New Approaches for the Economic Production of Rhamnolipid Biosurfactants from Renewable Resources

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Erklärung

Ich versichere, dass die hier vorliegende Dissertion mit dem eingereichten und genehmigten Prüfungsexemplar der Doktorarbeit übereinstimmt.

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Abstract

Rhamnolipids are versatile biosurfactants with excellent interfacial properties and additional ecological features such as biodegradability and biocompatibility. Furthermore, they display various biological activities including antimicrobial, antiviral and antifungal activity. Rhamnolipids are commonly produced biotechnologically with *Pseudomonas aeruginosa* in batch- or fed-batch cultivations whereas different substrates like plant oil, glycerol, sugars and even hydrocarbons can be employed. Although rhamnolipid production has been intensively studied since the 1980´s, rhamnolipids have not widely succeeded in substituting synthetic surfactants; rather their use is restricted to specific applications where biocompatibility is required. The main reason for this situation can be found in the high costs for synthesis and downstream processing of rhamnolipids. The development of new production processes is the key issue in overcoming these economic obstacles. Therefore, different aspects of the production process were addressed in this work, from the upstream to the downstream processing.

The first approach was the investigation of different glycerol based waste substrates as cost-saving alternatives to the substrate plant oil. Good results were obtained for crude glycerol from biodiesel manufacturing, although this substrate contains impurities from the harsh biodiesel manufacturing process. The specific productivity for crude glycerol was even higher than for pure glycerol. Thus, crude glycerol is a cost-saving alternative to the conventional substrate plant oil.

In-situ product removal represents another possibility to improve rhamnolipid production. It facilitates the purification of the rhamnolipids and reduces the foam problems associated with rhamnolipid production. Therefore, the second approach aimed at an integrated process with *in-situ* product removal of the rhamnolipids and immobilised cells. Different immobilisation methods were evaluated for this purpose. *P. aeruginosa*, however, was deemed inappropriate for immobilisation due to its high mobility which led to a cell leakage of the particles. Furthermore, due to low productivities of the immobilised cells and mass transfer problems, the integrated concept could not be realised.

The third approach was to search for a non-pathogenic production strain as the common production strain *P. aeruginosa* is pathogenic, which imposes essential safety implications for an industrial application. Therefore, eleven non-pathogenic strains which were already described as rhamnolipid producing strains were selected from literature; however, only limited information was available about rhamnolipid production of these strains: the structures of the generated rhamnolipids were mostly not determined and

few production processes at bioreactor scale were reported. In some cases, the rhamnolipids were not even quantified. Therefore, a shake flask screening with intensive analytics was carried out first to evaluate the rhamnolipid production of the non-pathogenic strains. Different media and substrates were tested. While three strains did not show rhamnolipid formation in shake flask, eight non-pathogenic strains were successful. However, rhamnolipid formation was very low in shake flasks. Therefore, the eight strains were transferred to bioreactor scale and cultivated in a 6-fold parallel bioreactor system. Two strains of the species *B. plantarii* were most successful in these cultivations giving relative high yields of biomass and rhamnolipid. Especially *B. plantarii* DSM 9509 is of interest because it was not yet described as rhamnolipid producer. Therefore, the production process with this strain was scaled up to 40 L scale to obtain higher amounts of rhamnolipid for purification and structure elucidation. This process yielded over 100 g of crude rhamnolipid extract. Different chromatographic methods were evaluated for the purification of the *B. plantarii* rhamnolipids: thick layer chromatography, fast centrifugal partition chromatography and column chromatography. A combination of two chromatographic steps was most effective for the purification of *B. plantarii* rhamnolipids. The structure of these rhamnolipids was elucidated by mass spectrometry and unconventional rhamnolipids were found. *B. plantarii* synthesises mainly RL-2,2_{14}, a rhamnolipid with two rhamnose moieties and two ß-hydroxy tetradecane acid moieties. These rhamnolipids are interesting for cleaning applications due to their longer hydrophobic chains. In summary, it was demonstrated that the non-pathogenic rhamnolipid producing species *B. plantarii* is a rewarding alternative to the conventional, pathogenic rhamnolipid production strain *P. aeruginosa*. However, further process optimisation has to be carried out to enhance rhamnolipid production with this strain.

In this work, new approaches for the production of rhamnolipids have been elucidated. Especially the utilisation of waste substrates like crude glycerol from biodiesel manufacturing, the application of the non-pathogenic production strain *B. plantarii* and the implementation of new rhamnolipid recovery methods like fast centrifugal partition chromatography can contribute to improved rhamnolipid production processes. Accordingly, the economic obstacles of rhamnolipids may eventually be overcome and rhamnolipids may find broader application.

Zusammenfassung

Rhamnolipide sind vielseitige Biotenside mit hervorragenden Tensideigenschaften und zusätzlichen ökologischen Eigenschaften wie biologische Abbaubarkeit und Biokompatibilität. Des Weiteren weisen sie zahlreiche biologische Aktivitäten wie antimikrobielle, antivirale und antifungale Aktivität auf. Rhamnolipide können biotechnologisch mit *Pseudomonas aeruginosa* in Batch- oder Fed-batch-Fermentationen hergestellt werden, wobei verschiedene Substrate wie Pflanzenöl, Glycerin, Zucker und sogar Kohlenwasserstoffe eingesetzt werden. Obwohl Rhamnolipidproduktion bereits seit den 1980ern intensiv untersucht wird, sind Rhamnolipide immer noch nicht konkurrenzfähig zu synthetischen Tensiden. Der Hauptgrund liegt in den hohen Synthese- und Aufreinigungskosten der Rhamnolipide. Neue Produktionsprozesse sind der Schlüssel, um diese Hindernisse zu überwinden. Deshalb befasst sich diese Arbeit mit verschiedene Aspekte der Rhamnolipidproduktion, vom den Produktionsstämmen bis zur Aufreinigung.

Der erste Ansatz war die Verwendung von glycerin-basierte Abfallsubstraten als kostengünstige Alternativen zu dem Standardsubstrat Pflanzenöl. Dabei konnten mit Rohglycerin aus der Biodieselherstellung gute Ergebnisse erzielt werden, trotzdem dieses Substrat Verunreinigungen aus der Biodieselherstellung enthält. Die spezifische Produktivität mit Rohglycerin war sogar höher als mit reinem Glycerin. Folglich ist Rohglycerin aus der Biodieselherstellung eine kostensparende Alternative zum Standardsubstrat Pflanzenöl.

In-situ Produktentfernung stellt eine weitere Möglichkeit zur Verbesserung des Rhamnolipidprozesses dar. Dadurch kann die Aufreinigung des Rhamnolipids erleichtert werden sowie die erheblichen Schaumprobleme bei der Rhamnolipidproduktion verringert werden. Deshalb zielte der zweite Ansatz auf die Entwicklung eines integrierten Prozesses mit *in-situ* Produktentfernung und immobilisierten Zellen. Dafür wurden verschiedene Immobilisierungsmethoden evaluiert, jedoch war *P. aeruginosa* aufgrund seiner hohen Mobilität ungeeignet für die Immobilisierung. Auch weil nur geringe Produktivitäten erzielt wurden und aufgrund von Massentransferproblemen konnte das integrierte Konzept nicht realisiert werden.

Der dritte Ansatz war die Suche nach einem nichtpathogenen Produktionsstamm da der herkömmliche Produktionsstamm *P. aeruginosa* gesundheitsgefährdend ist, was eine industrielle Anwendung des Stammes erheblich erschwert. Dafür wurden 11 nichtpa-

thogene Stämme aus der Literatur ausgewählt, für die bereits Rhamnolipidbildung beschrieben wurde. Jedoch war wenig über die Rhamnolipidbildung dieser Stämme bekannt: die Strukturen der gebildeten Rhamnolipide waren größtenteils nicht aufgeklärt und wenige Produktionsprozesse im Bioreaktorformat waren beschrieben. In einigen Fällen wurde das Rhamnolipid nicht mal quantifiziert. Deshalb wurde ein Schüttelkolbenscreening mit ausführlicher Analytik durchgeführt, um die Rhamnolipidbildung der Stämme zu beurteilen. Dabei wurden verschiedene Medien und Substrate untersucht. Während drei Stämme keine Rhamnolipidbildung im Schüttelkolben zeigten, waren acht der Stämme erfolgreich im Schüttelkolben. Allerdings war die Rhamnolipidbildung im Schüttelkolben sehr gering. Daher wurden die acht Stämme auf den Bioreaktormaßstab übertragen und in einem Sechsfachparallelreaktor kultiviert. Zwei Stämme der Spezies *B. plantarii* waren am erfolgreichsten und erzielten gutes Wachstum und relativ hohe Ausbeuten an Rhamnolipid. Einer der Stämme, *B. plantarii* DS 9509, ist besonders interessant, da er noch nicht als Rhamnolipidbildner beschrieben wurde. Daher wurde der Prozess mit diesem Stamm auf den 40 L-Maßstab übertragen, um größere Mengen Rhamnolipid für Aufreinigung und Strukturaufklärung zu gewinnen. Über 100 g Rohextrakt konnten gewonnen werden, die mit verschiedenen chromatographischen Methoden aufgereinigt wurden. Dabei wurden Dickschicht-Chromatographie, Flüssig-Flüssig-Chromatographie und Säulenchromatographie untersucht. Eine Kombination von zwei verschiedenen Säulenchromatographieschritten war am geeignetsten, um die Rhamnolipide von *B. plantarii* aufzureinigen. Die Struktur der gebildeten Rhamnolipide wurde mittels Massenspektrometrie aufgeklärt, wobei ungewöhnliche Rhamnolipide gefunden wurden. *B. plantarii* bildet vor allem RL-2,2_{14}, ein Rhamnolipid mit zwei Rhamnoseeinheiten und zwei ß-Hydroxy-Tetradecansäuren. Diese Rhamnolipide sind interessant für verschiedene Anwendungen. Es wurde in dieser Arbeit gezeigt, dass das nichtpathogene Bakterium *B. plantarii* eine aussichtsreiche Alternative zu dem konventionellen, pathogenen Rhamnolipid-Produktionsstamm *P. aeruginosa* ist, auch wenn eine weitere Prozessoptimierung nötig ist um die Rhamnolipid-Bildung dieses Stammes zu steigern.

In summary, it was demonstrated that the non-pathogenic rhamnolipid producing species *B. plantarii* is a rewarding alternative to the conventional, pathogenic rhamnolipid production strain *P. aeruginosa*. However, further process optimisation would have to be carried out to maximise rhamnolipid production with this strain.

In dieser Arbeit wurden verschiedene Ansätze zur Verbesserung von Rhamnolipidproduktionsprozessen untersucht. Besonders die Verwendung von Abfallsubstraten wie Rohglycerin aus der Biodieselherstellung, der Einsatz des nichtpathogenen Produktionsstammes *B. plantarii* und die Integration von neuen Aufreinigungsmethoden wie der Flüssig-Flüssig-Chromatographie können zu verbesserten Produktionsprozessen beitragen. Dadurch könnte die ökonomische Hürde der

Rhamnolipide möglicherweise überwunden werden, so dass Rhamnolipide breitere Anwendung finden könnten.

Content

1 Introduction

1.1 Biosurfactants

Surfactants are amphipathic molecules that are composed of a non-polar hydrophobic part attached to a hydrophilic head group which can be polar or ionic (see Figure 1). Due to this amphipathic character, they have an interfacial activity and tend to adsorb to interfaces, e.g. between water/oil or water/air.

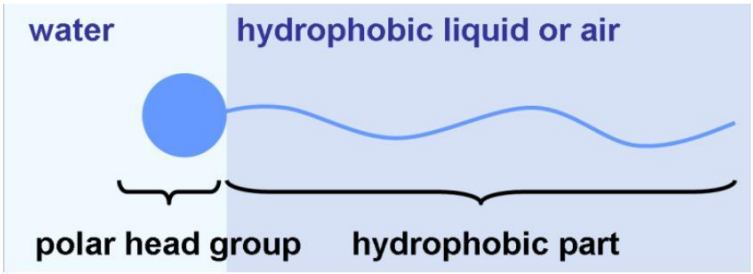


Figure 1: Structure of a surfactant

Surfactants aggregate in solution forming different structures, e.g. micelles, inverse micelles, vesicles or lamella (see Figure 2). In the micelle, the hydrophobic groups of the surfactant are directed towards the interior of the aggregates while the polar head groups are directed towards the solvent (Tadros 2005).

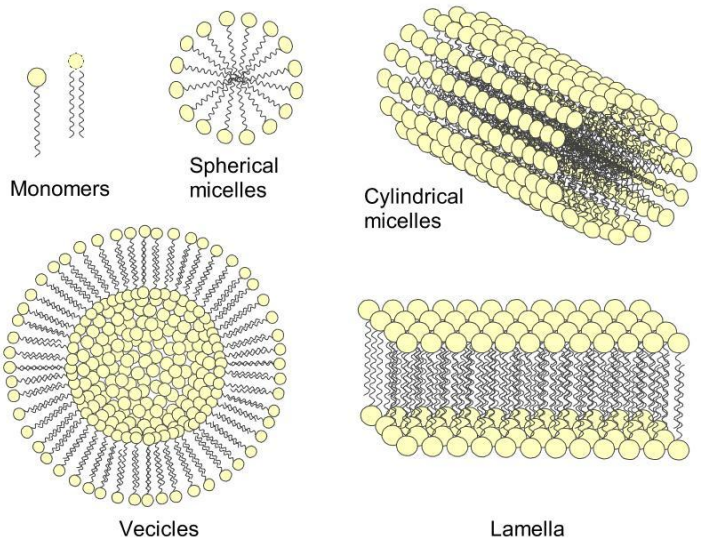


Figure 2: Aggregates of surfactant molecules in aqueous solution (Magario 2008)

A biosurfactant is a biologically produced surfactant. Of special interest are microbial biosurfactants which are produced by bacteria, fungi or yeasts as extracellular compounds. Rhamnolipids, which are the subject of this work, are commonly produced by the bacterium *Pseudomonas aeruginosa*.

Biosurfactants display a wide variety of molecular structures. The hydrophilic moiety of biosurfactants often contains a mono-, oligo- or polysaccharide, an amino acid, peptide or protein, whereas the hydrophobic part is composed of saturated or unsaturated fatty acids, hydroxy fatty acids or fatty alcohols (Lang 2002). Biosurfactants are classified according to Lang and Wullbrandt 1999 in:

- glycolipids (e.g. rhamnolipids, sophorolipids, trehalose lipids)
- lipoamino acids and lipopeptides (e.g. surfactin, liposan)
- polymers (e.g. lipoproteins, lipopolysaccharides)
- phospholipids, mono- and diglycerides, fatty acids

Surfactants can be derived from petrochemical or renewable resources. Originally manufactured from renewable resources like fats and oils, today a substantial amount of surfactants are of petrochemical origin (Deleu and Paquot 2004). The advantages of biosurfactants are:

- structural diversity
- good interfacial properties
- low aquatic toxicity (Poremba et al. 1991; Develter et al. 2007)
- biological activities covering antibiotics, fungicides, insecticides, antiviral and antitumoral agents, immunomodulators or specific toxins or enzyme inhibitors (Deleu and Paquot 2004)
- biodegradability (Poremba et al. 1991; Develter et al. 2007)
- production from renewable resources.

1.2 Rhamnolipids

Rhamnolipids are anionic glycolipids composed of L-rhamnose and ß-hydroxy fatty acids. The hydrophilic rhamnose moiety is attached by a glycosidic linkage to the lipid fatty acid tail. Rhamnolipids were first isolated from *P. aeruginosa* by Jarvis and Johnson in 1949 (Jarvis and Johnson 1949). Rhamnolipids are generated as a mixture of different structure variants. The main rhamnolipids of *P. aeruginosa* are rhamnolipid 1 (RL1, L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate) and rhamnolipid 3 (RL3,

L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate) (Syldatk et al. 1985b). RL1 is a mono-rhamnolipid with one rhamnose unit while RL3 contains two rhamnose units and is a di-rhamnolipid. Moreover, RL2 and RL4 are also produced by *P. aeruginosa* in minor amounts. These rhamnolipids contain only one ß-hydroxy fatty acid. The structures of the four major rhamnolipids of *P. aeruginosa* are shown in Figure 3.

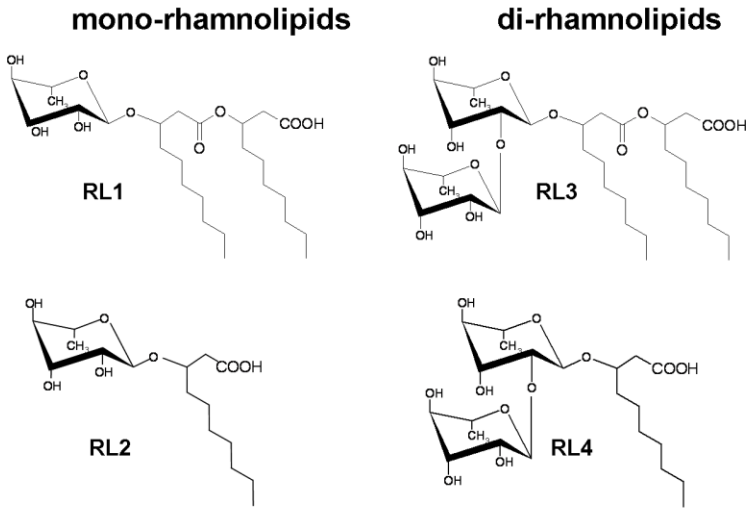


Figure 3: Structure of the rhamnolipids RL1 – 4 of *P. aeruginosa*

More than 20 other rhamnolipid structures are synthesized by *P. aeruginosa* in trace amounts (Déziel et al. 1999). These structures vary in the number of ß-hydroxy fatty acids (one or two), in the number of rhamnose units (one or two) and in the chain length and saturation of the ß-hydroxy fatty acids.

Rhamnolipids display strong interfacial activity (Syldatk et al. 1985b); for example, RL1 and RL3 reduce the surface tension of water from 72 to 31 mN/m. The critical micelle concentration is another important parameter for evaluation of the performance of a surfactant. The cmc is the concentration at which the surfactant monomers in a solution start to form micelles. The cmc of rhamnolipids is very low, about 20 mg/L for RL1 and RL3 in water. Expressed in molar concentrations, this is $3{,}96 \cdot 10^{-5}$ mol/L (RL1) resp. $3{,}07 \cdot 10^{-5}$ mol/L (RL3). In comparison, the cmc of sodium dodecyl sulphate, is much higher: $8{,}39 \cdot 10^{-3}$ (Tadros 2005)

Nomenclature of rhamnolipids

Different rhamnolipid nomenclatures are found in literature. In this work, the following nomenclature is used for rhamnolipids:

RL-X,Y_z stands for a rhamnolipid with x rhamnose units and y ß-hydroxy fatty acid chains of chain length z. Consequently, RL 1 is also termed RL-1,2_{10} and RL 3 is equivalent to RL-2,2_{10}.

1.2.1 Application of rhamnolipids

Biosurfactants are scarcely able to compete with established synthetic surfactants from an economical point of view because their production costs are currently too high. The production costs of biosurfactants, compared to synthetic compounds on the surfactant market, are at least 50 times higher, depending on the biosurfactant and its purity (Deleu and Paquot 2004). Lang and Wullbrandt estimated the production costs of rhamnolipids produced in 20 – 100 m^3 scale at about 5 – 20 US $/kg (Lang and Wullbrandt 1999). Compared with petrochemical bulk surfactants like ethoxylates or alkyl polyglycosides, ranging at 1 – 3 US $/kg, rhamnolipids are not competitive in this field.

However, applications of biosurfactants arise whenever the biological origin gives better biocompatibility and biodegradation. Furthermore, sustainability is gaining more and more importance today. As a consequence, the interest in rhamnolipids is increasing. Due to their interfacial and also pharmaceutical activity, rhamnolipids can be applied in many fields, for example:

Cleaning

Rhamnolipids have a high potential for eco-friendly cleaning solutions due to their excellent biodegradability and low aquatic toxicity (Develter et al. 2007).

Pharmaceutical applications

Rhamnolipids can be used for the stabilisation of water/oil formulations or, due to their antimicrobial activity, even as active pharmaceutical ingredients (Haba et al. 2003). They enhance wound healing (Stipcevic et al. 2006) and are effective against various dermatologic diseases (Piljac and Piljac 1995; Stipcevic et al. 2004).

Environmental applications

Rhamnolipids have been applied for the bioremediation of contaminated soils (Benincasa 2007). Due to their interfacial activity, rhamnolipids enhance the bioavailability of hydrocarbon contaminants in soils and allow a faster degradation of these compounds (Rahman et al. 2003). Rhamnolipids are also effective for the treatment of marine oil pollutions (Lang and Wullbrandt 1999).

Food industry

If licensed for food applications, rhamnolipids could be applied in food industry as stabilisers and emulsifiers, for example for bakery and confectionary products (Van Hae-

sendonck and Vanzeveren 2006). Furthermore, they are a source of rhamnose for the synthesis of flavours (Trummler et al. 2003).

1.3 Rhamnolipid production with *P. aeruginosa*

Pseudomonas aeruginosa is the common production organism for rhamnolipid production. *P. aeruginosa* is an aerobic, motile, gram-negative rod occurring primarily in water, soil and vegetation. However, *P. aeruginosa* is able to grow and survive in almost any environment. The bacterium is able to cause a multitude of human infections. While infections in immunocompetent patients are rare, immunocompromised patients, such as cystic fibrosis or AIDS patients, are particular susceptible to opportunistic infections with *P. aeruginosa* (Van Delden and Iglewski 1998). Therefore, the organism is viewed as opportunistic and classified in the Biosafety level 2 according to the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (DSMZ). *P. aeruginosa* is able to produce a wide variety of virulence factors, including rhamnolipids, lipopolysaccharides, flagella, type IV pili, proteases, exotoxins, pyocyanins, exopolysaccharides, type III secretion, etc. (Kiil et al. 2008).

P. aeruginosa synthesises rhamnolipids as an extracellular biosurfactant. Besides the interfacial activity, rhamnolipids also display antimicrobial, antiviral, antifungal, mycoplasmacidal, algicidal, zoosporicidal, antiamoebal and hemolytic activity (Soberón-Chávez et al. 2005). According to Soberón-Chávez et al. 2005, the putative physiological functions of the rhamnolipids are:

- **Solubilization and uptake of hydrophobic substrates** due to the interfacial activity
- **Contacting of hydrophobic surfaces** due to the interfacial activity
- **Virulence factor:** Due to the interfacial and haemolytic activity, rhamnolipids contribute to tissue invasion of *P. aeruginosa,* e.g. in the lung (Van Delden and Iglewski 1998).
- **Defence from other microorganisms** due to the wide antimicrobial activity.

1.3.1 Biosynthesis

The biosynthesis of rhamnolipids in *P. aeruginosa* proceeds in two principal steps: first, precursors are synthesised and, subsequently, the linkage of the precursors takes place (Soberón-Chávez et al. 2005). An overview of the biosynthesis of rhamnolipids by *P. aeruginosa* is given in Figure 4. The intermediates of the rhamnolipid biosynthesis are also employed for other biosynthetic pathways, for example for the biosynthesis of lipopolysaccharides (LPS), polyhydroxy alkanoates (PHA) and 4-hydroxy-2-alkylquinolines (HAQ).

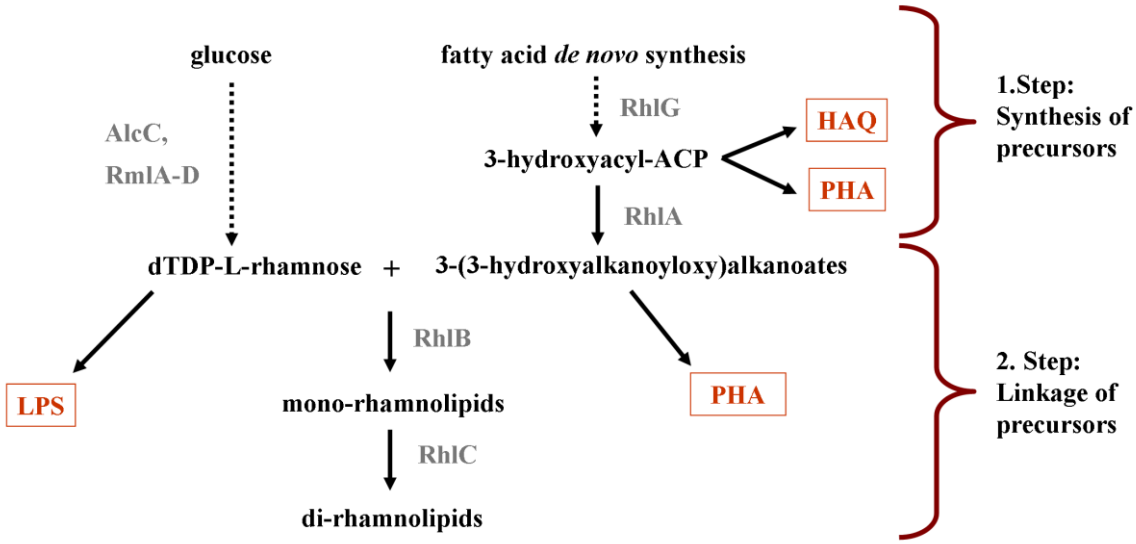


Figure 4: Rhamnolipids biosynthesis pathway of *P. aeruginosa* (according to Soberón-Chávez et al. 2005)

The precursors for the hydrophobic and the hydrophilic moiety of the rhamnolipids are dTDP-L-rhamnose and 3-(3-hydroxyalkanoyloxy)alkanoate. They are derived from the central metabolism; the activated rhamnose coming from glucose and the hydroxy fatty acids from a fatty acid *de novo* synthesis, starting with Acetyl-CoA. The rhamnosyl-transferase RhlA links the two hydroxy fatty acids to a dimer.

In the second step, the precursors dTDP-L-rhamnose and di-hydroxydecanoic acid are linked by the rhamnosyltransferase RhlB to mono-rhamnolipids. The mono-rhamnolipids can then be converted to di-rhamnolipids by the enzyme RhlC which catalyses the addition of the second rhamnose unit.

The *rhlA* and *rhlB* genes are arranged as an operon and are clustered with *rhlR* and *rhlI*, which encode proteins involved in their transcriptional regulation through the quorum sensing mechanism described below. In contrast, the *rhlC* gene is not linked to other *rhl* genes and forms an operon with a gene whose function is not known. This operon is regulated at the transcriptional level in a similar manner as *rhlAB* (Rahim et al. 2001).

The rhamnolipid formation in *P. aeruginosa* is regulated by a cell density dependent regulation system called quorum sensing (QS) which allows the bacteria to act in a co-ordinated cell density dependent manner (Ochsner and Reiser 1995). The QS system depends on the production of two autoinducers, butanoyl-homoserine lactone (C_4-HSL) and 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}HL). The autoinducers bind to specific transcriptional regulators to activate gene expression at high cell densities. Other extracellular virulence factors of *P. aeruginosa* have been shown to be regulated by quorum sensing signals as well (Pearson et al. 1997).

Rhamnolipids are secondary metabolites, and thus, their production coincides with the onset of the stationary phase. This is in agreement with the fact that transcription from the *rhlAB* promoter is primarily regulated by the cell density-dependent quorum sensing (Soberón-Chávez et al. 2005). Cultivations with *P. aeruginosa* resting cells showed an inhibition of the rhamnolipid formation upon feeding of a nitrogen source or the addition of multivalent cations, different nitrogen sources and EDTA (Syldatk et al. 1985a).

1.3.2 Production strains

Numerous rhamnolipid producing bacteria have been isolated, most of them belonging to the species *P. aeruginosa*. Depending on the required physiological roles of rhamnolipids, rhamnolipid producing bacteria can be found in and isolated from different environments. Many rhamnolipid producing microorganisms have been isolated from soil or water which is contaminated with hydrophobic compounds such as refinery wastes (Abalos et al. 2001; Rahman et al. 2002; Bodour et al. 2003; Benincasa 2007). Undisturbed sites, however, have also been successfully screened (Bodour et al. 2003). For isolation and screening of potential candidates, several techniques have been developed which are reviewed by Walter et al. 2008. Most of these screening techniques are directly based on the surface or interfacial activity of the culture supernatant, e.g. drop collapse assay, oil spreading assay or emulsification capacity assay.

Little attention is paid to other rhamnolipid producing organisms which do not belong to the species *P. aeruginosa*. These strains are reviewed in section 1.4.

The rhamnolipid product spectrum is dependent on the production strains, as has been shown for two different *P. aeruginosa* strains under identical cultivation conditions (Leitermann 2008). Therefore, the production stain should be carefully chosen, depending on the desired product spectrum.

Several attempts at the production of *Pseudomonas* rhamnolipids in heterologous hosts have been reported. Ochsner et al. 1995 cloned the *rhlAB* rhamnosyltransferase gene into various hosts, *P. fluorescens*, *P. oleovorans*, *P. putida*, and *E. coli*. The best rhamnolipid production of 60 mg/L was achieved with *P. putida* whereas no rhamnolipid production occurred in *E. coli.*

Cabrera-Valladares et al. 2006 succeeded in producing mono-rhamnolipids in *E. coli*. They found that the availability in *E. coli* of dTDP-L-rhamnose, a substrate of the rhamnosyltransferase RhlB, restricts the production of mono-rhamnolipids in *E. coli*. By coexpression of the *rhlAB* operon and the *rmlBDAC* operon which encodes the dTDP-L-rhamnose biosynthesis enzymes, they generated a rhamnolipid producing *E. coli* strain. The rhamnolipid concentration produced with this strain was 52,2 mg/L, lower than the *P. aeruginosa* level. In contrast to these findings, Wang et al. 2007 claim rhamnolipid production in *E. coli* expressing only *rhlAB*.

Cha et al. 2008 also reported the heterologous production of rhamnolipids in *P. putida*, using recombinant *rhlABRI* genes. A maximum of 7,2 g/L rhamnolipids was achieved.

1.3.3 Cultivation strategies

Various rhamnolipid production processes have been published, with different production strains, process strategies and substrates. The different approaches for rhamnolipid production are presented below.

As secondary metabolites, rhamnolipids are synthesised under growth limiting conditions, and their production coincides with the onset of the stationary phase (Lang and Wullbrandt 1999). This correlates with the fact that the production of rhamnolipids in continuous culture is favoured by low dilution rates below 0,15 h^{-1} (Guerra-Santos et al. 1984). According to this regulatory principle, all cultivation strategies for the production of rhamnolipids aim at limiting at least one medium component like the nitrogen source or multivalent ions. These limiting conditions can be maintained by the following cultivation strategies (Lang and Wullbrandt 1999):

1. (Fed)batch cultivation under growth-limiting conditions (e.g. Giani et al. 1997; Lee et al. 2004; Chen et al. 2007a)
2. Batch cultivation under resting cell conditions (e.g. Syldatk et al. 1985a)
3. Production with immobilized, resting cells (e.g. Siemann and Wagner 1993; Jeong et al. 2004)
4. Continuous cultivation under limiting conditions/with low growth rates (e.g. Guerra-Santos et al. 1984; Guerra-Santos et al. 1986; Reiling et al. 1986).

Substrates

Water-soluble or water-insoluble carbon sources have been utilised for rhamnolipid production and production processes with many different natural and petrochemical substrates have been published, for example with

- plant oils (e.g. Giani et al. 1997; Trummler et al. 2003),
- sugars (e.g. Guerra-Santos et al. 1984; Reiling et al. 1986; Lee et al. 2004),
- glycerol (e.g. Syldatk et al. 1985a; Chen et al. 2007a),
- hydrocarbons (e.g. Syldatk et al. 1985a; Syldatk et al. 1985b; Lee et al. 2004).

Waste substrates are also interesting substrates as they are usually less expensive than pure substrates. Furthermore, the utilisation of waste substrates contributes to a complete exploitation of resources and so to the sustainability of production processes. Different waste substrates have already been utilised for rhamnolipid production, for ex-

ample olive oil mill effluents (Mercadé et al. 1993), waste frying oils (Haba et al. 2000), soap stock (Benincasa 2007), whey and distillery waste (Dubey and Juwarkar 2001) or waste free fatty acids (Abalos et al. 2001).

Foaming

A general problem of rhamnolipid production is excessive foaming due to the aeration and agitation of the culture broth in the bioreactor. The dimension of this problem is illustrated in Figure 5. In this example, a rhamnolipid production process was carried out in a 40 L bioreactor with a maximum working volume of 30 L. The actual working volume had to be reduced to 15 L to manage foam formation. When excessive foaming started, the whole bioreactor was filled with foam.

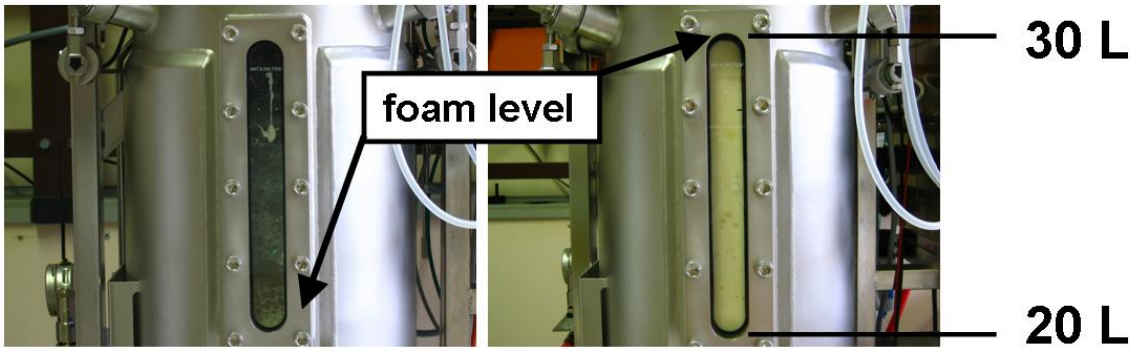


Figure 5: Rhamnolipid production in 40 L scale with 15 L working volume - left: at the beginning of cultivation (moderate foaming), right: at the end of cultivation (excessive foaming)

The generated foam can drain into the exhaust air conduct and block the exhaust air filter. This increases the risk of infection, diminishes the productivity and endangers the whole process. The working volume of the bioreactor is usually not completely exploited; rather, it has to be reduced substantially to handle foam formation. Moreover, mechanical and/or chemical foam control must be applied in rhamnolipid production processes. Conventionally, chemical antifoam is used, e.g. based on silicone oil, polyethylene glycol or polypropylene; however, the productivity of rhamnolipid production in *P. aeruginosa* decreases with increasing antifoam agent dosage (Leitermann 2008). *In-situ* product removal (ISPR) is another option to handle the foam problems during rhamnolipid production. Some ISPR techniques have already been integrated into rhamnolipid production processes. They are presented in chapter 1.3.

Production processes

An overview on published rhamnolipid production processes at bioreactor scale is given in Table 1. Besides the amount and type of the applied carbon source, the final rhamnolipid concentration (RL), the dry biomass concentration (DBM), the process time (for batch and fed-batch processes) respective dilution rate (for continuous processes) the following parameters are given in the table: the volumetric and specific productivity P_v and P_{sp}, the product/substrate yield $Y_{P/S}$ and the product/biomass yield $Y_{P/X}$.

Table 1: Production processes for rhamnolipids: $Y_{P/S}$: ratio of synthesized product to substrate employed. $Y_{P/X}$: ratio of synthesized product to final dry biomass. P_V: total product amount per volume of fermentation broth and process time. P_{sp}: total product amount per dry biomass and process time.

process	production strain	C-source [conc.] [g/L]	RL [g/L]	DBM [g/L]	$Y_{P/S}$ [-]	$Y_{P/X}$ [-]	process time [h]	P_{sp} [g/gh]	P_v [g/Lh]	reference
batch	*P. aeruginosa* DSM 2659	corn oil [40]	8,94	7	0,224	1,277	42	0,03	0,213	Hembach 1994
	P. aeruginosa DSM 7107	soybean oil [125]	78	-	0,62	-	167	-	0,47	Giani et al. 1997
	P. aeruginosa BYK-2 KCTC 18012P	fish oil [25]	17	5,3	0,68	3,21	216	0,015	0,08	Lee et al. 2004
	P. aeruginosa S2	glucose [40]	5,31	2,4	0,13	0,22	144	0,015	0,04	Chen et al. 2007b
batch with resting cells	*P. aeruginosa* DSM 2874	n-tetradecane [100]	13,2	3,6 – 4,1	0,132	3,66 -3,22	210	0,015 – 0,017	0,06	Syldatk et al. 1985a
batch with immobilised cells	*P. aeruginosa* DSM 2874	glycerol	-	1,96	0,12	-	> 20	0,017	0,033	Siemann and Wagner 1993
	P. aeruginosa BYK-2 KCTC 18012P	fish oil [10]	6	-	0,6	-	192	-	0,03	Jeong et al. 2004
fed-batch	*P. aeruginosa* DSM 2659	corn oil [84]	33,78	5	0,402	6,756	107	0,074	0,518	Hembach 1994
	P. aeruginosa DSM 7108	soybean oil [128 - 163]	95 - 112	-	0,74 – 0,69	-	216 - 264	-	0,44 – 0,42	Giani et al. 1997
	P. aeruginosa BYK-2 KCTC 18012P	fish oil [30,2]	22,7	6,1	0,75	3,72	264	0,014	0,09	Lee et al. 2004
	P. aeruginosa S2	glucose	6,06	2,62	-	2,31	195	0,012	0,03	Chen et al. 2007b
fed-batch with resting cells	*P. aeruginosa* DSM 2874	oleic acid or rapeseed oil [198]	40	48 (wet biomass)	0,2	-	288	-	0,14	Trummler et al. 2003
process	**production strain**	**C-source [g/L]**	**RL [g/L]**	**DBM [g/L]**	**$Y_{P/S}$ [-]**	**$Y_{P/X}$ [-]**	**D [h^{-1}]**	**P_{sp} [g/gh]**	**P_v [g/Lh]**	**reference**
continuous	*P. aeruginosa* DSM 2659	glucose [20]		2,4	0,05	-	0,135	0,056	0,134	Guerra-Santos 1985
	P. aeruginosa DSM 2659	glucose [30]	2,25	2,5	0,08	0,9	0,065	-	0,15	Reiling et al. 1986
	P. aeruginosa DSM 2659	corn oil [40]	4 - 18	7 – 7,5	0,182 – 0,459	-	0,025 – 0,1	0,052 – 0,1	0,451 – 0,691	Hembach 1994
continuous with cell retention/immobilisation	*P. aeruginosa* DSM 2659	glucose [20]	-	13,3	0,15	-	0,18	0,041	0,545	Gruber et al. 1993
	P. aeruginosa DSM 2659	corn oil [40]	-	-	0,484	-	0,1	0,056	2	Ochsner et al. 1996
	P. aeruginosa BYK-2 KCTC 18012P	fish oil [10]	4,3 – 5,8	10 (wet biomass)	0,43 – 0,58	-	0,018	-	0,08	Jeong et al. 2004

The scale of theses processes ranges from small laboratory scale, e.g. 1 L, up to industrial scale, e.g. 18,5 m^3. As process strategies, batch, fed-batch and continuous culture were applied and cell retention and immobilisation of the cells were also included. Different substrates such as glycerol, glucose, plant oils or hydrocarbons were applied as well as different production strains. Moreover, different analytical methods for the determination of the rhamnolipid content were used, e.g. HPLC (Hembach 1994), photometric methods like orcinol assay (Lee et al. 2004) or anthrone assay (Syldatk et al. 1985a) and even indirect determination by F_{cmc}-measurement (Guerra-Santos et al. 1984; Reiling et al. 1986). The indirect F_{cmc}-method is based on the fact that the surface activity is dependent on the concentration of the active compound. When the concentration of surfactant is decreased below the critical micelle concentration (cmc), for example by dilution, the surface activity is lost; which is expressed by increasing surface and interfacial tensions. This property is used for the estimation of biosurfactant concentration. The culture broth is diluted until the interfacial tension increases and the corresponding dilution factor (F_{cmc}) is determined. Increasing F_{cmc} values indicate the increasing concentration of active compounds.

Due to the mentioned differences in process design, cultivation strategy, medium, substrates and analytics, it is challenging to compare different published rhamnolipid production processes. However, the batch and fed-batch processes of Giani et al. 1997 achieved the best rhamnolipid production in terms of maximum rhamnolipid yield and specific productivity. A maximum product concentration of 78 – 112 g/L was reported. Unfortunately, insufficient technical details are given in this patent: for example no information on the applied analytical method for the rhamnolipid determination is presented.

Continuous processes for the production of rhamnolipids are very rewarding in terms of productivity; relatively high specific and volumetric productivities have been reported for continuous rhamnolipid production processes (see Table 1). However, few attempts have been made to promote this process strategy. The reason is probably that continuous processes are more complex in preparation, realization and process control and that they also have a higher risk of contamination of the culture.

Many attempts have been made with cell retention, immobilisation of the cells or cultivations with resting cells. Good results were obtained for some of these processes, but altogether, these strategies do not provide much extra benefit and a clear superiority of these processes to conventional processes with free/growing cells is not apparent. Moreover, these processes are more labor- and time-consuming, they require more equipment and comprise more process steps than simple batch or fed-batch processes.

1.3.4 Recovery of rhamnolipids

The recovery is the last step in the rhamnolipid production process. This step contributes strongly to the overall yield of the production process and thus to the overall production costs. Different recovery methods for rhamnolipids are presented below.

Solvent extraction

Solvent extraction is often applied for the first recovery of rhamnolipids from the culture supernatant. Different solvents or solvent mixtures, including ethyl acetate (Syldatk et al. 1985b; Schenk et al. 1995) or chloroform-methanol (2:1) (Mata-Sandoval et al. 1999) can be applied. However, ethyl acetate is commonly used. The extraction is preferentially performed at a pH of 2 – 3 as the rhamnolipids are protonated at this pH and, thus, soluble in hydrophobic organic solvents. In the following, a general recovery scheme for the purification of rhamnolipids is given (see Figure 6).

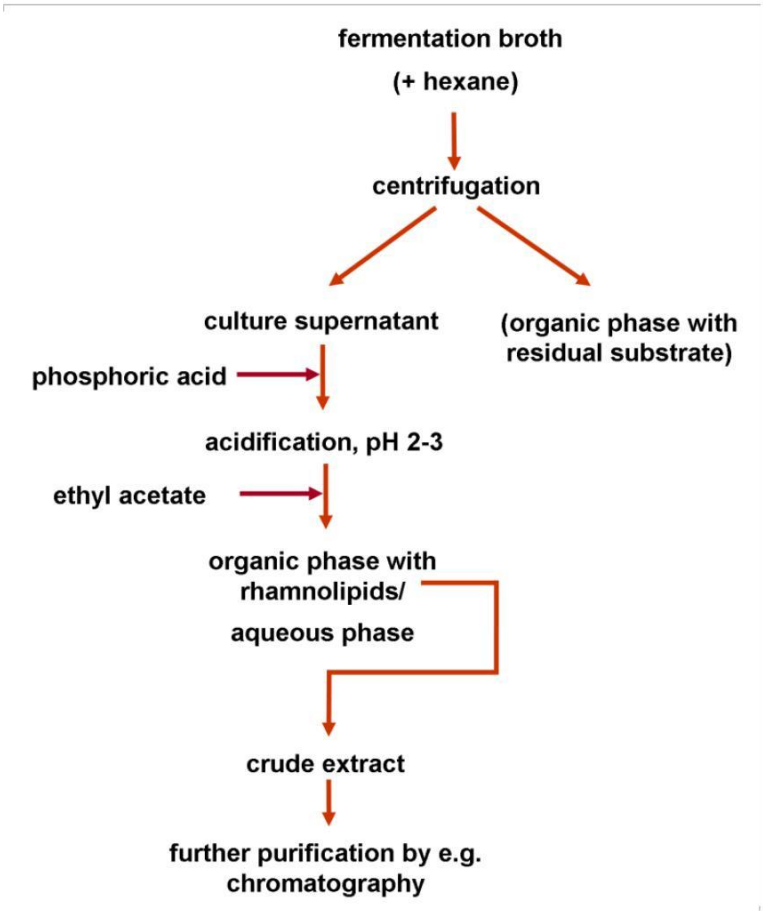


Figure 6: General recovery scheme for rhamnolipids

First, the fermentation broth is centrifuged to remove the cells. If plant oil or hydrocarbons were applied as substrates, they can be removed in the same step by extraction with hexane. The cell free culture supernatant is acidified to a pH of 2 – 3 and the protonated rhamnolipids are extracted with ethyl acetate. After evaporation of the organic

solvent, the crude extract can be further purified, for example by column chromatography with silica gel.

In addition, the following methods are applied for the purification of rhamnolipids:

Precipitation

Through acidification of the supernatant to a pH of 2 – 3, rhamnolipids pass to their protonated form, become insoluble in aqueous solution and precipitate. The precipitate is collected by centrifugation and then further processed (Mixich et al. 1997; Déziel et al. 1999; Wei et al. 2005; Monteiro et al. 2007). Mixich et al. 1997 claim a yield of the precipitation step of 90 – 99 % in a patent.

Filtration

Membrane filtration is another option for rhamnolipid enrichment. Rhamnolipid micelles can be retained by micro- and ultrafiltration (Mulligan and Gibbs 1990; Gruber 1991). 92 % rhamnolipid retention was obtained with a 10 kDa cutoff ultrafiltration membrane (Mulligan and Gibbs 1990) whereas only about 50 % retention was achieved with a 0,2 and 0,45 µm pore size membrane (Gruber 1991). Filtration is an attractive technique for *in-situ* product removal processes. However, the concept of integration of membranes into the rhamnolipid production process failed due to rapid membrane fouling, probably caused by exopolysaccharides and bacteria (Gruber 1991).

Foam fractionation

Another method for *in-situ* product removal of biosurfactants is foam fractionation. This technique uses the interfacial properties of rhamnolipids, namely the property to form stable foams. The rhamnolipid foam is conducted out of the reactor through an outlet in the top plate and collapsed by the addition of acid or shear forces. The rhamnolipids are enriched in the foam. Gruber and Matulovic used such an integrated concept with a foam fractionation column (Matulovic 1987; Gruber 1991). A drawback of this method is that the bacteria also adsorb to the foam interface, so that they are drained out of the reactor simultaneously (Gruber 1991). The enrichment of the biomass in the foam fraction is even more pronounced than the enrichment of rhamnolipids. Therefore, a retention or immobilization of the cells is necessary for a successful implementation of foam fractionation.

Chromatography

I Preparative TLC

Rhamnolipids can be purified by preparative thick-layer chromatography using silica gel with a solvent mixture of chloroform-methanol-acetic acid/water. The separated rhamnolipids are eluted from the silica gel with methanol or chloroform-methanol (Syldatk et al. 1985b; Déziel et al. 2000; Monteiro et al. 2007). The drawback of this

technique is that it has to be repeated several times to obtain pure rhamnolipids (Syldatk et al. 1985b) and that only relative small amounts of sample can be applied.

II Column chromatography

Normal-phase chromatography with silica gel is a standard method for rhamnolipid purification (Itoh et al. 1971; Schenk et al. 1995; Monteiro et al. 2007). The rhamnolipids are eluted for example with chloroform and methanol under isocratic conditions or with gradient elution.

Reversed-phase chromatography on a C_{18} column with a gradient of acetonitrile and water is another option (Mata-Sandoval et al. 1999; Heyd et al. 2008).

Ion exchange chromatography with DEAE sepharose can be applied as well (Reiling et al. 1986; Schenk et al. 1995). The rhamnolipids are eluted from the ion exchange resin by increasing salt concentrations.

Adsorption chromatography with hydrophobic Amberlite XAD-2 or XAD-16 polystyrene resin can be used for first rhamnolipid enrichment from the culture supernatant (Reiling et al. 1986; Matulovic 1987; Gruber 1991; Abalos et al. 2001; Haba et al. 2003). The rhamnolipids are eluted from the XAD resin for example with methanol. A drawback of this technique is that the XAD material absorbs hydrophobic and amphiphilic substances and, therefore, the adsorption of rhamnolipids to the XAD particles is rather unspecific.

A continuous rhamnolipid production process in a 50 L pilot plant with subsequent rhamnolipid recovery by 3 chromatographic steps (adsorption chromatography, ion exchange chromatography and again adsorption chromatography) was realised for several weeks (Reiling et al. 1986). The recovery of rhamnolipid in the first chromatographic step was above 75 %, whereas the recovery of the products from ion-exchange chromatography was over 90 %. An overall rhamnolipid recovery yield of 60 % was achieved.

***In-situ* product removal**

The term *in-situ* product removal (ISPR) covers methods for the removal of products by recovery methods that are integrated into the production process. In general this offers some advantages because inhibitory or toxic effects and product degradation can be overcome, and because the number of downstream processing steps can be reduced. Therefore, ISPR methods can increase the productivity and the yield of a production process. The following methods can be applied for ISPR: evaporation, extraction, permeation, immobilization of biocatalyst and precipitation. For the production of rhamnolipids, ISPR offers some significant advantages. The most important is the reduction of the pronounced foam formation. However, by now, all trials on *in-situ* product removal of rhamnolipids by filtration have been ineffective due to rapid fouling of the filtration

membranes (Gruber 1991; Dhariwal 2007). Another option is *in-situ* product removal by foam fractionation, as described above.

1.4 Non-pathogenic production strains

Conventionally, *P. aeruginosa* is utilised as production strain for rhamnolipid production. Nevertheless, rhamnolipid-producing bacteria have been found in other species and genera as well, most of them belonging to the closely related genera *Pseudomonas* and *Burkholderia*. Bacteria which were described in literature as rhamnolipid-producing bacteria are listed in Table 2. Altogether, up to now, rhamnolipid-producing bacteria have been found in the phyli proteobacteria, firmicutes (gram-positives) and actinobacteria. Most of the rhamnolipid producing bacteria are classified as non-pathogenic (Biosafety level 1) according to Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (DSMZ). A structure determination of the rhamnolipids that are produced by these bacteria has not been accomplished for the majority of these species. Moreover, for most bacteria, little information is available on the rhamnolipid production from a commercial point of view, for example on product yield, production processes etc.

Table 2: Overview on rhamnolipid producing bacteria

strain	Biosafety level	phylum	structure determination	reference
Pseudomonas aeruginosa, various strains	2	proteobacteria	HPLC-MS	e.g. Déziel et al. 1999
Pseudomonas chlororaphis NRRL B-30761	1	proteobacteria	HPLC-MS	Gunther et al. 2005
Pseudomonas putida ATCC 4359, CB100 and 21 BN	1	proteobacteria	GC of fatty acid part	Suzuki and Itoh 1972; Tuleva et al. 2002; Martínez-Toledo et al. 2006
Pseudomonas oleovorans ATCC 8062	1	proteobacteria	No	Suzuki and Itoh 1972
Pseudomonas cruciviae ATCC 21283	1	proteobacteria	No	Suzuki and Itoh 1972
Pseudomonas fluorescens 378 and ATCC 15453	1	proteobacteria	No	Suzuki and Itoh 1972; Persson et al. 1988; Fiechter 1992
Pseudomonas boreopolis ATCC 15452	1	proteobacteria	No	Suzuki and Itoh 1972
Pseudomonas stutzeri	1	proteobacteria	No	Janiyani et al. 1992
Burkholderia pseudomallei NCTC 10274	2	proteobacteria	GC-MS, NMR	Häußler et al. 1998
Burkholderia plantarii DSM 6535	1	proteobacteria	NMR	Andrä et al. 2006
Burkholderia glumae	1	proteobacteria	MS	Manso Pajarron et al. 1993
Tetragenococcus koreensis KCTC 3924^T	1	bacilli	No	Lee et al. 2005
Bacillus cereus 28 BN	2	bacilli	No	Tuleva et al. 2005
Renibacterium salmoninarum 27 BN	2	actinobacteria	No	Christova et al. 2004

Some of these strains produce exceptional rhamnolipids. While *P. aeruginosa* synthesises a mixture of mono- and di-rhamnolipids with hydroxy acyl moieties from C_8 up to C_{14}, the *Burkholderia* species produce only di-rhamnolipids with two rhamnose units and mainly C_{14} hydroxy acyl chains. For *B. glumae*, the following rhamnolipids were detected by GC-MS: RL-2,2_{14}, RL-2,$2_{12,14}$ and RL-2,$2_{14,16}$ (Manso Pajarron et al. 1993). The rhamnolipids RL-2,1_{14}, RL-2,2_{14} and RL-2,3_{14} were recorded for *B. plantarii* (Andrä et al. 2006) and only one rhamnolipid species was detected for *B. pseudomallei*: RL-2,2_{14} (Häußler et al. 1998). The exclusive formation of di-rhamnolipids seems to be a characteristic feature of the genus *Burkholderia*, as well as the preference of C_{14} chain length. A triacylated rhamnolipid (RL-2,3_{14}) has been reported for *B. plantarii*. Such triacylated rhamnolipids have not been described for any other species.

In contrast to the di-rhamnolipids of *Burkholderia* species, *P. chlororaphis* synthesises only mono-rhamnolipids with one rhamnose unit and two hydroxy acyl moieties (Gunther et al. 2005). Gunther et al. suggest that the lack of a *rhlC* gene homologue is the reason for this finding as RhlC catalyses the addition of the second rhamnose unit in *P. aeruginosa*. Rhamnolipids with unsaturated fatty acid moieties were found for *P. chlororaphis,* as was the case for *P. aeruginosa*. The major components of *P. chlororaphis* rhamnolipids are RL-1,$2_{12,10}$ and RL-1,$2_{12:1,10}$. The rhamnolipid composition of the other strains listed in Table 2 has not been investigated.

The genus *Pseudomonas* covers one of the most diverse and ecologically significant groups of bacteria. Members of the genus are found in large numbers in a wide range of environmental niches, such as terrestrial and marine environments, as well as in association with plants and animals. Bacteria of the genus *Pseudomonas* are gram-negative, rod-shaped bacteria with a size of 0,5 – 0,8 × 1 – 3 µm, motile by polar flagella, chemoorganotrophic and aerobic or facultative anaerobe (Palleroni 1981). The genus *Burkholderia* is quite young. It arose from the genus *Pseudomonas* and became classified as a new genus in 1992 based on 16S rRNA sequence analysis (Yabuuchi et al. 1992). Consequently, bacteria of this genus have similar characteristics as *Pseudomonas*: they are gram-negative, rod-shaped bacteria with polar flagella; they are facultative aerobe, chemoorganotrophic, and some species produce rhamnolipids.

Little is known about the biotechnological potential of the above mentioned strains as alternatives to the conventional production strain *P. aeruginosa*. Especially the non-pathogenic species are, however, of high commercial interest if sufficient rhamnolipid yields could be obtained.

1.5 Economics of rhamnolipid production

Up to now, microbial surfactants have not been widely successful in substituting synthetic surfactants; rather, their use is restricted to specific applications where biocompatibility is required. The main reason for this situation can be found in the high costs for production and downstream processing of biosurfactants (Gruber et al. 1993). According to Mulligan and Gibbs 1993, the economics of biosurfactant production are influenced by the following factors:

a) **Fermentation process**
   - **Raw materials**
   - **Productivity**

b) **Product recovery.**

The following aspects should be addressed to minimise the production costs of biosurfactants (Mulligan and Gibbs 1993):

a) **Optimisation of the fermentation process**, for example reactor design, operation conditions and production scale.

The cultivation strategy and the process conditions like aeration, agitation, pH and temperature should be carefully chosen to obtain optimum rhamnolipid production. Especially the nutrient limitation is of great importance for the regulation of rhamnolipid biosynthesis (see section 1.3.1). Another aspect that has to be considered is foaming (see section 1.3.3). Foam formation dramatically reduces the working volume of the bioreactor and even endangers the whole process if the foaming gets out of control. The volumetric productivity is decreased if the working volume of the bioreactor cannot be exploited completely.

- **Application of inexpensive raw materials** such as crude or waste materials.

The raw materials can make up to 10 – 50 % of the final product costs of biosurfactants (Mulligan and Gibbs 1993). Depending on the desired purity of the product, the production costs are principally influenced by either the raw material costs or by the purification costs. Rhamnolipids can be applied as fine chemicals, for example for pharmaceutics or cosmetics, or as bulk surfactants, for example for cleaning products. For high purity products, the product costs are affected mainly by the downstream processing. If high purity is not required, for example for bulk applications, the raw material costs can amount up to 50 % of the overall production costs. For batch rhamnolipid production processes for example, a $Y_{P/S}$ of 0,13 up to 0,68 has been reported (see Table 1). Consequently, between 1,5 and 7,7 times more substrate is needed than product is synthesized. Therefore, low-cost raw materials are important for an economic production process. In general, less pure materials are less expensive and usually tolerated by the microorgan-

isms. Crude materials like corn syrup, molasses or non-refined plant oils are promising carbon sources. Waste materials are also a good option because they are even less expensive than crude materials.

- **Increase of productivity** of the production process, for example by looking at the biosynthesis control, screening for overproducers or by genetic engineering.

Overproducing strains may be isolated from the environment, especially from hydrocarbon-contaminated sites by different isolation and screening techniques for biosurfactant producing microorganisms (Walter et al. 2008). Alternatively, overproducing strains can be generated by random mutagenesis or, more straightforward, by a directed mutagenesis by genetic engineering. Targets for metabolic engineering are the genes involved in the biosynthesis of the biosurfactant or genes for proteins that are involved in substrate uptake, side-reactions etc. The regulation of rhamnolipid biosynthesis must be addressed as well. However, insufficient information is available on the overall regulation of rhamnolipid biosynthesis.

b) **Optimisation of the recovery** with respect to yield and economics, for example with *in-situ*-methods.

Many factors influence the recovery costs of biosurfactants, e.g. the product concentration, the characteristics of the broth and of the product, the desired purity and the selected recovery methods (see section 1.3.4). Especially the broth composition is of concern as *P. aeruginosa* produces polysaccharides like alginate that constrain the purification processes. This phenomenon was observed, for example, for column chromatography by Reiling et al. 1986 who reported that clogging of the column during the loading procedure probably resulted from the fact that the cell-free culture liquid contained polysaccharide material. Recovery costs are significantly reduced if a separation of the individual rhamnolipid species in the product mixture is not necessary. In this case, expensive chromatography steps can be reduced or even omitted. A first recovery by precipitation is also very effective (see section 1.3.4) as it is a simple and inexpensive method requiring little equipment and no organic solvent. But little attention is paid to this method in current rhamnolipid research projects and solvent extraction is conventionally applied for the first recovery of rhamnolipids. *In-situ* product removal is also rewarding for rhamnolipid recovery because the amount of necessary recovery steps is reduced and, additionally, foam problems are decreased. However, all reports on ISPR of rhamnolipids by filtration or foam fractionation conclude that these techniques are unsuccessful (see chapter 1.3.4).

1.6 Problems and limitations of rhamnolipid production

Recently, there has been an increasing interest in rhamnolipids for cleaning, food, cosmetics, pharmaceutics and environmental applications as sustainability and environmental compatibility are gaining more and more importance. However, the overall establishment of rhamnolipids is impeded by a lack of availability of economic products. The production costs of rhamnolipids are too high for a broader market development. Hence, rhamnolipids are not competitive against conventional synthetic surfactants and, consequently, they represent only niche products. The economic competitiveness of rhamnolipids is mainly determined by the low productivity of rhamnolipid production processes resulting from low production rates and long process times for batch and fed-batch processes (see Table 1). Furthermore, the recovery of rhamnolipids is quite expensive. Another factor is the high safety level of the conventional production strain *P aeruginosa*. Moreover, the formation of side-products such as the polysaccharide alginate decreases the productivity and enhances viscosity. A kinematic viscosity of up to 45 mm^2/s was reported for a rhamnolipid production process with *P. aeruginosa*, whereas the starting viscosity was in the range of 2 – 5 mm^2/s (Leitermann 2008). Such a high viscosity can lead to severe problems in industrial scale processes concerning power input and mass transfer. Polysaccharides also complicate the product recovery (see section 1.5 b). Therefore, the formation of polysaccharides by rhamnolipid production strains should be minimized by process design or by genetic engineering.

A problem of research in the area of rhamnolipid production is that many different analytical methods are used, for example HPLC (Hembach 1994), photometric methods like orcinol assay (Lee et al. 2004) or anthrone assay (Syldatk et al. 1985a) and even gravimetric determination (Gunther et al. 2005) or indirect determination by F_{cmc}-measurement (Reiling et al. 1986, Guerra-Santos et al. 1984). This complicates the comparison of the different proposed rhamnolipid production processes and production strains.

1.7 Aim of the work

New production processes are the key issue in overcoming the economic obstacles of rhamnolipid production. Therefore, increased efforts must be made to address the mentioned problems of rhamnolipid production. This work tackles rhamnolipid production with different approaches by looking at all aspects of the production process, from the upstream to the downstream processing. The following aspects were investigated:

a) Plant oils and waste glycerol as raw materials (chapter 4.1)

Two different types of substrates were applied in this work: plant oils and waste glycerol. Plant oils are common substrates for rhamnolipid production. They were used as standard substrate.

Different waste substrates have already been studied for rhamnolipid production, for example olive oil mill effluents (Mercadé et al. 1993), waste frying oils (Haba et al. 2000) or waste free fatty acids (Abalos et al. 2001). Waste glycerol has not been examined as a substrate for rhamnolipid production yet although glycerol is a common substrate for rhamnolipid production with *P. aeruginosa* (Syldatk et al. 1985a; Chen et al. 2007a). Waste glycerol offers the advantages that it is a waste substrate from a renewable resource and less expensive than plant oils. Waste glycerol from biodiesel production and the pharma industry were investigated in this work. The price of crude glycerol from biodiesel manufacturing is about 500 €/ton and thus about 50 % cheaper than plant oil (ADM Biodiesel 2007).

But waste glycerol from biodiesel production contains impurities from the harsh biodiesel manufacturing, such as aldehydes, esters, halogenated compounds, chlorides, heavy metals and sulphates (ADM Biodiesel 2007). The aim of this working package was to verify if these substrates are suitable for rhamnolipid production or if the impurities affect or even inhibit bacterial growth and rhamnolipid production.

b) Immobilisation of *P. aeruginosa* with the aim of establishing an *in-situ* product removal process (chapter 4.2)

In-situ product removal offers some significant advantages for the production of rhamnolipids. The most important is the reduction of foam formation. However, all attempts at *in-situ* product removal of rhamnolipids by filtration have failed due to rapid fouling of the filtration membranes (Gruber 1991). The fouling of the inserted membrane could be prevented by immobilisation of the cells, as immobilisation separates the cells and the culture broth. Moreover, immobilised cells are necessary for *in-situ* product removal by foam fractionation because otherwise free cells are enriched in the foam and carried out of the reactor (Gruber 1991).

Immobilisation of microbial cells offers some additional advantages when compared to classical fermentation processes with free cells (Tramper 1989). The immobilised cells can be recycled and reused; moreover, a high cell density and thus a high volumetric productivity can be achieved. The purification of the desired product is facilitated and inactivation of fragile cells at interfaces can be prevented.

The aim of this working package was to develop a simple, efficient and economic immobilisation method for *P. aeruginosa* and a suitable cultivation process for the production of rhamnolipids with immobilised cells. The immobilised cells should be integrated into a production process with *in-situ* product removal by membrane or electro kinetically controlled deep filtration (see Figure 7). This part of the process was under development by project partners from the former upt GmbH, Saarbrücken, Germany.

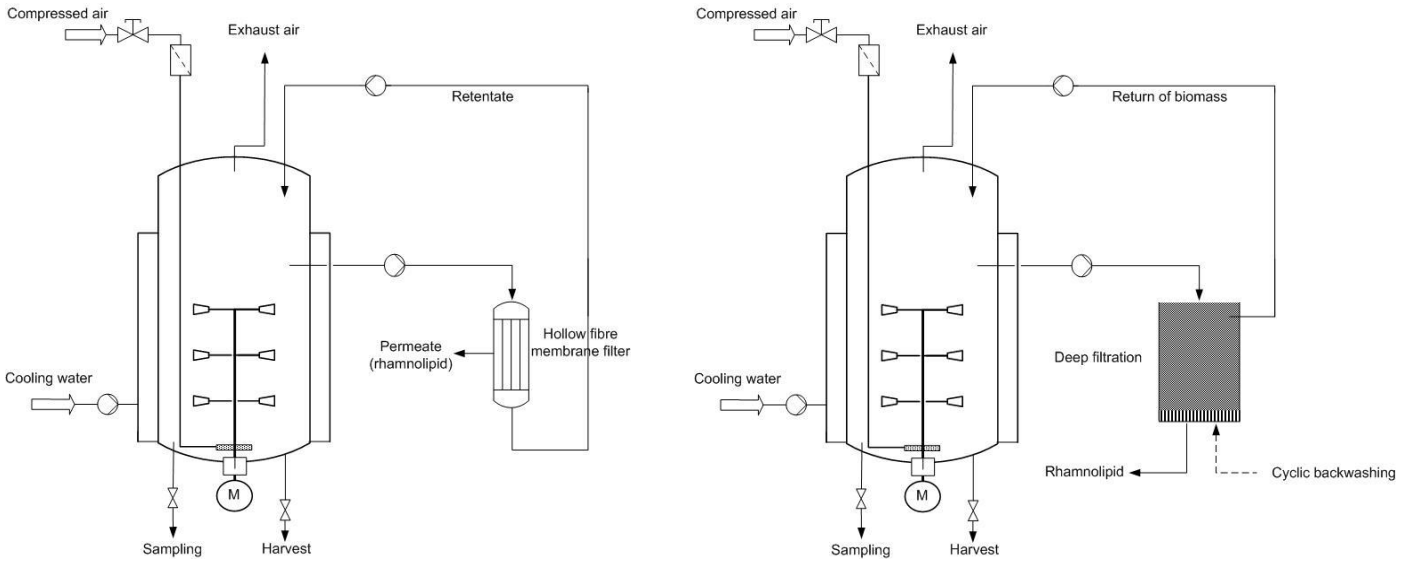


Figure 7: Process concept for the rhamnolipid production process with immobilised cells and ISPR by filtration (left) or electro kinetically controlled deep filtration (right)

The filtration step was planned with a hollow fibre membrane filter with a pore size of 10 µm. Immobilised cells with a diameter of at least 100 µm should be retained by the filter whereas the rhamnolipid micelles with a diameter of ≈ 1 µm are separated. The electro kinetically controlled deep filtration should be undertaken with a charged absorber material (Mavrov et al. 2001). An appropriate filter material would absorbs the immobilised cells whereas the rhamnolipid micelles pass the filter.

Immobilisation of microorganism or enzymes can be carried out by inclusion into particles or membranes or by coupling to a matrix (Buchholz et al. 2005). The common immobilisation techniques are illustrated in Figure 8.

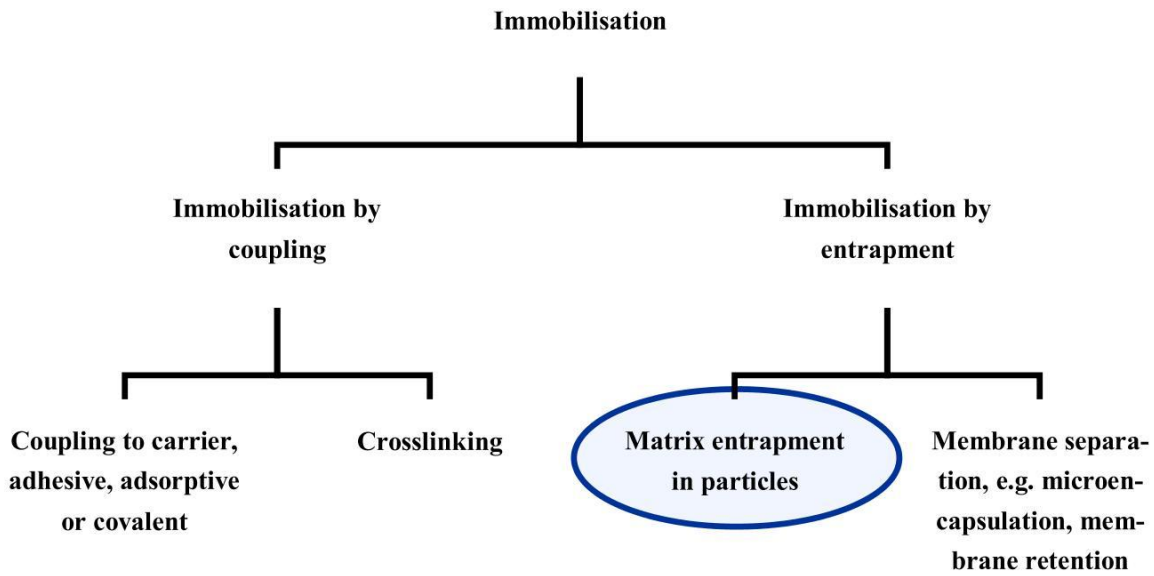


Figure 8: Methods for immobilisation of enzymes or microorganisms (modified from Buchholz et al. 2005)

In this work, immobilisation by matrix entrapment was chosen as preferred method because it is a gentle method and, thus, commonly used for immobilisation of whole microorganisms. Membrane retention is another option for retention of microorganisms, however, not adequate for *P. aeruginosa* because of the already mentioned rapid membrane fouling and blocking. Coupling methods are too aggressive for microbial cells and the binding is probably not strong enough for whole cells.

c) Non-pathogenic production strains as alternatives to the conventional, pathogenic production strain *P. aeruginosa* (chapter 4.3)

This approach aimed at finding an alternative, non-pathogenic production strain capable of producing sufficient amounts of rhamnolipids. The aim was to investigate if one of the non-pathogenic, rhamnolipid producing strains mentioned in chapter 1.4 is competitive to the standard production strain *P. aeruginosa*.

For most of the non-pathogenic rhamnolipid producing strains, a structure elucidation of the synthesised rhamnolipids has not yet been accomplished; only for *P. chlororaphis*, *B. plantarii* and *B. glumae* have the structures of the synthesised rhamnolipids been determined. Therefore, an intensive analysis of their rhamnolipid production had to be carried out to evaluate potential candidates. Moreover, little information was available on the rhamnolipid production from a commercial point of view, for example product yield, production process, production costs etc. Therefore, the successful candidates from first screening were further studied at bioreactor scale.

2 Material

2.1 Chemicals

All applied chemicals were of analytical grade and purchased from ROTH, with the exception of:

- agar, tryptone, yeast extract (Becton Dickinson, Germany)
- sunflower oil (food grade, "Gut & Günstig", Germany)
- olive oil (food grade, "ALDI Süd", Germany)
- $CaCl_2 \cdot 2H_2O$, $MnSO_4 \cdot H_2O$, $MnCl_2 \cdot 4H_2O$, $NH_4H_2PO_4$, $Ce(SO_4)_2$, $CoSO_4 \cdot 6H_2O$, $MgSO_4$, $NaMoO_4$ $2H_2O$, L-rhamnose, sodium-alginate, κ-caragenaan, xanthane, porcine gelatin, Gum Arabicum, meat extract, 4-bromo phenacyl bromide, triethyl ammonium, anisaldehyde, triethyl amine (Fluka, Germany)
- $BaCl_2$, 50 % glutaraldehyde (Merck, Germany)
- 5-methylresorcin monohydrate, protamine sulphate (Sigma-Aldrich, Germany)
- $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$ (Janssen Chimica, Switzerland)

2.2 Strains

P. aeruginosa DSM 2874 and DSM 7108 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

The following non-pathogenic microorganisms were investigated in this work: *P. chlororaphis* DSM 50083, *P. chlororaphis* NRRL B-30761, *P. putida* DSM 5232, *P. putida* DSM 12735, *P. oleovorans* DSM 1045, *P. cruciviae* NCIMB 10833, *B. glumae* DSM 9512, *B. glumae* PG1 (obtained from F. Rosenau, IMET, Jülich, Germany), *B. plantarii* DSM 9509, *B. plantarii* DSM 6535 and *T. koreensis* DSM 16501. These strains were classified as non-pathogenic by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (DSMZ).

2.3 Media

Saline contains 9 g/L NaCl in $H_2O_{VE.}$

LB-medium was applied according to Bertani 1951. The composition of the LB-medium is given in Table 3.

Table 3: Composition of LB-medium

compound	concentration [g/L]
tryptone	10
yeast extract	5
NaCl	10

The pH is adjusted to 7.

For **LB-agar**, 20 g/L agar is added to the LB-medium.

M3-medium (Matulovic 1987) is a medium for resting cell cultivation. It was used for cultivations with immobilised cells and for cultivations of *P. aeruginosa* with waste glycerol under resting cell conditions. For the latter cultivation, the medium is buffered with phosphate buffer. The composition of M3-medium is given in Table 4.

Table 4: Composition of M3-medium

compound	concentration [g/L]
NaCl	2,5
$CaCl_2 \cdot 2\ H_2O$	0,37
Na_2HPO_4 (only for cultivation with free cells)	0,15
NaH_2PO_4 (only for cultivation with free cells)	0,26
C-source (olive oil or glycerol)	20

The pH is adjusted to 6,8.

Giani-medium was applied according to Giani et al. 1997. This medium is used for cultivations in bioreactor. According to Leitermann 2008, a 10fold diluted medium is used for shake flask cultivation, whereas the oil content is not reduced. Cultivations with *P. aeruginosa* have shown that a diluted medium is favourable for rhamnolipid

production in shake flasks. The composition of the Giani-medium is given in Table 5 and Table 6.

Table 5: Composition of Giani-medium

compound	**concentration [g/L]**
$MgSO_4 \cdot 7\ H_2O$	0,5
KCl	1
$NaNO_3$	15
Na_2HPO_4	6,27
NaH_2PO_4	10,11
trace element solution	1 mL/L
C-source, e.g sunflower oil or glycerol	125

The pH is adjusted to 6,8.

Table 6: Composition of trace element solution for Giani-medium

compound	**concentration [g/L]**
trisodium citrate$\cdot 2\ H_2O$	2
$FeCl_3 \cdot 6\ H_2O$	0,28
$ZnSO_4 \cdot 7\ H_2O$	1,4
$CoCl_2 \cdot 6\ H_2O$	1,2
$CuSO_4 \cdot 5\ H_2O$	1,2
$MnSO_4 \cdot H_2O$	0,8

Kay´s minimal medium was applied according to Gunther et al. 2005. The composition of the medium is given in Table 7.

Table 7: Composition of Kay´s minimal medium

compound	**concentration [g/L]**
$NH_4H_2PO_4$	3
K_2HPO_4	2
$MgSO_4$	1
$FeSO_4$	0,0005
glucose	2

The pH is adjusted to 7.

Gunther-medium was modified from Gunther et al. 2005. The composition of the medium is given in Table 8.

Table 8: Composition of modified Gunther-medium

compound	**concentration [g/L]**
KH_2PO_4	0,7
Na_2HPO_4	0,9
$NaNO_3$	2
$MgSO_4 \cdot 7\ H_2O$	0,4
$CaCl_2 \cdot 2\ H_2O$	0,1
$FeSO_4 \cdot 7\ H_2O$	0,004
$MnSO_4 \cdot H_2O$	0,003
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	0,0012
trisodium citrate$\cdot 2\ H_2O$	0,05
C-source, e.g. sunflower oil or glucose	120

The pH is adjusted to 6,7.

Suzuki-medium was modified from Suzuki and Itoh 1972. The composition of the Suzuki-medium is given in Table 9 and Table 10.

Table 9: Composition of modified Suzuki-medium

compound	**concentration [g/L]**
$(NH_4)_2SO_4$	5
KH_2PO_4	2
$Na_2HPO_4 \cdot 2\ H_2O$	2,2
$NaSO_4$	1
yeast extract	3,5
trace element solution	50 mL/L
C-source, e.g. sunflower oil or glycerol	125

The pH is adjusted to 6,5.

Table 10: Composition of trace element solution for Suzuki-medium

compound	**mass [g] per 50 mL solution**
$MgSO_4 \cdot 7\ H_2O$	1
$FeSO_4 \cdot 7\ H_2O$	1
$MnSO_4 \cdot H_2O$	0,38
$ZnSO_4 \cdot 7\ H_2O$	0,01
sodium citrate$\cdot 2\ H_2O$	2
biotin	0,001

Zähringer-medium according to Zähringer et al. 1997 is used for cultivation of *B. plantarii* at bioreactor scale. For shake flask cultivation, the medium is diluted 5fold or 10fold whereas the concentration of the oil is not reduced. The composition of the medium is given in Table 11.

Table 11: Composition of Zähringer-medium

compound	**concentration [g/L]**
$NH_4H_2PO_4$	5
KH_2PO_4	3,5
K_2HPO_4	3,5
yeast extract	10
$MgSO_4 \cdot 7\ H_2O$	1
$CaCl_2 \cdot 2\ H_2O$	0,026
sunflower oil	75

The pH is adjusted to 6,5.

MRS-medium (DSMZ 2007) was modified for halophilic *T. koreensis* by adding sodium chloride. The composition of the medium is given in Table 12.

Table 12: Composition of modified MRS-medium

component	concentration [g/L]
casein peptone	10
meat extract	8
yeast extract	4
NaCl	10
K_2HPO_4	2
trisodium acetate·3 H_2O	8,3
diammonium hydrogen citrate	2
$MgSO_4$	0,2
$MnSO_4 \cdot H_2O$	0,055
sunflower oil or glucose	20

The pH is adjusted to 9.

M92-medium (DSMZ 2007) was modified for halophilic *T. koreensis* by adding sodium chloride. The composition of M92-medium is given in Table 13.

Table 13: Composition of modified M92-medium

compound	concentration [g/L]
soy peptone	30
yeast extract	3
NaCl	20

The pH is adjusted to 9.

GYP-medium (glucose-yeast-peptone) (DSMZ 2007) was modified for halophilic *T. koreensis* by raising the sodium chloride concentration. Plant oil was used instead of glucose. The composition of the GYP-medium is given in Table 14.

Table 14: Composition of modified GYP-medium

compound	**concentration [g/L]**
casein peptone	10
yeast extract	10
sodium acetate·3 H_2O	16,6
NaCl	10,1
$MgSO_4$·7 H_2O	0,2
$MnSO_4$·H_2O	0,0075
$FeSO_4$·7 H_2O	0,01
sunflower oil or glucose	20

The pH is adjusted to 9.

Trummler-medium was modified from Trummler et al. 2003. The composition of the medium is given in Table 15 and Table 16.

Table 15: Composition of modified Trummler-medium

compound	**concentration [g/L]**
$(NH_4)_2SO_4$	6
$MgSO_4$·7 H_2O	0,4
$CaCl_2$·2 H_2O	0,4
Na_2HPO_4·2 H_2O	7,59
KH_2PO_4	4,43
trace element solution	2 mL/L
carbon source	20

The pH is adjusted to 6,5.

Table 16: Composition of trace element solution for modified Trummler-medium

compound	**concentration [g/L]**
citrate	20,1
$FeCl_3$·$6H_2O$	16,7
$CoCl_2$·$6H_2O$	0,18
$ZnSO_4$·$7H_2O$	0,18
$CuSO_4$·$5H_2O$	0,16
$MnSO_4$·H_2O	0,1

Tuleva-medium was modified from Tuleva et al. 2002. The composition of the medium is given in Table 17.

Table 17: Composition of Tuleva-medium

compound	concentration [g/L]
$K_2HPO_4 \cdot 3\ H_2O$	4,8
KH_2PO_4	1,5
$(NH_4)_2SO_4$	1
yeast extract	0,1
$MgSO_4$	0,2
$CaCl_2 \cdot 2\ H_2O$	0,002
$MnCl_2 \cdot 4\ H_2O$	0,0004
$NiCl_2 \cdot 6\ H_2O$	0,0004
$ZnSO_4 \cdot 7\ H_2O$	0,0004
$FeCl_3 \cdot 6H_2O$	0,0002
$Na_2MoO_4 \cdot 2\ H_2O$	0,0002
citric acid	0,35
sunflower oil or glucose	20

The pH is adjusted to 7,2.

CTAB agar was prepared according to Siegmund and Wagner 1991. Glycerol, glucose or sunflower oil were utilized as carbon sources. If plant oil is used as carbon source, the oil droplets are stabilized with 1 g/L Gum Arabicum.

Crude glycerol (Concerine CD 80) and glycerol pharma grade were obtained from ADM Biodiesel, Hamburg, Germany. The composition of crude glycerol is given in Table 18.

Table 18: Composition of Concerine CD 80 (crude glycerol from ADM Biodiesel)

compound	content (m/m)
glycerol	81,9 %
water	12,8 %
NaCl	5,3 %
methanol	0 %
MONG (Matter of Organic Non Glycerol)	< 1,5 %

Furthermore, the crude glycerol contains traces of impurities from the harsh biodiesel manufacturing, for example, aldehydes, esters, halogenated compounds, chlorides, heavy metals and sulphates (ADM Biodiesel 2007).

Waste glycerol from passion fruit oil, linseed oil and fish oil was obtained from KD Pharma, Bexbach, Germany. These waste products are side-products from the manufacturing of ω-3-faty acids from natural oils.

Solutions for immobilisation

Immobilisation in calcium-alginate:

Matrix: 20 g/L sodium-alginate in H_2O_{VE}, dissolved at 80 °C

Hardener: 20 g/L $CaCl_2 \cdot 2H_2O$ in H_2O_{VE}

Storage solution: 5 g/L $CaCl_2 \cdot 2H_2O$ in H_2O_{VE}

Immobilisation in barium-alginate:

Matrix: 17 g/L sodium-alginate in H_2O_{VE}, dissolved at 80 °C

Hardener: 12,22 g/L $BaCl_2$ in H_2O_{VE}

Crosslinker: 5 g/L protamine sulphate in H_2O_{VE}

Immobilisation in barium-alginate/xanthan:

Matrix: 7,5 g/L sodium-alginate in H_2O_{VE}, dissolved at 80 °C

3 g/L xanthan in saline

Hardener: saturated barium alginate solution: 437,5 g/L $BaCl_2$ in H_2O_{VE}

Crosslinker : 5 g/L protamine sulphate in H_2O_{VE}

Immobilisation in κ-caragenaan:

Matrix: 30 g/L κ-caragenaan in H_2O_{VE}, dissolved at 80 °C

or 15 g/L κ-caragenaan in H_2O_{VE}, dissolved at 80 °C

Hardener: 23,82 g/L KCl in H_2O_{VE}

Immobilisation in gelatin:

Matrix:	200 g/L porcine gelatin in H_2O_{VE}, dissolved at 60 °C
Hardener:	7,36 g/L $CaCl_2 \cdot 2H_2O$ in H_2O_{VE}
Crosslinker:	50 % glutaraldehyde diluted 1:25 in H_2O_{VE}

Immobilisation in gelatin-alginate:

Matrix:	200 g/L porcine gelatin in H_2O_{VE}, dissolved at 60 °C
	30 g/L sodium-alginate in H_2O_{VE}, dissolved at 80 °C or
	20 g/L sodium-alginate in H_2O_{VE}, dissolved at 80 °C
Hardener:	7,36 g/L $CaCl_2 \cdot 2H_2O$ in H_2O_{VE}
Crosslinker:	50 % glutaraldehyde diluted 1:25 in H_2O_{VE}

2.4 Solutions for analytics

TLC

Mobile phase:

The composition of the mobile phase for TLC analytics is given in Table 19.

Table 19: Composition of mobile phase for TLC

compound	volume [mL]
chloroform	650
methanol	150
acetic acid (conc.)	2

Staining of fatty acid moiety:

The ß-hydroxy fatty acid part of the rhamnolipids was stained with the solution given in Table 20.

Table 20: Composition of staining solution for fatty acid part

compound	amount
ammonium (IV) molybdate tetrahydrate	1,05 g
cerium (IV) sulphate	0,5 g
sulfuric acid (conc.)	15,5
water	250 mL

Staining of rhamnose moiety:

The rhamnose moiety of the rhamnolipids was stained with anisaldehyde (see Table 21).

Table 21: Composition of staining solution for rhamnose part

compound	volume [mL]
anisaldehyde	1
sulfuric acid (conc.)	2
acetic acid (conc.)	100

HPLC

Derivatisation:

Solution A: 40 mM 4-bromo phenacyl bromide in acetonitrile

Solution B: 20 mM triethyl ammonium in acetonitrile

Mobile phase:

Solvent A: 95 % deionised water, 5 % acetonitrile

Solvent B: 5 % deionised water, 95 % acetonitrile

Orcinol assay

Orcinol reagent:

The orcinol reagent is composed of 0,19 % 5-methylresorcin in 53 % sulfuric acid. Therefore, 55,2 mL of conc. sulfuric acid (96 %) are added to 44,8 mL of water, and subsequently, 21,8 mg of 97 % 5-methylresorcin are dissolved in the acid.

Rhamnose solutions for calibration:

For the calibration of the orcinol assay, L-rhamnose solutions with 0,01 – 0,1 g/L rhamnose in 0,1 M phosphate buffer pH 7 were used.

3 Methods

3.1 Microbiological methods

Storage of microorganisms

Microorganisms were stored at – 20 °C with 15 % glycerol as cryo preservative.

Measurement of biomass concentration

The optical density (OD) of a culture was measured photometrically at 580 nm. The optical density was not measured if oil was applied as substrate because the oil droplets interfere with the photometric measurement. The wet and dry biomass concentrations were calculated from the OD using the following correlations:

Table 22: OD-biomass correlations

strain	OD/WBM	OD/DBM
P. aeruginosa	0,333	2,087
P. chlororaphis	0,171	1,87
P. putida	1,187	1,65
P. oleovorans	0,183	2,06
P. cruciviae	0,253	2,34
B. glumae	0,148	2,38
B. plantarii	0,148	2,38
T. koreensis	0,152	2,69

An example for the calculation for *P. aeruginosa* is given below:

Determination of wet biomass: $$WBM = \frac{OD_{580}}{0{,}333}\left[\frac{g}{L}\right]$$

Determination of dry biomass: $$DBM = \frac{OD_{580}}{2{,}087}\left[\frac{g}{L}\right]$$

3.2 Cultivations

Cultivations were performed at shake flask scale and at bioreactor scale (see Figure 9). Two different bioreactor systems were applied, depending on the task. Parallel cultivations were performed in the 6-fold parallel bioreactor system and larger cultivations were performed in the 40 L bioreactor.

Figure 9: Applied cultivation systems, top: 500 mL shake flasks, bottom left: 0,6 L 6-fold parallel bioreactor system, bottom right: 40 L bioreactor

Shake flask

Precultures were grown in baffled shake flasks overnight at 30 °C or 37 °C and 120 – 130 rpm in LB-medium or Kay's minimal medium (*P. chlororaphis*).

The main cultivations were performed in 500 mL baffled shake flasks with 100 mL medium. The standard medium for *P. aeruginosa* shake flask cultures was 1/10 diluted Giani-medium, according to Leitermann 2008. The medium was diluted to achieve nutrient limitation in shake flask culture. The flasks were inoculated with 20 mg DBM of a growing culture and incubated 8 – 9 days at 120 – 130 rpm and 30 °C or 37 °C. *P. chlororaphis* was incubated at 23 °C according to Gunther et al. 2005. At least two samples were taken during each cultivation.

Cultivations with waste and crude glycerol were carried out with *P. aeruginosa* DSM 7108 under resting cell conditions or with growing cell. The cells were cultivated in 500 mL baffled shake flasks with 100 mL M3 medium (resting cells) or 100 mL 1/10 Giani-medium (growing cells). 2 g glycerol (resting cells) respective 12,5 g glycerol (growing cells) were applied. For resting cells, 3 g of wet biomass were inoculated and for growing cells 20 mg dry biomass. The cells were incubated for 8 – 9 days at 120 rpm. Where extensive foaming started, the agitation was reduced to 105 rpm to prevent wetting and clogging of the cotton filter. The substrate concentration was adjusted for waste glycerol and crude glycerol according to the glycerol concentration of these substrates. Crude glycerol from ADM Biodiesel contained 81,9 % of glycerol. The glycerol content of the waste glycerol was determined with an enzymatic kit (see chapter 3.3).

Immobilised cells were cultivated under resting cell conditions in unbuffered M3-medium in shake flasks. The pH was corrected manually during the cultivation.

Parallel bioreactor cultivations

The promising strains from the shake flask cultivations were then cultivated in a 6fold parallel bioreactor system (Sixfors, Infors AG, Switzerland). The cultivations were performed at least in duplicate. The total volume of the parallel bioreactors was 600 mL and the working volume was set to 300 mL (non-pathogenic strains). The working volume had to be reduced to 200 mL for *P. aeruginosa* to handle excessive foaming. The cultivation conditions were 30 °C, 800 rpm and an aeration rate of 20 L/h. *P. chlororaphis* was cultivated at 23 °C according to Gunther et al. 2005. Two mechanisms were applied for foam control. First, a self-constructed mechanical foam separator with the shape of a 4-bladed Rushton turbine was installed on the stirrer shaft in the head space. This foam separator performs optimally at 1100 rpm; accordingly, the stirrer was set to 1100 rpm and the aeration rate to 6 L/h when extensive foaming started. Second, the antifoam agent Contraspum A4050 (Zschimmer & Schwartz, Germany) was fed upon contact with an antifoam probe. The pH was corrected with 4 M H_3PO_4 and 4 M NaOH (25 % NH_3 for *B. plantarii*). The cultivations were run for 8 – 9 days and 1 – 2 samples were taken each day. For cultivations according to Giani et al. 1997, Giani-medium was used and an additional oil feed of 125 g/L was dosed after 40 h of process time. Furthermore, trace elements were fed after 0, 20, 40, 70 and 120 h of process time.

Table 23: Process parameters for cultivation in parallel bioreactor

parameter	**value**
working volume	200 or 300 mL
pH	depending on medium
T	30 °C (23 °C for *P. chlororaphis*)
stirrer speed	800 – 1100 rpm
aeration rate	20 – 6 NL/h (= 1 – 0,3 vvm)
process time	8 – 9 days

Cultivation in 40 L bioreactor

B. plantarii DSM 9509 was cultivated in a 40 L Biostat Cplus bioreactor (Sartorius, Melsungen, Germany). The pH was corrected with 4 M H_3PO_4 and 25 % NH_3. Foaming was detected with a foam probe and the mechanical foam separator FUNDAFOM, which was installed in the top plate of the reactor, was activated when foaming started.

Table 24: Process parameters for cultivation of *B. plantarii* in 40 L Biostat Cplus bioreactor

parameter	**value**	
	first cultivation	**second cultivation**
working volume	12 L	18,2 L
pH	6,5	6,5
T	30 °C	30 °C
pO_2 set point	20 %	20 %
stirrer speed	350 – 550 rpm	207 – 284 rpm
aeration rate	1 – 3 NL/min (= 0,08 – 0,25 vvm)	5,5 – 18,2 NL/min (= 0,3 – 1 vvm)
process time	120 h	160 h

The dissolved oxygen level was regulated by the stirrer speed. The stirrer speed range for the 40 L bioreactor was scaled up from the cultivation from 0,6 L parallel bioreactor as follows:

First cultivation:

The stirrer speed was calculated for constant peripheral speed $v_{periphery}$.

$$v_{periphery} = 2 \cdot \pi \cdot N \cdot d_R$$

For two different scales A and B, the stirrer speed N can be calculated as follows:

$$N_A = \frac{N_B \cdot d_{R,B}}{d_{R,A}}$$

Second cultivation:

The stirrer speed was calculated for constant power input (P/V). The power input P in a stirred tank reactor is $Ne = \frac{P}{N^3 d_R^5 \rho}$.

The following assumptions were made:

1) turbulent flow ($Re > 10^4$), so that the Newton number Ne is constant, and $P \sim N^3 d_R^5 \rho$.
2) $\frac{P_1}{V_1} = \frac{P_2}{V_2}$
3) $V = d_R^3$

For two different scales A and B, the following equation results: $N_A = \sqrt[3]{\frac{N_B^3 \cdot d_{R,B}^2}{d_{R,A}^2}}$

The culture broth of the second 40 L cultivation was harvested and subjected to purification. First, the cells were removed by centrifugation (20 min at 7500 rpm and 4 °C). The cell free supernatant (15 L) was extracted two times with 13 L in of hexane in total. The aqueous phase was acidified with H_3PO_4 to a pH of 2 – 3. The protonated rhamnolipids were then extracted from the aqueous phase three times with 9 L of ethyl acetate in total and the organic phase was collected and evaporated to gain the crude extract.

3.3 Analytics

Sample preparation

In shake flasks, samples from the cultivations were taken at day 5 and day 9 of cultivation. 1-2 samples were taken daily during bioreactor cultivation. If plant oil was used as carbon source, the samples were first extracted with hexane to remove the residual plant oil. Hexane was added 1:1 (v:v). After mixing, the aqueous phase, the hydrophobic phase and the biomass are separated by centrifugation, for example at 4700 rpm and 4 °C for 10 min. The upper hexane phase was used to determine the oil concentration and the biomass pellet for the determination of the dry biomass. The lower, aqueous phase was subjected to further rhamnolipid analysis, for example by HPLC, TLC or orcinol assay. Consequently, the aqueous phase was transferred into a new flask, acidified with 1 % (v:v) of 85 % H_3PO_4 and the protonated, nonpolar rhamnolipids were extracted with ethyl acetate. A complete working scheme for analytics is shown in Figure 10.

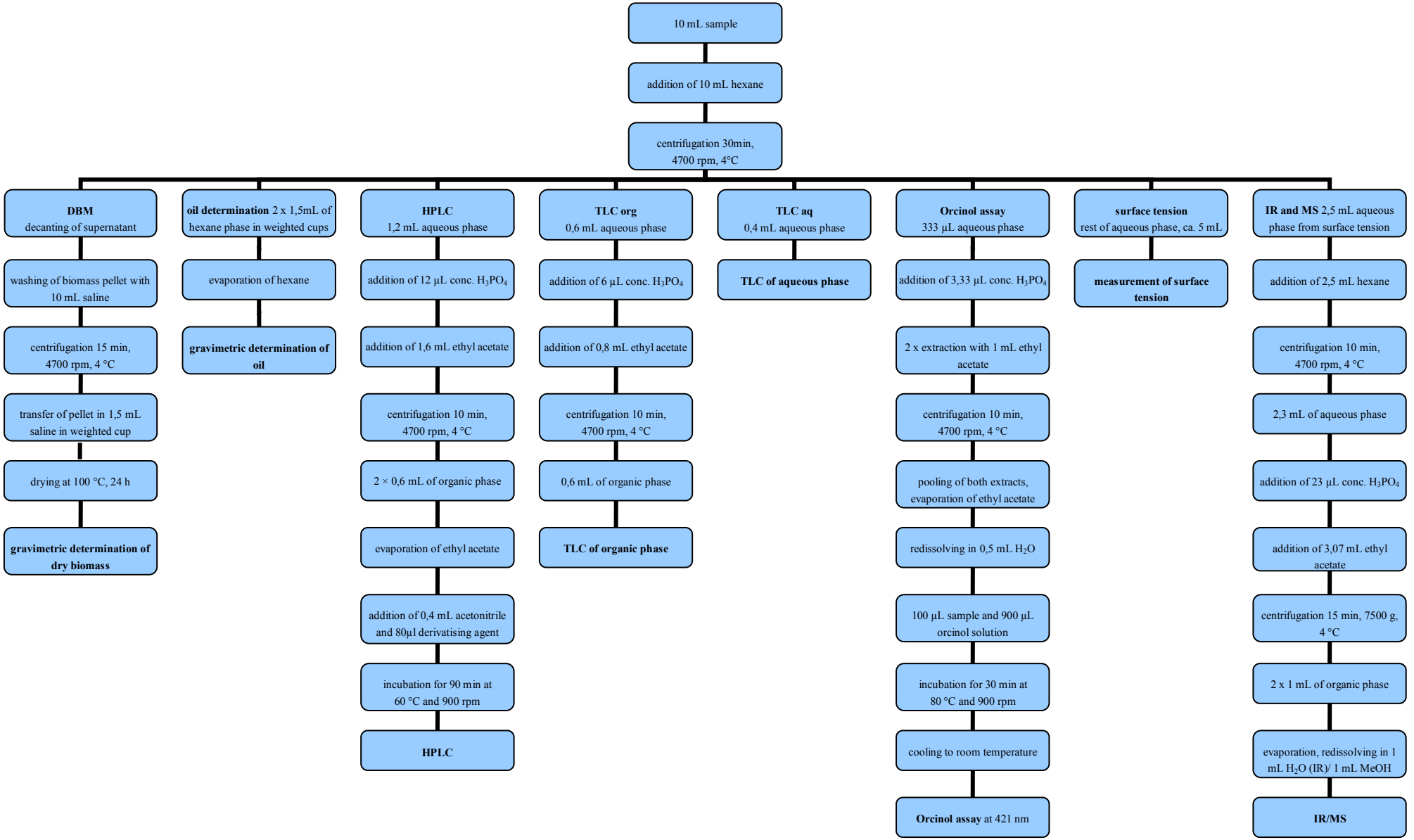


Figure 10: Working scheme for analytics

Measurement of oil concentration

Oil concentration was measured gravimetrically. Therefore, an aliquot of the hexane phase was transferred into a dried, pre-weighted tube. The organic solvent was evaporated and the residual oil was weighted.

Measurement of dry biomass (DBM)

The dry biomass was also measured gravimetrically. The biomass pellet, obtained after the first centrifugation step, was washed with sterile saline. Following a second centrifugation step, the biomass pellet was transferred to a dried, pre-weighted tube. The dry biomass concentration was determined after evaporation of the water at 100 °C for 24 h.

Measurement of glycerol concentration

Glycerol concentration was measured with an enzymatic kit for the determination of glycerol containing three enzymes; a glycerokinase, a pyruvate kinase and a lactate dehydrogenase (r-Biopharm, Darmstadt, Germany).

High performance liquid chromatography

HPLC was performed according to Schenk et al. 1995. The main rhamnolipids of *P. aeruginosa*, namely RL1 and RL3, could be quantified with this method whereas no standard for the other rhamnolipids of *P. aeruginosa* or other rhamnolipid producing strains was available. Pure RL1 and RL3 were used for calibration. Pure RL3 was obtained from the former Hoechst AG (Frankfurt, Germany). Pure RL1 was produced by enzymatic cleavage of pure RL3 and further purification by silica gel chromatography (Magario et al. 2008). The ethyl acetate from the rhamnolipid samples was evaporated and the sample was redissolved in acetonitrile. The rhamnolipids were then derivatised to phenacyl ester derivatives with 4-bromophenacyl bromide and triethyl ammonium (see Figure 11). Subsequently, equal volumes of the derivatising solutions A and B were mixed and 1 part of the solution was added to 5 parts of the sample. The sample was incubated at 60 °C for 90 min.

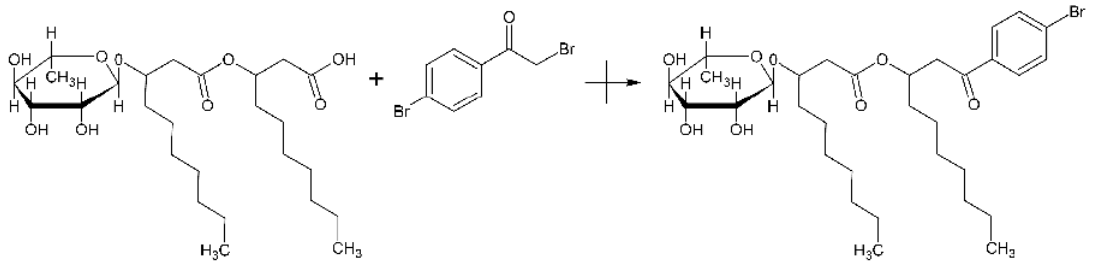


Figure 11: Reaction scheme of derivatisation of RL1 with 4-bromophenacyl bromide

The HPLC was performed on an Agilent 1100 Series HPLC equipped a hydrophobic Supelcosil LC-18 column with 5 µm particle size. Water and acetonitrile were used as mobile phase, with the following gradients:

0 – 4 min:	70 % solvent B
4 – 14 min:	70 % to 100 % solvent B
14 – 28 min:	100 % solvent B
28 – 33 min:	100 % solvent B to 70 % solvent B
33 – 38 min:	70 % solvent B

Solvent A: 5 % acetonitrile, 95 % water; Solvent B: 95 % acetonitrile, 5 % water

The chromatography was performed at 25 °C with a flow rate of 0,8 mL/min. The effluent was monitored with an UV detector at 265 min.

Emulsification

The emulsification of the plant oil was observed for first evaluations of the surface activity of the non-pathogenic strains. If plant oil was used as carbon source, the emulsification of the oil was evaluated visually.

Surface tension measurements

The surface tension σ of culture supernatant was measured at 23 °C with the digital tensiometer K10T from Krüss (Hamburg, Germany) and the Wilhelmy plate method (Wilhelmy 1863). If plant oil was used for the cultivation, this was extracted with hexane before the measurement. The Wilhelmy plate and the sample reservoir were cleaned with acetone and flamed before every measurement in order to prevent contamination of the samples.

Thin layer chromatography (TLC)

Rhamnolipid samples, for example aqueous culture supernatant, organic crude extract or other rhamnolipid samples, were analysed by thin layer chromatography. The TLC was performed according to Syldatk et al. 1985b; thereby, the rhamnolipids are separated on a TLC plate according to their hydrophobicity. A rhamnolipid standard was prepared from Jeneil JBR 425 (Jeneil Biosurfactants Company, Saukville, United States). JBR 425 contains the rhamnolipids 1 – 4 of *P. aeruginosa*. For the standard, 85 µL of JBR 425 were suspended in 1 mL of 0,1 M sodium phosphate buffer, pH 7, and acidified with 10 µL of concentrated phosphoric acid. This mixture was extracted with 1,333 mL of ethyl acetate and this ethyl acetate phase was applied as TLC standard. Silica60-coated plates were used for TLC and the solvent system was chloroform:methanol:acetic acid (65:15:2). The sugar moieties were stained with anisaldehyde (anisaldehyde:sulphuric acid:glacial acetic acid 0,5:1:50), the fatty acid moieties were stained with ammonium molybdate/cerium sulphate (0,42 % w/v ammonium molybdate

and 0,2 % w/v cerium(IV) sulphate in 6,2 % sulfuric acid). The yellow colour was developed by heating with a fan.

Orcinol assay

The orcinol assay is a method for direct assessment of the amount of sugars in a sample. It was originally developed by Chandrasekaran and BeMiller 1980 and performed according to the modifications of Tuleva et al. 2002. The extracellular glycolipid concentration was evaluated in duplicate by measuring the concentration of rhamnose in the organic extract. 333 µL of culture supernatant were acidified with 3,33 µL of 85% phosphoric acid and then extracted twice with 1 mL of ethyl acetate. The ethyl acetate fractions were pooled, evaporated and then resolved in 0,5 mL of H_2O. 900 µL of orcinol solution (0,19 % orcinol in 53 % H_2SO_4) was added to 100 µL of this sample. The samples were incubated at 80 °C for 30 min. After cooling to room temperature, the OD_{421} was measured. The rhamnose concentrations were calculated from a standard curve prepared with L-rhamnose and expressed in rhamnose equivalents RE [mg/mL]. For *P. aeruginosa* rhamnolipids, the rhamnose equivalents are usually multiplied by the factor three to obtain the rhamnolipid concentration (Mercadé et al. 1993; Abalos et al. 2002; Nitschke et al. 2005). This factor represents the correlation rhamnolipid/rhamnose and considers the proportion of rhamnose in the mono-rhamnolipid RL1 and the di-rhamnolipid RL3 and the ratio of RL1 and RL3 themselves. In the present work, rhamnose was also used for calibration because pure rhamnolipids of the non-pathogenic strains were not available. The results of the orcinol assay were not multiplied with the factor three so that the results of all strains can be compared, even if the rhamnolipid composition of the strain is not known.

CTAB agar

The CTAB agar method is a rapid screening method for the detection of anionic biosurfactants (Siegmund and Wagner 1991). As carbon source, sunflower oil, glycerine and glucose were applied. Gum Arabicum was added to the medium to stabilize the oil in the aqueous phase. 1 g/L Gum Arabicum is added to the oil and a part of the medium and then the mixture is homogenised with a sonicator. The suspension is then added to the rest of the medium before sterilisation. To strengthen the visual effect of the CTAB method, small wells were melted into the agar surface with the heated point of a glass stick or pipette. The cultures were then placed in the wells and incubated at 30 °C for 1 – 2 days and then stored at 4 °C for further colour development. The test was considered as positive if a dark blue halo is formed around the colonies.

IR

Infrared spectroscopic measurements of culture supernatant were performed on a Bruker Tensor 27 (Bruker Optics, Ettlingen, Germany) with the FTIR-ATR technique.

Spectra were recorded from 4000 – 800 cm^{-1} at a resolution of 4 cm^{-1} and represent the average of 64 scans. A characteristic glycolipid spectrum displays C-H stretching bands of CH_2 and CH_3 groups in the region of 3000 – 2700 cm^{-1}, a carbonyl stretching band, which is characteristic for ester compounds, at 1745 cm^{-1} and a fingerprint region from 1500 – 800 cm^{-1}, where characteristic bands for sugars are located (Christova et al. 2004).

MS

Mass spectrometry of the organic extracts of *B. plantarii* (see chapter 4.3.2) was performed with an API 4000 tandem-mass spectrometer (Applied Biosystems, Foster City, United States). The sample was solved in methanol/10 mM ammonium acetate (1:1) and injected by a pump with 0,6 mL/h. Analytes were ionised by electro spray ionisation in a turbo ion spray interface by – 4500 V in negative mode and a temperature of 400 °C. The experiments were performed in MCA mode, which means that 5 spectra are summated. The measurement range was from 300 – 1200 amu.

Mass spectrometry of the purified rhamnolipid of *B. plantarii* (see chapter 4.3.4) was performed on a Finnigan MAT 90 mass spectrometer (70 eV) (Thermo Fisher Scientific Inc., Waltham, United States) as electron impact mass spectrometry (EI). The molecular fragments are quoted as the relation between mass and charge (m/z), the intensities as a percentaged value relative to the intensity of the base signal (100%). The abbreviation [M+] refers to the molecular ion.

NMR

^{1}H-NMR spectra of the purified rhamnolipid of *B. plantarii* were recorded on a Bruker AVANCE 500 (400 MHz) spectrometer (Bruker Optics, Ettlingen, Germany) as solutions in $CDCl_3$. Chemical shifts are expressed in parts per million (ppm, δ) downfield from tetramethylsilane (TMS) and are referenced to $CHCl_3$ (7.26 ppm) as internal standard. ^{13}C-NMR spectra were recorded on a Bruker AVANCE 500 (125 MHz) spectrometer as solutions in $CDCl_3$.

3.4 Immobilisation of *P. aeruginosa* DSM 2874

3.4.1 Production of biomass for immobilisation

P. aeruginosa DSM 2874 was grown in 3 L baffled shake flasks with 1 L LB medium at 37 °C and 120 rpm. The medium was inoculated with 1 mL of a preculture in the exponential phase (about 20 g/L WBM). Cells for immobilisation were harvested in the exponential phase by centrifugation at 7500 rpm for 15 min and washed twice with sterile saline. The biomass was then resuspended in sterile saline to a concentration of 1 g

WBM/L. This suspension was subsequently utilised either for further immobilisation or for cultivation with free cells as reference to the immobilised cells.

3.4.2 Immobilisation by matrix entrapment

The immobilisation of *P. aeruginosa* DSM 2874 was realised by entrapment into gel particles. The liquid gel matrix (for example sodium alginate) was mixed with the cells so that an optimal cell load of the particles of 30 % resulted, according to Matulovic 1987. From this mixture, small gel particles are formed by a drop forming procedure. The droplets then fall into an appropriate hardening solution (e.g. calcium chloride) and solidify. A scheme of this procedure is shown in Figure 12. An immobilisation apparatus was used to generate the small droplets. This apparatus is essentially a spray nozzle with a diameter of 0,8 mm, generating small liquid droplets by pressure and a surrounding air stream and a reservoir for the matrix-cell-mixture (Matulovic 1987).

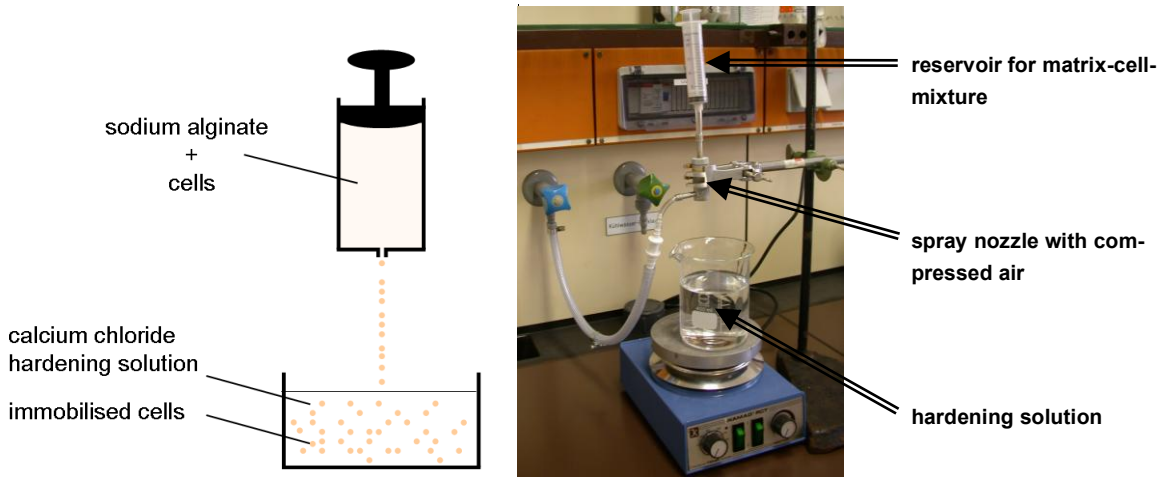


Figure 12: Scheme of immobilisation procedure for entrapment immobilisation (left) and experimental setup (right)

The immobilisation methods were tested in advance without cells and the immobilisation parameters were modified to improve the immobilisation procedure and the stability of the particles.

The hardening mechanism of the applied matrix materials is ionotropic gelation (alginate, κ-caragenaan) or sol-gel conversion (gelatin). In some cases further stabilisation of the particles was carried out by drying or by treatment with the crosslinkers protamine sulphate or glutaraldehyde.

Immobilisation in calcium-alginate:

Cells were immobilized according to Matulovic 1987. The harvested cells were mixed with the sodium-alginate-matrix before the matrix-cell-mixture was filled into the im-

mobilisation apparatus and dropped into the calcium-crosslinker-solution. After 30 min hardening in the crosslinker solution, the pearls were washed twice with saline.

A part of the calcium-alginate pearls was further treated to improve the stability of the pearls. For this, the pearls were dried on filter paper according to Vorlop and Klein 1981 at 20 °C for 3 h or at 40 °C for 4,5 h.

Immobilisation in barium-alginate

The immobilisation method in barium-alginate was modified from Orive et al. 2003. The cells were mixed with 1,7 % sodium-alginate-matrix and dropped into the barium-crosslinker solution. After hardening for 30 min, the pearls were washed twice with sterile saline.

A part of the pearls was further hardened for 30 min with the crosslinker 2, protamine sulphate and then again washed twice with sterile saline.

Immobilisation in barium-alginate/xanthane

This method was modified from Vigo et al. 2004. The saturated barium-solution is added to the xanthane solution to a final concentration of 20 mM of Ba^{2+}. Then, the cells are added. This mixture is dropped into the 0,75 % sodium-alginate solution, the pearls are hardened for 30 min and barium-alginate encapsulated xanthan particles were obtained. The pearls were further hardened with protamine sulphate for 30 min and again washed twice with saline.

Immobilisation in κ-caragenaan

The immobilisation method of Brodelius and Nilsson 1980 was applied. Due to rapid hardening of the gel, the κ-caragenaan concentration was reduced from 3% to 1,5 %. But even at that concentration, the κ-caragenaan could not be processed with the immobilisation apparatus.

Consequently, the gel was poured into sterile agar dishes. To harden the gel, it was laid in 0,3 M KCl solution and then cut into cubes of about 2×2×2 mm.

Immobilisation of *P. aeruginosa* was not performed with this method due to the rapid hardening of the gel even with the pouring method.

Immobilisation in gelatin/gelatin-alginate

The immobilisation method in gelatin and gelatin-alginate was performed according to Brodelius and Nilsson 1980. For gelatin-alginate, the gelatin-solution was mixed 1:1 with 3 % alginate solution or 1:2 and 1:3 with 2 % alginate solution. Gelatin and gelatin-alginate in different concentrations could not be processed with the immobilisation apparatus. Therefore, pouring of the gel into sterile agar dishes was performed. The cells were mixed with sterile gelatin-solution and poured into sterile petri dishes. The

mixture was allowed to cool for 2 h at RT, then 1 h at 4 °C. Subsequently the gel was treated with 2 % glutaraldehyde for 30 min, washed twice with saline and cut into cubes of about 2×2×2 mm.

3.4.3 Cultivation of immobilised *P. aeruginosa*

10 g of the particles with immobilised *P. aeruginosa* DSM 2874 with a cell load of 30 % were cultivated in 100 mL M3-medium for 9 days at 30 °C and 90 rpm in a baffled shake flask. As carbon source, 20 g/L olive oil or glycerol was applied. The pH of the cultivation in the unbuffered M3-medium was corrected several times during the cultivation with 1 M NaOH or 1M HCl. Seven samples were taken during the cultivation period. The rhamnolipid concentration of the supernatant was determined by HPLC, the optical density of the supernatant was determined photometrically and the cell leakage was calculated therefrom:

$$\text{cell leakage}\,[\%] = \frac{\text{biomass in supernatant}\,[\text{g}]\,\text{per}\,100\,\text{ml}}{\text{biomass in particles}\,[\text{g}]\,\text{per}\,100\,\text{ml}} \cdot 100$$

The cell leakage could only be determined for the cultures with glycerol as the oil droplets interfere with the photometric biomass measurement.

Free cells were cultivated as positive controls with the same cell concentration (3 g DBM in 100 mL M3-medium).

3.5 Downstream processing of rhamnolipids

The downstream processing of harvested culture broth starts according to the extraction protocol for analytics (see chapter 3.3) with the extraction of the plant oil with hexane and centrifugation. After separation of the cells and the supernatant, the supernatant is acidified with H_3PO_4 to a pH of 2 – 3 and the rhamnolipids are extracted thereof with ethyl acetate. The ethyl acetate extract is evaporated and the crude extract is gained. This crude extract was further processed by the following methods.

3.5.1 Thick layer chromatography

Thick layer chromatography was performed with 2 mm Silica60-covered plates (43 – 60 µm silica gel) and the solvent system chloroform:methanol:acetic acid (65:15:2) (Syldatk et al. 1985b). 3 – 9 mL of rhamnolipid extract from *B. plantarii* DSM 6535 was charged on each plate. After separation of the rhamnolipids, the edges of the plate were stained with anisaldehyde (see chapter 2.4) to visualize the rhamnolipid front. The rhamnolipid front was scratched off the plate with a knife and the silica gel was pestled. The rhamnolipids were extracted from the silica gel with different solvents, namely ethyl acetate, methanol, methanol/chloroform (1:1) and isopropanol.

3.5.2 Column chromatography

All column chromatography steps were performed with Silica60, 43 – 60 µm (230 – 400 mesh) and with crude extract of *B. plantarii* DSM 9509. The elution fractions were collected with a fraction collector and analysed by TLC and staining with anisaldehyde.

Primary cleaning

For a coarse purification, a column chromatography under isocratic conditions with the mobile phase chloroform:methanol:acetic acid (65:15:2) was performed. 5 mL of crude extract were loaded on a column of 473 mL (44 cm × 3,7 cm).

Alternatively, a column chromatography step with sequential elution, modified from Andrä et al. 2006, was performed. 0,48 g of crude extract was loaded on the column of 185 mL (59 cm × 2 cm). The following solvent systems were used for elution: fraction 1: chloroform; fraction 2: chloroform: methanol (95:5); fraction 3: chloroform: methanol (90:10); fraction 4: chloroform: methanol (80:20); fraction 5: chloroform: methanol (60:40); fraction 6: chloroform: methanol (50:50) and fraction 7: methanol. Four bed volumes of each solvent system were used for elution.

Fine cleaning

The fine purification of the rhamnolipid fractions from the first chromatographic step was performed as flash chromatography under isocratic conditions with 30:3:1 (chloroform:methanol:acetic acid) according to Bauer et al. 2006. 180 mg of rhamnolipid from the primary cleaning were dissolved in chloroform and loaded on a column of 170 mL (24 cm ×3 cm). The rhamnolipid fractions were pooled after the chromatography and the solvent was evaporated. The acetic acid was stripped from the mixture to prevent acid hydrolysis of the rhamnolipids following evaporation of the organic solvents. Therefore, an equal volume of toluene was added to the rhamnolipid fractions several times during evaporation.

3.5.3 Fast centrifugal partition chromatography (FCPC)

Fast centrifugal partition chromatography is a chromatographic technique applicable for analytic and preparative purpose. The technique uses a liquid-liquid biphasic system without solid support to maintain the stationary phase. Essentially, a CPC instrument is a series of channels linked in cascade by ducts and aligned in cartridges or disks in a circle around a rotor. When the rotor is set in motion, the assembly is subjected to a constant centrifugal field. Two immiscible liquids are used as mobile and stationary phase, the mobile phase being percolated through the stationary phase by a pump and the centrifugal field. Due to the liquid nature of the solvents, the upper or the lower phase of the biphasic system can be selected as mobile or stationary. In the ascending mode, the lighter phase is percolated through the heavier one in a direction opposite to

the centrifugal field (from the periphery to the centre of the rotor in each channel), and in descending mode the heavier phase is percolated through the lighter one in the direction of the centrifugal field (from the centre to the periphery of the rotor in each channel).

The advantage of FCPC is that the operation costs are lower, compared to column chromatograph; elution requires 10 times less solvent and the stationary phase can be easily recycled (Marchal et al. 2003).

The FCPC purification experiments were performed with a FCPC200 from Kromaton (Angers, France), kindly provided by Alphacrom OHG, Langenau, Germany. The rotor had a capacity of 200 mL. The experimental setup is shown in Figure 13.

Figure 13: FCPC setup with pump and detector (left-hand side) and FCPC device (right-hand side)

Two solvent systems were chosen for the purification experiments:

1. Ethyl acetate:water (1:1)
2. Hexane:ethyl acetate:methanol:water (5:1:3:5)

For solvent system 1, the nonpolar phase was selected as stationary phase (descending mode); for solvent system 2, the polar phase was selected as stationary phase (ascending mode).

100 mg crude extract of *B. plantarii* DSM 9509 were applied for each purification run. The elution fractions were collected with a fraction collector and analysed by TLC. After elution, the stationary phase was drained out of the rotor by changing the pump mode from ascending to descending or vice versa.

4 Results and Discussion

4.1 Waste glycerol substrates

The reduction of production costs is a fundamental requirement for a broader competitiveness of rhamnolipids on the (bio)surfactants market. The application of crude glycerol from biodiesel manufacturing as substrate can contribute to this aim because it is half as expensive as the standard substrate plant oil. Glycerol is a common substrate for rhamnolipid production (see section 1.3.3). Four glycerol based waste substrates were investigated in this work: crude glycerol from biodiesel production (ADM Biodiesel, Germany) and waste glycerol from linseed oil, passion fruit oil and fish oil (KD Pharma, Germany). The latter ones are derived from the production of ω-3-fatty acids from natural oils. The glycerol content of the glycerol substrates from KD Pharma was determined by an enzymatic assay in order to ensure that an equal amount of glycerol was used for the cultivations. The glycerol content of the substrates is given in Table 25.

Table 25: Glycerol content of waste and crude glycerol

substrate	glycerol content w/w [%]
crude glycerol (ADM Biodiesel)	81,9 (specification of ADM Biodiesel)
waste glycerol from linseed oil (KD Pharma)	55,0
waste glycerol from passion fruit oil (KD Pharma)	62,3
waste glycerol from fish oil (KD Pharma)	56,9

The applicability of the glycerol substrates for rhamnolipid production was then evaluated. *P. aeruginosa* DSM 7108 was cultivated with crude and waste glycerol as substrate in shake flasks. Two cultivation strategies were chosen: cultivation with growing cells and cultivation with resting cells. The cells were cultivated under resting cell conditions (in buffered M3 medium) and as growing cells (in 1/10 Giani-medium). The substrate concentration was adjusted according to the glycerol content of the waste substrates and pure glycerol was applied as reference substrate.

The results of these cultivations are given in Table 26. Similar rhamnolipid yields were obtained for pure glycerol and crude glycerol from biodiesel production. Up to 1,5 g/L rhamnolipid were produced whereas higher rhamnolipid concentrations were achieved

with resting cells than with growing cells. The reason for this finding is probably that higher cell concentrations are present in the resting cell cultivations and that the rhamnolipid biosynthesis by *P. aeruginosa* is favoured at high cell densities (see chapter 1.3.1).

These first investigations demonstrated that crude glycerol from biodiesel manufacturing is a very promising substrate because the rhamnolipid yields were similar to those of pure glycerol. The impurities from the harsh biodiesel manufacturing (see chapter 1.7 b) do not hinder cell growth and rhamnolipid synthesis.

The waste glycerol substrates from linseed oil, passion fruit oil and fish oil from KD Pharma were not appropriate for rhamnolipid production with *P. aeruginosa* DSM 7108. Rhamnolipid production occurred only with resting cells, and even for resting cells, the rhamnolipid yields were extremely low, compared to pure glycerol. A maximum of 0,18 g/L rhamnolipid was achieved with glycerol from linseed oil. The reason for the low productivity with these substrates is probably that inhibitory substances are present in the waste substrates, on relic from the prior manufacturing process, as the waste glycerol substrates are side-products from the production of ω-3-fatty acids from natural oils. Due to these negative results, the application of waste glycerol from linseed oil, passion fruit oil and fish oil was not further pursued.

Table 26: Results of cultivations of *P. aeruginosa* DSM 7108 with crude and waste glycerol, final rhamnolipid concentrations are given in [g/L] (cultivation at 30 °C and 120 rpm, quantification of rhamnolipid by HPLC)

cultivation conditions	**substrate**				
	glycerol	**crude glycerol**	**glycerol from linseed oil**	**glycerol from passion fruit oil**	**glycerol from fish oil**
growing cells (Giani-medium)	0,28 +/- 0,17	0,25 +/- 0,36	0,0 *	0,0 *	0,0 *
resting cells (Giani-medium)	**1,17 +/- 0,03**	**1,08 +/- 0,07**	0,18 +/- 0,26	0,03 +/- 0,04	0,04 +/- 0,02
resting cells (M3-medium)	**1,35 +/- 0,66**	**1,47 +/- 0,06**	0,01 +/- 0,01	0,0	0,0

* shake flask cultivation at 37 °C except of 30 °C

The highest rhamnolipid production of this set of experiments was achieved with crude glycerol and resting cells. The disadvantage of resting cell processes at bioreactor scale is that they are complex, laborious and expensive. They proceed in two steps. In the first step, the biomass is produced and harvested and in the second step, the biomass is util-

ised for production. Consequently, a simple cultivation process with growing cells would be favourable. However, significantly lower rhamnolipid concentrations were obtained with growing cells, probably due to lower biomass concentrations. The cultivations with growing cells also showed a high variance; therefore, the experiments with crude glycerol and growing cells were repeated and the final biomass concentrations were additionally determined. The mixing speed was increased to 150 rpm to promote bacterial growth and sunflower oil was chosen as second reference substrate.

The results of these cultivations are illustrated in Figure 14. The highest biomass concentration of 3,8 g/L was achieved with sunflower oil whereas the highest rhamnolipid yield of 0,31 g/L was obtained for crude glycerol. The highest specific productivity of 2,2 mg/gh was also obtained for crude glycerol.

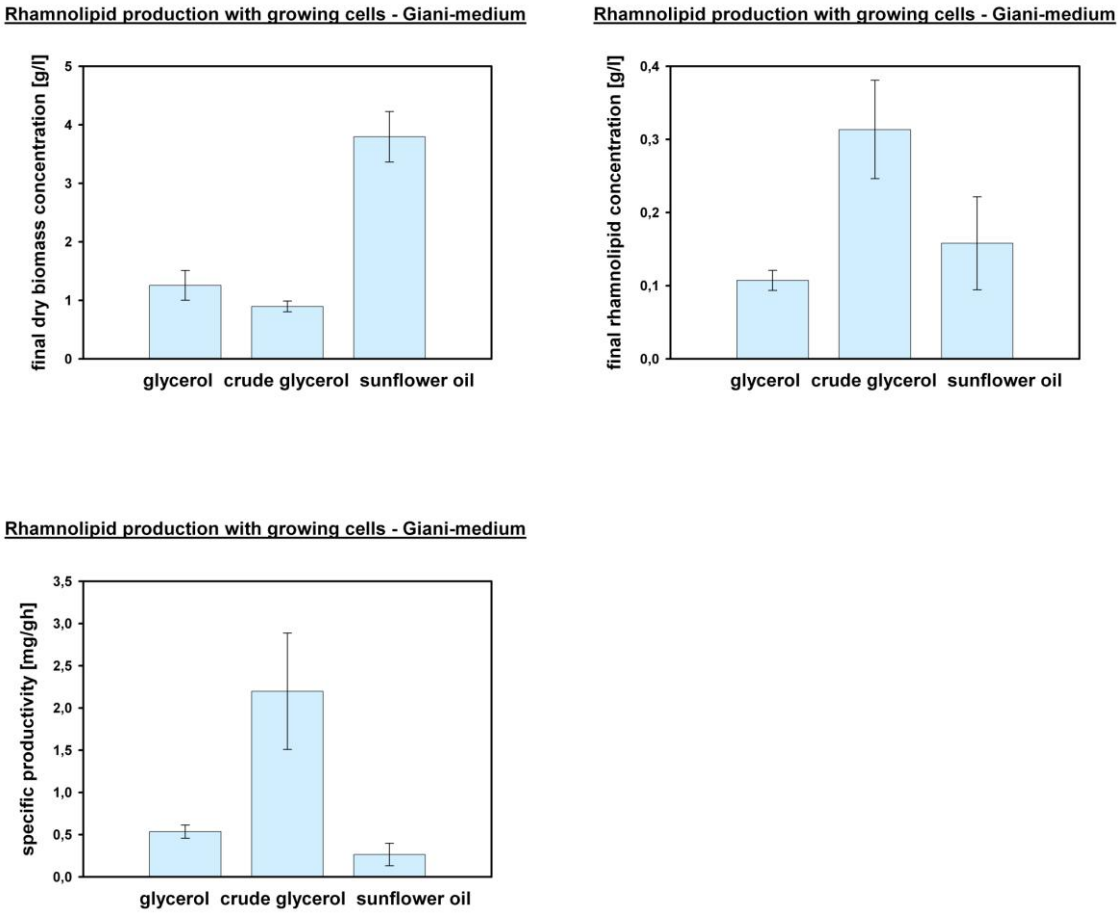


Figure 14: Results of cultivations of *P. aeruginosa* DSM 7108 as growing cells with crude glycerol (cultivation at 30 °C and 150 rpm in 1/10 Giani-medium, quantification of rhamnolipid by HPLC)

Conclusions

It was demonstrated that the waste substrates from linseed oil, passion fruit oil and fish oil from KD Pharma were not appropriate for rhamnolipid production with *P. aeruginosa* DSM 7108. They probably contain too many inhibitory impurities from the prior manufacturing process.

Crude glycerol from biodiesel production, on the other side, is a suitable substrate for rhamnolipid production with *P. aeruginosa*. Impurities from the biodiesel manufacturing do not hinder rhamnolipid production. In contrast, the rhamnolipid production with crude glycerol was even higher than the production with pure glycerol. This confirms that crude glycerol from biodiesel production is a promising low-cost waste substrate. The utilisation of crude glycerol could contribute to a reduction of the production costs of rhamnolipids so that rhamnolipids become competitive on the surfactant market. Therefore, the process with crude glycerol and growing *P. aeruginosa* cells should be further optimised and transferred to bioreactor scale.

4.2 Immobilisation of *P. aeruginosa*

The aim of this approach was to establish an *in-situ* product removal process for rhamnolipid production with immobilised cells and integrated membrane or deep filtration (see chapter 1.7). The *P. aeruginosa* cells have to be immobilised for this approach because free cells tend to block membranes integrated in bioreactors very quickly (Gruber 1991; Dhariwal 2007). Immobilisation by encapsulation was chosen as the immobilisation method because it is a gentle immobilisation method commonly applied for the immobilisation of whole cells.

Different immobilisation materials for encapsulation, namely calcium-alginate, barium-alginate, barium-alginate/xanthane, gelatin, gelatin/alginate and κ-caragenaan, were evaluated in pretests without cells. The feasibility of the immobilisation process with the immobilisation apparatus and the stability of the resulting particles were addressed in these pretests. The immobilisation parameters, such as matrix concentration, hardener concentration and hardening time were modified in order to improve the immobilisation procedure. In addition, two crosslinker, protamine sulphate and glutaraldehyde, were applied to enhance the stability of the particles. Drying of the calcium-alginate particles was also conducted to improve the stability. Suitable immobilisation matrices that could be processed with the immobilisation apparatus were then chosen for immobilisation experiments with *P. aeruginosa* DSM 2874. Immobilisation in gelatin was performed by the pouring and cutting method, as the material could not be processed with the immobilisation apparatus.

Particles with a size of about 70 – 900 µm were generated by the immobilisation apparatus. The particles were cultivated under resting cell conditions with olive oil or glycerol as carbon source. Free cells were cultivated as reference whereas an equal amount of biomass was applied for cultivations with immobilised and free cells. The cell leakage of the particles at the end of the cultivation was determined by measuring the final biomass concentration (OD) of the supernatant. This was only performed for the cultivations with glycerol as plant oil droplets interfere with the photometric measurement. Table 27 summarises the results of all cultivations with immobilised and free cells. The best results of each cultivation type in terms of rhamnolipid yield are highlighted in bold.

Table 27: Results of cultivations with free and immobilized *P. aeruginosa* DSM 2874; maximum rhamnolipid concentrations are given in [g/L], cell leakage (in brackets) is given in percent (cultivation at 30 °C and 120 rpm in unbuffered M3-medium, quantification of rhamnolipid by HPLC)

Cultivation type, substrate	**calcium-alginate I**	**calcium-alginate II**	**calcium-alginate III**	**barium-alginate/ protamine sulphate**	**gelatin/ glutaraldehyde**
Free cells, glycerol	0,72	-	-	0,32	**2,16**
Free cells, oil	0,47	-	-	**1,03**	0,49
immobilized, glycerol	0,66 (31 %)	0,61 (19 %)	**1,24** (31 %)	0,44 (66 %)	(1,07) (unstable)
immobilized, oil	0,33	0,37	0,58	**0,85**	(1,33) (unstable)
immobilized & post treatment, glycerol	-	drying I 0,63 (17 %)	drying II **1,85** (24 %)	protamine sulphate 1,00 (37 %)	glutaraldehyde (0,50) (unstable)
immobilized & post treatment oil	-	0,45	**0,82**	**0,82**	(0,64) (unstable)

A maximum of 1,9 g/L rhamnolipid was achieved with immobilised cells for calcium-alginate and glycerol whereas up to 2,2 g/L of rhamnolipid were obtained with free cells and glycerol. Significantly lower rhamnolipid yields were obtained for plant oil: 0,9 g/L for immobilised cells and 1 g/L for free cells.

All particles exhibited a high cell leakage of 17 – 66 %. The cell leakage was slightly reduced to 17 – 37 % by treatment with crosslinker or by drying of the particles, but this is still unacceptably high for a production process. It is unclear if the measured biomass in the culture supernatant resulted completely from a cell leakage of the particles or if growth of some free cells in the supernatant contributed to the high biomass concentrations in the supernatant.

Details for each immobilisation technique are presented and discussed below.

Immobilisation in calcium-alginate

Cultivation I: The rhamnolipid yield of calcium-alginate immobilised cells was comparable to the yield for free cells for both substrates, but the cell leakage of the immobilised cells was very high (31 %).

Cultivation II: The calcium-alginate particles were dried at 20 °C for 2 h to improve the stability and to reduce the cell leakage. This led to a weight reduction of 13 %. The cell leakage was reduced to 17 %. But the rhamnolipid yield was still in the same range as for free cells and immobilised cells without drying.

Cultivation III: The particles were dried at 40 °C for 4,5 h in this set of experiments. This led to a reduction of 40 – 48 % of weight and an increased rhamnolipid production of up to 1,24 g/L. The reason for the increase in rhamnolipid production is probably that the cell load was enhanced due to the extensive drying. The cell leakage remained very high (24 %).

Immobilisation in barium-alginate

Immobilisation in barium-alginate was carried out next. A part of the barium-alginate particles was further treated with the crosslinker protamine sulphate. The barium-alginate particles were very instable without crosslinking and they exhibited a cell leakage of 66 %. The cell leakage could be reduced by protamine sulphate treatment, but it was with 37 % still very high. The rhamnolipid production of barium-alginate immobilised cells was in the mid range, from 0,4 up to 1 g/L; at least for glycerol the rhamnolipid yield was higher than for free cells.

Immobilisation in barium-alginate/xanthane

In the pretests, the xanthane-cell-mixture could not be processed with the immobilisation apparatus because the resulting particles were unstable and clumped. Therefore, the matrix was processed manually through a syringe with a 0,9 mm capillary. However, no instantaneous gelification occured and the particles clumped and aggregated. They were too instable and broke when taken out of the alginate solution into the crosslinker solution. Therefore, the concentration of the crosslinker Ba^{2+} was increased from 20 mM up to 50 mM, but the pearls remained unstable. For this reason, no cultivation was carried out with this type of immobilisation.

Immobilisation in κ-caragenaan

The immobilisation method with κ-caragenaan was examined in pretests. The matrix-cell-suspension hardened very fast and blocked the immobilisation apparatus so that the material could not be processed with the immobilisation apparatus. Modifications of the matrix composition and warming of the matrix to 60 °C did not diminish the problem.

Not even the pouring method could be carried out due to the rapid hardening of the gel. Therefore, this method could not be applied for the immobilisation of *P. aeruginosa*.

Immobilisation in gelatin

Pretests were also used to test immobilisation in gelatin matrix. Gelatin and gelatin-alginate were tested in different concentrations, but they could not be processed with the immobilisation apparatus because no instantaneous hardening occurred. The immobilisation in gelatin was therefore performed with the pouring and cutting method and a part of the particles was further hardened by glutaraldehyde. A rhamnolipid concentration of up to 1,3 g/L was obtained with in gelatine immobilised cells. However, all gelatine particles dissolved completely during the cultivation. The reason is probably that *P. aeruginosa* possesses gelatinolytic activity (Palleroni 1981). Consequently, the material gelatin is inappropriate for immobilisation of this species. The degraded gelatin probably also served as an additional substrate which explains the high rhamnolipid yields of these cultivations.

Conclusion

The highest rhamnolipid concentration of 2,2 g/L was obtained with free cells and glycerol. For oil as substrate, a maximum product titer of 1,0 g/L could be obtained with free cells. The rhamnolipid production of all immobilised cells was below the maximum production with free cells, with the exception of the cultivations with gelatin immobilisation. However, these particles were instable and the gelatin probably served as additional substrate.

Furthermore, most particles displayed a high cell leakage of 17 – 66 %. Thus it is unclear if the rhamnolipids were synthesised more by the immobilised cells or more by the free cells in the culture supernatant.

The immobilisation of the cells is very laborious and expensive because several additional process steps, such as production of biomass, harvest of biomass and immobilisation of biomass are required. But this extra effort was not justified by any extra productivity. In contrast, the generated particles also exhibited a poor stability and an excessive cell leakage. This complicates the ISPR approach by filtration and a reuse of the cells. The high mobility of *P. aeruginosa* is probably the reason for the high cell leakage. *P. aeruginosa* is equipped with flagella and pili, and, therefore, capable of three different motilities: twitching on solid surfaces, swarming on semisolid surfaces and swimming in liquids (Caiazza et al. 2005). Another possible reason is that the rhamnolipids themselves may facilitate the leakage of the cells by altering the interfacial characteristics between the particle and the cells. It seems that *P. aeruginosa* is inappropriate for immobilisation by matrix entrapment. Membrane retention, which would be another

option for cell retention, is also unsuitable for *P. aeruginosa* because the membranes are instantaneously blocked by the cells and the polysaccharides (Gruber 1991; Dhariwal 2007).

A further drawback of the immobilisation technique is the low volumetric productivity because of the limited diffusional transport of nutrients and product through the particles in which the cells are immobilised (Gruber et al. 1993). This phenomenon was also observed in this work as lower product yields were obtained for plant oil than for glycerol.

However, many attempts have been made at immobilisation of *P. aeruginosa* for the production of rhamnolipids. For example, alginate/polyvinyl alcohol was examined as matrix for *P. aeruginosa* immobilisation and rhamnolipid production (Jeong et al. 2004). This immobilisation matrix was prone to cell leakage as well. Matulovic also reported a significant cell release during a cultivation with *P. aeruginosa* immobilised in calcium-alginate particles (Matulovic 1987). A dry biomass concentration of up to 2,5 g/L was present in the supernatant during the rhamnolipid production process. Consequently, due to stability problems, mass transfer problems for hydrophobic substrates and the lack of a significant increase in rhamnolipid production, the immobilisation approach is not advantageous. *P. aeruginosa* is not suitable for immobilisation with the studied entrapment immobilisation techniques and the advantages of ISPR can not be exploited in combination with these methods. Therefore, the immobilisation approach was not further pursued and the integrated processes with immobilised cells and ISPR by filtration or electro kinetically controlled deep filtration could not be realised.

4.3 Non-pathogenic production strains

This aim of this working package was to identify a suitable non-pathogenic rhamnolipid production strain which could become a non-pathogenic alternative to the common production strain *P. aeruginosa*. The non-pathogenic strains were selected from the literature (see chapter 1.4). For most of the investigated strains, little information was available on the structure of the synthesised rhamnolipids. Therefore, a screening with intensive analytics had to be carried out to investigate the rhamnolipid production of theses strains. Furthermore, little information was available on cultivation conditions, product yields and production processes from a commercial point of view. Therefore, successful candidates from the shake flask screening had to be subjected to further studies on rhamnolipid production at bioreactor scale.

4.3.1 Screening in shake flasks

The non-pathogenic strains were selected from Table 2. Some of the listed strains were not accessible to public and therefore not included in this study. In this case, other strains of the species were selected. Most of the selected strains belong to the genera *Pseudomonas* or *Burkholderia* and, thus, to the phylum proteobacteria. One bacterium of the phylum bacillus, namely *Tetragenococcus koreensis*, was investigated as well.

The non-pathogenic strains were first studied in shake flasks with different media and substrates. The first cultivations were carried out with 1/10 Giani-medium, which is an adequate medium for rhamnolipid production by *P. aeruginosa* in shake flasks. As substrate, the corresponding substrates from literature were chosen. *P. aeruginosa* DSM 7108 was cultivated as reference. The following analytics were performed to monitor biosurfactant formation in the culture supernatant: emulsification of the plant oil, CTAB agar assay, surface tension measurement, TLC with staining of the sugar and the fatty acid moiety, HPLC, IR, orcinol assay and determination of dry biomass.

The Giani-medium was inappropriate for rhamnolipid production for most of the strains. Therefore, corresponding media from literature and different substrates were evaluated subsequently. As substrates, glucose, glycerol and sunflower oil were then applied. The applied media and substrates for each strain are summarised in Table 28, the best medium being highlighted in bold. The cultivations with the best medium for each strain were repeated.

Table 28: Overview on applied cultivation media and substrates for shake flask screening of non-pathogenic strains

strain	**media**						
P. aeruginosa DSM 7108	**1/10 Gia, 12,5 g oil**						
P. chlororaphis DSM 50083	1/10 Gia, 12,5 g glu	Gun, 2 g glu	Gun, 2 g oil	**Gun, 12 g oil**	**Gun, 12 g oil**		
P. chlororaphis NRRL B-30761	-	Gun, 2 g glu	Gun, 2 g oil	**Gun, 12 g oil**	**Gun, 12 g oil**		
P. putida DSM 5232	1/10 Gia, 12,5 g glu	Tul, 2 g glu	Tul, 2 g oil	**1/10 Gia, 12,5 g oil**	1/5 Gia, 12,5 g oil	**1/10 Gia, 12,5 g oil**	
P. putida DSM 12735	1/10 Gia, 12,5 g glu	Tul, 2 g glu	Tul, 2 g oil	**1/10 Gia, 12,5 g oil**	1/5 Gia, 12,5 g oil	**1/10 Gia, 12,5 g oil**	
P. oleovorans DSM 1045	1/10 Gia, 12,5 g oil	1/10 Gia, 12,5 g gly	Suz, 12,5 g gly	**Suz, 12,5 g oil**	**Suz, 12,5 g oil**		
P. cruciviae NCIMB 10833	1/10 Gia, 12,5 g oil	1/10 Gia, 12,5 g gly	Suz, 12,5 g gly	**Suz, 12,5 g oil**	**Suz, 12,5 g oil**		
B. glumae DSM 9512	**1/10 Gia, 12,5 g oil**	1/10 Gia, 2 g gly	1/5 Gia, 12,5 g oil	Tru, 20 g oil	**1/10 Gia, 12,5 g oil**		
B. glumae PG1	**1/10 Gia, 12,5 g oil**	1/10 Gia, 2 g glu	1/5 Gia, 12,5 g oil	Tru, 20 g oil	**1/10 Gia, 12,5 g oil**		
B. plantarii DSM 9509	1/10 Gia, 12,5 g oil	1/10 Zäh, 0,75 g oil	**1/10 Zäh, 7,5 g oil**	1/5 Zäh, 7,5 g oil	**1/10 Zäh, 7,5 g oil**		
B. plantarii DSM 6535	1/10 Gia, 12,5 g oil	1/10 Zäh, 0,75 g oil	**1/10 Zäh, 7,5 g oil**	1/5 Zäh, 7,5 g oil	**1/10 Zäh, 7,5 g oil**		
T. koreensis DSM 16501	1/10 Gia, 12,5 g glu	M92	GYP, 2 g glu	MRS, 2 g glu	GYP, 2 g oil	**MRS, 2 g oil**	**MRS, 2 g oil**

Gia = Giani-medium, Gun = Gunther-medium, Tul = Tuleva-medium, Suz = Suzuki-medium, Tru = Trummler-medium, Zäh = Zähringer-medium, glu = glucose, gly = glycerol, substrate per 100 mL of medium

The 1/10 Giani-medium was an appropriate medium for rhamnolipid formation for only four of the non-pathogenic strains: both *P. putida* and both *B. glumae* strains. For the other strains, the corresponding media from the literature were favourable. Plant oil was the best substrate for all strains. The results of the best cultivation for each strain are given in Table 29, the given values representing the average of two cultivations. The strains with the best results in terms of rhamnolipid production are highlighted. These strains displayed especially positive TLC results.

Table 29: Results of shake flask cultivations of non-pathogenic strains with best cultivation conditions for each strain, given are final surface tension and surface tension of medium (in brackets), max. RE value, final DBM, R_f values of TLC, R_t of HPLC whereas peaks with similar R_t to *P. aeruginosa* are highlighted, CTAB agar assay results and IR results (+ means that IR spectrum was glycolipid-like) (cultivation at 120 rpm and 30 °C)

strain	Cultivation conditions	analytics							
		Emulsifi-cation	σ_{final} [mN/m]	Orcinol assay RE_{max} [mg/L]	DBM_{final} [g/L]	TLC R_f [-]	HPLC R_t [min]	CTAB agar	IR
P. aeruginosa DSM 7108	1/10 Giani + 12,5 g oil	++	29,2 (72,0)	0,356	7,2	0,65 (RL1); 0,48 (RL2); 0,31 (RL3); 0,14 (RL4)	**14,7** (RL1); **13,9** (RL2), **12,7** (RL3); **10,1** (RL4)	++ (oil) + (glucose)	+
P. chlororaphis DSM 50083	Gunther-Medium + 12 g oil, 23 °C	+++	28,6 +/- 0,0 (72,4)	0,011 +/-0,008	2,4 +/- 0,1	0,68	9,3; **10,3**; 12,0; **14,0**; 15,6; 19,9; 23,6	++ (oil) ++ (glucose	+
P. chlororaphis NRRL B-30761	Gunther-Medium + 12 g oil, 23 °C	+++	29,3 +/- 2,0 (72,4)	0,007 +/- 0,005	2,2 +/- 0,1	0,58	8,2; **9,9**; 11,6; **13,5; 14,0**; 15,8; 19,9; 23,1; 23,5	++ (oil) ++ (glucose)	+
P. putida DSM 5232	1/10 Giani + 12,5 g oil	++	42,5 +/- 7,4 (72,0)	0,010 +/- 0,005	1,7 +/- 0,1	-	7,8; 8,2; 19,9; 23,2	+ (glucose)	+
P. putida DSM 12735	1/10 Giani + 12,5 g oil	+	38,3 +/- 4,8 (72,0)	0,008 +/- 0,006	3,9 +/- 3,2	-	8,2; 9,0; 9,5; **9,8**; 12,0; **13,5**; 13,9; 23,6; 24,7	+ (oil)	-
P. oleovorans DSM 1045	Suzuki-Medium + 12,5 g oil	++	48,5 +/- 0,1 (53,4)	0,010 +/- 0,001	4,5 +/- 1,2	-	8,2; 8,5; **10,6**; 11,6; 23,2	+ (glucose)	-
P. cruciviae NCIMB 10833	Suzuki-Medium + 12,5 g oil	+++	47,4 +/- 4,0 (53,4)	0,014 +/- 0,004	3,3 +/- 1,2	-	8,2; 7,3; 7,8; 9,2	+ (oil)	-
B. glumae DSM 9512	1/10 Giani + 12,5 g oil	+++	33,4 +/- 1,1 (72,0)	0,058 +/- 0,037	1,3 +/- 1,0	0,46	8,6; 11,0; 12,0; **13,2; 13,4**; 15,7; 16,0; 16,2; 18,6; 19,2; 23,6	+ (glucose)	+
B. glumae PG1	1/10 Giani + 12,5 g oil	+++	34,4 +/- 2,3 (72,0)	0,021 +/- 0,004	1,6	0,50	7,6; 11,9; **13,2**; 17,5; 20,7; 23,6; 24,6	++ (oil) + (glucose)	+
B. plantarii DSM 9509	1/10 Zähringer + 7,5 g oil	+++	34,5 +/- 1,1 (64,7)	0,010 +/- 0,008	2,1 +/- 0,4	0,55	**13,5**; 15,8; 16,2; 19,9; 23,1; 23,6; 29,2	++ (oil) + (glucose)	+
B. plantarii DSM 6535	1/10 Zähringer + 7,5 g oil	+++	35,6 +/- 2,0 (64,7)	0,008 +/-0,006	2,1 +/- 0,6	0,56	**14,4**; 15,9; 19,9; 23,1; 23,6; 29,2	+ (oil)	+
T. koreensis DSM 16501	MRS + 2 g oil	++	40,1 +/- 8,1 (47,1)	0,018 +/- 0,001	2,4 +/- 0,8	-	7,3; 9,1; 9,6	-	+

The results of each analytical method are presented in the following.

Emulsification/surface tension measurement: The supernatants of *P. chlororaphis*, *B. glumae* and *B. plantarii* exhibited the best surface activity. The surface tension σ of the medium was reduced to about 29, 34 and 35 mN/m, respectively. This correlates with the fact that the plant oil was very well emulsified in these cultivations. *P. putida* showed a good surface activity as well and reduced the surface tension of the medium to 43 respective 38 mN/m.

Orcinol assay: The highest rhamnose equivalent of 0,36 g/L was measured for *P. aeruginosa*. The non-pathogenic production strains reached significant lower values of only 0,01 – 0,06 g/L. But it must be considered that theses strains have not yet been optimised for rhamnolipid production whereas rhamnolipid production with *P. aeruginosa* has been studied for decades. Thus, higher yields could be obtained by further optimisation of the medium composition and culture conditions. Furthermore, microbial production in shake flask is usually much lower than in bioreactor because process control, pH-control and better oxygen-supply are present in bioreactors and thus, higher biomass concentrations are achieved. That means that better results could be obtained by cultivation in bioreactor scale.

Dry biomass concentration: *P. aeruginosa* reached a maximum biomass concentration of 7,2 g/L whereas the non-pathogenic strains obtained significant lower biomass concentrations of 1,3 to 4,5 g/L. The better growth of *P. aeruginosa* culture might be an explanation for the higher RE values of *P. aeruginosa*, because more biomass can produce more rhamnolipid. Furthermore, the biosynthesis of rhamnolipids in *P. aeruginosa* is favoured at high cell densities (see chapter 1.3.1).

Thin layer chromatography: The TLC method separates the rhamnolipids according to their hydrophobicity. Consequently, the rhamnolipids of *P. aeruginosa* appear on the TLC plate in this order: RL1 (R_f = 0,65), RL2 (R_f = 0,48), RL3 (R_f = 0,31) and RL4 (R_f = 0,14). The RL1 of *P. aeruginosa* is very hydrophobic as it contains one rhamnose unit and two fatty acid units (RL-1,2_{10}) whereas the RL4 of *P. aeruginosa* (RL-2,1_{10}) is the less hydrophobic rhamnolipid. The TLC analysis of the supernatants respective organic extracts of the non-pathogenic strains displayed spots for both *P. chlororaphis*, both *B. glumae* and both *B. plantarii* strains. An example is shown in Figure 15. No specific spots were obtained for the other non-pathogenic strains.

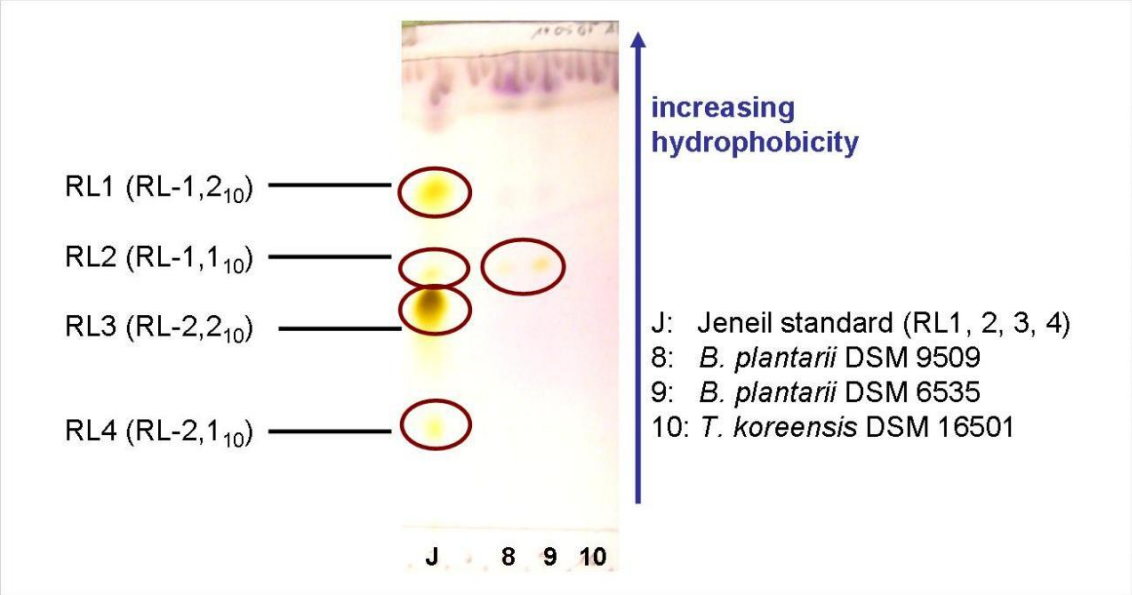


Figure 15: TLC of organic extracts of three non-pathogenic strains, staining with anisaldehyde

HPLC: The organic extracts of the non-pathogenic strains were derivatised with 4-bromo phenacyl bromide and then analysed by the HPLC method of *P. aeruginosa*. With this method, the rhamnolipids RL1 – 4 of *P. aeruginosa* can be identified by their corresponding retention times. However, the rhamnolipid composition of most of the non-pathogenic strains is not known, and so it is unclear where they will appear in the chromatogram. Therefore, the HPLC analysis can only indicate rhamnolipid production of the non-pathogenic strains. Peaks in the chromatogram were detected for all non-pathogenic strains; however, these peaks could also relate to fatty acids or mono-/di-glycerides, which are generated by the cleavage of the carbon source plant oil. These substances are also present in the organic extracts and they are probably also derivatised by 4-bromo phenacyl bromide, due to their acid function.

However, significant HPLC peaks were detected for some of the non-pathogenic strains. Peaks with a similar retention time to the rhamnolipids RL1 – 4 of *P. aeruginosa* (up to +/- 0,7 min) were found for of both *P. chlororaphis*, *B. glumae* and *B. plantarii* strains, one *P. putida* strain and *P. oleovorans*. In some cases, this corresponds to the results of the TLC analysis, where putative analogues to RL1 or RL2 were detected for *P. chlororaphis*, *B. glumae* and *B. plantarii*.

CTAB agar assay: The formation of a dark blue halo around a colony on the CTAB agar indicates the production of an anionic biosurfactant (Siegmund and Wagner 1991). The CTAB agar assay is considered as positive if large, dark blue halos are formed around the colonies; as weak positive if just a small halo is formed; and as strong positive if a large halo is formed. Large blue halos were observed for both *P. chlororaphis*

strains, *B. plantarii* DSM 9509 and *B. glumae* PG1. Two positive examples are shown in Figure 16.

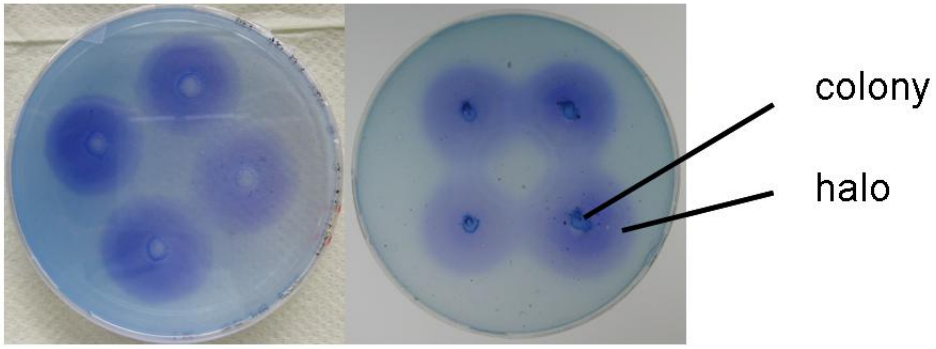


Figure 16: CTAB agar assay, left: *B. plantarii* DSM 9509, right: *P. chlororaphis* NRRL B-30761

Some of the strains with negative results did not grow on the agar, probably due to inhibitory substances in the agar medium (e.g. CTAB, methylene blue). Therefore, the negative results might be false negatives because the strains did not grow on the agar and not because they did not produce an anionic biosurfactant. Furthermore, fatty acids and mono- or di-glycerides can also lead to the formation of blue halos around the colonies because these substances are also anionic surfactants. They are generated by the lipolytic cleavage of the plant oil, which was applied, besides glucose, as substrate for the agar plates. This corresponds to the fact that for some strains, larger halos were obtained for oil as substrate than for glucose. Consequently, the CTAB agar assay can only provide a hint on rhamnolipid production.

IR: With the exception of *P. oleovorans*, *P. cruciviae* and both *P. putida* strains, all culture supernatants exhibited characteristic glycolipid spectra under infrared spectrometry. C-H stretching bands of CH_2 and CH_3 groups were observed in the region of 3000 – 2700 cm^{-1}. A carbonyl stretching band, which is characteristic for ester compounds, was found at 1745 cm^{-1}. In the fingerprint region from 1500 – 800 cm^{-1}, where characteristic bands for sugars are located, several bands were found. As an example, the IR spectra of the supernatants of *B. plantarii* DSM 6535 at day 4 and 8 are given in Figure 17. The IR signal increases with cultivation time in this example.

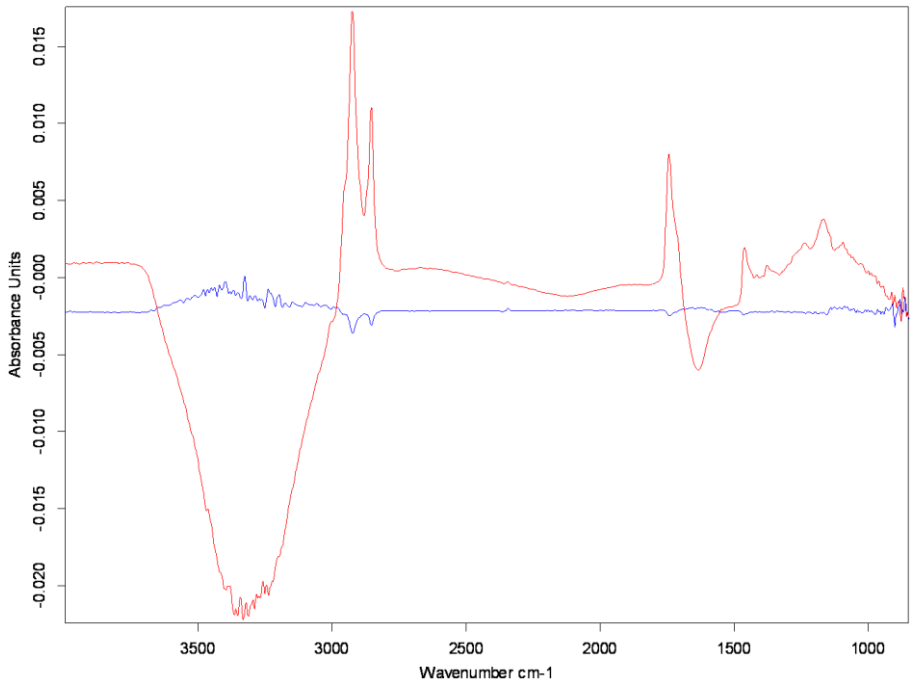


Figure 17: IR spectrum of culture supernatant of *B. plantarii* DSM 6535; blue line: day 4, red line: day 8

MS: Further analysis of the culture supernatants of the most successful strains was performed by mass spectroscopy. No specific masses were detected, probably because the rhamnolipid concentrations were too low. Therefore, this analytical method should be repeated with samples from bioreactor cultivations with higher rhamnolipid content.

Discussion

Different analytical methods were applied in the shake flask screening for assessment of rhamnolipid formation of the non-pathogenic strains. Some of these methods are specific for rhamnolipids while others are general methods for the detection of (anionic) biosurfactants or glycolipids. The observation of emulsification, the measurement of surface tension and the CTAB agar assay are general methods for surfactants, so that they only indicate rhamnolipid formation. These assays can be influenced by fatty acids or mono-/di-glycerides from the plant oil substrate. The IR spectrometry method, the orcinol assay and the TLC with staining of sugars and fatty acids are specific for glycolipids, but they also can not prove the existence of rhamnolipids. For a structural proof, MS or NMR experiments of (pure) rhamnolipids must be performed. Mass spectrometric analysis of the culture supernatants of the most promising strains was performed; but without success because the rhamnolipid concentrations in shake flask cultures were too low. Therefore, the shake flask screening with the applied methods only gives an indication on rhamnolipid formation; it can not prove the existence of rhamnolipids.

The results of each non-pathogenic strain are discussed in detail in the following.

P. chlororaphis:

In the present work, two different *P. chlororaphis* strains were investigated, *P. chlororaphis* NRRL B-30761 and DSM 50083. Both strains delivered positive results in terms of rhamnolipid formation in the shake flask screening, especially from TLC and IR analytics. Spots with R_f values of 0,54 respective 0,58 were detected on the TLC plates for both strains. These R_f-values could correlate to RL 2 of *P. aeruginosa* (RL-1,2_{10}) or to a rhamnolipid similar to RL 3 of *P. aeruginosa* (RL-2,2_{10}) with a more hydrophobic fatty acid moiety. Thus, the spots could relate to the rhamnolipids RL-1,$2_{12,10}$ or RL-1,$2_{12:1,10}$ which were already described for *P. chlororaphis* NRRL B-30761 by Gunther et al. 2005. This could not be verified by mass spectrometry due to the low rhamnolipid concentrations. Gunther et al. 2005 determined the structure of the rhamnolipids by HPLC/MS and they detected mono-rhamnolipids with two ß-hydroxy fatty acids of varying chain length, mainly RL-1,$2_{12,10}$ and RL-1,$2_{12:1,10}$. Gunther et al. 2005 also investigated *P. chlororaphis* DSM 50083, but they did not detect rhamnolipid formation by this strain. This is in contrast to the results of the present shake flask screening, where both strains performed similar.

P. putida:

The two selected *P. putida* strains performed less positive in the shake flask screening and there was little evidence of rhamnolipid formation by these strains. Especially the TLC did not show any specific spots. However, rhamnolipid production by *P. putida* has been described for different strains (Suzuki and Itoh 1972; Tuleva et al. 2002, Martínez-Toledo et al. 2006). Tuleva et al. 2002 reported a rhamnolipid concentration of 1,2 g/L RE for *P. putida* 21BN in shake flask, whereas Martínez-Toledo et al. 2006 reached a maximum rhamnolipid concentration of 0,062 g/L with *P. putida* CB100 in shake flask. Suzuki et al. 1972 presented no results of rhamnolipid production by *P. putida*. Tuleva et al. 2002 quantified the synthesised rhamnolipid by orcinol assay whereas Martínez-Toledo et al. 2006 measured the rhamnolipid concentration by hydrolysing the rhamnolipids and further analysis of the hydrophilic and hydrophobic compound by gas chromatography. They detected rhamnolipids with fatty acid chains from $C_{18:2}$ up to $C_{22:0}$. Tuleva et al. 2002 did not determine the structure of the rhamnolipids.

However, these two publications on rhamnolipid production with *P. putida* can hardly be compared with the present shake flask cultivations because different strains, media and cultivation conditions were applied. Martínez-Toledo et al. 2006 used a mineral salts medium with glucose and corn oil, while Tuleva et al. 2002 utilised a mineral salts medium with hexadecane or glucose. Unfortunately, the strains which were utilised by Tuleva et al. 2002 and Martínez-Toledo et al. 2006 are not accessible to public.

P. oleovorans* and *P. cruciviae:

There was little evidence of rhamnolipid production by *P. oleovorans* or *P. cruciviae* in the shake flask screening although the culture conditions and medium from literature were applied. The only difference was that plant oil and glycerol were applied as substrate instead of n-paraffin. Neither were specific spots observed on the TLC plates, nor a specific absorption in IR spectrometry. *P. oleovorans* and *P. cruciviae* are claimed in a patent as rhamnolipid producers (Suzuki and Itoh 1972), but no information on cultivation conditions, production processes or structure of the rhamnolipids is given in that patent. Due to the results of the shake flask screening, *P. oleovorans* and *P. cruciviae* are not considered as rewarding non-pathogenic alternatives to the conventional production strain *P. aeruginosa*.

B. glumae:

The two *B. glumae* strains delivered good results in the shake flask screening: the supernatants had a low surface tension; specific spots were detected on TLC and specific bands were found by IR spectrometry. Therefore, the two *B. glumae* strains are considered as interesting non-pathogenic candidates.

Rhamnolipid formation by *B. glumae* (synonym *P. glumae*) has already been reported by Manso Pajarron et al. 1993. They detected the following rhamnolipid species: RL-2,2_{14}, RL-2,$2_{12,14}$ and RL-2,$2_{14,16}$. Unfortunately, no information on the applied strain, culture medium or culture conditions is given in that publication.

B. plantarii:

Andrä et al. 2006 reported rhamnolipid production with *B. plantarii* DSM 6535. They found three different rhamnolipids with two rhamnose units and C_{14} ß-hydroxy fatty acids: RL-2,1_{14}, RL-2,2_{14} and RL-2,3_{14}. Remarkably, these rhamnolipids were isolated from the biomass and not, as usual, from the supernatant. This means they were intracellular metabolites. It was not confirmed if these rhamnolipids are also excreted and thus, present in the culture broth as extracellular metabolites.

In the present work, both *B. plantarii* strains achieved good results: they showed good surface activity, specific spots on TLC and specific bands in IR spectrometry. The TLC spots of the supernatant of the two *B. plantarii* strains were located at a R_f value of 0,56. The rhamnolipid RL-2,2_{14} which was described for *B. plantarii* DSM 6535 is more hydrophobic than RL 3 (RL-2,2_{10}) of *P. aeruginosa* and should have a higher R_f-value, so the spot with a R_f value of 0,56 could relate to RL-2,2_{14} or a similar rhamnolipid. This could not be confirmed by the performed mass spectrometry due to the low rhamnolipid concentrations. Altogether, the two investigated *B. plantarii* strains are very promising non-pathogenic rhamnolipids production strains and for the first time, a production of extracellular rhamnolipid by two different *B. plantarii* strains was recorded.

T. koreensis:

No rhamnolipid formation could be detected by TLC, HPLC or CTAB agar assay for *T. koreensis* in the present work. The other analytical methods also showed few positive results concerning rhamnolipid formation. Altogether, there is little evidence of rhamnolipid formation by *T. koreensis* with the applied culture conditions and analytics. However, rhamnolipid formation has already been described in literature for *T. koreensis* by Lee et al. 2005. They analysed the rhamnolipids by reversed phase HPLC and detected them with a UV detector; a mixture of rhamnolipids was applied as standard. Unfortunately, the structure of the synthesised rhamnolipid has not been investigated and no details on cultivation conditions are given in the publication.

Summary

Six of the non-pathogenic strains from the shake flask screening are very interesting candidates in terms of rhamnolipid formation: *P. chlororaphis* DSM 50083, *P. chlororaphis* NRRL B-30761, *B. glumae* DSM 9512, *B. glumae* PG1, *B. plantarii* DSM 9509, and *B. plantarii* DSM 6535. Overall, these strains showed the best results for rhamnolipid production in shake flask; consequently, they are regarded as the most promising candidates for further investigations.

4.3.2 Cultivation in parallel bioreactor

The best strains from the shake flask screening were then cultivated at bioreactor scale. The aim was to investigate their potential for rhamnolipid production at bioreactor scale and to obtain more rhamnolipid material for analysis and further purification. Little information was available for the non-pathogenic strains concerning production processes in bioreactor format but significantly higher yields of a bioproduct can usually be obtained in bioreactors than in shake flasks. In bioreactors, the cultivation conditions, such as like pO_2 or pH can be measured and controlled and the oxygen supply is enhanced due to air sparger and stirrer. Consequently, higher biomass concentrations can be achieved than in shake flask and this can also enhance rhamnolipid formation. Therefore it was very important to investigate the non-pathogenic rhamnolipid production strains at bioreactor scale. In addition to the six best strains from the shake flask screening, two more strains were chosen for cultivation in bioreactors, namely the two *P. putida* strains. Although these strains achieved less positive results in shake flask screening and especially no spots on TLC, they were also cultivated in bioreactor with the objective that better cultivation conditions in bioreactor could lead to better rhamnolipid production and because rhamnolipid production by *P. putida* has been reported in literature for different *P. putida* strains.

The eight selected strains were cultivated in the parallel bioreactor system Sixfors from Infors AG, Switzerland. The vessels have a total volume of 0,6 L. The bacteria were

cultivated under the same cultivation conditions as in the best shake flask cultivations. The medium concentration for *B. glumae*, *P. putida* and *B. plantarii* was elevated from 1/10 Giani-medium respective 1/10 Zähringer-medium to full Giani- respective Zähringer-medium, the full media being adequate for bioreactor cultivations. *P. aeruginosa* DSM 7108 was cultivated as reference. The results of these cultivations are given in Table 30. The values represent the average of at least two identical cultivations.

Table 30: Results of cultivations of non-pathogenic strains in parallel bioreactor, given are minimum surface tension and surface tension of medium (in brackets), max. DBM concentration, R_f values of TLC and max. RE values

strain	**medium**	**σ_{min} [mN/m]**	**max. DBM [g/L]**	**TLC [R_f]**	**Orcinol assay: max. RE [g/L]**
P. aeruginosa DSM 7108	Gia	n.d.	12,8 +/- 0,7	RL 1 (0,62) & RL 3 (0,40)	4,652 +/- 1,025
P. chlororaphis DSM 50083	Gun	30,1 +/- 1,3 (72,4)	6,2 +/- 1,1	-	0,013 +/- 0,002
P. chlororaphis NRRL B-30761	Gun	30,1 +/- 1,3 (72,4)	10,3 +/- 1,0	-	0,069 +/- 0,077
P. putida DSM 5232	Gia	n.d.	3,6 +/- 0,6	-	0,029 +/- 0,009
P. putida DSM 12735	Gia	n.d.	3,9 +/- 0,5	-	0,037 +/- 0,011
B. glumae DSM 9512	Gia	31,5 +/- 1,2 (73,1)	6,6 +/- 4,8	-	0,169 +/- 0,005
B. glumae PG1	Gia	28,4 +/- 0,3 (73,1)	7,9 +/- 2,8	-	0,695 +/- 0,742
B. plantarii DSM 9509	Zäh	27,1 +/- 1,1 (59,2)	51,6 +/- 4,0	0,35	0,210 +/- 0,084
B. plantarii DSM 6535	Zäh	26,6 +/- 1,1 (59,2)	26,2 +/- 3,3	0,35	0,164 +/- 0,107

Gia = Giani-medium + 125 g/L sunflower oil and additional oil feed of 125 g/L after 40 h, Gun = Gunther-medium + 120 g/L sunflower oil, Zäh = Zähringer-medium + 75 g/L sunflower oil

The highest rhamnolipid yield of 4,7 g/L RE was achieved with *P. aeruginosa*. The two *B. plantarii* strains were the best non-pathogenic strains: they showed good growth and relative high rhamnolipid concentrations (about 0,2 g/L RE). Moreover, they were the only non-pathogenic strains with positive TLC results. The cultivations with *P. chlororaphis*, *B. glumae* and *P. putida* were less successful because these strains grew slowly, reached only relative low RE values an especially because no specific spots were detected on TLC plates. Figure 18 shows a representative cultivation of each non-pathogenic strain.

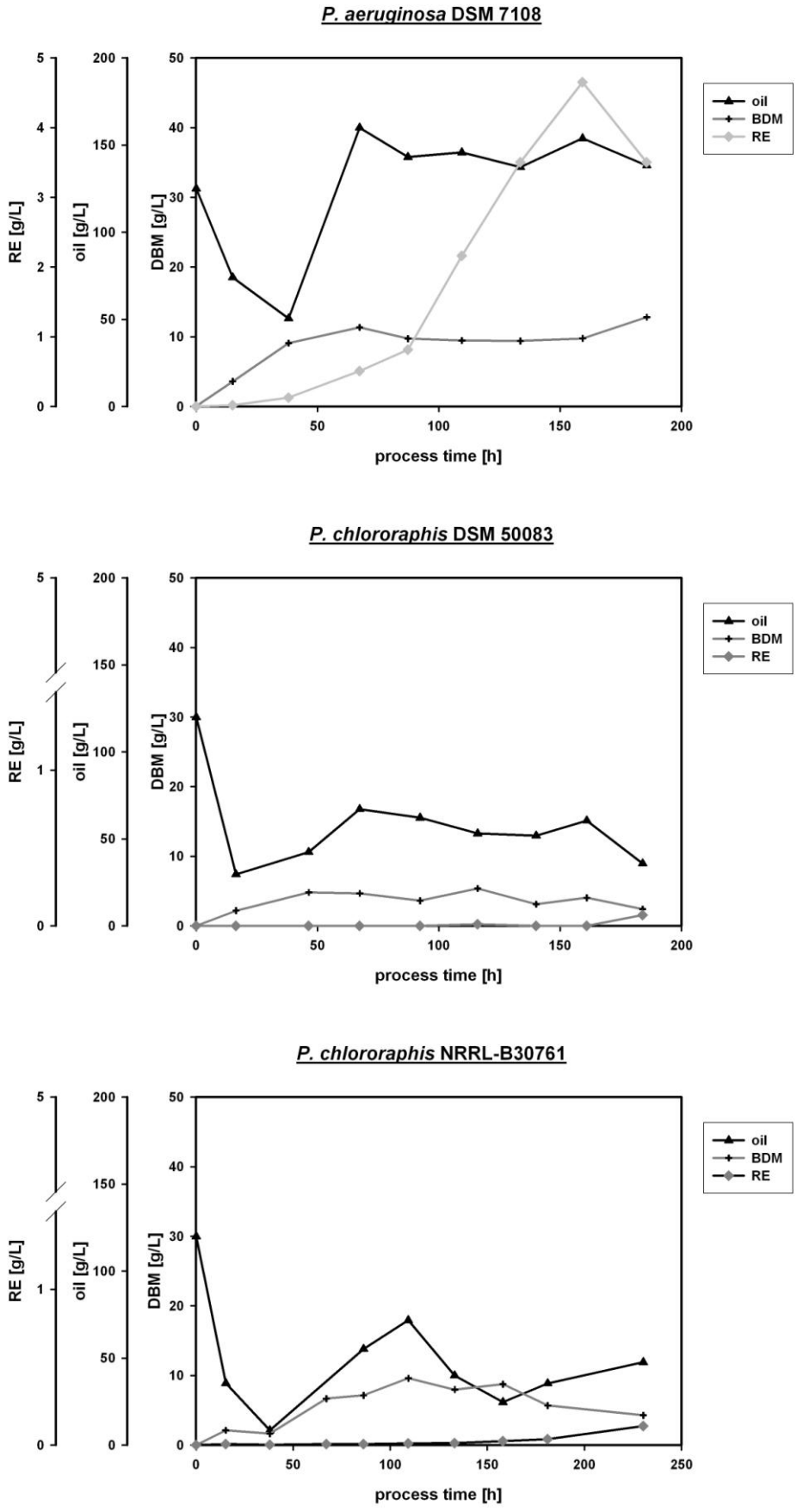


Figure 18: Cultivations of non-pathogenic strains in parallel bioreactor

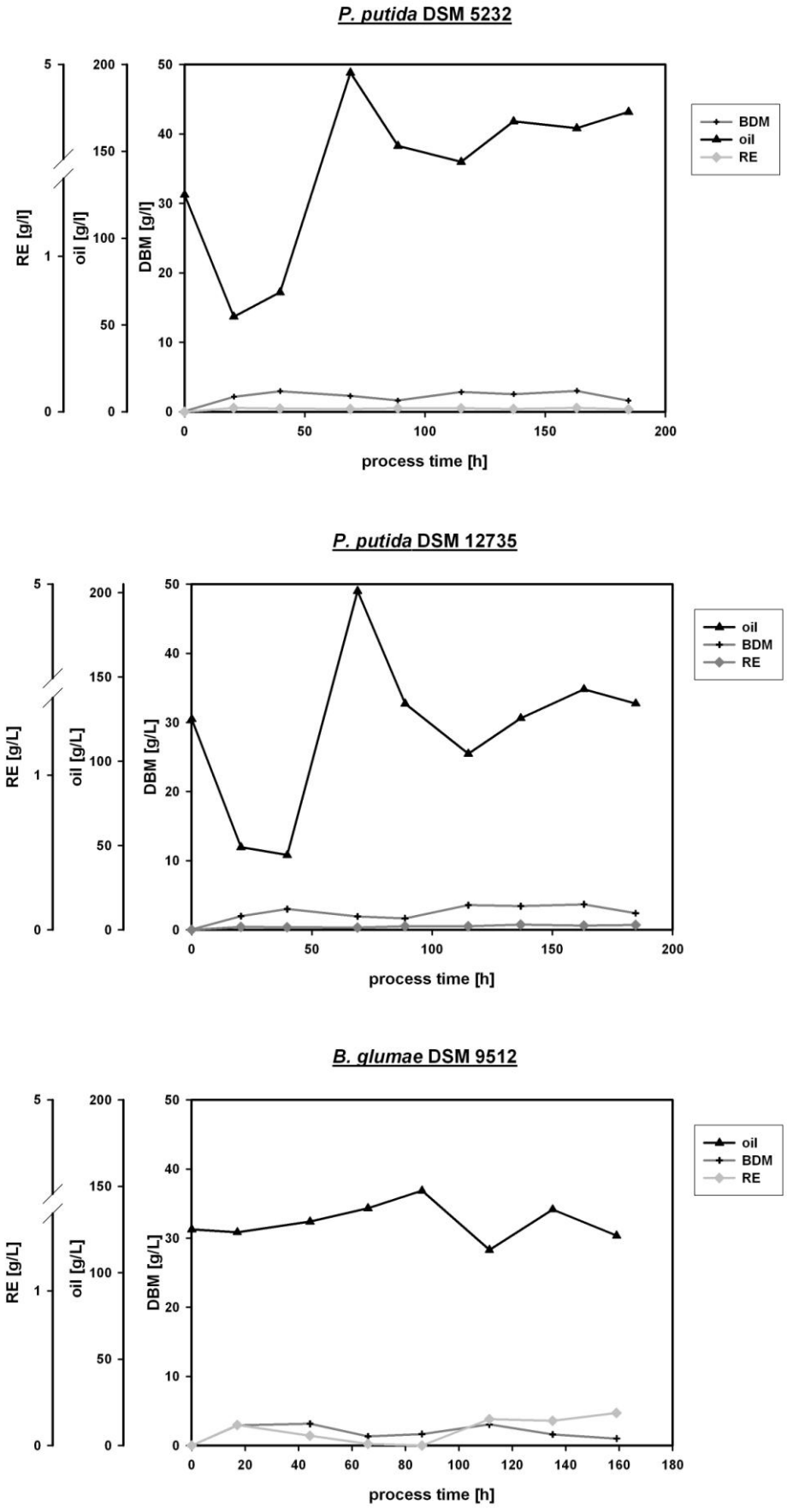


Figure 14 (continued): Cultivations of non-pathogenic strains in parallel bioreactor

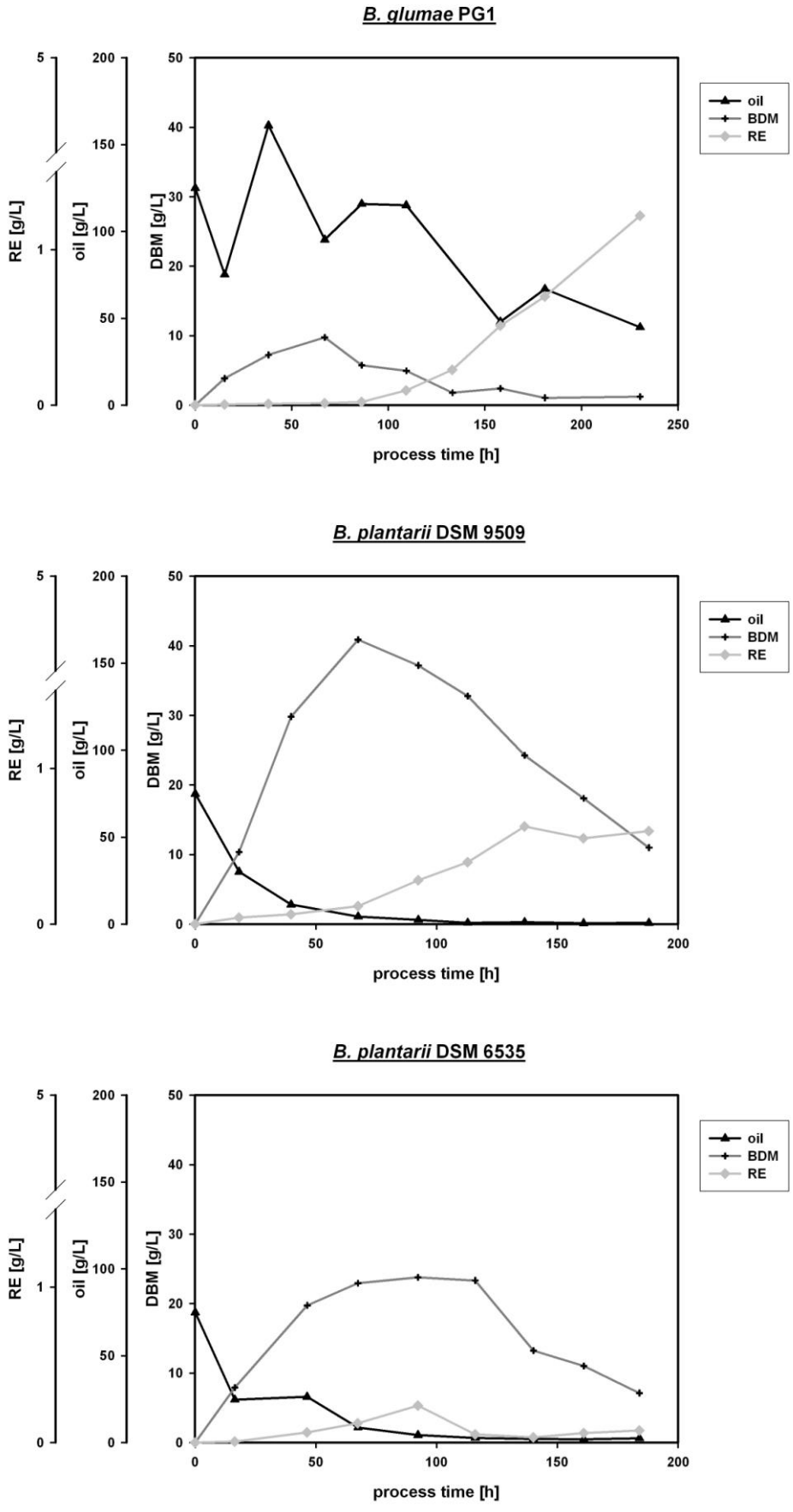


Figure 14 (continued): Cultivations of non-pathogenic strains in parallel bioreactor

The results of all strains are presented and discussed in detail in the following.

***P. aeruginosa* DSM 7108**

The standard rhamnolipid production strain *P. aeruginosa* DSM 7108 was cultivated as reference. The rhamnolipid concentration was determined with the orcinol assay and with the HPLC method for *P. aeruginosa* rhamnolipids RL1 and RL3. The HPLC measurement was performed at day 4 and 8 of the cultivations and a maximum of 16,5 +/- 2,2 g/L rhamnolipid was measured at day 8 (188 h process time) whereas only max. 4,7 g RE/L was measured with the orcinol assay. The strong discrepancy between the orcinol assay results and the HPLC results is illustrated in Figure 19.

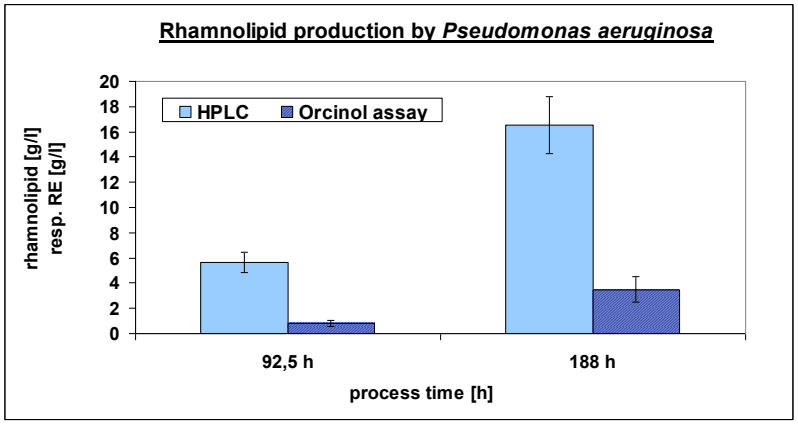


Figure 19: Comparison of rhamnolipid measurement by HPLC and orcinol assay

In this study, standards of RL1 and RL3 of *P. aeruginosa* were available whereas no standards for the other, non-pathogenic strains existed. The orcinol assay is often calibrated with pure rhamnose as standard if pure rhamnolipid is not available. The rhamnolipid concentration is then calculated by multiplying the rhamnose concentration by the factor three (e.g. Mercadé et al. 1993; Abalos et al. 2002; Nitschke et al. 2005). This factor represents the correlation of rhamnolipid/rhamnose and considers the proportion of rhamnose in the mono-rhamnolipid RL1 and the di-rhamnolipid RL3 and the ratio of RL1 and RL3 themselves. In the present work, rhamnose was used for calibration of the orcinol assay because pure rhamnolipids of the non-pathogenic strains were not available. The results of the orcinol assay were not multiplied with the factor three because the structure and composition of the rhamnolipid(s) of most of the non-pathogenic strains is not known. Therefore, the rhamnolipid concentrations measured by the orcinol assay are much lower than the HPLC results. The approach of taking the factor three has another disadvantage as the ratio of RL3 to RL1 changes during the production process (Leitermann 2008). Therefore, the results of the orcinol assay for *P. aeruginosa* cannot be multiplied with this factor if they are to be compared with the results of the

non-pathogenic strains. This is the reason for the strong variance of the HPLC and orcinol assay results for *P. aeruginosa*.

P. chlororaphis

The two *P. chlororaphis* strains reached low biomass concentrations and very low rhamnolipid concentrations of only 0,01 resp. 0,07 g RE/L. Additionally, no rhamnolipid spots were detected by TLC. Therefore, the parallel bioreactor cultivations of *P. chlororaphis* are not considered as successful. The two *P. chlororaphis* strains are no potent rhamnolipid production strains, at least not with the applied cultivation conditions, although they achieved quite good results in shake flaks. However, rhamnolipid production by *P. chlororaphis* NRRL-B 30761 has been reported by Gunther et al. 2005. They achieved up to 1 g/L rhamnolipid with the same strain, medium and temperature, but with glucose as substrate and in static shake flasks. Therefore, it might be advantageous to modify the cultivation conditions and perhaps even change to static culture. However, static cultures have the disadvantage that gas, energy and material exchange are extremely slow and, thus, the whole metabolism is decelerated. This leads to very long process times and diminishes the productivity. Therefore, rhamnolipid production with these strains was not pursued.

P. putida

The two *P. putida* strains performed even worse in parallel bioreactor than *P. chlororaphis*. They grew very slowly, consumed little of the oil and produced almost no rhamnose equivalents. Only 0,03 resp. 0,04 g RE/L were detected. Very weak yellow spots were detected on TLC plates for one of the triplicate cultivations of each strain. But these spots were only visible after concentrating the sample 10fold. Therefore, the two *P. putida* strains cannot be considered as interesting non-pathogenic alternatives, at least not with the applied cultivation conditions. The cultivations with *P. putida* in shake flask were also not successful. *P. putida* was cultivated in parallel bioreactor with the objective that better cultivation conditions enhance the productivity; this aim could not be achieved. Rhamnolipid production by *P. putida* in shake flask culture has already been reported (Tuleva et al. 2002; Martínez-Toledo et al. 2006); however, the strains that were applied in these publications are not accessible to public. Nevertheless, other *P. putida* strains might be more successful as well as a modification and optimisation of cultivation conditions and process parameters.

B. glumae

Better results were obtained for *B. glumae* than for *P. chlororaphis* and *P. putida*. At least 0,17 respective 0,70 g RE/L were measured by the orcinol assay. Although relative high rhamnose equivalent values were obtained, no yellow rhamnolipid spots were detected on TLC plates. Moreover, the high RE value for *B. glumae* PG1 has an extremely

high standard deviation, coming from two completely different cultivations. One cultivation yielded 1,22 g RE/L whereas the second one yielded only 0,17 g RE/L so that the positive result of the first cultivation could not be reproduced. The high RE value could be erroneous, for example because the sample was contaminated with polysaccharides that are produced by most of the strains. These polysaccharides tend to accumulate during the sample extraction at the interface of the supernatant and the hexane phase. The samples can then easily be contaminated with polysaccharides during the subsequent procedure and the polysaccharides would be recorded by the orcinol assay as well.

The two *B. glumae* strains are not considered as promising non-pathogenic rhamnolipid production strains because the TLC results were negative although they produced significant amounts of rhamnose equivalents. Therefore, rhamnolipid production with these strains was not pursued.

B. plantarii

The two *B. plantarii* strains are the most successful non-pathogenic strains: they grew very well, consumed the oil completely and generated relatively high rhamnose equivalents of 0,21 resp. 0,16 g/L. Moreover, they were the only non-pathogenic strains whose supernatant displayed large spots on the TLC plates. This correlates to the fact that the cultivations with *B. plantarii* were the only cultivations with excessive foam formation so that antifoam had to be feeded several times.

Very high biomass concentrations of up to 52 g/L respective 26 g/L were obtained with the two *B. plantarii* strains; *P. aeruginosa*, for example, reached only 13 g/L. The high biomass concentrations could be explained to some extent by the accumulation of poly-ß-hydroxybutyrate (PHB). The formation of PHB was already reported for *B. plantarii* by Azegami et al. 1987. PHB is a polyester of ß-hydroxybutyrate and produced by a variety of bacterial species (Chen and Wu 2005). They are synthesised by bacteria as storage compounds for energy and carbon, normally in the presence of excess carbon with at least one nutrient essential for growth, such as nitrogen, phosphorus, sulphur or oxygen present in limiting concentration (Anderson and Dawes 1990). In the later phase of the cultivation, the biomass decreases. This decrease could be explained by a consumption of the storage compound PHB by the cells when starvation starts. This correlates with the fact that the decrease of biomass begins when the oil is depleted.

In addition to the results of Andrä et al. 2006 that found rhamnolipids as intracellular component of *B. plantarii* DSM 6535 and that extracted the rhamnolipids from the biomass, rhamnolipids are found in this work as extracellular metabolites of two *B. plantarii* strains, DSM 6535 and DSM 9509. This is a new finding and very advantageous for an industrial application as is reduces the amount of recovery steps and thus the production costs greatly.

The rhamnolipid production of all non-pathogenic strains in the parallel bioreactor is summarised in Figure 20. This figure shows clearly that the rhamnolipid production of *P. aeruginosa* is far beyond the productivity of all non-pathogenic strains. But it must be considered that the rhamnolipid production with *P. aeruginosa* has been studied and optimised for many years whereas the non-pathogenic strains are a new field. The two *B. plantarii* strains are considered as the most promising non-pathogenic rhamnolipid production strains because of the positive TLC results although the two *B. glumae* strains obtained high RE values as well. Therefore, the two *B. plantarii* strains were chosen for further investigations, purification of rhamnolipids and structure elucidation.

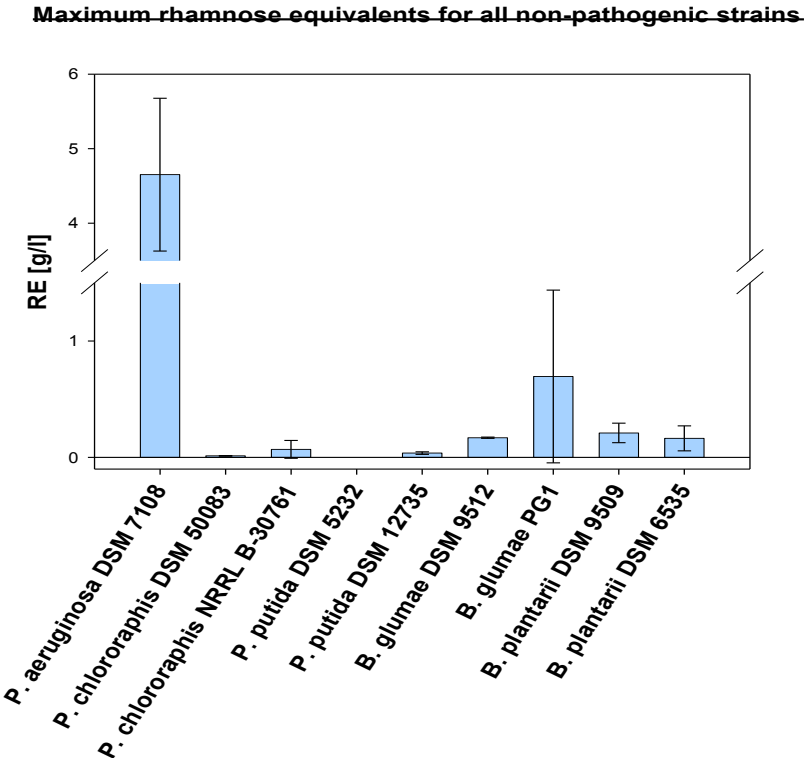


Figure 20: Comparison of rhamnolipid production of all non-pathogenic strains in parallel bioreactor

Further investigation of *B. plantarii* in parallel bioreactor

Two additional items were investigated in Sixfors parallel bioreactor. First, the effect of an additional oil feed corresponding to the Giani-process was examined because the oil was completely consumed by *B. plantarii* after 70 h of process time. An oil feed of 75 g/L sunflower oil was added after 40 h of process time in order to improve the rhamnolipid production. Second, the pH corrective NH_3 (aq) was replaced by NaOH because NH_3 (aq) is hazardous and tends to outgassing. The cultivations were performed with the same cultivation conditions as the former *B. plantarii* cultivations. The results of these cultivations are summarised in Table 31.

Table 31: Results of *B. plantarii* cultivations with additional oil feed and NaOH as pH corrective, given are max. DBM, max. RE and process time of RE maximum (cultivation with Zähringer-medium at 30 °C)

strain	oil-feed	pH corrective	max. DBM [g/L]	max. RE [g/L]	…at process time [h]
B. plantarii DSM 9509	-	NH_3	51,5 +/- 1,0	0,363 +/- 0,133	76,9 +/- 13,3
B. plantarii DSM 9509	+	NH_3	40,5 +/- 8,2	0,068 +/- 0,05	149,7 +/- 13,4
B. plantarii DSM 9509	+	NaOH	17,9	0,076	66,0
B. plantarii DSM 6535	-	NH_3	26,2 +/- 3,3	0,175 +/- 0,089	100,8 +/- 12,0
B. plantarii DSM 6535	+	NH_3	27,2 +/- 3,0	0,321 +/-0,255	171,6 +/- 17,6
B. plantarii DSM 6535	+	NaOH	15,9	0,052	86,2

Effect of additional oil-feed: Although the variance between the duplicate cultivations with and without additional oil feed was high, a tendency becomes clear: the maximum rhamnolipid concentration is reached later in the process if additional plant oil is fed. The maximum rhamnolipid yield is delayed about 70 h for both *B. plantarii* strains. Moreover, for *B. plantarii* DSM 9509, a higher rhamnolipid yield is obtained without an additional oil feed (0,36 g/L) than with (0,07 g/L). For *B. plantarii* DSM 6535, the additional oil feed is advantageous in terms of maximum rhamnolipid yield: the maximum rhamnolipid concentration was raised from 0,18 to 0,32 g/L. However, this advantage is diminished by the fact that this maximum rhamnolipid concentration is reached only at the very end of the production process, when the oil concentration decreases (see Figure 21). Additionally, the duplicate cultivations diverged strongly in this case so that a high standard deviation resulted. While one cultivation yielded 0,50 g RE/L, the second process yielded only 0,14 g RE/L.

Although the additional oil feed did not lead to the desired increase in rhamnolipid production it is very interesting because it reveals some interesting insights into the regulation of rhamnolipid formation by *B. plantarii*. The rhamnolipid biosynthesis of *B. plantarii* seems to be regulated in a different way than the regulation of rhamnolipid biosynthesis in *P. aeruginosa*. While a cell-density dependent regulatory principle applies for *P. aeruginosa* (Ochsner and Reiser 1995), the oil concentration seems to have more influence on rhamnolipid formation by *B. plantarii*. The biomass concentration of

B. plantarii DSM 6535 was very high during the cultivation with an additional oil feed, but the rhamnolipid formation started just when the oil was almost completely depleted.

Effect of NaOH as pH corrective: The second item that was investigated was the replacement of NH_3 by NaOH. This approach was not successful. Cell growth and rhamnolipid production were diminished extremely with NaOH as pH corrective. Only 18 g/L respective 16 g/L of biomass were obtained and a maximum of 0,08 g/L respective 0,05 g/L of rhamnolipid were formed. The nitrogen supply by the NH_3 seems to be essential for bacterial growth and rhamnolipid formation in this case, although nitrogen is present in the medium as ammonium and yeast extract.

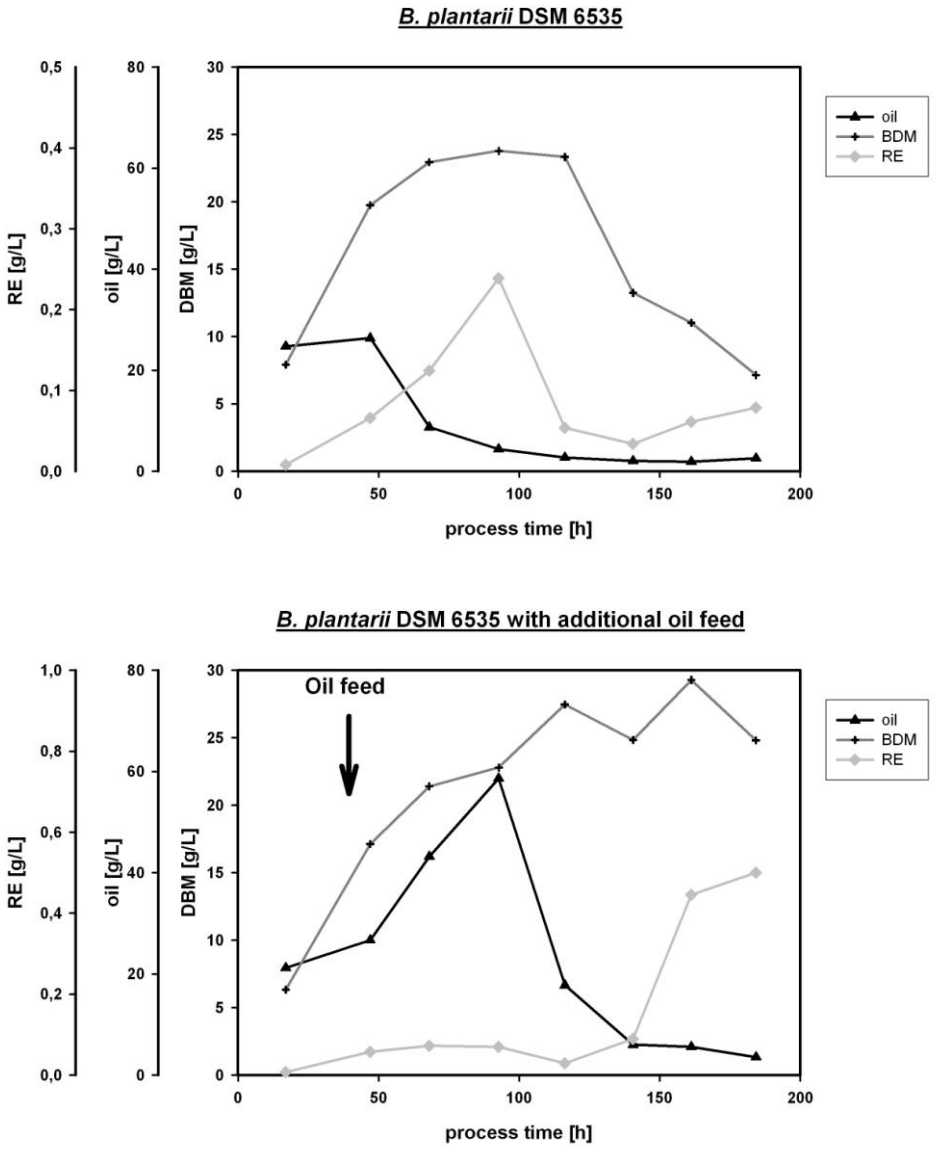


Figure 21: Cultivation of *B. plantarii* DSM 6535 without and with additional oil feed

Structure elucidation of *B. plantarii* rhamnolipids

The structure of the *B. plantarii* rhamnolipids was determined by mass spectrometry of the organic extracts of both *B. plantarii* strains. A Q1 scan of the organic extracts revealed similar structures and one dominant peak at about 762 m/z for both strains. This mass corresponds to a di-rhamnolipid with 2 C_{14} ß-hydroxy fatty acids (RL-2,2_{14}). Furthermore, two minor peaks with 734 respective 790 m/z were found. They correspond to a di-rhamnolipid with a C_{12} and a C_{14} ß-hydroxy fatty acid (RL-2,$2_{12,14}$) respective a C_{14} and a C_{16} ß-hydroxy fatty acid (RL-2,$2_{14,16}$) (see Figure 22).

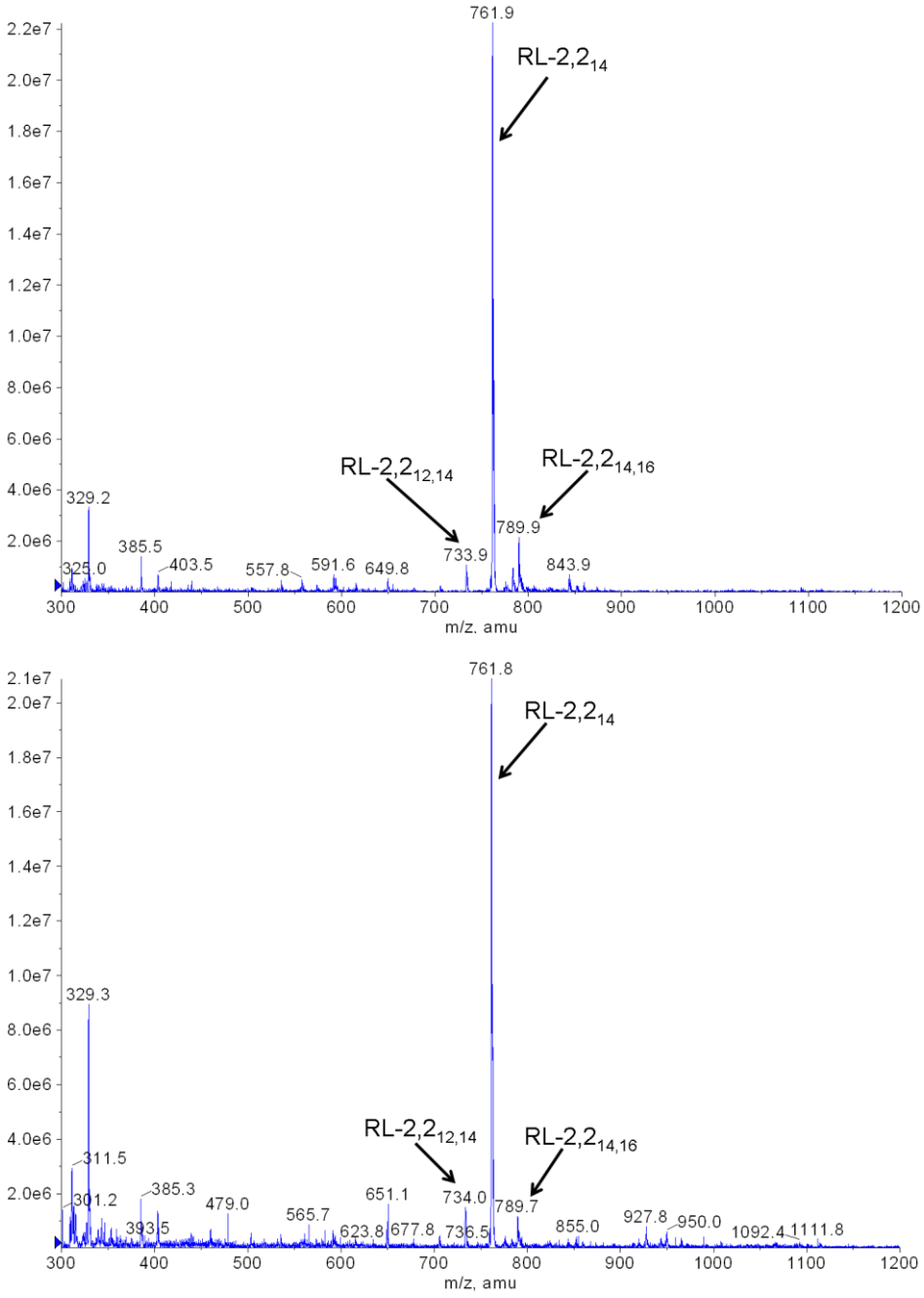


Figure 22: MS of extract of *B. plantarii* DSM 6535 (top) and DSM 9509 (below)

A fragmentation (Q2 scan) of the dominant peak of 762 m/z confirmed the assumption, that the related structure is RL-2,2_{14} as the corresponding fragmentation peaks for the corresponding mono-rhamnolipid, the C_{14} fatty acid and the rhamnose were detected (see Figure 23).

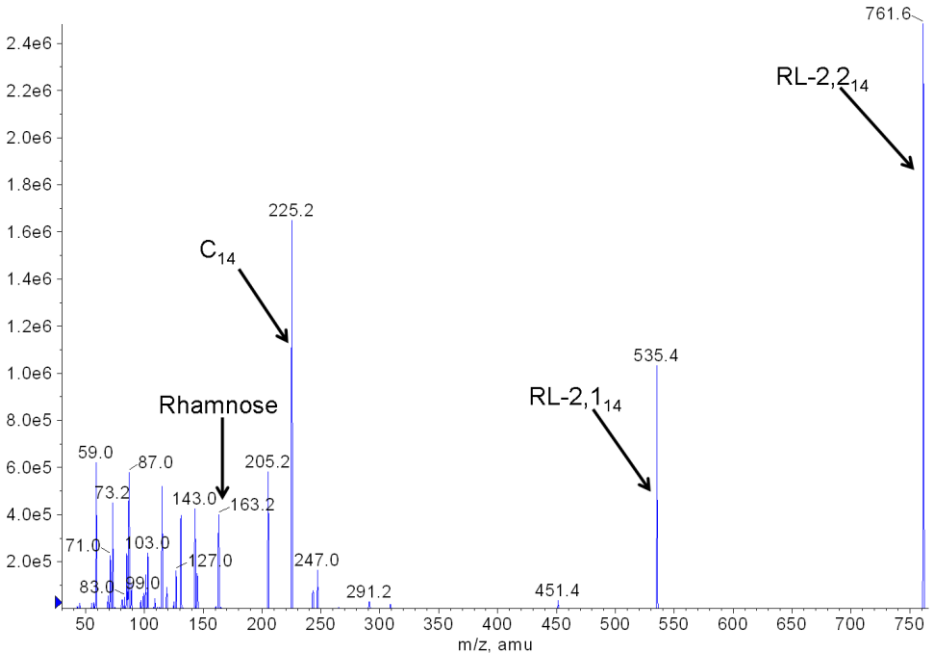
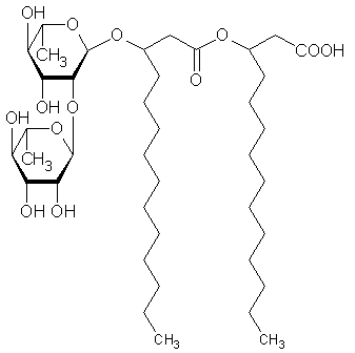


Figure 23: MS of extract of *B. plantarii* - Fragmentation of 762 m/z

Andrä et al. 2006 also detected the rhamnolipid RL-2,2_{14} in *B. plantarii* DSM 6535. Moreover, they found two additional rhamnolipids: RL-2,1_{14} and RL-2,3_{14}. These structures were not found in the present work; however, two other structures were detected in minor amounts: RL-2,$2_{12,14}$ and RL-2,$2_{14,16}$. This is in contrast to the findings of Andrä et al. 2006 and could relate to the fact that the samples were prepared differently. While Andrä et al. 2006 extracted the rhamnolipids from the biomass and purified them by silica gel chromatography, the rhamnolipids were extracted from the supernatants in this work and they were not further purified. Another explanation would be that different rhamnolipid congeners exist intracellular and extracellular.

Interestingly, the same rhamnolipids that were detected for *B. plantarii* in this work, RL-2,2_{14}, RL-2,$2_{12,14}$ and RL-2,$2_{14,16}$, were also reported for *B. glumae* (Manso Pajarron et al. 1993). Unfortunately, no information on the applied strain, culture medium or culture conditions is given in that publication.

The structure of the rhamnolipid RL-2,2_{14} of *B. plantarii* is given in Figure 24.



RL-2,2$_{14}$

Figure 24: Structure of *B. plantarii* rhamnolipid RL-2,2$_{14}$

4.3.3 Scale up of *B. plantarii* DSM 9509 cultivation to 40L bioreactor

The non-pathogenic rhamnolipid production strain *B. plantarii* DSM 9509 is of special interest because this strain has not previously been described as a rhamnolipid production strain, although it was also investigated by Gunther et al. 2005. High rhamnolipid yields of up to 0,36 g/L were obtained with this strain in the parallel bioreactor cultivations. Therefore, further investigations were carried out with this strain. Higher amounts of pure rhamnolipid of this strain were necessary for purification, structure elucidation and preparation of standards; consequently, a scale-up of the production process to the 40 L Cplus bioreactor (Sartorius, Melsungen, Germany) was carried out. The process parameters filling volume, aeration rate and stirrer speed were adopted from former cultivations of *P. aeruginosa* in the same bioreactor (see Table 32) because a good foam control could be obtained for *P. aeruginosa* with these parameters.

The process course of the cultivation of *B. plantarii* DSM 9509 in 40 L scale is shown in Figure 25. The cells grew well and reached up to 30 g/L of dry biomass. The oil was completely consumed after 90 h. However, almost no rhamnolipid formation occurred and only 0,01 g/L of rhamnolipid were synthesised. It seems that the process parameters that were adequate for *P. aeruginosa* were inappropriate for *B. plantarii*. The reason is probably that the gas exchange was insufficient because *B. plantarii* reached up to 4times higher biomass concentrations than *P. aeruginosa*. The pO_2 of the culture fell below the set point of 20 % during a long period; it decreased even up to 0 %. The pO_2 could not be regulated to the set point because an increase in stirrer speed or aeration rate leads to excessive foaming and endangers the whole process. Consequently, the concentration of CO_2 in the exhaust air mounted up to 10 %. High dissolved CO_2 concentrations can lead to an inhibition of bacterial cultures as was described by Geraats 1994 for a *Pseudomonas alcaligenes* cultivation. In this case, a significant productivity loss of over 50 % was observed for a lipase production process when 5 % or 10 % of CO_2 was added to the incoming air.

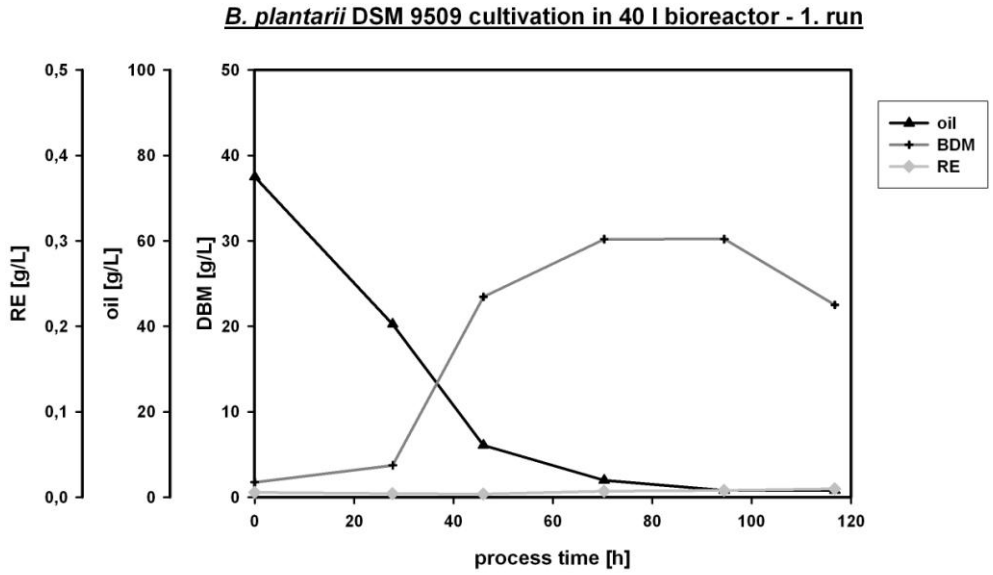


Figure 25: Cultivation of *B. plantarii* DSM 9509 in 40 L bioreactor - first run

Consequently, the process strategy was modified to improve oxygen supply and carbon dioxide removal. The process parameters aeration, mixing and filling volume were directly adopted from the parallel bioreactor cultivations of *B. plantarii* and scaled up to the 40 L bioreactor. A higher filling volume, a higher aeration rate and a decreased stirrer speed resulted, compared to the first run (see Table 32). The stirrer speed was calculated from the 0,6 L parallel bioreactor for constant volumetric power input.

Table 32: Process parameters for the cultivation of *B. plantarii* DSM 9509 in 0,6 L and 40 L bioreactor

	0,6 L	40 L, first run	40 L, second run
reactor volume	0,65 L	39,5 L	
working volume	0,3 L	12 L	20 L
relative filling (working volume/total volume)	ca. 0,5	ca. 0,3	ca. 0,5
reactor geometry (height/diameter)	2,65 (18 cm/6,8 cm)	2,61 (70 cm/26,8 cm)	
aeration rate	1 – 0,3 vvm (1,38 – 0,41 m/h)	0,25 – 0,08 vvm (0,80 – 0,27 m/h)	1 – 0,3 vvm (5,32 – 1,60 m/h)
stirrer speed	800 – 1100 rpm	350 – 550 rpm	207 – 284 rpm (P/V = const.)
max. rhamnolipid concentration (RE)	up to 0,36 g/L	0,01 g/L	0,15 g/L
max. DBM	50 g/L	30 g/L	35 g/L

This approach was more successful and up to 0,15 g/L of rhamnolipid were formed whereas the maximum biomass concentration was almost similar to the first run (35 g/L dry biomass). The CO_2 concentration in the exhaust air did not exceed 1 %. The process is shown in Figure 26.

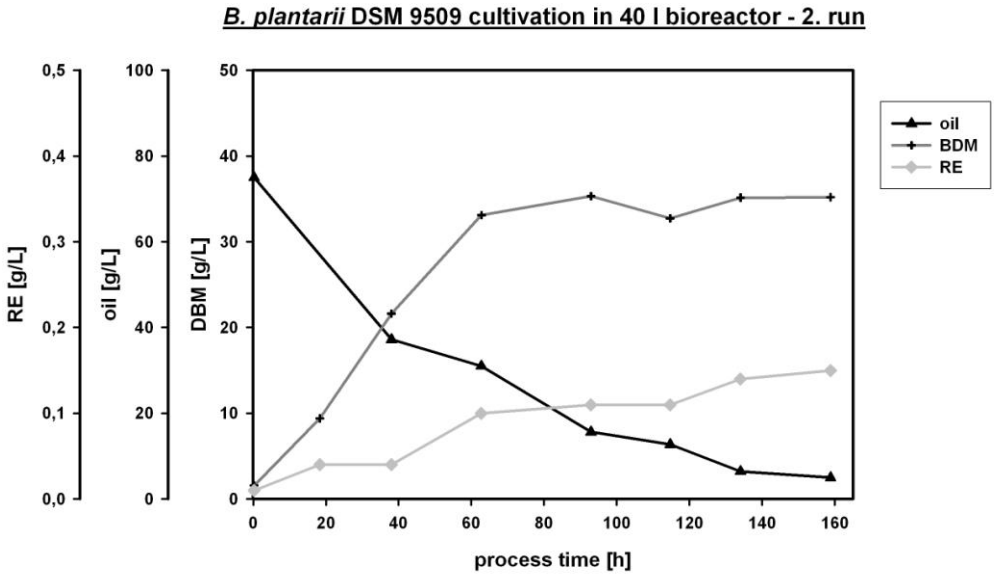


Figure 26: Cultivation of *B. plantarii* DSM 9509 in 40 L bioreactor - second run

However the rhamnolipid yield in 40 L scale was significantly lower than the yield in the 0,6 L scale, where maximum 0,36 g/L of rhamnolipid were achieved with *B. plantarii* DSM 9509. Further optimisation of the process including medium composition, process parameters and process control would be necessary to increase the rhamnolipid yield of this process. Although the product concentration of parallel bioreactor cultivations could not be reached in the 40 L bioreactor, the final rhamnolipid amount was enough for purification of a larger amount of the rhamnolipid of *B. plantarii* DSM 9509.

The culture broth was processed according to the general recovery scheme of hexane extraction, acidification and ethyl acetate extraction (see chapter 3.2) and 104,1 g of brownish, oily and viscous *B. plantarii* DSM 9509 crude extract were gained. The rhamnolipids were further purified by chromatographic methods. Crude extract of *B. plantarii* DSM 6535 from the parallel bioreactor cultivations was also applied for purification.

4.3.4 Purification of rhamnolipid of *B. plantarii*

Different chromatographic methods were employed for the purification of *B. plantarii* rhamnolipids from the crude extract. The crude extract is composed mainly of rhamnolipid and hydrophobic impurities. These impurities could be mono-/di- or triglycerides derived from the plant oil or other hydrophobic compounds from the bacteria like phospholipids. A TLC of the crude extract of *B. plantarii* DSM 9509 illustrates the composition of the crude extract (see Figure 28, left-hand side).

Thick layer chromatography

Purification of the rhamnolipids of *B. plantarii* DSM 6535 was first addressed with the simple and rapid thick layer chromatography method (see chapter 3.5.1). After development of the plates, the rhamnolipids were extracted from the silica gel with different solvent systems: A: ethyl acetate, B: methanol, D: methanol/chloroform (1:1) and E: isopropanol. The elution of the rhamnolipids from the Silica-gel was favoured with isopropanol and methanol/chloroform (see Figure 27). However, TLC analysis of the eluted rhamnolipids revealed that the extract still contained some hydrophobic impurities.

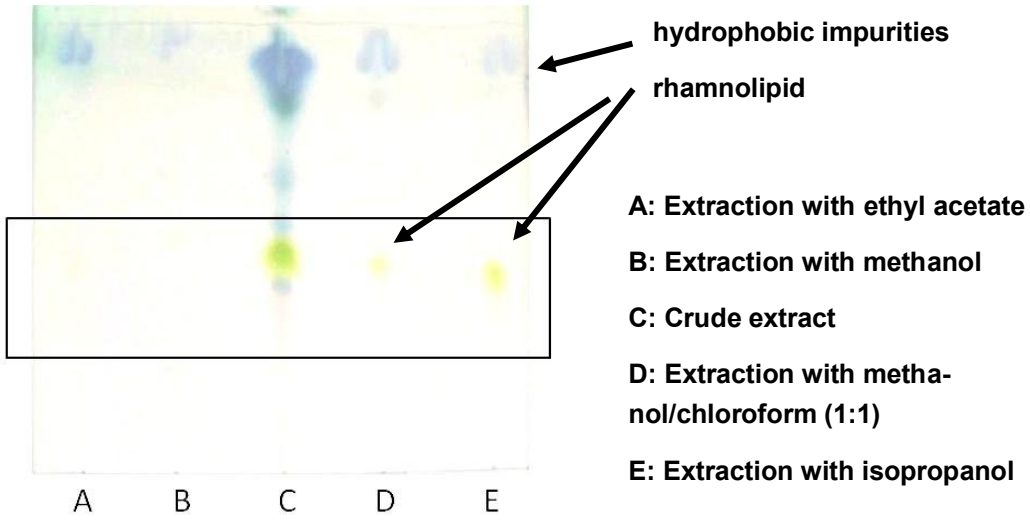


Figure 27: Results of thick layer chromatpgraphy of *B. plantarii* DSM 6535 extract, extraction of the Silica gel with different solvent systems, analysis by TLC

The procedure would have to be repeated several times to obtain pure rhamnolipid. This leads to product loss in every purification step. Moreover, the amount of sample which can be handled with this method is limited. Therefore, the method was considered as ineffective and it was not further pursued.

Fast centrifugal partition chromatography (FCPC)

Fast centrifugal partition chromatography is a chromatographic technique applicable for analytic and preparative purpose. The technique uses a liquid-liquid biphasic system instead of a solid stationary phase and a liquid mobile phase. Two immiscible liquids are used as mobile and stationary phase, the mobile phase being percolated through the stationary phase by a pump and the centrifugal field (see chapter 3.5.3).

FCPC has been successfully applied for analytic and preparative purposes for different substances (Marchal et al. 2003; Bérot et al. 2007), but rhamnolipids have not yet been

purified by this method. Thus, the aim of these investigations was to explore if FCPC is a suitable method for the purification of rhamnolipids. The FCPC instrument was kindly supplied by Alphacrom OHG, Langenau, Germany.

A first FCPC purification run was performed with solvent system 1 (ethyl acetate:water, 1:1). Ethyl acetate was chosen as mobile phase (ascending mode). 100 mg of crude extract of *B. plantarii* DSM 9509 were injected whereas a maximum of up to 5 g sample can be loaded on the rotor. Almost no separation of the rhamnolipid and the impurities was achieved with this solvent system. The rhamnolipid and the impurities eluted simultaneously with the mobile phase ethyl acetate. The elution fractions are shown in Figure 28. On the left side, the crude extract with the rhamnolipid and the impurities is shown.

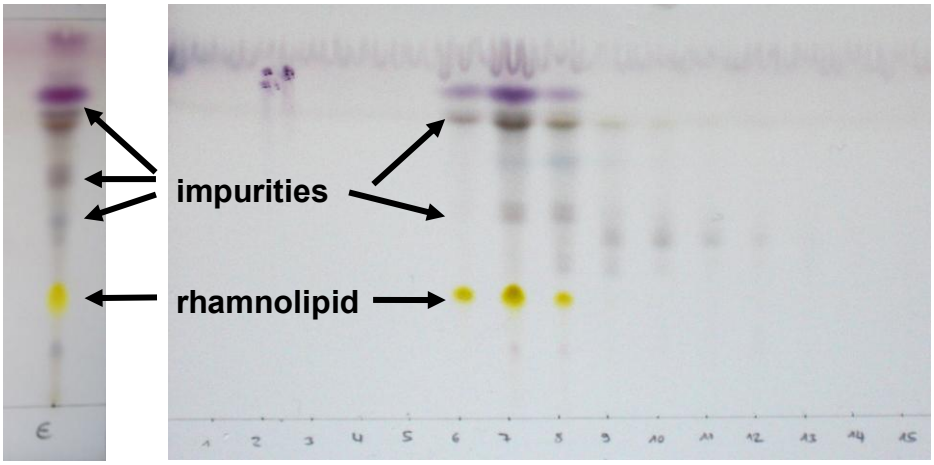


Figure 28: Result of first FCPC run, left: crude extract E, right: elution fractions

Therefore, the solvent system was modified to achieve a better distribution of the substances in the two phases and, thus, better purification results. For solvent system 2, hexane was added to the nonpolar phase to increase the hydrophobicity of this phase and methanol was added to the polar phase to decrease the polarity of the polar phase. The operation mode was changed to descending mode so that the polar phase becomes the mobile phase. This procedure improved the purification result and already quite pure rhamnolipid was obtained (see Figure 29). The impurities eluted with the hydrophobic phase while the rhamnolipids stayed in the stationary, hydrophilic phase and eluted when changing into descending mode so that the former stationary phase drained out.

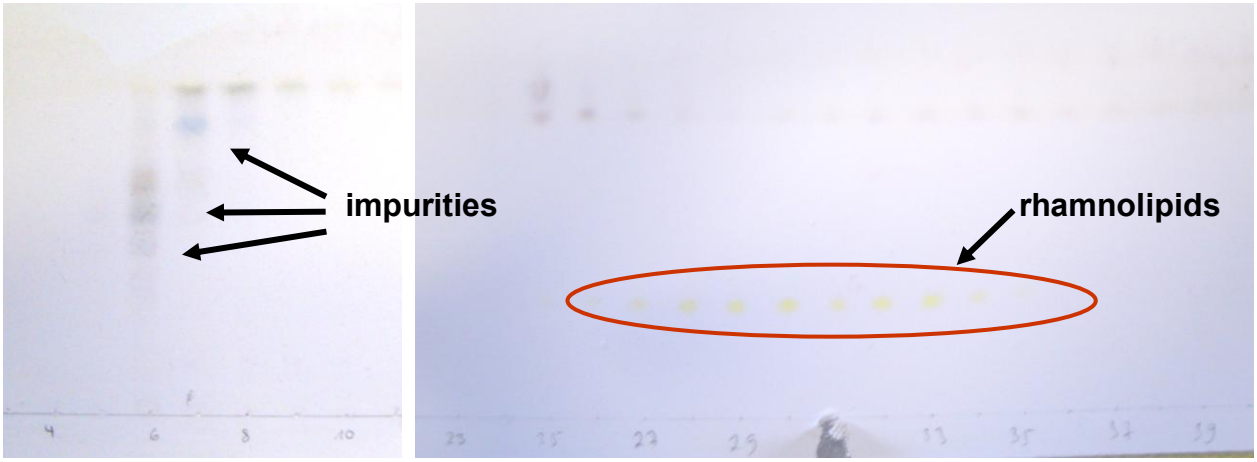


Figure 29: Result of second FCPC purification run, left: elution of impurities, right: elution of rhamnolipid

Good purification results were obtained after only two trials. The advantage of FCPC is that the operation costs are lower, compared to column chromatography because the elution needs 10 times less solvent and the stationary phase can be easily recycled (Marchal et al. 2003). For each FCPC run, 840 mL of solvent were consumed whereas the loading capacity of the rotor is 5 g sample and only 100 mg of crude extract were applied in these experiments due to a shortage in crude extract. Consequently, 50 times more sample could be processed with the same solvent consumption. Moreover, the solvent can be recycled. Therefore, this purification method is regarded as very promising for the purification of rhamnolipids. It should be further optimised to enhance the performance.

Column Chromatography

Isocratic

Column chromatography was carried out to purify the rhamnolipids of *B. plantarii* DSM 9509. A column chromatography with isocratic mode and the solvent system chloroform:methanol:acetic acid (65:15:2) was conducted first. The purification result was not satisfactory as the rhamnolipids did not elute in a sharp peak. They eluted simultaneously to the hydrophobic impurities (data not shown), so that no purification was achieved. Gradient elution was performed next as the isocratic mode was not appropriate for the purification.

Gradient

A second chromatography with sequential elution with chloroform and methanol was run with the same crude extract. The method was modified from Andrä et al. 2006. The elution starts with pure chloroform and subsequently, the methanol ratio is raised up to 100 % methanol (see section 3.5.2). This chromatography removed the majority of hy-

drophobic impurities. The hydrophobic impurities eluted in the second fraction (chloroform/methanol 95:5) whereas the rhamnolipids eluted in the fourth fraction (chloroform/methanol 80:20). However, MS and NMR analysis of the rhamnolipid fractions still showed some impurities (data not shown).

Isocratic II

As the rhamnolipid was still impure, the pooled rhamnolipid fractions of the gradient chromatography were further purified by a second chromatographic step according to Bauer et al. 2006. Isocratic conditions were applied and the solvent system was chloroform:methanol:acetic acid 30:3:1. The structure of the purified rhamnolipid was confirmed by NMR and mass spectrometry and RL-2,2_{14} was detected. NMR spectroscopic data were in accordance with literature values (Bauer et al. 2006). The following molecular fragments were detected by MS: m/z (EI) 801 (M + K+, 100%), 785 (M + Na+, 58%). A TLC of the pure rhamnolipid RL-2,2_{14} is shown in Figure 30.

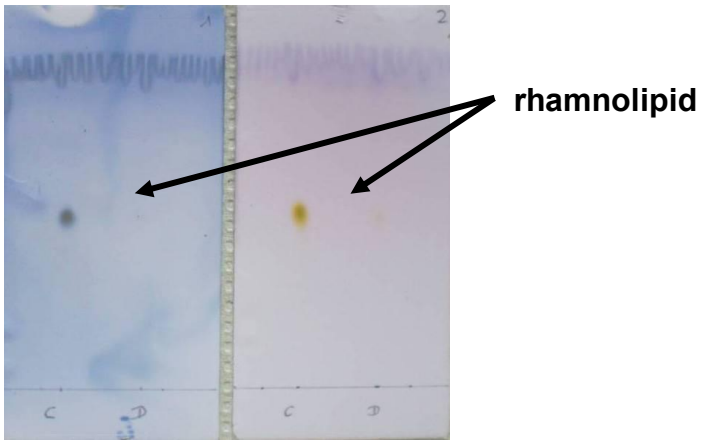


Figure 30: TLC of rhamnolipid after second chromatographic step, left: stained with Cer-molybdate, right: stained with anisaldehyde

Summary:

Column chromatography in two steps with a sequential elution according to Andrä et al. 2006 and a second chromatography with isocratic conditions (Bauer et al. 2006) is a feasible method for the purification of *B. plantarii* rhamnolipids. This technique could be further optimised to reduce organic solvent consumption and to enhance efficiency.

It was demonstrated that FCPC is another suitable method for the purification of *B. plantarii* rhamnolipids, although this method would have to be further optimised as well.

A purification technique for rhamnolipids which requires no organic solvent would be even more advantageous. Therefore, rhamnolipid recovery by precipitation or crystallisation should be further addressed. The rhamnolipids can be precipitated directly from

the culture broth by acidification and collected by centrifugation (Mixich et al. 1997; Déziel et al. 1999; Wei et al. 2005; Monteiro et al. 2007). Mixich et al. 1997 even claim a recovery yield of up to 98 % with this simple technique. The rhamnolipids could then be further purified, for example by crystallisation.

4.4 Conclusions

New production processes are the key issue in overcoming the economic obstacles of rhamnolipid production. Therefore, different aspects of the production process were addressed in this work, from the upstream to the downstream processing.

Waste substrates

The reduction of production costs is fundamental for a broader competitiveness of rhamnolipids on the (bio)surfactants market. The application of crude glycerol from biodiesel manufacturing as substrate can contribute to this aim because it is half as expensive as the standard substrate plant oil. It was demonstrated in this work that crude glycerol from biodiesel production can be applied for rhamnolipid production with growing cells and resting cells. The impurities from the biodiesel manufacturing did not hinder cell growth and rhamnolipid production. A high productivity was gained with this substrate and the specific rhamnolipid productivity was even higher than for pure glycerol. Consequently, this approach should be further pursued and transferred to bioreactor scale.

Recovery/Immobilisation/ISPR

The recovery of the rhamnolipids is a very important factor for an economic rhamnolipid production process. In this work, different recovery methods were studied: immobilisation/ISPR, FCPC and column chromatography.

Column chromatography with different solvent systems is commonly applied for the purification of rhamnolipids. In this work, the rhamnolipids of *B. plantarii* were purified by a combination of two chromatographic steps. The disadvantage of column chromatography is that large amounts of organic solvents are required. A recovery without expensive and harmful organic solvents would be highly desirable to reduce both production costs and environmental burden.

Rhamnolipids can also be purified by FCPC, as was shown in this work. Good purification results were obtained already after two trials. The advantage of this method is that the solvent consumption is much lower than for column chromatography. The solvent consumption can be reduced up to 10 times (Marchal et al. 2003). Further modifications of the applied solvent system would be necessary to optimise rhamnolipid recovery with this method.

The immobilisation approach was not successful due to stability problems of the particles and mass transfer problems for the hydrophobic substrate plant oil. Furthermore, no increase in rhamnolipid production was gained with this technique. It was demonstrated that *P. aeruginosa* is not suitable for an immobilisation by matrix entrapment due to its high mobility. Therefore, the integrated processes with immobilised cells and ISPR by

filtration or electro kinetically controlled deep filtration could not be realised. Furthermore, the immobilisation technique is very complex and laborious and therefore not cost effective. The applicability and competitiveness with traditional technology seem to be a general problem of ISPR approaches: "The proposed ISPR processes have mostly been too complex and consequently not cost effective. …very few processes have been transferred to an industrial scale" (Stark and von Stockar 2003). Another option for rhamnolipid production with ISPR would be membrane retention, but this technique is also not suitable for *P. aeruginosa* processes due to the rapid blocking of the membranes by cells and polysaccharides (Gruber 1991; Dhariwal 2007).

Another possibility for *in-situ* product removal of rhamnolipids might be continuous extraction with an appropriate solvent. The challenge of this approach would be to select the extraction solvent so that rhamnolipids are extracted selectively whereas the other compounds, for example hydrophobic substrates, remain in the medium. Nevertheless, this could be a rewarding approach.

Rhamnolipid recovery by acid precipitation should also be further addressed. It is a simple batch-wise recovery method for rhamnolipids and a yield of up to 98 % was reported in a patent (Mixich et al. 1997). Although it is such a simple and effective method, it is rarely applied for the recovery of rhamnolipids. The advantage of precipitation is that no organic solvents are necessary, and, with the exception of acid, no other chemicals are required and the precipitate can easily be recovered by centrifugation.

Non-pathogenic production strains

Rhamnolipid formation was reported for some non-pathogenic strains but little information was given on the product spectrum of these strains or on production processes with these strains. Furthermore, the data from literature are difficult to compare because different analytical methods were applied for the quantification of rhamnolipids of the non-pathogenic strains. In some cases, the rhamnolipids were not even quantified (e.g. Suzuki and Itoh 1972; Janiyani et al. 1992). Therefore, the rhamnolipid production of 11 non-pathogenic strains was evaluated in this work: *B. plantarii* was the most successful candidate. A maximum rhamnolipid yield of 0,3 – 0,4 g RE/L was achieved with the two *B. plantarii* strains in parallel bioreactor. Both strains synthesise interesting rhamnolipids with longer chain length than the common production strain *P. aeruginosa*. The major component is RL-2,2_{14} with two rhamnose units and two C_{14} ß-hydroxy fatty acid chains. The long chain length makes this rhamnolipid very interesting for cleaning applications. In addition to the findings of Andrä et al. 2006, extracellular rhamnolipid was detected for *B. plantarii* which is very advantageous for industrial application. Furthermore, rhamnolipid production was detected for two different *B. plantarii* strains. The strain *B. plantarii* DSM 9509 is of special interest because until now it has not been described as rhamnolipid producing strain.

However, an extensive process optimisation has to be carried out to further increase the rhamnolipid production of *B. plantarii* which is still much below the rhamnolipid production of *P. aeruginosa*. Process strategy, cultivation condition, medium composition and process control should be addressed to enhance the rhamnolipid yield. Furthermore, biotechnological tools like mutagenesis, genetic engineering or systems biology could be useful to improve rhamnolipid production of this species. The regulation of the rhamnolipid biosynthesis of *B. plantarii* should also be explored to maximise rhamnolipid production with *B. plantarii*.

5 Outlook

Since the beginning of detergent manufacturing, several generations of surfactants have been developed. In the next generation, biosurfactants will possibly have a significant market share as they combine several useful features: structural diversity, excellent surface active properties, low toxicity, antibiotic activity, biodegradability and production from renewable resources.

Although the production of rhamnolipids has been studied intensively since the 1980´s, rhamnolipids are still not widely successful in substituting synthetic surfactants. The main reason for this situation can be found in the high costs for synthesis and downstream processing of rhamnolipids. Thus, the reduction of production costs is fundamental for a broader competitiveness of rhamnolipids on the (bio)surfactants market. The metabolic engineering of the common production strain *P. aeruginosa*, the utilisation of waste substrates, the application of non-pathogenic *B. plantarii* production strains and an improved rhamnolipid recovery by FCPC or precipitation could contribute to this goal.

Metabolic engineering is a possibility to increase the productivity of rhamnolipid production by *P. aeruginosa*. The rhamnolipid biosynthesis should be enhanced by genetic engineering. Especially the complexity of the *P. aeruginosa* genetic regulatory network involved in rhamnolipid production is an important issue for the construction of strains with enhanced rhamnolipid production. The biosynthesis of rhamnolipids is linked with other metabolic pathways and the precursors for rhamnolipid formation are derived from the central metabolism. Therefore, a high activity of the rhamnolipid biosynthesis enzymes RhlA, RhlB and RhlC is not sufficient if the precursors from the sugar and the lipid metabolism are not supplied in adequate amount. The overall regulation of rhamnolipid formation must be addressed by genetic engineering, the quorum sensing being of particular importance. Rhamnolipid biosynthesis should be tackled globally and a systems biology approach could be applied to optimise rhamnolipid production. The formation of the side product polysaccharide should be addressed by genetic engineering of *P. aeruginosa* as well because the production of high amounts of side products diminishes the productivity. Furthermore, high polysaccharide concentrations lead to an extreme increase of viscosity which complicates the production process and the rhamnolipid recovery (see chapter 1.6).

Nevertheless, the pathogenicity of the common production strain *P. aeruginosa* imposes severe safety implications for an industrial application of this strain. This obstacle can be overcome by using the non-pathogenic rhamnolipid production strain *B. plantarii*. This species offers the additional advantage of unconventional rhamnolipids with longer

chain length, compared to *P. aeruginosa*. These rhamnolipids are very interesting for new applications, for example in the cleaning sector. Thus, rhamnolipid production with non-pathogenic *B. plantarii* should be further pursued. The production process has to be optimised to enhance the rhamnolipid yield and factors like process conditions, medium composition and process control should be addressed. Furthermore, the biosynthesis pathway of *B. plantarii* rhamnolipids should be elucidated, as well as the regulation of rhamnolipid formation in *B. plantarii*. A directed metabolic engineering approach could then be applied to enhance rhamnolipid production of *B. plantarii*. A random mutagenesis approach could also be useful, although this approach is less straightforward. A problem of rhamnolipid production with *B. plantarii* is the accumulation of a storage substance, which is probably PHB. The formation of high amounts of this side-product diminishes the rhamnolipid productivity. Therefore, the formation of PHB should also be addressed by genetic engineering and process optimisation to achieve maximum rhamnolipid production and minimum PHB formation.

Another cost factor are the raw material costs which can amount up to 50 % of the overall production costs, depending on the desired purity of the product. Therefore, the application of inexpensive substrates like the crude glycerol from biodiesel manufacturing, which was investigated in this work, is fundamental for the development of economic rhamnolipid production processes. Furthermore, the product yield per substrate ($Y_{P/S}$) should be maximised to employ as little substrate as necessary.

The recovery of rhamnolipids also contributes greatly to the overall production costs of rhamnolipids. Rhamnolipid recovery is usually quite complex and many purification steps like cell separation, extraction steps and chromatographic steps are necessary. Therefore, new purification techniques, for example FCPC, should be implemented into the rhamnolipid recovery process. FCPC offers the advantage that the solvent consumption can be reduced up to 10 times, compared to column chromatography. Rhamnolipid recovery by acid precipitation should also be further addressed. It is a simple batch-wise recovery method for rhamnolipids and a yield of up to 98 % was reported in a patent. Although it is such a simple and effective method, it is rarely applied for the recovery of rhamnolipids.

Adding all optimisation approaches together, a remarkable potential for the reduction of rhamnolipid production costs becomes achievable and rhamnolipids will possibly be one of the next generation surfactants.

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7 Appendix

Abbreviations

ATCC	American Type Culture Collection
ATR	attenuated total reflection
B.	*Burkholderia*
DBM	dry biomass [g/L]
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
cmc	critical micelle concentration
CTAB	cetyl trimethyl ammonium bromide
EDTA	ethylenediaminetetraacetic acid
FCPC	fast centrifugal partition chromatography
FTIR	Fourier transform infrared spectroscopy
H_2O_{VE}	deionised water
HPLC	high performance liquid chromatography
IR	infrared
ISPR	*in-situ* product removal
KCTC	Korean Collection for Type Cultures
LB	Luria-Bertani-medium
MS	mass spectrometry
NaCl	sodium chloride
NCTC	National Collection of Type Cultures
OD_x	optical density at x nm [-]
P.	*Pseudomonas*
PHB	polyhydroxy butyrate
RL	rhamnolipid
RL-X,Y_z	rhamnolipid with x rhamnose units and y ß-hydroxy fatty acid chains of the chain length z
RE	rhamnose equivalents [g/L]

RT	room temperature
TLC	thin layer chromatography
UV	ultra violet
WBM	wet biomass [g/L]

Symbols

c	concentration [g/L]
$c_{x,max}$	maximum biomass concentration [g DBM/L]
d_R	stirrer diameter [m]
μ	specific growth rate [1/h]
N	stirrer speed [rpm]
Ne	Newton number [-]
P	power [Nm/s]
pH	negative decimal logarithm of the hydrogen ion activity in an aqueous solution
pO_2	dissolved oxygen [%]
P_v	volumetric productivity [g product/L·h]
P_{sp}	specific productivity [g product/g biomass·h]
Re	Reynolds number [-]
R_f	ratio of fronts
R_t	retention time [min]
ρ	density [kg/m^3]
σ	surface tension [mN/m]
T	temperature [°C]
v	velocity [m/s]
V	volume [L]
$Y_{P/S}$	yield [g product/g substrate]
$Y_{P/X}$	yield [g product/g dry biomass]

Units

amu	atomic mass unit
°C	grad Celsius
g	gramm
h	hour
L	liter
m	meter
M	molar (mol per liter)
min	minute
m/z	mass-to-charge ratio [-]
N	Newton
NL	norm liter
rpm	rounds per minute
s	second
vvm	aeration rate in volume per culture volume and minute [L/L·h]