approaches are in accordance with “X–omics” based systems biotechnology for strain improvement (Lee et al. 2005). This review summarizes current knowledge about RL biosynthesis and its QS and environmental factors dependent regulation with focus on production by *P. aeruginosa* and recombinant expression strategies. Exemplarily, different “X-omics” studies investigating the regulatory and metabolite network of RL synthesis will be presented. Finally, an integrative strategy for systems biotechnology driven engineering of economic RL production is presented.

4.3 Rhamnolipid biosynthesis and regulation in *P. aeruginosa*

In the following, the biological background for rhamnolipid synthesis is described in detail. Three major synthesis steps can be distinguished. The precursors, namely β-hydroxyalkanoyl-β-hydroxyalkanoyl-ACP (HAA-ACP) and dTDP-L-rhamnose, were shown to be synthesized *de novo* during the first two steps (Hauser and Karnovsky 1957; 1958; Burger et al. 1963; Zhu and Rock 2008). Finally two special rhamnosyltransferases sequentially catalyze the condensation of the precursors to mono- and di-RLs. The RL biosynthesis in *P. aeruginosa* is controlled by a cell-density depended QS system (Sullivan 1998). QS signals integrate into a global regulatory network of signal molecules (Williams and Camara 2009).

4.3.1 Biosynthesis pathway

A putative RL biosynthesis pathway for *P. aeruginosa* is given in Figure 4-2 (Burger et al. 1963; Ochsner et al. 1996; Kanehisa and Goto 2000; Rahim et al. 2001; Soberón-Chávez et al. 2005; Winsor et al. 2009; Abdel-Mawgoud et al. 2010a).

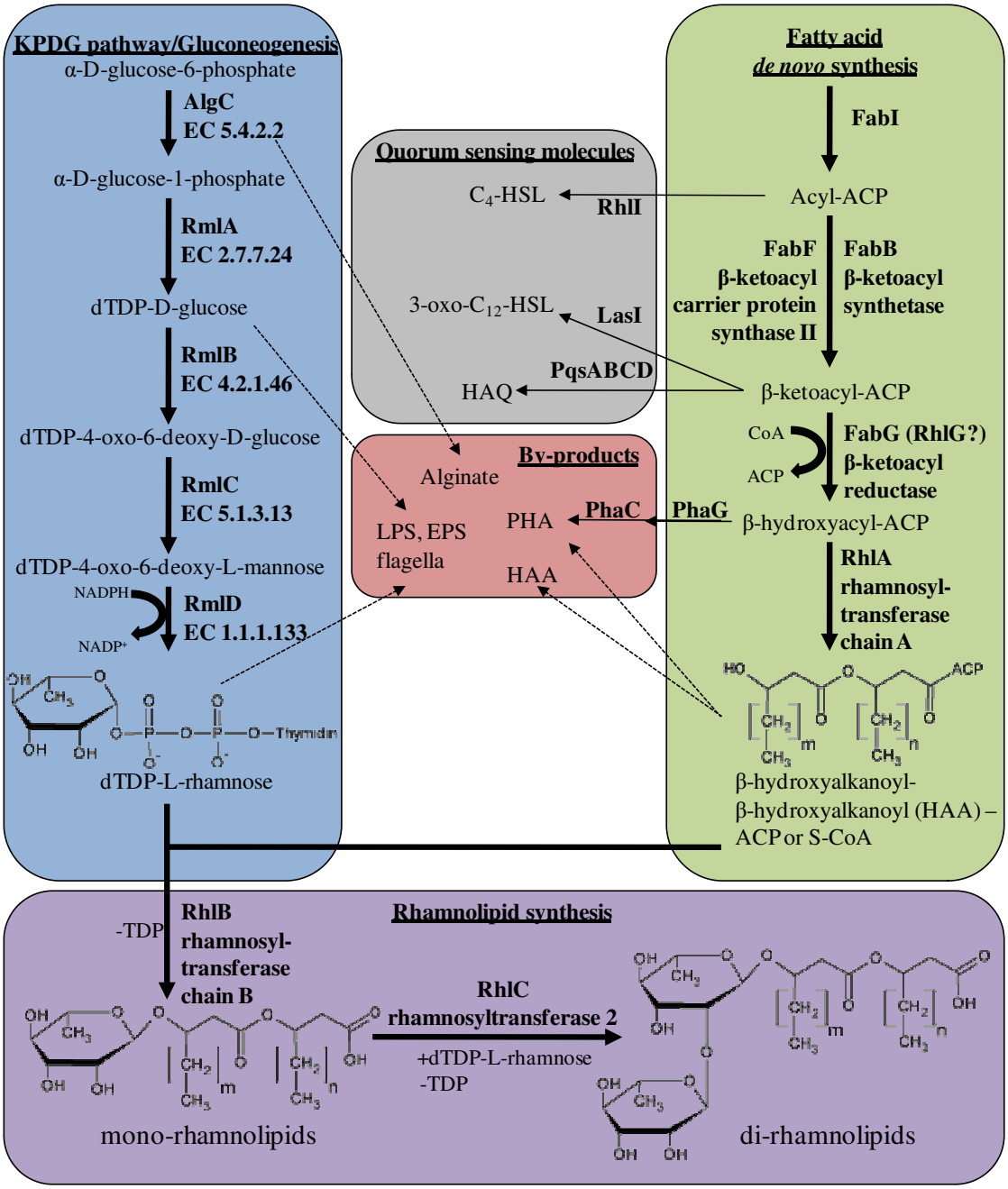

Figure 4-2. Putative rhamnolipid biosynthesis pathway adopted from Soberón-Chávez (2005), with updates. The pathway is based on the sequenced and fully annotated type strain *P. aeruginosa* PAO1 (Holloway 1955; Stover et al. 2000). The three major synthesis steps towards mono- and di-rhamnolipids and their connection to several by-products and QS molecules are illustrated (m,n = 4 – 8). The biosynthesis mechanism for rhamnolipids with only one β-hydroxyalkanoic acid moiety or unsaturated fatty acids is still unravelled (Soberón-Chávez et al. 2005; Abdel-Mawgoud et al. 2010a).

The dTDP-L-rhamnose precursor for the hydrophilic part directly can derive from both, the Entner-Doudoroff-Pathway and gluconeogenisis, respectively, and is synthesized by a subsequent conversion of D-glucose-6-phosphate by the phosphoglucomutase AlgC (Olvera et al. 1999; Pham et al. 2004) and the *rmlBDAC* operon gene products (Robertson et al. 1994; Rahim et al. 2000). The RL biosynthesis pathway is cross-linked with the formation of various polysaccharide species (Rahim et al. 2000; Byrd et al. 2009; Lindhout et al. 2009). The *de novo* synthesis of the hydrophobic[7] moiety occurs by classical fatty acid synthetases of type-II (FAS II) (Rehm et al. 2001; Abdel-Mawgoud et al. 2010a) which basically comprise the *fab* gene products (Hoang and Schweizer 1997; 1999). The syntheses of the fatty acid moiety, of N-acyl homoserine lactones (AHL) and of 4-hydroxyl-2-alkylquinolens (HAQ) are closely linked to each other (Schaefer et al. 1996; Hoang and Schweizer 1997; 1999; Deziel et al. 2004; Bredenbruch et al. 2005). Even though RhlG was reported to be an active β-ketoacyl reductase (Campos-García et al. 1998), FabG was 2000-fold more active in carrying out the same NADPH-dependent reduction of β-ketoacyl-ACP to β-D-hydroxyacyl-ACP (Miller et al. 2006). Further structural and biochemical studies by Zhu and Rock (2008) affirmed that RhlG is not involved. The β-hydroxyalkanoic acid (C_{4+m} for mono- and C_{4+m}-C_{4+n} for the respective di-fatty acids, with m,n = 4 - 8) precursors can also be integrated in poly(3-polyhydroxy-alkanoate) (PHA) granules (Pham et al. 2004). PHA synthesis is directly connected via the transacylase PhaG (Rehm et al. 2001) and recent findings show that C_{4+m} are secreted and later retaken-up for RL or PHA synthesis (Choi et al. 2011). RhlAB (rhamnosyltransferase 1) catalyzes the formation of either HAA-ACP or –CoA (Rehm et al. 1998; Maier and Soberón-Chávez 2000; Lepine et al. 2002; Déziel et al. 2003; Zhu and Rock 2008) and of mono-RLs (Ochsner et al. 1994a). Both chains of RhlAB are encoded and co-expressed within the *rhlABRI* regulon, which is under control of AHL- dependent QS (Sullivan 1998). Subsequently a fraction of the mono-RLs is converted to di-RLs by rhamnosyltransferase 2 (RhlC) through transfer of a second rhamnosyl-group (Rahim et al. 2001). Till now, there is no detailed information about the exact mechanisms that lead to the secretion of the rhamnolipids to the extracellular space. However, the fact that RhlA is probably an inner-membrane bound protein (Rahim et al. 2001) that functions for a synchronized

[7] hydrophilic was changed to hydrophobic

rhamnolipid synthesis and transport. In contrast, the RL production by other bacteria is a younger field of research with many uncertainties which was recently reviewed in detail (Toribio et al. 2010).

4.3.2 Cell-density dependent regulation

The AHL- dependent (or *las/rhl*-) system is mainly responsible for RL biosynthesis regulation (Ochsner and Reiser 1995; Pearson et al. 1997) and integrates into the minor HAQ- dependent *Pseudomonas* quinolone signal (PQS) system (cf. Figure 4-3).

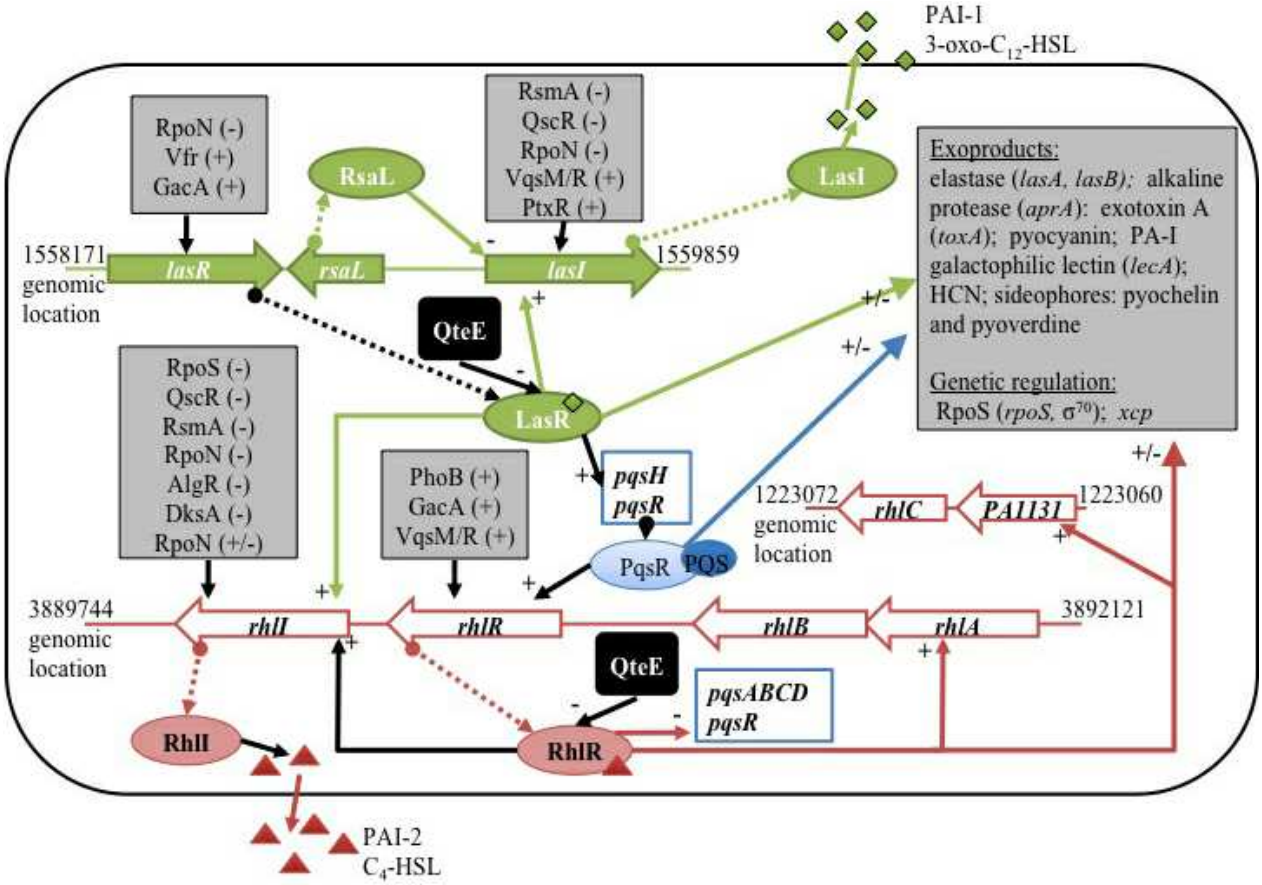

Figure 4-3. Overview of the *las/rhl*- QS system and the connection with the PQS- system (Ochsner and Reiser 1995; Sullivan 1998; Soberón-Chávez et al. 2005; Williams and Camara 2009; Abdel-Mawgoud et al. 2010a). The interaction of diverse regulatory elements has a deep impact on timing and strength of rhamnolipid biosynthesis gene expression. Functions and abbreviations are described in detail within the text. *Arrows* with *solid lines* indicate positive (+) or negative (-) regulatory effects. *Arrows* with do*tted line* and starting *circles* indicate gene expression. Genomic locations are related to the PAO1 genome from the *Pseudomonas* Genome Database (Winsor et al. 2009).

The AHLs mediate the cellular responsiveness towards environmental signals. First, the expression of *lasR* is induced via the transcriptional regulator Vfr (Albus et al.

1997) and the global activator protein (GacA) (Reimmann et al. 1997). The N-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}-HSL), also termed *P. aeruginosa* autoinducer 1 (PAI-1), is synthesized by the LasI protein. PAI-1 binds to LasR and thus positively regulates the *rhlABRI* operon (Medina et al. 2003). N-butanoyl-homoserine lactone (C_4-HSL or PAI-2) is then synthesized by RhlI. Bound to RhlR, PAI-2 induces the expression of the rhamnosyltransferase genes *rhlAB* and *rhlC* (Ochsner et al. 1994b; Pearson et al. 1997; Rahim et al. 2001). Autoinducer signalling between surrounding cells is regulated by a complex network of positive and negative feedback loops on the transcriptional and post-transcriptional level (Schuster and Greenberg 2007). Recently and for the first time, a quorum threshold expression element (QteE) was identified to control the AHL- dependent QS on protein level by destabilizing LasR. Because QteE can block QS when signal levels are high, this could provide a mechanism for individual cells to exert autonomous control over their QS regulons (Siehnel et al. 2010). However, while for *P. aeruginosa* the molecular regulation is already known in large part, recent studies propose a different regulation for *Burkholderia* sp. like *B. thailandensis* and *B. pseudomallei* (Dubeau et al. 2009). While *P. aeruginosa rhlAB* and *PA1131-rhlC* constitute two independently transcribed operons cross-linked by complex QS signalling, for *B. thailandensis and B. pseudomallei* an *rhlABC* operon was identified as independently functional duplicates on the genome. However, among the four completely annotated *P aeruginosa* strains, PA7 does not harbour the *PA1131-rhlC* operon at all (Winsor et al. 2009; Roy et al. 2010; Toribio et al. 2010).

4.4 Rhamnolipid production

According to Figure 4-1, among the most conceivable strategies for rhamnolipid production improvement is the use of *P. aeruginosa* wild-type strains and of homologous hosts like *P. putida*. In the next paragraphs 60 years of rhamnolipid production with *P. aeruginosa* and about 15 years of heterologous production of RL are summarized.

4.4.1 Rhamnolipid production and process design with *P. aeruginosa* strains

RLs are typical secondary metabolites and thus production strategies with *P. aeruginosa* wild-types mainly focus on limiting at least one media component like the N-source (Guerra-Santos et al. 1984; Mulligan and Gibbs 1989; Soberón-Chávez

et al. 2005), the P-source (Mulligan et al. 1989; Zhang and Miller 1992; Chayabutra et al. 2001) or trace elements (Manresa et al. 1991), however, not the C- source. Cultivation strategies described for rhamnolipid production implicate **(1)** batch (Syldatk et al. 1985b; Linhardt et al. 1989; Müller et al. 2010), **(2)** fed-batch (Lee et al. 2004; Chen et al. 2007a; Salwa et al. 2009), **(3)** continuous (Guerra-Santos et al. 1984; Reiling et al. 1986; Ochsner et al. 1996) and **(4)** integrated microbial/enzymatic processes (Trummler et al. 2003) and were recently discussed in more detail (Müller et al. 2011b). C- sources include water-soluble substrates like glycerol, glucose, mannitol and ethanol (Robert et al. 1989; Matsufuji et al. 1997; Sim et al. 1997) but water immiscible substrates or hydrocarbons lead to higher final RL concentrations (Syldatk and Wagner 1987; Ochsner et al. 1996; Trummler et al. 2003). N-sources reported are NO_3^-, NH_4^+, urea and amino acids containing supplements (Syldatk and Wagner 1987, Zhang and Miller 1992, Lee et al. 2004). It is interesting to note that the described processes for the RL production with *P. aeruginosa* range from 6 to 112 g/L and from 5 to 95 g/L of RL for batch and fed-batch, respectively. Generally, fed-batch processes are the reasonable strategy of choice for a broad range of microbial products (Lee et al. 1999; Wlaschin and Hu 2006). Most probably, the lack of understanding the complex RL production regulation during bioreactor cultivation is a major reason explaining why most of the described batch processes outperform the mainly heuristic fed-batch strategies. However, the best reported substrate conversion factor ($Y_{RL/S}$) of 0.75 g/g was obtained when a feed medium containing 25 g/L of fish oil and 0.01 % urea (*w/v*) was used with *P. aeruginosa* BYK-2 KCTC 18012P (Lee et al. 2004). Contrary, a patent claims up to 112 g/L RL when cultivating *P. aeruginosa* DSM7107 or DSM7108 (250 g/L soybean oil; 15 g/L $NaNO_3$) in batch (Giani et al. 1997). Repeatedly, nitrate was shown to be the best N-source for the induction of RL synthesis (Guerra-Santos et al. 1984; Manresa et al. 1991; Arino et al. 1996) but N-limiting conditions do not favour RL production per se (Soberón-Chávez et al. 2005). While some reports show that RL production starts after nitrogen exhaust (Ramana and Karanth 1989; Robert et al. 1989; Manresa et al. 1991), others show, that even though nitrogen was still present in excess, RL productivity was already high (Ochsner et al. 1996; Müller et al. 2011a). However, under batch cultivation, RL production was always highest during the transition towards a stationary phase (cf. Figure 4-4a + b).

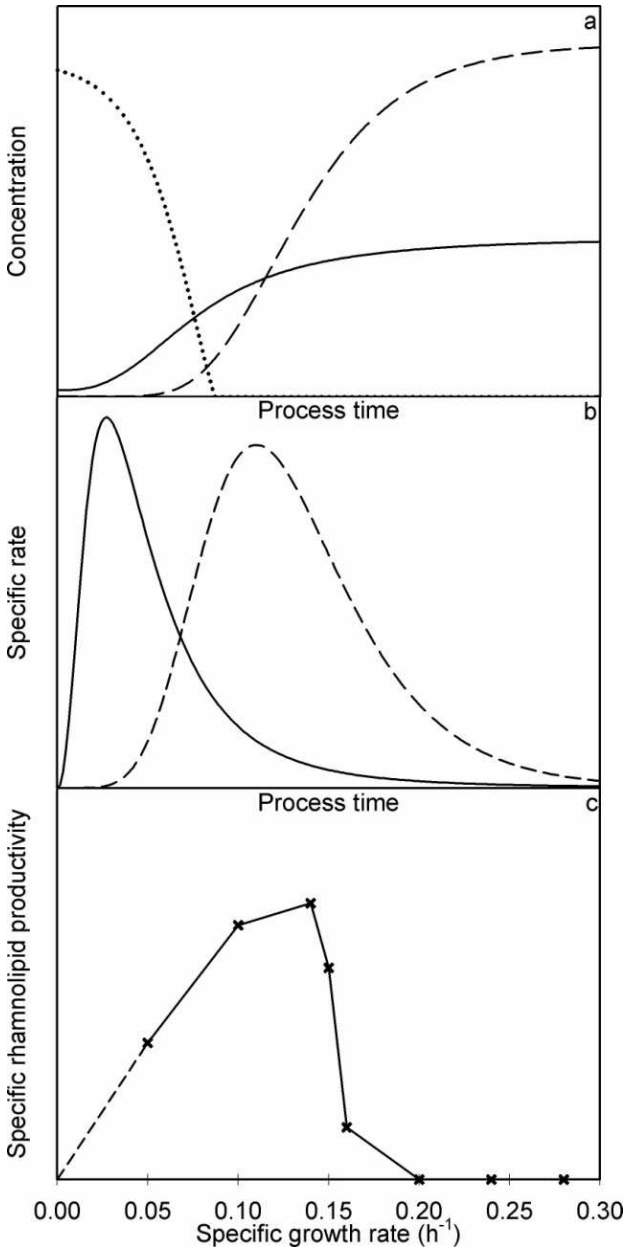

Figure 4-4. Characteristic graphs for rhamnolipids production. **a** Typical logistic course for biodrymass (*straight*) and rhamnolipid (*dashed*) concentrations and lapse of the N- source (*dotted*) (Müller et al. 2010). **b** Extracted courses of specific growth (*straight*) and specific rhamnolipid productivity (*dashed*) (Müller et al. 2011). **c** Correlation of specific growth and rhamnolipid productivity (Guerra-Santos et al. 1986). *Dashed line* represents the putative lapse for growth rates lower than 0.05 h^{-1}.

In the transition from the growth to a stationary phase, there is a decrease in RL production after the initial increase observed from the beginning on. However, while the increase can be explained by positively regulatory reasons (QS, RpoN, RpoS…, cf. Figure 4-3), the causes for the decrease are still not really understood. The central question which needs to be answered for further process optimization with *P. aeruginosa* wild-type strains is, why exactly the specific production decreases

during bioreactor cultivation and which process design strategies may help to avoid or decelerate this circumstance. Guerra-Santos (1986) investigated the relation between the dilution rate ($D = \mu$) and specific RL productivity ($q_{RL;spec}$) in continuous cultures of *P. aeruginosa* DSM 2659 (33 °C; pH 6.25, 20 g/L glucose). The data (cf. Figure 4-4c) showed highest $q_{RL;spec}$ at μ = 0.13 h^{-1}. However, the productivity was decreasing fast with little higher μ. Unfortunately the relevance of different cell culture densities, thus QS regulation, was not investigated in this study. Recently for *B. plantarii* DSM 9509^T growth associated RL production of up to 46 mg/L was observed during parallel bioreactor cultivation on glucose supplemented rich medium (Hörmann et al. 2010).

4.4.2 Recombinant production of rhamnolipids by heterologous hosts

Main candidates for the heterologous expression of the *P. aeruginosa rhl* genes were *E. coli* (Ochsner et al. 1994a; Cabrera-Valladares et al. 2006; Wang et al. 2007) and *P. putida.* (Ochsner et al. 1995; Cha et al. 2008). Ochsner et al. (1995) report final RL concentrations of 0.25 g/L with *P. fluorescence*, 0.6 g/L when using *P. putida* KT2442 and no RLs with *E. coli*. In contrast the expression of *rhlAB* in *E. coli* BL21 has lead to RL production (Wang et al. 2007). However, the highest yet reported heterologous RL production of 7.3 g/L was achieved with *P. putida* KCTC1067 expressing *rhlAB* operon and *rhlI* of *P. aeruginosa* EMS1 (Cha et al. 2008). Nevertheless, in this study the value of the produced biomass is lacking. All these studies aimed to over-express *rhlAB*, thus provoking mono-RL and HAA production. Cabreras-Valladares (2006) investigated recombinant *E. coli* strains which in addition to the *rhlAB* operon also expressed *rmlBDAC* (cf. Figure 4-2). By this strategy, they could achieve final yields of 120.6 mg/L of mono-RL and showed that the availability of L-rhamnose was a limiting factor in *E. coli*. Recombinant RL production can be one strategy to avoid the complex regulation mechanism and safety issues of pathogenic *P. aeruginosa*, but the fact, that both precursors are derived from central metabolic pathways makes it difficult to over-produce RLs in heterologous hosts (Ochnser et al. 1994, Ochsner and *Reiser* 1995). Additionally, by-products like PHA, which compete for the precursor pools, are also available in *P. putida* strains. Thus, a strategy for increase of the precursor pools and flux balancing should be followed in parallel for future recombinant approaches.

4.5 Systems biotechnological toolbox for RL production strain improvement

Microorganisms evolved to survive under natural environmental living conditions, and, are not prone for high production and accumulation of any sort of biomolecules. Thus for any product, e.g. RL, where new, economically competitive and environmentally friendly processes are demanded, effective up-to-date engineering strategies must be used to satisfy this demand (Kuhn et al. 2010). The most effective approach will probably be to take advantage of the recent advances in systems biology (Kitano 2002; Stephanopoulos et al. 2004; Otero and Nielsen 2010; Zhang et al. 2010) to decipher systematically the complex nature of RL synthesis regulation under production conditions by knowledge-based engineering instead of heuristics. The toolbox for this purpose is already available. With PAO1 (Holloway 1955) a completely sequenced (Stover et al. 2000) and annotated (Winsor et al. 2009) *P. aeruginosa* strain, the capability of which as a model strain for RL production was recently shown (Müller et al. 2010; Müller et al. 2011a), is at hand. PAO1 is the best studied *P. aeruginosa* strain for genetic regulation mechanisms (Potvin et al. 2008; Winsor et al. 2009) and most of the tools for systems biology are already established (Choi et al. 2007). However, RL production development can only benefit from this toolbox and advances in systems biology when a rational approach for their implementation is chosen during process development. Therefore, it will be necessary to integrate the qualitative description of the cellular function into the reaction and process level, as proposed for other applications by Zhang et al. (2006).

4.5.1 Revealing the regulatory network

The QS regulation is a complex network (cf. Figure 4-3) depending on the interaction between DNA, RNA and proteins. Information from the genome of *P. aeruginosa* PAO1 identified 550 transcriptional regulators and 24 putative sigma factors (Potvin et al. 2008). On the genetic level, transcriptional regulators like the regulator of secondary metabolites (RsmA), interfere with the QS regulation (Williams and Camara 2009). The alternative sigma factor RpoN is associated to nitrogen metabolism and respective binding-sites are reported for *rhlR* (Medina et al. 2003), *rhlAB operon* and the *PA1131-rhlC* operon (Rahim et al. 2001). PhoB is a response regulator, and stimulates *rhlR* expression under phosphate- limiting conditions via the HAQ- dependent QS (Jensen et al. 2006). Parallel starvation of C- and N-sources

could also induce RL production in spite of a *las*-QS mutation via the regulation of the alternative sigma factor RpoS (Van Delden et al. 1998). To assess the active components of such a genetic network, quantitative comprehension on mRNA level will certainly be the method of choice (Herrgard et al. 2004). Microarray analysis of *P. aeruginosa* QS regulons revealed an impact of environmental factors like medium composition, oxygen availability and temperature on QS regulated genes (Wagner et al. 2003). Wagner et al. (2003) classified > 10 % of the *P. aeruginosa* PAO1 genome as QS regulated and showed that the regulation was growth phase dependent. The *rhlAB* operon was over 100-fold expressed in the early stationary phase when QS was induced (Wagner et al. 2003). However, *PA1131-rhlC* was only induced 7.4-fold from the mid-logarithmic to the early stationary phase. A common subset of 53 genes was detected being QS-induced under aerobic and anaerobic planktonic, and biofilm growth conditions (Wagner et al. 2004). Interestingly also 222 genes were identified being repressed by QS including many nitrate respiration associated genes. Another microarray analysis showed that genes that were differentially expressed with respect to iron were very consistent between cells grown complex (D-TSB) or mineral salt (M9) medium. One of the few exceptions was the *rhlAB* operon, which is controlled by QS and RpoN (Ochsner and Reiser 1995), and 80-fold iron regulated in M9 but constitutive in D-TSB medium (Ochsner et al. 2002). For biofilms growing under iron limitation the RL expression was shifted to the initial stages of biofilm formation (Glick et al. 2010). In contrast, the transcriptome analysis of the *P. aeruginosa* PAO1 response to iron revealed no effect on RL synthesis (Palma et al. 2003). Swarming motility in *P. aeruginosa* was shown to be dependent on RL and HAA excretion for the purpose of wetting and chemotactic-like stimuli (Déziel et al. 2003; Caiazza et al. 2005; Tremblay et al. 2007). Interestingly, transcriptome analysis of *P. aeruginosa* PA14 showed that *rhlAB* and *rhlC* are down-regulated in swarm tendril tips, and on the other hand, *rhlAB* is higher expressed in cells from the swarm centre than from the tendril tips, suggesting that RLs are primarily produced from cells at the centre of a swarming colony (Tremblay and Deziel 2010). These results highlight the presence of distinct bacterial sub-populations in swarming colonies and probably can be assigned to planktonic cultivations were stochastic segregation of sub-populations may have dramatic influence on the overall productivity (Kussell and Leibler 2005; Delvigne et al. 2009). These effects have not

been analysed yet for bioreactor cultivations while rhamnolipid production, but are most probably of importance.

4.5.2 Revealing the metabolic network

The proteome of a cell reveals the active metabolic network and reflects the final stage of the cellular information flow as a response to certain environmental conditions (Han and Lee 2006). The extracellular proteome of the stationary phase from *P. aeruginosa* PAO1 and various QS- mutants (e.g. Δ*rhl,* Δ*las,* Δ*vfr*) was compared by 2-DE (Nouwens et al. 2003). Here, the most abundant extracellular proteins were elastase (LasB), LasA protease and a putative peptidase (PA2939). Interestingly, the Lipase (LipA) was found to be *rhl*- QS (Reimmann et al. 1997) regulated but seemed to be repressed by the *las*- QS system (Nouwens et al. 2003). A similar study was conducted for the QS- regulated proteins in *B. glumae* (Goo et al. 2010). Recently, a comparative proteome analysis of *P. aeruginosa* PA1 was done investigating the differences of the intracellular proteome after the cultivation of RL producing and non-producing cells (Reis et al. 2010). RL production was either induced or suppressed by variation of media conditions. A total of 21 differentially expressed proteins could be identified by MALDI-TOF/TOF MS. Under the RL favouring conditions, proteins involved in the response to oxidative stress, secretion pathways, QS and other metabolisms were mainly differentially expressed. However, neither RL biosynthesis-related nor extracellular proteins were analysed in this study. A closer connection to the phenotype of a cell is its metabolome; the set of all metabolites in the cell (Fiehn 2002). Intracellular metabolite pools directly represent the physiological condition of the cell and should help to identify metabolic “bottle-necks” of RL production. For *P. aeruginosa* PAO1 and TBCF10839, a basic core metabolism was identified with glutamate being a dominant metabolite (Frimmersdorf et al. 2010). It was shown that the metabolome was mostly defined by the growth substrate rather than the strain-specific genetic background. Frimmersdorf et al. (2010) found by GC/MS analysis 243 components and could identify 144 thereof. The chemical diversity and range of concentrations of the components is challenging for the scientist, and adequate standards are not always available or are unstable. Additionally, fast and adequate sampling procedures are a must (van Gulik 2010). Footprint and fingerprint analysis by NMR of *P. aeruginosa* PAO1 growing either as

biofilm or planktonic revealed that the qualitative metabolism in a spent medium (footprint) appeared similar for both modes of growth, while intracellularly, (fingerprint) the cells displayed marked differences in their spectra profiles (Gjersing et al. 2007). Once the skeletal structure of a metabolic network is composed the distribution can be identified by ^{13}C- based metabolic flux analysis (Sauer 2006) and related methods (Blank and Kuepfer 2010). The estimation of metabolite fluxes will play a key role in engineering strains with higher RL yields and productivities e.g. by avoidance of by-products. PHA-synthase negative PAO1 mutants are still capable of RL production but accumulate more alginate than the wild-type (Pham et al. 2004). Comparative ^{13}C NMR analysis of *P. aeruginosa* PAO1 and PA14 were done to estimate the fluxes of hydrophilic or hydrophobic C- sources to RLs and the by-products PHA and HAA (Choi et al. 2011). The ratio of di- to mono-RL was shown to be C- source dependent and RL-negative mutants accumulated higher PHA than in the wild-type strains. PHAs of medium chain length are RpoN regulated (Hoffmann and Rehm 2005), and can directly derive from incorporation of the C- source, as was shown with octanoic-1-^{13}C acid (Choi et al. 2011). Interestingly, PHA-negative mutants did not result into higher β-hydroxyfatty acid precursor flux towards RLs, however, the production seemed to be very tightly regulated at the transcriptional level via QS and environmental conditions (Choi et al. 2011).

4.5.3 Mathematical description, simulation and experimental design

Rational engineering needs detailed mathematical description and simulation of the microbial behaviour in the bioreactor (Endy and Brent 2001). Typically, growth and RL curves show logistic behaviour (cf. Figure 4-4 a) under the limiting conditions in batch cultivation (Manresa et al. 1991; Ramana et al. 1991; Babu et al. 1996; Müller et al. 2010; Müller et al. 2011a). Corresponding to the limiting character of such cultivations it is possible to mathematically describe the growth by logistic or modified Gompertz equations (Zwietering et al. 1990; Chen et al. 2007b; Wu et al. 2008; Müller et al. 2010). This approach allowed to extract the specific growth rate (μ) and the specific RL productivity ($q_{RL;spec}$) for *P. aeruginosa* PAO1 and DSM 7108, and *Pseudomonas sp.* DSM 2874 during batch cultivations (cf. Figure 4-4 b). The complex biological context, however, does not allow a complete reflection on the simulation and modelling level. Nevertheless, for a significant reduction of

experiments, statistical experimental design methods should be implicated into the optimization process (Kennedy and Krouse 1999). A 2^4 full factorial, central composite rotational design and response surface modelling method (RSM) was used to enhance RL and biomass production by *P. aeruginosa* AT10, with soybean oil, $NaNO_3$, phosphate and iron concentrations as critical components (Abalos et al. 2002). Maximum biomass of 12.06 g/L was achieved with 50 g/L soybean oil, 9 g/L $NaNO_3$, 7 g/L phosphate and 13.7 mg/L iron source. In contrast, the maximum RL concentration of 18.66 g/L was achieved with 50 g/L soybean oil, 4.6 g/L $NaNO_3$, 1 g/L phosphate and 7.4 mg/L iron source. Another central composite experimental design and surface response analysis was used to optimize the concentrations of residual soybean oil (RSO), NH_4NO_3 and brewery residual yeast (De Lima et al. 2007). Finally, the best results found for rhamnose concentration was 22 g/L RSO, 5.7 g/L NH_4NO_3 and 11 g/L of residual brewery. However, to further optimize the composition of a production medium, shake flasks experiments were designed by RSM (Wu et al. 2008) leading to an improvement by 47 % when compared with the starting conditions. Even though RSM experimental design was an effective tool for improvement of RL production media and showed to reduce the number of experiments significantly, all the studies were conducted in shake flask experiments. However, for realistic simulations of process-like conditions, small parallel bioreactor and microtiter plate-based microreactors are already available (Weuster-Botz 2005; Duetz 2007). The use of such technologies should further improve the space of potential process optimization. Although relatively simple kinetic models have been used for the design, operation and optimization of bioprocesses, it is doubtful whether biological systems can be described by input and output parameters only, while the whole-cell catalyst itself is treated as a “black-box” (Katagiri 2003).

4.6 Conclusions and perspectives

Traditional rhamnolipid process development mainly included random mutagenesis, screening methods and heuristic process development (cf. Figure 4-1). Although similar methodologies have already proven to be commercially successful, e.g. for penicillin (Otero and Nielsen 2010), introduced mutations are mostly not clear and can include unwanted side effects occurring in later stages of optimization. By traditional rhamnolipid process development, the mechanistic understanding leading

to desired yields or phenotypes on process or cellular level, respectively, is minimal and thus starting points for further improvement are missing. Looking back at the last 60 years of RL research the conclusion is, that in this case traditional strain improvement and process development has not succeeded. A system-wide analysis of growth and RL production regulation (cf. Figure 4-5) has the potential to reveal several unknown interactions between genes, proteins, and metabolites, thus facilitating rational process engineering strategies (Vemuri and Aristidou 2005), either with *P. aeruginosa* strains or heterologous hosts. Excessive high-throughput "X-omics" analysis and predictive computational modelling or simulation can be combined to generate new knowledge through iterative modification of an *in silico* model and experimental design (Lee et al. 2005). Nearly all areas of the so called "systems biotechnology" approach have already been applied to reveal RL regulatory and metabolite network interactions as well as to construct simple response models or mathematical descriptions of RL production. However, the comprehensive understanding of a bioprocess should not be restricted to the quantitative description of cellular functions but, in addition, include analysis and evaluations on the biocatalyst and process level (Kuhn *et* al. 2010). Thus the integration of engineering and biology is more profoundly needed (Wang et al. 2009). Quantitative metabolite data on process level should help to reconstruct the biochemical network (Feist et al. 2009) and thus facilitate the removal or reduction of by-products like PHA, which may account for more than > 50% of the cell dry weight (Fernández et al. 2005; Costa et al. 2009). Global transcription machinery engineering also proved to be a valuable alternative to traditional methods of strain improvement (Alper et al. 2006; Wang et al. 2009). Additionally, detailed mathematical description of the biological systems may combine kinetic information with stoichiometric models and integrate into an engineering design cycle of *measure*, *model* and *manipulate* (Bujara and Panke 2010) as shown in Figure 4-5. On one side, the fact that the precursors for RL synthesis are active dTDP- sugars and ACP- fatty acid molecules makes metabolomics and fluxomics difficult. However, on the other side only 2- 3 enzymatic steps (cf. Figure 4-2) are needed to constitute these comparable simple secondary metabolites from the primary metabolism. RLs are promising secondary metabolites whose production could be optimized by an integrated rational whole-cell biocatalyst and bioprocess design methodology, termed systems biotechnology.

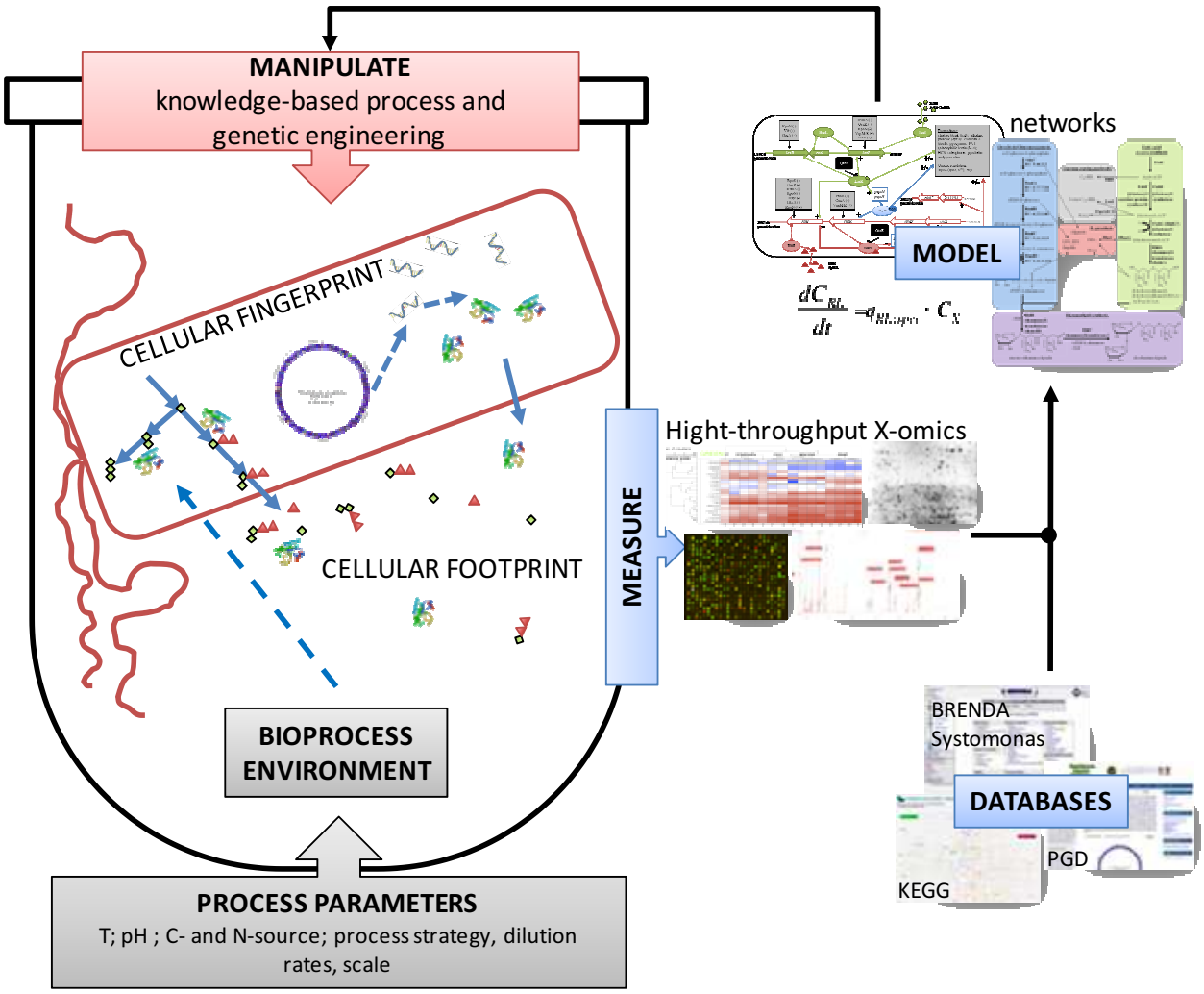

Figure 4-5. Integrative systems biotechnology approach for the reconstruction of the metabolic and regulatory networks of rhamnolipid biosysnthesis. In parallel to conventional process variable *measures* the cellular foot- (exrectome) and fingerprint (intracellular) are analysed by technology-driven high-throughput X-omics methods, like transcriptomics, proteomics and metabolomics. In combination with the available genomic information from databases and software tools the regulatory and metabolic networks are reconstructed and *in-silico* flux *models* can be created. Detailed mathematical description of the biological system combines kinetic information with stoichiometric *models* integrating into an engineering design cycle of *measure, model and manipulate*. The obtained biological information can be used for production strain improvement by genetic *manipulations* (e.g. flux distribution, removal of by-products, engineering of the transcription machinery, and implementation of missing genes, like long-chain fatty acid ACP ligase and phosphofructokinase) and advanced process controlling strategies. Iteratively the design cycle process is done till previously set objectives like higher productivity, substrate yields, ease of downstream processing, process robustness and ecological or economical goals have been achieved (Lee et al. 2005; Feist et al. 2009; Wang et al. 2009; Kuhn et al. 2010; Otero and Nielsen 2010).

The decoupling of the bacterial growth from the rhamnolipid production regulation will play a key role in this approach. This could be done by genetic alteration of *P. aeruginosa* strains or the development of respective heterologous hosts. Also the genetic engineering of production strains, which could directly integrate fatty acids from the media into rhamnolipids, should be considered. The challenge will be to develop a process where: **(1)** high amounts of biomass are produced in relatively short time, **(2)** rhamnolipid is produced with constantly high specific production rates, **(3)** inhibiting product concentrations are avoided e.g. by *in situ* product removal (ISPR) and **(4)** maximum possible substrate conversion rates ($Y_{RL/S}$) are achieved. Table 4-1 shows the energetically possible maximum substrate conversion rates for the most important substrates for rhamnolipid production based on ATP balance of the *P. aeruginosa* PAO1 metabolic pathway (Kanehisa and Goto 2000; Winsor et al. 2009). Hereby it was assumed that the producing cells spend all the biochemical energy for the product formation. Compared with current market prices of 1 - 3 USD/kg of alkylpolyglycosides (APG) (Alibaba 2011), which are chemically synthesized from vegetable oil or sugars, one has to conclude that with the help of the discussed engineering methods the economically viable rhamnolipid production process is in the not-so-distant future.

Table 4-1. Overview of the possible production costs per kilogram of rhamnolipid based on the theoretically maximum substrate conversion rates. Substrate costs reflect current market prices (Halai 2010; Onvista 2011).

Substrate	substrate costs (€/ton)	theoretical[a] Y_{max} (g_{RL}/g_S)	reported Y (g_{RL}/g_S)	production[b] costs (€/kg)
Sugar	420	0.35	0.15 (Chen et al. 2007a)	1.2
Glycerine (crude - vegetable)	200 – 500	0.41	0.21 (Syldatk et al. 1985a)	0.5 – 1.2
Vegetable oil	800 – 900	0.89	0.74 (Giani et al. 1996)	0.9 – 1.0
Fatty acids (rubber grade)	80 - 100	0.89	-	0.1

[a]maximum substrate conversion rate based on ATP balancing of the metabolic pathways of *P. aeruginosa* PAO1 (Kanehisa and Goto 2000; Winsor et al. 2009)
[b]reflects the substrate related production costs per kg of produced rhamnolipid

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5 Conclusion and outlook

Rhamnolipids are versatile surfactants based on renewable substrates produced by *Pseudomonas aeruginosa*. *P. aeruginosa* PAO1 has the potential to produce rhamnolipids in high enough quantities to match with well-established production strains like DSM 7108 and DSM 2874. PAO1 not only seems to be an appropriate model, but surprisingly could be shown to display the potential as a strain of choice for further biotechnological rhamnolipid production development. A platform batch process was established, thus leaving space for higher productivities and yields with PAO1 in future studies. In short, the main findings can be summarized as the following:

- The sequenced type strain *P. aeruginosa* PAO1 is capable to produce high quantities of rhamnolipid and thus is an appropriate model for rhamnolipid production development (cf. Chapter 2)
- PAO1 was proven to display the potential as a strain of choice for further biotechnological rhamnolipid production development (cf. Chapter 2)
- A robust, reproducible batch platform process was established (cf. Chapter 2)
- The often stated general presumption that nitrogen exhaust initiates the rhamnolipid production was disproved for bioreactor cultivations with three different strains (cf. Chapter 3)
- The secondary metabolite character of the rhamnolipid production rate was quantitatively shown for three different strains (cf. Chapter 3)
- Specific rhamnolipid production rates and growth rates during rhamnolipid production in bioreactor environment were quantified and intensively evaluated for the first time (cf. Chapter 3)
- It became obvious that the qualitative procedures during rhamnolipid production regulation are well understood, while quantitatively understanding is still missing (cf. Chapter 4)
- The question of quantitative understanding of the rhamnolipid production regulation and metabolism was explicitly posed for the first time (cf. Chapter 4)

Rhamnolipids are promising biosurfactants which over-production could be optimized by an integrated rational whole-cell biocatalyst and bioprocess design methodology, termed systems biotechnology. Additionally, detailed mathematical description of the biological systems may combine kinetic information with stoichiometric models and integrate into an engineering design cycle of *measure*, *model* and *manipulate*. With the help of the investigated model strain PAO1 and the proposed systems biotechnological methodology the economically viable rhamnolipid production process is in the not-so-distant future.

The decoupling of the bacterial growth from the RL production regulation will play a key role in this approach. This could be done by genetic alteration of *P. aeruginosa* strains, “alternative” non-pathogenic natural RL producers, or the development of respective heterologous hosts. *P. aeruginosa* PAO1 could be used as a reference organism to quantitatively characterise the rhamnolipid production metabolism using genetic engineering and omics examination, and to use it as a guide for efficient recombinant production in non-pathogenic strains like *Pseudomonas fluorescens*.

The aim of further process and genetic engineering should be a two-stage process with:

(1) Relatively high amounts of biomass in short time in the first stage
(2) Constantly high specific RL production rates ($q_{RL;spec}$) in the second stage
(3) Cost effective downstream processing dependent on final product needs
(4) Optimal substrate conversion rates and economics ($Y_{RL/S}$)
(5) Avoidance or control of quorum sensing regulation

6 Appendix

6.1 Abbreviations and symbols

2-DE	2-D electrophoresis
3-oxo-C_{12}-HSL	N-3-oxo-dodecanoyl-homoserine lactone
ACP	acyl carrier protein
AHL	N-acylhomoserine lactone
APG	alkylpolyglycoside
AQ	2-alkylquinolone
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
b	logistic shape parameter
B.	*Burkholderia* or *Bacillus*
BDM	biodrymass
BDM	biodrymass (g/L)
C/N	carbon to nitrogen ratio (g/g or mol/mol)
C_4-HSL	N-butanoyl-homoserine lactone
CER	carbon-dioxide evolution/exhaust/emission rate (mmol/L·h)
cf.	confer; compare
c_i	concentration of component *i* (mol/L or g/L)
CMC	critical micelle concentration
-C_n	fatty acid or hydroxyfatty acid with *n* C-atoms
CoA	coenzyme A
conc.	concentration
C-source/-limitation	carbon source/limitation
D	dilution rate (h^{-1})
DNA	deoxyribonucleic acid
DOE	design of experiments
DSM	German Collection of Microorganisms Deutsche Sammlung von Mikroorganismen
DSMZ	German Collection of Microorganisms and Cell Cultures Deutsche Sammlung von Mikroorganismen und Zellkulturen
dTDP	deoxythymidine 5'-diphosphate
E.	*Escherichia*
e.g.	exempli gratia; for example

EC	enzyme commission number
EGAS-L	exhaust gas analyzing system
EOR	enhanced oil recovery
EPA	Environmental Protection Agency
EPO	European Patent Office
EPS	exopolysaccharide
FAB	fast atom bombardment
FAS	fatty acid synthetase
FDA	Food and Drug Administration
FNR	Fachagentur Nachwachsende Rohstoffe e.V.
FTIR	Fourier transform infrared spectroscopy
GC	gas chromatography
HAA	3-(3-hydroxyalkanoyloxy)alkanoate
HAQ	4-hydroxy-2-alkylquinoline
HPLC	high-pressure liquid chromatography
IR	infrared
ISO	International Organization for Standardization
ISPR	*in situ* product removal
KCTC	Korean Collection of Type Cultures
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KPDG	2-keto-3-deoxy-6-phosphogluconate
L1	safety level 1 according to German Infection Protection Law
L2	safety level 2 according to German Infection Protection Law
LB	lysogeny broth
LC	liquid chromatography
LPS	lipopolysaccharide
m/m	mass per mass (g/g)
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MEOR	microbial enhanced oil recovery
MFCS	mass flow controller system
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MW	molecular weight (g/mol)
n/n	amount of substance per amount of substance (mol/mol)
NADPH/$NADP^+$	nicotinamide adenine dinucleotide phosphate

NMR	nuclear magnetic resonance
N-source/limitation	nitrogen source/limitation
OD_X	optical density at X nm (-)
OECD	Organisation for Economic Cooperation and Development
OUR	oxygen uptake rate (mmol/L·h)
P.	*Pseudomonas*
P. a.	*Pseudomonas aeruginosa*
PAI	*Pseudomonas* autoinducer
PGD	*Pseudomonas* Genome Database
pH	$-\log_{10}$ of $[H^+]$
PHA	polyhydroxyalkanoate
pK_a	$-\log_{10}$ of the acid dissociation constant (K_a)
pO_2	dissolved oxygen (%)
PQS	*Pseudomonas* quinolone signal
P_{RL}	specific rhamnolipid productivity (g/(g·h))
P-source/-limitation	phosphor source/limitation
PTCC	Persian Type Culture Collection
P_v	volumetric productivity per woking volume (g/(L·h))
$q_{RL,spec}$	specific rhamnolipid productivity (g/(g·h))
QS	quorum sensing
Rha	L-rhamnose
Rha-C_m-C_n	mono-rhamnolipid with hydroxyfatty acid moieties containing m and n C-atoms
rhamnolipid 1 Rha-C_{10}-C_{10}	α-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate
rhamnolipid 2 Rha-C_{10}	α-L-rhamnopyranosyl-3-hydroxydecanoate
rhamnolipid 3 Rha-Rha-C_{10}-C_{10} Rha_2-C_{10}-C_{10}	α-L-rhamnopyranosyl-(1-2)-α-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate
rhamnolipid 4 Rha-Rha-C_{10}	α-L-rhamnopyranosyl-(1-2)-α-L-rhamnopyranosyl-3-hydroxydecanoate
Rha-Rha-C_m-C_n Rha_2-C_m-C_n	di-rhamnolipid with hydroxyfatty acid moieties containing m and n C-atoms
RhlAB	rhamnosyltransferase 1
RhlC	rhamnosyltransferase 2
r_i	production or consumption rate of component *i* (g/(L·h))

RL	rhamnolipid
RNA	ribonucleic acid
rpm	revolutions per minute
RQ	respiratory quotient; CER/OUR (mmol/mmol)
RSM	response surface modelling
RSO	residual soybean oil
SCER	specific carbon-dioxide evolution rate per biodrymass (mmol/g·h)
SDS	sodium dodecyl sulphate
SOUR	specific oxygen uptake rate per biodrymass (mmol/g·h)
sp. spec.	species
spp.	species pluralis
T	temperature (°C) or absolute temperature (K)
t	cultivation or process time (h)
t_d	cell doubling time (h)
USD	US dollar
UV/vis	ultraviolet and visible spectrum
V	volume (L)
v/v	volume per volume (L/L)
V_L	working volume (L)
w/v	weight per volume (g/L)
$Y_{i/j}$	substrate conversion rate/coefficient/yield of component *i* to *j* (g/g)
$\alpha C_{10:1}$	α-decenoic acid
γ_{min}	minimum interfacial tension (mN/m)
σ^{54}	alternative sigma factor RpoN
σ_{min}	minimum surface tension (mN/m)
σ^{S}	alternative sigma factor RpoS
μ	specific growth rate (h^{-1})

6.2 Formulae

General mass balance

$$\frac{dM}{dt} = \frac{d[\int c_i dV]}{dt} = F_{\text{in}} \times c_{\text{in}} - F_{\text{out}} \times c_{\text{out}} + \int r_i \times dV \rightarrow \text{in} \left(\frac{\text{g}}{\text{h}}\right) \qquad \text{(Equation 5–1)}$$

with

M	= total mass (g)
t	= time (h)
$F_{\text{in/out}}$	= flow *in/out* of the system (L/h)
$c_{\text{in/out}}$	= concentration of component *i* in the *in/out* flow (g/L)
V	= reaction volume (L)
r_i	= production or consumption rate of component *i* (g/(L·h))

In this work mainly batch cultivations were done. Thus for most of the components $F_{\text{in}} = F_{\text{out}} = 0$. However, this is not true for CO_2 and O_2, respectively. The mass balance for batch cultivation is:

$$\frac{dM}{dt} = r_i \times V_{\text{L}} \rightarrow \text{in} \left(\frac{\text{g}}{\text{h}}\right) \qquad \text{(Equation 5–2)}$$

with

V_{L}	= working volume (L)
r_i	= production or consumption rate of component *i* (g/(L·h))

V_{L} is expected to be constant in the case of batch cultivation and thus the general concentration balance of a component *i* is:

$$r_i = \frac{\Delta M}{V_{\text{L}} \Delta t} = \frac{\Delta c_i}{\Delta \text{t}} \rightarrow \text{in} \left(\frac{\text{g}}{\text{L·h}}\right) \qquad \text{(Equation 5–3)}$$

with

V_{L}	= working volume (L)
r_{i}	= production or consumption rate of component *i* (g/(L·h))
Δc_{i}	= produced or consumed amount of component *i* (g/L)
Δt	= reaction time (h)

Cellular growth

The general balance (cf. Equation 5–3) can be applied to biomass growth and leads together with the Monod kinetics to the following Equation 5–4 for the specific growth rate (μ) and Equation 5–5 for the doubling time (t_d):

$$\mu = \frac{r_x}{c_x} = \frac{\ln\left(\frac{c_{x,t_2}}{c_{x,t_1}}\right)}{\Delta t_{2,1}} \rightarrow \text{in } \left(\frac{1}{h}\right) \qquad \text{(Equation 5–4)}$$

$$t_d = \frac{\ln 2}{\mu} \; and \; t_{d,min} = \frac{\ln 2}{\mu_{max}} \rightarrow \text{in (h)} \qquad \text{(Equation 5–5)}$$

with

c_x	= BDM (g/L)
r_x	= growth rate (g/(L·h))
μ	= specific growth rate (h^{-1})
t_d	= doubling time (h)
$t_{d,min}$	= minimal doubling time (h)

Fitting of biodrymass with logistic growth

$$c_x(t) = c_{x,0} + \frac{\Delta c_{x,max}}{1+\left(\frac{t}{t^*_{c_x}}\right)^b} \rightarrow \text{in } \left(\frac{g}{L}\right) \qquad \text{(Equation 5–6)}$$

with

c_x (t)	= BDM at cultivation time t (g/L)
$c_{x,0}$	= BDM at t = 0 h after inoculation (g/L)
$\Delta c_{x,max}$	= maximum additionally to $c_{x,0}$ produced BDM (g/L)
t	= cultivation time (h)
$t^*_{c_x}$	= cultivation time when ½ $\Delta c_{x,max}$ is reached (h)
b	= logistic shape parameter (-)

Fitting of rhamnolipid concentration with logistic lapse

$$c_{RL}(t) = \frac{c_{RL,max}}{1+\left(\frac{t}{t^*_{c_{RL}}}\right)^b} \rightarrow \text{in } \left(\frac{g}{L}\right) \qquad \text{(Equation 5–7)}$$

with

c_{RL} (t)	= RL concentration at cultivation time t (g/L)
$\Delta c_{RL,max}$	= maximum produced RL (g/L)
t	= cultivation time (h)
$t^*_{c_{RL}}$	= cultivation time when ½ $\Delta c_{RL,max}$ is reached (h)
b	= logistic shape parameter (-)

Specific rhamnolipid production rate

$$q_{\mathrm{RL,spec}} \approx \frac{\Delta c_{\mathrm{RL},t_{2,1}}}{\Delta t_{2,1} \times \bar{c}_{\mathrm{x},t_{1,2}}} = \frac{r_{\mathrm{RL}}}{\bar{c}_{\mathrm{x},t_{1,2}}} \rightarrow \text{in } \left(\frac{\mathrm{g}}{\mathrm{g \cdot h}}\right) \qquad \text{(Equation 5–8)}$$

with

$q_{\mathrm{RL,spec}}$ = specific RL production rate (g/(g·h))
$\Delta c_{\mathrm{RL},t_{2,1}}$ = produced RL between t_1 and t_2 (g/L)
$\Delta t_{2,1}$ = cultivation time difference between t_1 and t_2 (h)
$\bar{c}_{\mathrm{x},t_{1,2}}$ = mean value of BDM between t_1 and t_2 (g/L)
r_{RL} = RL production rate (g/(L·h))

Biomass by the means of nitrogen consumption

$$c_{\mathrm{x,N}}(t) = Y_{\mathrm{x/N}} \times \left(c_{\mathrm{N},t_0} - c_{\mathrm{N},t}\right) = \frac{\left(c_{\mathrm{N},t_0} - c_{\mathrm{N},t}\right)}{Y_{\mathrm{N/x}}} \rightarrow \text{in } \left(\frac{\mathrm{g}}{\mathrm{L}}\right) \qquad \text{(Equation 5–9)}$$

with

$c_{\mathrm{x,N}}(t)$ = BDM theoretically produced from consumed nitrogen (g/L)
$Y_{\mathrm{x/N}}$ = nitrogen conversion coefficient (g/g)
$Y_{\mathrm{x/N}}$ = BDM from nitrogen coefficient (g/g)
c_{N,t_0} = nitrogen concentration at t = 0 h (g/L)
$c_{\mathrm{N},t}$ = nitrogen concentration at cultivation time t (g/L)

Biodrymass from nitrogen coefficient

$$Y_{\mathrm{N/x}} = \left|\frac{r_{\mathrm{N}}}{r_{\mathrm{x}}}\right| = \left|\frac{\Delta c_{\mathrm{N}}}{\Delta c_{\mathrm{x}}}\right| \rightarrow \text{in } \left(\frac{\mathrm{g}}{\mathrm{g}}\right) \qquad \text{(Equation 5–10)}$$

with

$Y_{\mathrm{N/x}}$ = BDM from nitrogen coefficient (g/g)
r_{N} = nitrogen consumption rate (g/(L·h))
r_{x} = growth rate (g/(L·h))
Δc_{N} = consumed nitrogen (g/L)
Δc_{x} = produced BDM (g/L)

Product yield coefficient/substrate conversion rate

$$Y_{\mathrm{P/S}} = \left|\frac{r_{\mathrm{P}}}{r_{\mathrm{S}}}\right| = \left|\frac{\Delta c_{\mathrm{P}}}{\Delta c_{\mathrm{S}}}\right| \rightarrow \text{in } \left(\frac{\mathrm{g}}{\mathrm{g}}\right) \qquad \text{(Equation 5–11)}$$

with

$Y_{\mathrm{P/S}}$ = substrate conversion rate (g/g)
r_{P} = production rate (g/(L·h))
r_{S} = substrate consumption rate (g/(L·h))
Δc_{P} = produced product (g/L)
Δc_{S} = consumed substrate (g/L)

Rhamnolipid yield coefficient

$$Y_{\mathrm{RL/S}} = \left|\frac{r_{\mathrm{RL}}}{r_{\mathrm{S}}}\right| = \left|\frac{\Delta c_{\mathrm{RL}}}{\Delta c_{\mathrm{S}}}\right| \rightarrow \text{in } \left(\frac{\mathrm{g}}{\mathrm{g}}\right) \qquad \text{(Equation 5–12)}$$

with

$Y_{\mathrm{RL/S}}$	= substrate to RL conversion rate (g/g)
r_{RL}	= RL production rate (g/(L·h))
r_{S}	= substrate consumption rate (g/(L·h))
Δc_{RL}	= produced RL (g/L)
Δc_{S}	= consumed substrate (g/L)

Volumetric productivity

$$P_{\mathrm{V}} = \frac{c_{\mathrm{RL,max}}}{t_{\mathrm{RL,max}}} \rightarrow \text{in } \left(\frac{\mathrm{g}}{\mathrm{L{\cdot}h}}\right) \qquad \text{(Equation 5–13)}$$

with

P_{V}	= volumetric productivity (g/(L·h))
$c_{\mathrm{RL,max}}$	= total produced RL (g/L)
$\Delta t_{\mathrm{RL,max}}$	= cultivation time where $c_{\mathrm{RL,max}}$ is reached (h)

Equations for exhaust gas analysis

$$\mathrm{OUR} = q_{\mathrm{O_2}} = \frac{\dot{V}_{\mathrm{G}}^{\alpha} \times p_{\mathrm{tot}}}{V_{\mathrm{L}} \times R \times T^{\alpha}} \times \left(y_{O_2}^{\propto} - \frac{1 - y_{O_2}^{\propto} - y_{CO_2}^{\propto}}{1 - y_{O_2}^{\omega} - y_{CO_2}^{\omega}} \times y_{O_2}^{\omega}\right) \rightarrow \text{in } \left(\frac{\mathrm{mmol}}{\mathrm{L{\cdot}h}}\right) \qquad \text{(Equation 5–14)}$$

$$\mathrm{CER} = q_{\mathrm{CO_2}} = \frac{\dot{V}_{\mathrm{G}}^{\alpha} \times p_{\mathrm{tot}}}{V_{\mathrm{L}} \times R \times T^{\alpha}} \times \left(y_{CO_2}^{\omega} \times \frac{1 - y_{O_2}^{\propto} - y_{CO_2}^{\propto}}{1 - y_{O_2}^{\omega} - y_{CO_2}^{\omega}} - y_{CO_2}^{\alpha}\right) \rightarrow \text{in } \left(\frac{\mathrm{mmol}}{\mathrm{L{\cdot}h}}\right) \qquad \text{(Equation 5–15)}$$

with

OUR	= oxygen uptake rate (mmol/(L·h))
CER	= carbon-dioxide exhaust/evolution/emission rate (mmol/(L·h))
α	= index for inlet gas
ω	= index for exhaust gas
$\dot{V}_{\mathrm{G}}^{\alpha}$	= gasflow rate (L/h)
p_{tot}	= ambient pressure (bar)
V_{L}	= working volume (L)
R	= ideal gas constant ((bar·L)/(mol·K))
T^{α}	= absolute temperature (K)
y_i	= molar fraction of the gas component *i*

Equations for specific exhaust gas analysis

$$\mathrm{SOUR} = \frac{q_{O_2}}{c_x} = \frac{\mathrm{OUR}}{c_x} \rightarrow \text{in } \left(\frac{\mathrm{mmol}}{\mathrm{g \cdot h}}\right) \qquad \text{(Equation 5–16)}$$

$$\mathrm{SCER} = \frac{q_{CO_2}}{c_x} = \frac{\mathrm{CER}}{c_x} \rightarrow \text{in } \left(\frac{\mathrm{mmol}}{\mathrm{g \cdot h}}\right) \qquad \text{(Equation 5–17)}$$

$$\mathrm{RQ} = \frac{\mathrm{CER}}{\mathrm{OUR}} = \frac{\mathrm{SCER}}{\mathrm{SOUR}} \rightarrow \text{in } \left(\frac{\mathrm{mmol}}{\mathrm{mmol}}\right) \qquad \text{(Equation 5–18)}$$

with

SOUR	= specific OUR per BDM (mmol/(g·h))
SCER	= specific CER per BDM (mmol/(g·h))
c_x	= BDM (g/L)
RQ	= respiratory quotient (mmol/mmol)