RHAMNOLIPID BIOSURFACTANT PRODUCTION FROM GLYCEROL: NEW METHODS OF ANALYSIS AND IMPROVED DENITRIFYING FERMENTATION

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RHAMNOLIPID BIOSURFACTANT PRODUCTION FROM GLYCEROL: NEW METHODS OF ANALYSIS AND IMPROVED DENITRIFYING FERMENTATION

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ABSTRACT

Glycerol is generated in large quantities as the major byproduct of biodiesel manufacturing. Developing new processes requiring large volumes of glycerol is essential for a sustainable and profitable biodiesel production. Rhamnolipids are high-value effective biosurfactants that can be produced by *Pseudomonas aeruginosa* from glycerol. These biosurfactants have been recognized for their immense potential in bioremediation and enhanced oil recovery. Industrial production of rhamnolipids is still a challenge especially under aerobic conditions because of the highly foaming nature of the culture broth and the complex regulatory mechanisms involved. Foaming in aerobic rhamnolipid fermentation appears extremely fast and is too stable to be handled by common foaming control methods. An alternative approach to avoid foaming problems is to use a denitrifying fermentation, taking advantage of the ability of *P. aeruginosa* to perform nitrate respiration. Isolates of glycerol-utilizing *P. aeruginosa* were obtained from soil samples of a biodiesel production plant. With an improved methylene blue/cetyl trimethylammonium bromide (CTAB) agar plate’s method, the highest rhamnolipid producers from glycerol were identified. *P. aeruginosa* E03-40 and *P. aeruginosa* PAO1 were selected as the production strains for batch denitrifying studies. Successful cell growth and rhamnolipid production were obtained with glycerol as substrate under denitrifying fermentation conditions. Higher cell concentrations, using a more controlled nitrate addition better matching the respiration needs of the culture, were attained from
the NAD(P)H fluorescence signal monitoring. The foaming problems associated with commonly used aerobic rhamnolipid fermentations were also avoided using the denitrifying approach in which nitrate (instead of oxygen) was employed as electron acceptor for respiration.

In comparison to the free-cell fermentation, immobilized systems were also evaluated in this study under denitrifying conditions. Immobilized systems relying on oxygen as electron acceptor have been previously investigated but oxygen transfer limitation presents difficulties for continuous rhamnolipid production. The immobilized approach based on a hollow fiber set-up eliminated the transfer limitation problems and was found suitable for a continuous rhamnolipid production using glycerol as carbon source.

Another aspect that was addressed in this study was directed towards the development of a robust analysis for rhamnolipid quantification with the similar aim of large scale production of rhamnolipids. The currently used methods are tedious and laborious. A fast method for rhamnolipid analysis can significantly enhance the strain selection, metabolic engineering, and process development for improved production. A qualitative method was proposed earlier to differentiate rhamnolipid producing and non-producing strains using agar plates containing methylene blue and CTAB. By systematically investigating the complexation of rhamnolipids and methylene blue, with and without the presence of CTAB, a rapid and simple method for rhamnolipid analysis was developed. The method was successfully applied to the batch samples obtained from the denitrifying fermentations used during this project.
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CHAPTER I

INTRODUCTION

1.1. Overview and significance

Exhaustion of fossil fuels and the harmful effects of their emissions to the environment is a worldwide concern. The use of alternative fuels from renewable resources is now a reality. Biodiesel is one of the most viable alternative fuels that could be used as a blending component or a direct substitute for diesel fuel in vehicle engines. Biodiesel is a mixture of monoalkyl esters of long chain fatty acids (FAME) produced from domestic renewable resources such as vegetable oils via transesterification of oils and fats with simple alcohols [1, 2].

At present, the European Union is the primary producer of biodiesel in the world with an estimated production of about 5.7 million metric tons in 2007 [3]. The United States of America is the second producer with a production that increased from 7300 tons in 2000 to an estimated 0.9 million tons in 2006 [4]. In addition, the biodiesel production capacity is expected to keep increasing in the future years. The capacity of Europe grew by 55% in 2008 reaching more than 16 million tons and it is estimated to reach more than 20 million by 2009 [5]. Unfortunately, the biodiesel industry generates large quantities of glycerol (glycerin) as a major byproduct that will soon emerge as an abundant resource or a waste. For every 9 kg of biodiesel produced, about 1 kg of a crude glycerol is generated.
as a byproduct [6]. The demand for glycerol in the United States is not large enough to use all the excess glycerol that the biodiesel market will produce. In fact, the price of crude glycerol has decreased by more than ten fold in the last couple of years [7]. Consequently, there is an urgent need to find innovative ways for the economic conversion of glycerol to value-added products. Among other applications, glycerol can be used as an inexpensive substrate in industrial microbiology. For example, 1,3-propanediol, which can be used as a monomer to produce polyesters, polyethers, and cyclic compounds, has already been obtained from different bacterial strains using glycerol as carbon source [8-10]. Other compounds such as succinic acid, propionic acid, ethanol, and citric acid have also been successfully produced from glycerol as a feedstock for new industrial fermentations [4].

In order to improve the overall economics of the biodiesel process by avoiding the possibility of glycerol becoming an industrial waste, this project explored the utilization of glycerol as a carbon source to produce potentially high-volume, high-valued biosurfactants. Surfactants are one of the most important organic compounds widely used in almost all industries, i.e. food, cosmetic, personal care, household, agrochemical, painting and coating, textile, dyes, polymer, etc. As a result, every year millions of tons of surfactants (equivalent to billions of dollars) are commercialized in the world. The major consumers of this market are North America with 35%, followed by Asia–Pacific with 29%; and Western Europe with 23% [11]. Looking for more environmentally friendly processes in contrast to traditionally produced surfactants based on petroleum feedstock, extensive research has been done on biosurfactants in the last few decades. Biosurfactants are natural surfactants produced by certain microorganisms primarily
when living on water-immiscible substrates [12, 13]. Like their synthetic counterparts, biosurfactants can reduce the surface and interfacial tension, resulting in excellent detergency as well as emulsifying, foaming and dispersing properties. Furthermore, they are biodegradable and non-toxic, and they have improved environmental compatibility and effectiveness in a wide range of temperature and pH [14]. These characteristics make biosurfactants attractive for environment-friendly processes especially in the food, pharmaceutical, and oil industries. Among the various species of biosurfactants, much work has been done on the rhamnolipids produced by fermentations of *Pseudomonas aeruginosa* strains [15]. Rhamnolipids reduce the interfacial tension of water/kerosene systems from 43 to < 1 mN/m [16] and the surface tension of water from 72 to < 30 mN/m [17]. Rhamnolipids contain one or two molecules of rhamnose linked to one or two molecules of β-hydroxyalkanoic acid. The applications vary according to the structure; however, these biosurfactants have been recognized for their immense potential in bioremediation [18] and enhanced oil recovery [19]. As a particular example, rhamnolipids were compared to the pressurized water approach in the removal of Exxon Valdez spilled oil at Alaskan gravel [20]. Rhamnolipids were found to successfully release oil to a significantly greater extent (2 to 3 times) than water alone from contaminated gravel.

The types of rhamnolipids obtained can be affected by the bacterial strain, the culture conditions, the nature of carbon source, the concentrations of nitrogen and other compounds in the medium, as well as the fermentation strategy. Rhamnolipids can be produced with conventional batch fermentation [13, 21], continuous culture [22, 23], and processes using immobilized cells [24, 25] and/or resting cells [24]. Nonetheless,
industrial production of biosurfactants faces the obstacles of high production costs and low production rates as compared to the synthetic surfactants. Recent efforts have been directed to improve the yield with genetically engineered strains and use of low-cost feedstock or agricultural byproducts as substrates [18, 26, 27].

In particular, the cost of the industrial production of rhamnolipids is high because of the highly foaming nature of the fermentation broth and the complex quorum-sensing systems that regulate the cell growth and metabolism. The foaming in aerobic rhamnolipid fermentation appears extremely fast and is too stable to be handled by common foaming control methods [28]. An alternative fermentation technology based on the ability of *P. aeruginosa* to perform denitrification has been previously studied [13]. Among all anaerobic respiration mechanisms, denitrification is energetically favorable and the major product is nitrogen (N₂) gas. The feasibility of rhamnolipid production under denitrifying conditions, using a phosphorus limited medium with palmitic acid as the carbon substrate had been demonstrated [13]. This approach was found to completely eliminate the problems associated with the severe foaming nature of the rhamnolipid broth [13, 23] since nitrate instead of oxygen was used by the cells for respiration. However, in that report the specific productivity under denitrification was about one third of that under aerobic conditions. The difficulties and challenges related to the denitrifying fermentation technology were assessed in this project in order to improve the rhamnolipid productivity via this method. The fluorescence of intracellular NADH and NADPH (NAD(P)H), which are the reduced forms of nicotinamide adenine dinucleotide phosphate (NAD(P)) coenzymes, was used to monitor the shift of microbial electron-accepting mechanisms during denitrifying conditions. These coenzymes are generally
present in all living cells and serve as the major intermediate electron and hydrogen carriers in cellular metabolism [29]. The advantage of this method relies on the fact that the reduced forms of this coenzymes (NAD(P)H) are highly fluorescent with an excitation and emission maxima of approximately 340 and 460 nm, respectively. On the other hand, their oxidized counterparts NAD(P)+, are not. The online NAD(P)H fluorescence monitoring allowed a more accurate supply of the nitrate required by the cells for respiration via denitrification. With this method, successful cell growth and rhamnolipid production in liquid culture were obtained using glycerol as a carbon source.

Another aspect that was addressed in this study was directed towards the development of a robust analysis for rhamnolipid quantification with the similar aim of making possible the large scale production of rhamnolipids. Rhamnolipids are currently analyzed by either analytical methods (i.e. high performance liquid chromatography coupled with mass spectrometry (HPLC-MS)), or by colorimetric assays for carbohydrates (i.e. anthrone, phenol, and orcinol). Although analytical methods provide detailed structural information of the rhamnolipids, the equipment involved is considerably expensive and preliminary purification steps are required. The colorimetric methods comprise the extraction of rhamnolipids from the aqueous sample into an organic solvent followed by acid hydrolysis to separate the rhamnose from the lipid portion of the molecule. The sugar is then quantified spectrophotometrically after the reaction with anthrone, phenol, or orcinol. These methods are not only tedious, laborious, and operationally hazardous because of the strong acid involved, but are also indirect: rhamnose, instead of the whole rhamnolipid molecule, is actually detected. Additionally, the accurate determination of rhamnolipid concentration requires additional steps for
characterization and determination of the ratio between the sugar and the lipid moieties in the rhamnolipids mixture.

A fast and simple method for rhamnolipid analysis could significantly enhance all the aspects related to the development of a better process for rhamnolipid production. A semi-quantitative method using agar plates containing methylene blue and cetyl trimethylammonium bromide (CTAB) was proposed earlier [30]. On those agar plates, the colonies producing rhamnolipids were surrounded by dark blue halos and, ideally, the areas of the halos could be correlated with the rhamnolipid concentrations [30]. However, this method was not quantitative and extremely difficult to apply when selecting among strains producing similar amounts of rhamnolipid, especially when the colonies produced pigments that hinder the blue halo formation. To better understand and improve the method, the complexation of rhamnolipids with methylene blue and/or CTAB was also investigated in this project and a simple and yet quantitative method was developed for rhamnolipid analysis of liquid samples. The method was successfully applied to the batch samples obtained from the denitrifying fermentations carried out in this project.

1.2. Scope of the research

This research was focused on the improvement and application of the microaerobic denitrifying technology to obtain higher cell and rhamnolipid concentrations for enhanced rhamnolipid production. This study also examined the use of glycerol for the production of this high-valued biosurfactant with the presumed goal of improving the economics of the biodiesel industry. Isolates of glycerol-utilizing P. aeruginosa were obtained from soil samples of a biodiesel production plant. The
methylene blue/CTAB agar plate method was improved as part of the study and later used to identify and select the highest rhamnolipid producer strains from glycerol. *P. aeruginosa* E03-40 and *P. aeruginosa* PAO1 were selected for being high rhamnolipid producers with low pigment formation and used afterwards in the denitrifying rhamnolipid fermentations.

With the aim of overcoming the problems associated with conventional methods for rhamnolipid quantification, a new analysis for rhamnolipid determination of liquid samples was also developed in this project. This method was based on the investigation of the complexation by ion-pairing between anionic rhamnolipids and cationic methylene blue and CTAB. The method was verified with the results obtained from the commonly used anthrone reaction technique and was effectively applied to the analysis of batch denitrifying fermentation samples.

By studying different nitrate addition strategies and the effect of high osmotic pressure conditions, successful cell growth and rhamnolipid production under denitrifying conditions were obtained using glycerol as carbon source. However, the cell concentration was found to stop increasing even when there was no limitation from the medium components. The possible reasons for this behavior were further investigated. Higher cell concentrations, with a more controlled nitrate addition that matched better the respiration needs of the culture, were attained from the information obtained with the NAD(P)H fluorescence signal. This higher cell concentration can increase the rhamnolipid volumetric productivity of this process. On the other hand, the inhibitory effect of the spent medium (collected after removing the cells by centrifugation) from a common denitrifying fermentation was confirmed. Additionally, glycerol was also found
to promote the production of other compounds besides rhamnolipids under these anaerobic conditions.

As a comparison to the free cell fermentation, immobilized systems were also explored in this study using nitrate as electron acceptor. Immobilized systems relying on oxygen as electron acceptor have been previously investigated by other researchers [31] but oxygen transfer limitation presented difficulties for continuous rhamnolipid production. The immobilized approach based on denitrification eliminated the transfer limitation problems and was found suitable for a continuous rhamnolipid production using glycerol as carbon source.

1.3. Objectives

The main focus of this project was on the utilization of biodiesel byproduct glycerol to efficiently produce rhamnolipids under denitrifying conditions. Despite the demonstrated feasibility of the microaerobic denitrifying technology, increasing the rhamnolipid productivity under denitrifying conditions involved many challenges. Among them, low cell concentrations as well as lack of an online nitrate concentration monitoring and control system were identified as the main obstacles of this technology. These difficulties directly translated to low rhamnolipid concentrations. Additionally, the lack of a simple method that could be rapidly used to quantify rhamnolipids in liquid fermentation samples and for large scale production was also recognized and addressed in this study.
Accordingly, the following specific objectives were identified to advance the denitrifying fermentation process as a competitive technology for rhamnolipid production:

1) Select, via thorough screening, *P. aeruginosa* strains favorable in glycerol metabolism and high rhamnolipid (biosurfactant) production;

2) Develop a new fast and simple quantitative method for rhamnolipid detection;

3) Obtain higher cell and rhamnolipid concentrations using the advanced microaerobic denitrifying technology and glycerol as carbon source.

1.4. Structure of the dissertation

The dissertation was divided into two parts: (1) the development of a new method for the quantitative determination of rhamnolipids in liquid fermentation samples, and (2) the improved production of rhamnolipids under denitrifying conditions using glycerol as carbon source.

The essential literature background on biosurfactants and more specifically on rhamnolipids (properties, applications, synthesis, and current state of its production) was included in Chapter II. Also given in that same chapter were an overview of the current analytical methods for rhamnolipid determination as well as a description of the denitrification process and its potential application to rhamnolipid production. The last part of Chapter II was a brief summary on the glycerol synthesis from biodiesel production and the importance of finding new ways for its usage.

In Chapter III, a general description of the materials, microorganisms, and methods used throughout this study was included. Chapter IV and Chapter V focused on
the first part of the project and covered the investigation of the complexation mechanism between methylene blue/CTAB and anionic rhamnolipids. In particular, Chapter IV revisited the methylene blue/CTAB agar plate method for improved understanding and application to the identification of rhamnolipid producing strains. With the information obtained on the roles of methylene blue and CTAB as well as the ion-pair formation, Chapter V described the development of a new method for rhamnolipid quantification applicable to liquid fermentation samples.

The second part of the project comprised Chapter VI and Chapter VII. The production of rhamnolipids under denitrifying conditions is described in those chapters. Chapter VI focused on free-cell systems and improvement of the denitrifying fermentation technology towards higher rhamnolipid productivity. In Chapter VII, two kinds of immobilized-cell systems, based on either a dialysis tube or a hollow fiber cartridge, were evaluated for rhamnolipid production. Finally, Chapter VIII summarized all the conclusions obtained from this research work and the recommendations for future studies.
CHAPTER II
LITERATURE BACKGROUND

2.1. Biosurfactants

Surfactants are molecules that contain both a hydrophobic and a hydrophilic moiety and that partition at interfaces between liquids with different polarity and hydrogen bonding. Therefore, surfactants are capable of lowering the surface and interfacial tension affecting the way other molecules behave at interfaces and in solution [32]. These properties make surfactants extremely useful in many industries for a vast number of applications involving emulsification, detergency, wetting, foaming, dispersing or solubilization.

Because of their unique properties, natural surfactants, also known as biosurfactants, have received extensive attention in the past years as promising replacements of synthetic surfactants. Biosurfactants are not only biodegradable and non-toxic but they also have all the advantages of chemically produced surfactants. In addition, some biosurfactants have shown more effective and specific surface-active properties than many conventional synthetic surfactants, providing new possibilities for industrial applications. Biosurfactants have a tremendous potential in environmental protection and pharmaceutical and cosmetic production processes as well as in petroleum, biopesticides, agriculture, and food industries [33].
Biosurfactants are generally microbial metabolites that are excreted to the extracellular media or incorporated in the cell wall mainly to facilitate the diffusion of a hydrocarbon substrate into the cell [34]. Their hydrophobic moiety is either a long-chain fatty acid, a hydroxyl fatty acid, or an \(\alpha\)-alkyl-\(\beta\)-hydroxy fatty acid while the hydrophilic moiety can be a carbohydrate, an amino acid, or a cyclic peptide phosphate [34]. Many microorganisms are able to produce biosurfactants particularly when living on water-immiscible substrates. For example, when growing on hydrocarbons as the carbon source, some bacteria and yeasts excrete ionic surfactants that emulsify the hydrocarbon substrate in the growth medium. *Pseudomonas* and *Torulopsis* species are well known to excrete extracellular glycolipids, especially rhamnolipids [15, 35-37] and sophorolipids [38-40], respectively. There are also microorganisms that are capable of changing the structure of their cell wall. For instance, *Candida lipolytica* and *Candida tropicalis* produce cell wall-bound lypopolysaccharides when growing on n-alkanes [34].

Biosurfactants are categorized generally by their chemical composition and microbial origin. The major classes of biosurfactants are peptides, glycolipids, lipopeptides, fatty acids, phospholipids, and polymeric and particulate biosurfactants [41, 42]. The most commonly isolated and best studied groups of biosurfactants are the glycolipids and phospholipids. Glycolipids are carbohydrates with long-chain aliphatic acids or hydroxyl aliphatic acids. The most studied glycolipid surfactants are sophorolipids and rhamnolipids which are excreted to the growth medium by the cells [34].

Currently, biosurfactants are not as widely use as synthetic surfactants due to the considerably higher costs involved in their production. Using inexpensive medium
components and enhancing the biosynthesis efficiency are key research points to reduce biosurfactant production costs.

2.2. Rhamnolipid biosurfactant

Certain species of *Pseudomonas* are known to produce large amounts of the glycolipids rhamnolipids, consisting of one or two molecules of rhamnose linked to a β-hydroxyalcanoic acid or a chain of two β-hydroxyalcanoic acids joined by an ester bond. Figure 2.1 shows the structures of the rhamnolipids most commonly found in the producing fermentation processes.

Figure 2.1. Common types of rhamnolipids found in *Pseudomonas* species: (a) Rha-C10-C10, (b) Rha-C10, (c) Rha-Rha-C10-C10, (d) Rha-Rha-C10.
The two most abundant rhamnolipids found in fermentation broths are rhamnosyl-
β-hydroxydecanoyl-β-hydroxydecanoate (Rha-C10-C10), a mono-rhamnolipid; and
rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C10-C10), a
di-rhamnolipid [15]. However, several homologue molecules with other fatty acid
chains, differing in chain length and/or extent of saturation, have been identified, for
instance, using high-performance liquid chromatography coupled with mass spectrometry
(HPLC-MS) [36, 43, 44]. Lepine et al. [45] further showed that for the isomeric
rhamnolipids with an ester chain of two β-hydroxyalcanoic acids of different lengths (for
example, Rha-C10-C8 and Rha-C8-C10) found in culture broths of *Pseudomonas
aeruginosa*, the compound with the shorter β-hydroxyalcanoate linked to the sugar (for
example, Rha-C8-C10) was at least three times more abundant than the other compound
(for example, Rha-C10-C8) [45]. In addition, if the longer β-hydroxyalcanoate was
unsaturated, the rhamnolipid with the shorter β-hydroxyalcanoate adjacent to the sugar
was more than 20 times more abundant than its isomeric counterpart [45].

Rhamnolipids always have a free carboxylic acid; consequently, the surface
activities, solubility and other properties and applications of rhamnolipids are sensitive to
pH.

2.2.1. Properties

Like their synthetic counterparts, rhamnolipids can reduce surface and interfacial
tensions, resulting in excellent detergent, emulsifying, foaming, and/or dispersing
properties. For instance, rhamnolipids reduce the interfacial tension of water/kerosene
systems from 43 to < 1 mN/m [16] and the surface tension of water from 72 to < 30 mN/m [17].

Among other properties, the critical micelle concentration (CMC) and the hydrophilic/lipophilic balance (HLB) are commonly used to characterize a surfactant. The CMC is the surfactant concentration at which micelles are formed, usually indicating the minimum concentration for surfactant efficacy. Low CMC properties have been reported for different rhamnolipid molecules: 5 mg/L for the di-rhamnolipid Rha-Rha-C10-C10, and 40 mg/L for the mono-rhamnolipid Rha-C10-C10 [17]. Rhamnolipids with shorter fatty acid chains, such as Rha-C10 and Rha-Rha-C10 (Figure 2.1) have larger CMC values of about 200 mg/L.

The HLB is an empirical value that is used to describe the hydrophobicity or hydrophilicity of the surfactant [46]. In general, the lower the HLB number (i.e. < 7) the more lipophilic/hydrophobic is the compound and vice versa. Higher HLB numbers particularly confer detergent, solubilizing, and emulsifying properties. The HLB of a rhamnolipid produced by *P. aeruginosa* UG2 was estimated to be 24.1 using group contributions and 17.0 using a correlation of HLB with CMC for sodium carboxylic acids [46]. Another study using *P. aeruginosa* AT10 reported a HLB value of 10.07 [47].

For easy comparison, the surface tension and CMC values for the two most common glycolipids biosurfactants are summarized in Table 2.1. Rhamnolipids appear to be more effective than sophorolipids. This higher surface-activity of rhamnolipids is presumably due to the clearer separation of hydrophobic and hydrophilic moieties in rhamnolipids, as described earlier in section 2.2. The properties of two common synthetic surfactants, alkylate dodecylbenzene and sodium dodecyl sulfate, are also
included in Table 2.1 for comparison. Both of the biosurfactants are significantly more surface active than the two synthetic surfactants.

Table 2.1. Comparison of some properties of glycolipid biosurfactants and synthetic surfactants.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Min. Surface Tension (mN/m)</th>
<th>CMC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sophorolipids</td>
<td>33-37</td>
<td>10- &gt; 200</td>
</tr>
<tr>
<td>Rhamnolipids</td>
<td>26-29</td>
<td>5-200</td>
</tr>
<tr>
<td>Detergent alkylate dodecylbenzene</td>
<td>47</td>
<td>590</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>37</td>
<td>2000-3000</td>
</tr>
</tbody>
</table>

The pKa of rhamnolipids is an important property to be considered because their surface activities, solubility, and applications are sensitive to pH. The pKa of a monorhamnolipid mixture in water measured by potentiometric titration was determined to be 4.28 ± 0.16 and 5.50 ± 0.06 for concentrations below and above the CMC of this rhamnolipid, respectively [48]. In this same study, $^1$H NMR titration and spectrochemical titration using ATR-FTIR (attenuated total reflection infrared Fourier transform spectroscopy) were also used to measure the pKa of the monorhamnolipid. These analyses resulted in a value of 4.39 ± 0.06 and 4.84 ± 0.05, respectively. The first value was measured at a concentration close to the CMC and the latter, at a concentration higher than the CMC.
2.2.2. Applications

Rhamnolipids are biodegradable, non-toxic to human, and can be produced from renewable resources [14, 49]. These properties make these glycolipids attractive for more environment-friendly usages of surfactants. Many applications have also been proposed and/or demonstrated in the food, cosmetic, and pharmaceutical industries [17, 50, 51].

Due to their excellent effectiveness in solubilizing and emulsifying hydrocarbons, rhamnolipids are proven to be one of the most effective surfactants for bioremediation and enhanced oil recovery [18, 19, 52, 53]. They are ideal surfactants for cleaning oil spills, oil emulsification, and in breaking industrially derived oil-in-water and water-in-oil emulsions [54]. As a particular example, rhamnolipids were compared to the pressurized water approach in the removal of Exxon Valdez spilled oil at Alaskan gravel [20]. Rhamnolipids were found to successfully release oil from contaminated gravel to a significantly greater extent (2 to 3 times) than water alone.

There are a large number of research publications in which rhamnolipids have been tested for the biodegradation as well as washing of soils contaminated with gasoline and other hydrocarbons. Neto Doumit et al. [55], for example, demonstrated that an aqueous rhamnolipid solution was 3.2-fold more efficient than water in leaching organic material from the soil. Additionally, biodegradation experiments with an initial octadecane concentration of 1.5 g/L showed that 20% of the octadecane was mineralized in the presence of 300 mg rhamnolipid/L, compared with only 5% octadecane mineralization when no biosurfactant was added [56]. Rahman et al. [57, 58] has demonstrated the bioremediation of n-alkanes in petroleum sludge using rhamnolipids
and different bacterial consortia: C8-C11 alkanes were completely degraded and alkanes in the range of C12-C21 were degraded up to 83-98%.

The use of rhamnolipids for the successful treatment of soil contaminated with heavy metals has also been confirmed [59-62]. These studies have shown that when using rhamnolipids a remarkable removal of cadmium, nickel, lead, and chromium, among other metals, was attained in addition to a better performance when compared to tap water and other synthetic surfactants.

In the petroleum industry, primary pumping methods only recover about 30% of crude oil from the field. Addition of surfactants is one of the techniques used to enhance oil recovery from the ground. The addition of surfactants lowers the surface and interfacial tensions of the oil in the reservoir, facilitating oil flow and penetration through pores in the reservoir during water, steam or fire flooding recovery operations. Rhamnolipids can be applied as agents for microbial enhanced oil recovery (MEOR) like replacement of synthetic surfactants or as co-surfactants due to their excellent phase behavior at low concentrations and their potential to achieve low interfacial tensions [19].

Other applications of rhamnolipids are in the pesticide, food, and cleaning industries owing to the antibacterial, anti-fungal, mycoplasmacidal, and antiviral activities of this biosurfactant [63-65]. A biofungicide containing rhamnolipids formulated to prevent plant pathogenic fungi has been approved by the FDA for use in fruit, vegetables, and legume crops [63]. Furthermore, these types of formulations are considered nonmutagenic and of low toxicity. An additional application that is already being commercialized by Ecover is in household detergent formulations advertised as ecological cleaning products [66]. Different patents involving several rhamnolipids
containing formulations as cleaning and biocide agents have also been granted in recent years [67-69].

Rhamnolipids can also be potentially used to obtain fine chemicals such as rhamnose. This sugar is commercially used as a starting material for high-quality flavor compounds and synthesis of some organic compounds [50]. Rhamnose is currently produced by extracting quercitrin from oak bark, naringin from citrus peels, or rutin from oak bark or other plants. This process generates considerable amounts of toxic wastes and involves corrosive materials [13]. On the other hand, rhamnolipids can be easily hydrolyzed to produce rhamnose.

2.3. Rhamnolipid Production

Production of rhamnolipids is a unique characteristic of the opportunistic pathogen *Pseudomonas aeruginosa*. While some isolates of the non-pathogenic *P. chlororaphis* [70] and *P. alcaligenes* [71], and the pathogenic *Burkholderia pseudomallei* [72] have also been reported to produce rhamnolipids, the product concentrations (or yields) obtained in the fermentations using *P. aeruginosa* are so far significantly higher than those from the other species. Various *P. aeruginosa* strains, for examples, DSM 2874 [73], ATCC 9027 [74], ATCC 10145 [36], and UG2 [75], have been reported as good producers of rhamnolipids.

*Pseudomonas aeruginosa* is a gram-negative non-spore forming bacterium found in many different habitats, including water, soil, and plants [15]. The natural ability of *P. aeruginosa* to survive and grow in a wide range of environmental conditions has contributed to its success as an opportunistic pathogen. In the year 2000, the complete
genome of this microorganism was sequenced [76] and it was found to include a large number of genes involved in the catabolism, transport and interchange of organic compounds as well as four potential chemotaxis systems. These findings suggest an incredible environmental adaptability which possibly explains its resistance to antibiotic treatment [77] and its success in chronically infecting the lungs of cystic fibrosis patients [78]. *P. aeruginosa* is generally found in aerobic environments, but it is able to grow under denitrifying conditions [13] and has been shown to form biofilms under oxygen depleted microaerobic conditions [79]. This bacterium has been recognized not only for its versatile metabolic capability but also for its ability to use compounds that are not easily degradable to other organisms, including aliphatic and aromatic hydrocarbons, fatty acids, insecticides and other environmental pollutants [12, 80, 81].

Rhamnolipids can be obtained with batch fermentation [13, 21], continuous culture [22, 23], and in the processes using immobilized cells [24, 25], solid-state [82], and/or resting cells [24]. Since rhamnolipids are predominantly reported as secondary metabolites over-produced by cultures under certain nutrient limitation, the fermentation strategies for rhamnolipid production commonly involve designs of growth-limiting conditions or employ resting cells. In these cases, rhamnolipid levels sharply increase as a consequence of the limitation of one or more components in the medium.

Chayabutra and Ju [36] demonstrated the production of rhamnolipids when the culture reaches the stationary phase of growth due to limitation of nitrogen or another essential nutrient. Optimal pH and temperature for rhamnolipid production under aerobic conditions and in continuous culture were found to be from 6.2 – 6.4 and 32°C - 34°C, respectively [83]. Chayabutra et al. [13] also confirmed that rhamnolipid production is
sensitive to pH. At a lower pH of 6.0, the specific rhamnolipid production rate was approximately 4 mg rhamnose per g of cells (dry weight) per hour [mg rhamnose/(g CDW-h)], while at a higher pH of 6.5, it increased to 12.5 mg rhamnose/(g CDW-h).

Rhamnolipid production is not only affected by the bacterial strain selected, and changes in pH and temperature, but also by the carbon and nitrogen sources, other nutrient concentrations in the medium, dissolved oxygen (DO) concentration, and the design or approach of the fermentation process.

A large number of carbon sources such as glycerol, ethanol, glucose, and corn oil can be used for rhamnolipid production [17, 84-86]. The rhamnolipid production from different carbon sources varies significantly: ~1.2 g/L using corn oil [87], 1.5 g/L using glucose [88], 6.9 g/L using glycerol [89], and 45 g/L using rapeseed oil [73]. For comparison, Table 2.2 shows some examples of the rhamnolipid concentration attained with different strains and carbon substrates. Additionally, the production yield ($Y_{P/S}$ product to substrate) was also estimated for each referred case to demonstrate better the variations in the production.

\textit{Pseudomonas} species can also use hydrocarbons for growth in aerobic conditions. The rhamnolipid concentrations obtained are usually higher when $n$-alkanes and vegetable oils are used as the carbon source as compared to when water-soluble carbon substrates are used [17]. However, in the absence of oxygen, hydrocarbons cannot be degraded by these microorganisms, at least not effectively for industrial production purposes.
Table 2.2. Rhamnolipid production by *P. aeruginosa* strains using different substrates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Rhamnolipid (g/L)</th>
<th>Yield -Y_{P/S} (%)*</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSM2659</td>
<td>Glucose</td>
<td>1.5</td>
<td>8.2</td>
<td>[88]</td>
</tr>
<tr>
<td>UW-1</td>
<td>Glucose</td>
<td>1.2</td>
<td>2^a</td>
<td>[90]</td>
</tr>
<tr>
<td>UW-1</td>
<td>Canola oil</td>
<td>11</td>
<td>18</td>
<td>[90]</td>
</tr>
<tr>
<td>DS10-129</td>
<td>Glycerol</td>
<td>1.77</td>
<td>22</td>
<td>[27]</td>
</tr>
<tr>
<td>DS10-129</td>
<td>Soybean oil</td>
<td>4.31</td>
<td>54</td>
<td>[27]</td>
</tr>
<tr>
<td>GL1</td>
<td>Glycerol</td>
<td>3.5</td>
<td>11.7</td>
<td>[91]</td>
</tr>
<tr>
<td>PAO1</td>
<td>Babassu oil</td>
<td>0.5^b</td>
<td>5</td>
<td>[89]</td>
</tr>
<tr>
<td>PAO1</td>
<td>n-hexadecane</td>
<td>0.32^b</td>
<td>3.2</td>
<td>[89]</td>
</tr>
<tr>
<td>PAO1</td>
<td>Glycerol</td>
<td>1.72^b</td>
<td>17.2</td>
<td>[89]</td>
</tr>
<tr>
<td>MM1011</td>
<td>Corn oil</td>
<td>1.2</td>
<td>2</td>
<td>[87]</td>
</tr>
<tr>
<td>LBI</td>
<td>Olive oil</td>
<td>5.4</td>
<td>21.6</td>
<td>[92]</td>
</tr>
<tr>
<td>LBI</td>
<td>Sunflower soapstock</td>
<td>12</td>
<td>48</td>
<td>[92]</td>
</tr>
<tr>
<td>LBI</td>
<td>Soybean oil</td>
<td>4.8</td>
<td>19.2</td>
<td>[92]</td>
</tr>
<tr>
<td>LBI</td>
<td>Sunflower oil</td>
<td>4.9</td>
<td>19.6</td>
<td>[92]</td>
</tr>
<tr>
<td>GS-3</td>
<td>Molasses and CSL</td>
<td>0.6^b</td>
<td>1.6</td>
<td>[93]</td>
</tr>
<tr>
<td>47T2</td>
<td>Frying oils</td>
<td>6.75^b</td>
<td>34</td>
<td>[94]</td>
</tr>
<tr>
<td>AT-10</td>
<td>Soybean oil wastes</td>
<td>9.5</td>
<td>19</td>
<td>[95]</td>
</tr>
<tr>
<td>GS9-119</td>
<td>Soybean oil</td>
<td>1.75</td>
<td>21.9</td>
<td>[27]</td>
</tr>
<tr>
<td>GS9-119</td>
<td>Glycerol</td>
<td>1.66</td>
<td>20.7</td>
<td>[27]</td>
</tr>
<tr>
<td>DSM 2874</td>
<td>Rapeseed oil</td>
<td>40</td>
<td>40</td>
<td>[73]</td>
</tr>
</tbody>
</table>

* Estimated assuming complete depletion of the carbon source
^a Estimated after 7 days of cultivation
^b Calculated assuming a 1:2.5 ratio rhamnose to rhamnolipids
Recent efforts to improve the yield of rhamnolipids have also been directed towards genetically engineered strains and the use of low-cost feedstock or agricultural byproducts, such as vegetable oils and the residue from the vegetable oil refinery, as substrates [18, 26, 27, 96, 97]. Zhu et al. [98] and Haba et al. [94], for example, investigated the rhamnolipid production using waste frying oil as the sole carbon source. Zhu et al. [98] obtained concentrations of up to 20 g/L of rhamnolipids after 5 days of cultivation in a 50 L tank. De Lima et al. [96] successfully replaced glucose and yeast extract with 22 g/L of waste fried soybean oil and 11.5 g/L of residual brewery yeast, obtaining approximately 6.8 g/L of rhamnolipids (considering a 1:2.5 ratio of rhamnose to rhamnolipids).

Currently, the industrial production of rhamnolipids faces significant obstacles in high production costs and low production rates as compared to the production of synthetic surfactants and certain other microbial products, including sophorolipids. One particular process challenge is caused by the fast and stable foam generated in aerobic *P. aeruginosa* fermentations [99]. Rhamnolipids, with their free carboxylic acid group (Figure 2.1), and other lipidic metabolites produced by *P. aeruginosa* render the fermentation broth extremely foaming at the near neutral pH optimal for the bacterial fermentation. (The foaming has been found much more reduced at pH lower than 5.5.)

Foaming is common in aerobic fermentations. Mechanical foam breakers and chemical antifoaming agents have been developed for controlling the foaming in many fermentation processes. Unfortunately, the foaming in aerobic rhamnolipid fermentation appears extremely fast and is too stable to be handled by these methods [28]. In addition, chemical antifoams can have adverse effects on the downstream recovery and
purification processes besides the considerable extra costs associated with the large amounts of antifoams required to match the rhamnolipids produced along the fermentation process. Different strategies have been attempted to break the foams. For example, Wu and Ju [99] used an external vessel to hold and collapse the overflowed foams, with automatic acid addition if necessary. The obtained broth was pumped back into the fermentor after the foam was broken down. Siemann and Wagner used a similar setup for their process employing immobilized cells [28]. However, even with this setup, the foaming was still difficult to control and the aeration rate had to be significantly lowered. This caused oxygen limitation and compromised cell viability as well as rhamnolipid productivity.

An alternative to the conventional aerobic fermentation approach relies on taking advantage of the capability of \textit{P. aeruginosa} to perform denitrification as a complementary or alternative respiration route. The denitrification approach can completely eliminate the problems associated with the severe foaming nature of rhamnolipid broth [13, 23]. A description of the basis of the denitrification-based fermentation technology is presented in more detail in section 2.6.

2.4. Biosynthesis Pathway and Regulation

For \textit{P. aeruginosa}, rhamnolipid production is not only important for the assimilation of hydrophobic carbon sources, but it is also involved in cell motility [100] and in biofilm formation [101, 102].

The synthesis of the hydroxyalkanoate used specifically in rhamnolipid synthesis is controlled by the enzyme RhlG, whose encoding gene \textit{rhlG} is homologous to the \textit{fabG}
gene in the general fatty acid synthesis [103]. *rhlAB* and *rhlC* are known to encode for rhamnosyltransferases which are the enzymes that catalyze the transfer of one (or two) rhamnose from TDP-L-rhamnose to β-hydroxyalkanoate to form rhamnolipids. Figure 2.2 illustrates the biosynthetic pathway of rhamnolipid production by *P. aeruginosa*.

Figure 2.2. Rhamnolipid biosynthetic pathway from glycerol. Modified from Soberon-Chavez et al. [15] and Zhu and Rock [104].
There are three sequential steps involved in this process: synthesis of the fatty acid dimer moiety of rhamnolipids, synthesis of the sugar moiety (dTDP-L-rhamnose), and the reactions catalyzed by RhlB and RhlC to yield mono and di-rhamnolipids [15]. This process is regulated at the transcriptional level by at least two interactive quorum-sensing systems las and rhl (Figure 2.3). Quorum sensing is the cell-to-cell signaling mechanism that *P. aeruginosa* uses to regulate virulence gene expression in a cell density dependent manner. The quorum sensing systems involve the production of particular N-acyl homoserine lactones known as autoinducers (AIs: PAI-1 and PAI-2 in Figure 2.3). As the bacterial concentration increases, AI concentrations build up in the growth medium. Each AI binds to its specific recipient proteins (LasR or RhlR), forming an AI–protein complex that activates and/or regulates various genes, including the *rhlAB* and *rhlG* genes for rhamnolipid synthesis [105]. Note that the AI-protein complexes also activate the *rhlI* and *lasI* genes; thus, “auto-inducing” the AI syntheses themselves.

In the *las* system, PAI1 (N-(3-oxododecanoyl) homoserine lactone) is first synthesized by LasI (an AI synthase). Subsequently, PAI1 binds with the *lasR*-encoded transcriptional activator, LasR [74]. In the same way, the *rhl* system controls the synthesis of the second AI, PAI2 (N-butyryl homoserine lactone). The enzyme that catalyzes this reaction is the AI synthase RhlI. PAI2 binds to the transcriptional activator protein RhlR forming the RhlR-PAI2 complex which controls the expression of *rhlAB* and *rhlG*. The two quorum-sensing systems are not independent (Figure 2.3). For instance, the production of PAI2 is not only promoted by the RhlR-PAI2 complex but also by the LasR-PAI1 complex from the *las* quorum sensing system [105].
Figure 2.3. Two known quorum sensing systems (las and rhl) in *P. aeruginosa* involved in rhamnolipid synthesis [74].

The kinetics of PAI2 synthesis and degradation in batch fermentation of *P. aeruginosa* using the wild type PAO1 and its *rhlI*(−) and *rhlR*(−) mutants were studied by Chen et al. [105]. The *rhlI*(−) and *rhlR*(−) mutants were found to produce insignificant amounts of rhamnolipids as compared to the wild-type *P. aeruginosa* PAO1. To study the PAI2 degradation kinetics, the AI-bearing supernatant was collected from a batch culture of PAO1 and added to two systems for comparison: one system contained the washed cells of *rhlI*(−) mutant collected from a continuous culture maintained at the dilution rate of 0.025 h\(^{-1}\); the other system was cell free. The use of *rhlI*(−) cells was to ensure no PAI2 synthesis occurred during the experiment so that the degradation could be examined apart from biosynthesis. The results indicated that the PAI2 degradation was predominantly cell-associated. While the autoinducer concentration decreased
insignificantly in the cell-free systems (especially when considering the range of data fluctuation caused by the sensitive bioassay), clear degradation was observed in the systems containing rhlI(−) cells.

PAI2 concentrations were next followed along a batch fermentation of the wild type PAO1 and the rhlR(−) mutant. The stationary-phase decay of PAI2 in the wild type was slower than that in the rhlI(−) mutant and different PAI2 concentration profiles during the growth phase of the wild type and the rhlR(−) mutant were observed. The mutant’s growth-phase autoinducer production was slower, yielding a maximum PAI2 concentration about one third of those from the wild-type PAO1. Since the two cultures had similar profiles of cell growth, the results implied that the auto-induction mechanism enabled a three-fold higher rate of autoinducer synthesis.

This work was then extended by Chen et al. [74] to evaluate the effect of PAI2 on specific rhamnolipid productivity, using the rhlI(−) mutant cultures added with various autoinducer concentrations. A model was developed accordingly to link the essential features of the rhl quorum-sensing system to the observed rhamnolipid production profiles. As expected, higher added PAI2 concentrations gave not only higher initial synthesis rates but also longer induced synthesis.

2.5. Rhamnolipid quantification

An accurate determination and quantification of rhamnolipids is essential for research on strain selection, metabolic engineering, and process development for improved rhamnolipid production. Rhamnolipids are currently analyzed by analytical, indirect, or colorimetric methods.
2.5.1. Analytical methods

Analytical methods available for rhamnolipid determination are chromatographic techniques such as thin layer chromatography (TLC), and high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) [106]. TLC is typically used to determine the rhamnolipid composition in culture broth extracts. Normal or reversed phase TLC has resulted in good separation of rhamnolipid mixtures based on either the number of sugars in the molecule (i.e. monorhamnolipids or dirhamnolipids) or the length of the fatty acid alkyl chain [44, 107, 108]. However, quantitative determination of rhamnolipids using TLC is not accurate and large amounts of sample are needed (compared to the amounts required in other analyses).

On the other hand, HPLC-MS not only separates completely different rhamnolipid species but also provides detailed structural information and quantification of the rhamnolipids present in the sample. In this technique, reversed-phase silica columns and electrospray ionization in negative mode are usually used for best performance [44, 45]. Even though rhamnolipid analysis with this technique is very precise, the equipment is very expensive and preliminary purification steps are required to remove salts and other impurities that can affect the rhamnolipids ionization in the MS. Additionally, the manipulation during the purification can account for losses of analyte that could also affect the quantification [106].

More recently, ATR-FTIR was also proposed as an alternative method for the determination and quantification of rhamnolipids in aqueous solutions [109]. Although this technique also relies on the availability of expensive equipment, it was found to be
suitable for the rapid analysis of rhamnolipids with good reproducibility. However, the accuracy of the method still needs to be improved and the method standardize.

2.5.2. Indirect methods

Indirect methods for rhamnolipid analysis rely on the properties of rhamnolipids as a biosurfactant and consist in measuring the surface tension that will vary with changing rhamnolipids concentration. Common equipment to evaluate surface and interfacial tension such as tensiometers and goniometers, and techniques like the pendant drop and the ring method have been used [110, 111]. These techniques usually use a calibration curve with pure rhamnolipids as a standard to correlate the surface tension to total rhamnolipid concentration. The availability of the equipment as well as the interference from other surface-active compounds and the variability of the surface tension with each rhamnolipid species are the major disadvantages of these methods.

2.5.3. Colorimetric methods

Among the colorimetric methods, anthrone [13, 112], phenol - sulfuric acid [112], and orcinol [113] are commonly used [12, 99, 114-116]. These methods involve the extraction of rhamnolipids from the aqueous sample into an organic solvent, followed by acid hydrolysis to separate the rhamnose from the lipid portion of the molecule. The sugar is then quantified spectrophotometrically after reaction with anthrone, phenol, or orcinol.

Even though these methods are still frequently used for rhamnolipid quantification, they are tedious, laborious, and operationally hazardous because of the
strong acid involved. They are also indirect: rhamnose, instead of the rhamnolipids, is actually detected and the accurate determination of rhamnolipid concentration requires additional steps for establishing the ratio between the sugar and the lipid moieties in the mixture (that could change during the production). Moreover, several solvents, inorganic salts (such as NaCl), carbonyl or oxidizing compounds, and proteins could also interfere with the measurement [106].

2.5.4. Methylene blue - cetyl trimethylammonium bromide agar plates

The increased interest in rhamnolipid production and applications warrant the development of fast and effective methods for screening the strains’ rhamnolipid productivity. For this purpose Siegmund and Wagner [30] proposed a semi-quantitative method using agar plates containing methylene blue and cetyl trimethylammonium bromide (CTAB) (SW plates). The method relies on the property of anionic surfactants to form insoluble ion pairs with cationic substances such as methylene blue or CTAB. Being an anionic biosurfactant, rhamnolipids can form a complex with these cationic compounds that could be identified by the formation of dark blue areas on the agar plates. Accordingly, rhamnolipid-producing strains can be recognized by the dark blue halos formed around the colonies and, ideally, the amounts of rhamnolipids produced could be correlated with the areas of the halos [30]. This method is specific for glycolipids and has been used for strain selection and screening studies [70, 117], particularly for differentiating rhamnolipid producers from non-producers. However, the method is often not quantitative enough for selecting the top producer among multiple producing strains, especially when many of them also produce other pigments that interfere with the blue
halo formation or detection. In addition, the halo diameter can be easily influenced by factors such as different bacterial cell growths, cultivation times, and filling levels of the agar plates.

2.6. Rhamnolipid Production under Denitrifying Conditions

Aeration and/or agitation are the mechanisms typically used in microbial fermentations to provide the interfacial transfer of O2 from gas bubbles to the aqueous media. Oxygen supply by surface aeration alone is generally not sufficient to provide the oxygen requirement for processes employing high cell concentrations for high volumetric productivity. Submerged aeration is used instead, providing air from the bottom of the fermentor which bubbles up through the fermentation broth. By breaking the large bubbles into fine ones, the agitation also increases the gas-liquid interfacial area available for oxygen transfer to take place. Unfortunately, these methods cannot be readily employed for rhamnolipid production because of the highly foaming nature of the broth.

The production of energy in the absence of oxygen can also be accomplished by anaerobic respiration. Anaerobic or anoxic conditions correspond to those in which microorganisms perform fermentation without external terminal electron acceptors or uses chemicals other than O2 as terminal electron acceptors (i.e. anaerobic respiration). The most common examples of electron acceptors different from O2 are nitrate (NO3−), sulfate (SO42−), sulfur (S0), carbonate (CO32−), etc. Facultative aerobes, which are organisms that use oxygen but that are capable of using anaerobic methods of energy production, commonly used nitrate as an alternative electron acceptor.
*P. aeruginosa* is a facultative aerobe, capable of performing aerobic and anaerobic respiration, for the latter, in the presence of nitrate and/or nitrite as alternative electron acceptors. The anaerobic nitrate-based respiration is known as denitrification. Nitrate is highly water soluble. It can be easily supplied to the growth media; thus, eliminating the vigorous, high-shear agitation required in conventional fermentors for generating fine bubbles. Since aeration is also not essential in denitrification-based fermentation, the aeration rate can be adjusted to avoid uncontrollable foaming problems.

In the denitrification process, nitrate is first converted to nitrite which is then reduced to nitrogen (N₂). This process is energetically favorable and provides N₂ gas as the principal end product [118, 119]. Four main reduction steps are involved in the denitrification mechanism [120, 121]:

\[
\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2
\]

Although not explored in industrial fermentation processes prior to the work developed in this laboratory, denitrification has been used in biological wastewater treatment for many years [122].

The mechanism and regulation of electron flow and ATP synthesis under denitrifying conditions is considered to be similar to the process of aerobic respiration. However, it has been recognized that different spectra of cytochromes and enzymes are required for anaerobic respiration. Two nitrate reductase enzymes involved in the first step of nitrate reduction, from nitrate to nitrite, have been identified in denitrifying bacteria [123]. One of them is a respiratory membrane-bound enzyme and the other one
is periplasmic. The roles of each of these enzymes may change with the microorganism. For example, in *P. denitrificans*, the periplasmic nitrate reductase is expressed under both aerobic and anaerobic conditions; nevertheless, under anaerobic conditions, the membrane-bound enzyme is more active than the periplasmic during nitrate reduction. In this case, the periplasmic enzyme is believed to be used for the transition from aerobic to anaerobic respiration or to dissipate the excess reducing power. In contrast, other studies with *Pseudomonas sp.* strain G-179 showed that the periplasmic nitrate reductase is required for the first step of denitrification [123] while the membrane-bound enzyme has secondary functions.

Even though it has been found that *P. aeruginosa* encodes both the periplasmic and the membrane-bound enzyme, the latter serves as the major catalyst of anaerobic nitrate respiration [79]. Additionally, this enzyme was found to be essential in *P. aeruginosa*’s anaerobic growth in conditions similar to those in biofilm environments [124]. The membrane-bound nitrate reductase is a molybdenum-containing enzyme in which the catalytic site is oriented toward the cytoplasm generating nitrite at the inner face of the membrane [120]. The role of the periplasmic nitrate reductase is not clear but it is believed to be used to balance cellular oxidation-reduction processes when the reducing power is high and oxygen concentrations are low [124].

Once nitrite is produced, another enzyme called nitrite reductase is responsible for the next reducing step to nitric oxide. Unlike nitrate reductases, nitrite reductases are periplasmic enzymes. After nitric oxide is produced, the nitric reductase and nitrous oxide reductase further reduce the nitric oxide and nitrous oxide to nitrous oxide and nitrogen (N₂), respectively. The enzymes involved in the dissimilative nitrate reduction
process are generally repressed by O₂ and are synthesized only under anaerobic or anoxic conditions. Nonetheless, denitrification under aerobic conditions has been reported [125, 126]. For example, a *P. aeruginosa* ATCC 9027 culture was found to perform denitrification even at dissolved oxygen concentrations higher than 1 mg/L when grown in continuous culture using glucose as the carbon source [23].

Besides the effect of oxygen on the mechanism of denitrification, Thomas et al. studied the effect of pH on denitrification using *Pseudomonas* species [127]. A pH range from 4.0 to 8.8 was examined and it was found that the maximum denitrification rate took place at pH around 7.0 – 7.5. At low pH values, such as 4.0, the nitrogen oxide reductases, especially that reducing N₂O, were progressively inhibited, resulting in a decrease of the overall denitrification rate.

The effect of different carbon sources (i.e. palmitic acid, stearic acid, oleic acid, linoleic acid, glycerol, vegetable oil, and glucose) on rhamnolipid production has also been previously studied [13]. All of these substrates were able to support the growth of *P. aeruginosa* ATCC 10145 under both aerobic and denitrifying (anaerobic) conditions. However, under magnesium-limited conditions, rhamnolipid production was only detected when hexadecane, palmitic acid, or stearic acid was used as the carbon substrate. On palmitic acid and stearic acid, the growth of the bacterium was slower probably because these were solid substrates that were not readily available to the cells.

Rhamnolipid production in aerobic fermentations has typically been done in nitrogen-source-limited media. Since *P. aeruginosa* can use nitrate as the N-source for growth, the media may need to be designed with other limiting nutrients. The rhamnolipid production in systems limited by nitrogen (N), iron (Fe), magnesium (Mg),
phosphorus (P), sulfur (S), and calcium (Ca) sources, respectively was investigated [13]. P limitation was found to give 4 to 5 times higher specific rhamnolipid productivity than N limitation. S limitation was comparable to N limitation in supporting rhamnolipid production while Mg limitation gave much poorer results. Ca and Fe were not effective for limiting cell growth.

The productivity with palmitic acid as substrate was about three times higher under the aerobic condition than under the denitrifying condition. As for the effect of C substrates, the aerobic productivity from palmitic acid was lower than that from hexadecane. The former was about 72% of the latter under N limitation and 82% under P limitation. Furthermore, P limitation was about four to five times more effective than N limitation, with either hexadecane or palmitic acid as the substrate.

The feasibility of rhamnolipid production under denitrifying conditions, using a phosphorus limited medium with palmitic acid as the carbon substrate was also demonstrated by Chayabutra et al. [13]. Although the specific rhamnolipid productivity under denitrification was about one third of that under aerobic conditions, the process did not encounter the problems of foaming and respiration limitation.

2.7. NAD(P)H fluorescence for monitoring rhamnolipid denitrifying fermentation

NADH and NADPH are the reduced forms of nicotinamide adenine dinucleotide phosphate (NAD(P)) coenzymes. These coenzymes are generally present in all living cells and serve as the major intermediate electron and hydrogen carriers in cellular metabolism [29]. The reduced forms of this coenzymes (NAD(P)H) are highly fluorescent with an excitation and emission maxima of approximately 340 and 460 nm,
respectively. On the other hand, their oxidized counterparts NAD(P)+, are not. The fluorescence intensity from NAD(P)H can then be related to the kinetic balance between the rates of NAD(P)H generation (by catabolism, i.e. NAD(P)H oxidation) and consumption (by respiration and anabolism, i.e. NAD(P)+ reduction) [128]. This intensity is extremely sensitive to the change in cellular electron-accepting mechanisms as shown in Figure 2.4, where the cyclic nature of NAD(P) coenzymes is illustrated [23].

NAD(P)+ is reduced to NAD(P)H during substrate catabolism. NADPH participates mainly as the reducing power in biosynthetic reactions (anabolism). In contrast, NADH is directly involved in ATP formation during respiratory metabolism being oxidized back to NAD+. The NAD(P) cycle for nitrate respiration is very similar to that for aerobic respiration, with NO3− replacing the role of oxygen. In anaerobic fermentation, the oxidation NAD(P)H to NAD(P)+ is coupled with the reduction of an organic compound that is formed during catabolism.

Figure 2.4. Cyclic nature of coenzymes NAD(P)H and NADH. Adapted from Ju et al. [129].
Accordingly, a fluorometer following the fluorescence intensity of intracellular NAD(P)H has been effectively applied to obtain information on biological activity and cellular metabolism of microbial fermentations and biological waste water treatment processes [23, 128, 130, 131].

Although, the signal obtained with a fluorometer is very complex due to intrinsic turbidity and variability of the biological broth and the relatively broad excitation and emission bandwidths employed by the equipment, it is sensitive enough to detect the changes in cellular metabolism. It could also be a useful indicator of the bacterial activity under microaerobic conditions. For instance, the fluorescence signal of resting cultures of *Escherichia coli* was monitored by a NAD(P)H fluorometer, clearly responding to the changes in culture condition from anoxic to aerobic conditions and to different low DO [129]. The NAD(P)H fluorescence signal displayed instant step changes upon a shift of microbial electron-accepting mechanism. As a result, the fluorescence level was highest under strictly anaerobic condition, followed by the anaerobic-denitrifying condition, and lowest under fully aerobic condition. Additionally, and as seen in Figure 2.4, the signal also responds to nitrate and carbon source changes [132]. This technique can also be potentially incorporated into large-scale industrial operations to provide better process control.

Chen et al. [23] studied the denitrification of *P. aeruginosa* using glucose as carbon source and an on-line fluorometer for NAD(P)H (including both NADH and NADPH). In this study, continuous cultures of *P. aeruginosa* ATCC 9027 were maintained at different dissolved oxygen (DO) to investigate the effect of DO on cell metabolism and respiratory mechanisms. The strain used in that study performed aerobic
denitrification even at relatively high DO (1 to 1.3 mg/L). The mechanism of aerobic
denitrification was believed to function as a system of electron acceptance that was
supplementary to or competitive with aerobic respiration. As expected and because of the
higher energy (ATP) yield of aerobic respiration compared to that of denitrification, the
steady-state cell concentration considerably increased as DO increased from 0 mg/L
(fully denitrifying conditions) to about 0.6 mg/L. With respect to the rhamnolipid
concentration, the profile showed an increase followed by a decrease that peaked at a
very low DO (0.1 mg/liter) under minimal aeration. Further investigation of the effect of
low DO on rhamnolipid production is still required.

2.8. Biodiesel byproduct glycerol

Biodiesel is nowadays one of the most important alternative fuels in the world. It
is produced from vegetable oils or animal fats mainly in a transesterification reaction
with an alcohol (usually methanol) in the presence of a catalyst (usually a base) [133].
The product of this reaction is the corresponding alkyl ester of the fatty acid mixture that
is found in the oil or fat reactant (Figure 2.5).

Biodiesel is renewable, biodegradable, non-toxic, and basically free from sulfur,
polycyclic aromatic hydrocarbons, and metals. The main current drawback of the
biodiesel production is the high price when compared to the petroleum based diesel.
However, many efforts are being successfully directed to using less expensive feedstocks
such as waste oils to improve the economics of the process [134].
Figure 2.5. Transesterification reaction to produce biodiesel (and glycerol as byproduct).

One of the major concerns with the biodiesel industry is not the production of the fuel itself but the large amounts of glycerol generated as the major byproduct of the reaction (Figure 2.5). In general, for every 9 kg of biodiesel produced, about 1 kg of a crude glycerol is generated [6]. Glycerol is widely used in the food industry and in pharmaceutical and medical formulations (prescription drugs, cosmetics, toothpastes, and ester gums). It is also used as raw material for urethane foams and synthetic resins [134] and can be used as emulsifier, softener agent, stabilizer, and wetting agent for bakery products [135]. As the biodiesel market increases, large amounts of glycerol are also being produced. However, the demand for glycerol in the world is not large enough to use all the excess glycerol. As a result, new uses and applications for glycerol are required to avoid its disposal as an organic loading to the environment and to improve the economics of the biodiesel industry as a whole.

The use of glycerol in fermentation processes to obtain higher valued products is one of the alternatives that have been proposed. 1, 3-Propanediol [136, 137], lipids [138],
β-carotene [139], and a mixture of succinic acid, butanol, ethanol, and hydrogen [140] had already been obtained by fermentation processes using glycerol as carbon source.
CHAPTER III
MATERIALS AND METHODS

3.1. Microorganisms and medium

The microorganisms used in this study were *P. aeruginosa* PAO1, *P. aeruginosa* ATCC 9027, and 8 other *P. aeruginosa* strains isolated from the soil samples of a biodiesel production plant (West Central Cooperative) in Ralston, Iowa: *P. aeruginosa* E03-36, E03-40, F04-63, F04-67, H05-03, H05-25, H05-45, and ST55. The cultures were maintained in frozen stocks of Tryptic Soy Broth medium (Sigma-Aldrich, St. Louis, MO) and 15% glycerol. The cultures were usually activated by incubation in a rotatory shaker (Innova 4080, New Brunswick Scientific Co.) at 34°C and 280 rpm using 10 mL of a 30 g/L Tryptic Soy Broth solution.

The medium used for the methylene blue/CTAB agar plate assays (SW agar plates) and for growth of *P. aeruginosa* cultures before plating was the one recommended by Siegmund and Wagner [15]: 20 g/L of glucose or glycerol, 0.7 g/L KH₂PO₄, 0.9 g/L Na₂PO₄, 2 g/L NaNO₃, 0.4 g/L MgSO₄•H₂O, 0.1 g/L CaCl₂•2H₂O, and 2 mL of a trace element solution containing 2 g/L FeSO₄•7H₂O, 1.5 g/L MnSO₄•H₂O and 0.6 g/L (NH₄)₆Mo₇O₂₄•4H₂O. The cultures were grown in the liquid medium at 34°C for 24 h before being transferred onto the agar plates. The methylene blue/CTAB agar plates were
prepared by adding 0.2 g of CTAB and 0.005 g of methylene blue to 1 L of the medium described above.

*P. aeruginosa* PAO1 and *P. aeruginosa* E03-40 were selected to conduct the batch fermentation experiments as they were found to be high rhamnolipid producer strains on methylene blue/CTAB agar plates. The medium used for batch fermentations typically contained glycerol as carbon source, 2 g/L KH$_2$PO$_4$, 0.3 g/L Mg$_2$SO$_4$$\cdot$7H$_2$O, 0.03 g/L CaCl$_2$$\cdot$2H$_2$O, 0.1 g/L FeSO$_4$$\cdot$7H$_2$O, 1.5 g/L NaCl, 0.03 g/L MnCl$_2$$\cdot$4H$_2$O and 2 mL of a trace element solution containing 0.08 g/L FeCl$_3$$\cdot$6H$_2$O, 0.75 g/L ZnSO$_4$$\cdot$7H$_2$O, 0.08 g/L CoCl$_2$$\cdot$6H$_2$O, 0.075 g/L CuSO$_4$$\cdot$5H$_2$O, 0.75 g/L MnSO$_4$$\cdot$H$_2$O, 0.15 g/L H$_3$BO$_3$, and 0.05 g/L Na$_2$MoO$_4$. NH$_4$Cl and NaNO$_3$ concentrations were adjusted depending on the experiment.

3.2. Experimental setup and equipment

A description of the experimental setup and equipment related to the different parts of the project will be described accordingly in the next subsections.

3.2.1. Improved rhamnolipid detection on methylene blue/CTAB agar plates

Following the improved procedure of Gunther et al. [70], shallow wells were cut on the agar plate surface using the heated tip of a 5-mL Eppendorf pipette. Ten (10) μL of the inoculum were added into each well. The plates were incubated for 48 h at 34 °C and then stored at 4 °C for at least 24 h (typically 48 h). In this study the halo edges (and multilayered structures) were found to be much more discernible when the plates were lighted on a fixed light source such as that of a dissecting microscope (Olympus SZX12...
ILLB100) or a UV transilluminator (at 312 nm, Fisher Scientific). An image analysis software was used to determine the halo diameters after taking pictures of the plates with a digital camera (Olympus C-4000 Zoom) connected to the UV transilluminator or a digital camera (Olympus DP71) connected to the dissecting microscope.

3.2.2. Batch fermentations for rhamnolipid production

The experiments were carried out either in glass fermentors or in shaker flasks using a shaker incubator (Innova 4080, New Brunswick Scientific Co.) at 32 ºC and 280 rpm. A diagram of the experimental setup typically used for the batch fermentations in glass fermentors is shown in Figure 3.1. These batch experiments were carried out in either a 2-L glass fermentor or 3-L glass BioFlo 110 modular benchtop fermentor (New Brunswick Scientific Co.) containing 0.8 L and 1 L of medium, respectively. An automatic pH control, with acid/base (HNO₃/NaOH) addition, was used to keep the pH at 7.1 ± 0.1. The pH control was achieved by using a set of pH probe (Mettler Toledo) and controller (pH/ORP controller Model 5656-00, Cole-Parmer Instrument Co.). Temperature was maintained at 32°C ± 0.2 using a heating tape connected to a temperature controller (Omega CN 370, Omega Engineering, Inc.) or, in the case of the 3-L fermentor, using the temperature control included in the unit which is connected to an external heating blanket.

Dissolved oxygen (DO) concentration was measured by an optical micro-sensor PST3 (PreSens Precision Sensing GmbH) that was attached on the inner wall of the fermentor. The micro-sensor contains an oxygen-sensitive dye immobilized in a silicone matrix which is added onto a flexible transparent polyester foil. The oxygen
concentration of the broth in contact with the silicone matrix was then acquired by projecting the tip of an optical fiber to the sensor spot from outside through the wall. On the other side, the optical fiber was connected to an oxygen meter Fibox 3-trace v3 (PreSens Precision Sensing GmbH) which was connected to a computer for data recording.

Figure 3.1. Experimental set-up for batch rhamnolipid denitrifying fermentations.

The NADH fluorometer (BioGuide System, BioChem Technology, Inc.) was set up on the side of the fermentor and it stayed in contact with the broth through an optical
well. This online fluorometer was designed for monitoring the fluorescence of intracellular NAD(P)H, with excitation wavelength of 340 ± 20 nm and emission wavelength of 400 – 480 nm. The fluorescence intensity was recorded as NFU (Normalized Fluorescence Unit) using FERMAC (fermentation monitoring, analysis, and control systems) software also obtained from BioChem Technology, Inc.

Surface aeration was provided by an air pump which outlet passed through a rotameter (flow rate typically set at 0.2 L/min) followed by a sterile 0.22 μm filter. Sterile concentrated solutions of glycerol and NaNO₃ (and if necessary NH₄Cl) were pumped into the fermentors using a Traceable® timer controller (Control Company) that allowed repeatedly turning the pumps (Masterflex L/S, Cole Parmer Instrument Co.) on or off at a unique time interval that was varied according to the needs of the culture. Samples were taken periodically from the fermentor and typically used to measure cell, glycerol, protein, nitrate (and nitrite), ammonia, and rhamnolipid concentrations.

3.3. Analytical methods

Samples taken from the fermentor (or shaker culture flasks when applicable) were first centrifuged to separate the cells from the medium (supernatant). The pelletized cells were kept to measure the cell concentration and the supernatant was used to measure the concentrations of carbon source and nitrogen sources, as well as NO₃⁻-N (and NO₂⁻-N), rhamnolipid, and extracellular protein.
3.3.1. Cell concentration measurement

After centrifugation the cell pellet was washed with distilled water and centrifuged again. The water was carefully removed and the cells were lysed with a 0.2 N NaOH solution followed by heating at 95°C for 20 minutes. Standard Bradford analysis was carried out to quantify the intracellular protein using a diagnostic kit (Bio-Rad Protein Assay kit II, Bio-Rad Laboratories), with absorbance measured at 595 nm with a Shimadzu UV/Vis spectrophotometer (Model 1601). The protein content was then converted to the cell dry-weight concentration according to a calibration curve developed for this purpose. For determination of dry cell weight, the washed cells collected by centrifugation were dried to constant weight at 90 °C (for ~24 h).

3.3.2. Glycerol concentration measurement

Glycerol concentration was usually analyzed with a Shimadzu LC-10A HPLC system equipped with a refractive index detector (RID-10A). Two different columns were used interchangeably to separate the supernatant components from glycerol: a Supelcosil LC-NH2 column (5 μm, 25 cm x 4.6 mm, Supelco) with a mixture of 3:1 (v/v) acetonitrile:water as mobile phase, and a Supelcogel H column (25 cm x 4.6 mm, Supelco) with 0.1% H₃PO₄ solution as mobile phase. The flow rates were 1 mL/min and 0.14 mL/min, respectively. A linear curve from 0.5 to 10 g/L of glycerol was obtained as the calibration curve for any of the columns. The samples were diluted accordingly for the concentrations to be within the calibration range.
3.3.3. Ammonia, nitrate, and nitrite concentration measurement

For a rapid determination, NH$_4$+\(^{-}\)-N, NO$_3$-\(^{-}\)-N, and NO$_2$-\(^{-}\)-N concentrations were estimated using Hach test strips (Hach Company) for ammonia (0 – 6 ppm), nitrate (0 – 50 ppm), and nitrite (0 – 3 ppm), respectively. The samples were diluted with distilled water to match the range of concentrations specified in each test kit.

Additionally, a more exact measurement of ammonia, and nitrate concentrations was carried out when necessary using an ammonia electrode. Using this method, the ammonium concentrations could be accurately measured in a wide range from 1–1000 mg/L of NH$_4$+\(^{-}\)-N. The nitrate (and nitrite) in the sample were reduced to ammonia using titanous chloride and measured using the ammonia electrode as well. For this case, the sample was properly diluted to match a narrower concentration range of 1 – 20 mg/L. Nitrite concentration was determined separately and the difference between this measured value and the nitrite concentration was taken as the NO$_3$-\(^{-}\)-N in the sample.

Nitrite is analyzed spectrophotometrically at 543 nm with a Shimadzu UV/Vis spectrophotometer (Model 1601) after a reaction with 0.1 mL of a sulfonamide reagent for 2 – 8 minutes in the dark followed by 0.1 mL of a N-1 (naphthyl)-ethylenediamine dihydrochloride reagent reacting for 10 minutes.

3.3.4. Rhamnolipid analysis

The sample pH was first adjusted to 2.3 ± 0.2 using 1 N HCl. These aqueous samples were then extracted either with 5-fold volume of chloroform (for methylene blue-based analysis) or with 4-fold volume of ethylacetate (for colorimetric anthrone analysis).
3.3.4.1. Methylene blue analysis

A detailed description of the quantitative analysis for rhamnolipid determination developed in this project is included in Chapter V. A brief description of the procedure is presented here: 4 mL of the chloroform extract are carefully removed and put in contact with a freshly prepared methylene blue solution containing 200 μL of the 1 g/L methylene blue reagent, and 4.9 mL of distilled water. The pH of this methylene blue aqueous solution is adjusted to 8.6 ± 0.2 by adding the 50 mM borax buffer (~15μL). After being vigorously mixed for 4 minutes, the samples were left to stand for 15 minutes. The chloroform phase was transferred into a quartz cuvette and the absorbance was measured at 638 nm with a Shimadzu UV/Vis spectrophotometer (Model 1601) against a chloroform blank. The absorbance values were converted to rhamnolipid concentrations using a calibration curve established by applying the same procedure to standard rhamnolipid solutions of different concentrations.

3.3.4.2. Anthrone analysis

The ethylacetate extract was first air dried to remove the solvent. The remaining lipids were redissolved in 0.05 M NaHCO3 followed by addition of the anthrone reagent (2 g/L of anthrone in concentrated sulfuric acid). The reaction mixture was maintained at 95 °C for 16 minutes, and then the absorbance was measured at 625 nm with the UV/Vis spectrophotometer. A calibration curve using rhamnose (Sigma-Aldrich) as the standard was developed to allow conversion of the absorbance to the rhamnose concentration.
3.3.5. Extracellular protein

Standard Lowry analysis was carried out to quantify the extracellular protein content in the supernatant using a diagnostic kit (Bio-Rad RC DC Protein Assay Kit II, Bio-Rad Laboratories), with absorbance measured at 750 nm with a Shimadzu UV/Vis spectrophotometer (Model 1601). A calibration curve using different concentrations (0.2 g/L to 1.4 g/L) of bovine serum albumin was performed to convert the absorbance values to protein concentration.

3.3.6. Alginate assay

Alginate was first precipitated with 3 volumes of ethanol (200 proof absolute dehydrated) and drop-wise addition of concentrated potassium chloride (KCl) solution. The precipitate was collected by centrifugation at 9,000 rpm for 20 minutes, the supernatant was discarded and the precipitate resuspended in water. Alginate was then quantified by the borate-carbazole method. Sodium alginate was used as the standard to determine the calibration curve by making several dilutions of a 2 g/L sodium alginate solution in saline (10 - 100 mg/L). Briefly, 90 μL of the standards and samples (diluted accordingly with saline to match the linear range of the calibration curve) were layered on top of 3 mL of a sulfuric acid - 0.1% borate reagent. After vigorously mixing the samples, 90 μL of a 0.1% carbazole solution (prepared in ethanol) were added and the solutions mixed again. The samples were heated at 55 ºC for 30 minutes. The absorbance was then measured at 530 nm with a Shimadzu UV/Vis spectrophotometer (Model 1601).
The sulfuric acid - 0.1% borate reagent was prepared using a stock borate solution (4 M $\text{BO}_3^{-3}$ prepared from $\text{H}_3\text{BO}_3$ and added KOH until borate dissolved in solution) in concentrated sulfuric acid.

3.3.7. Rhamnolipid standard characterization

A rhamnolipid sample obtained from Ecochem Canada Ltd., was analyzed using HPLC-MS (HP 1100 series + Bruker Daltonics Esquire LC MS$^n$ analyzer) to determine the types of rhamnolipids present. A mixture of tetrahydrofuran/acetonitrile and water was used as mobile phase in a ratio of 55:45 (v/v), with a flow rate of 0.8 mL/min and a Supelco C-18 column (2 mm×15 cm). Two dominant rhamnolipids (L-Rhamnosyl-$\beta$-hydroxydecanoyl-$\beta$-hydroxydecanoate, and L-Rhamnosyl L-rhamnosyl-$\beta$-hydroxydecanoyl-$\beta$-hydroxydecanoate) were found in the sample, with relative peak areas of MS total ion intensity of 48.4% (Type 1) and 51.6% (Type 2). Their molecular weight was approximately 504 and 650, respectively.
CHAPTER IV

IMPROVED DETECTION OF RHAMNOLIPID PRODUCTION ON METHYLENE BLUE/CTAB AGAR PLATES

4.1. Introduction

As described in Chapter II, a fast method for rhamnolipid analysis can significantly enhance the strain selection, metabolic engineering, and process development for improved rhamnolipid production. A simple semi-quantitative method using agar plates containing methylene blue and CTAB was proposed earlier by Siegmund and Wagner [30]. On such agar plates, the colonies producing rhamnolipids were surrounded by dark blue halos and, ideally, the areas of the halos could be correlated with the rhamnolipid concentrations [30]. This method has been used to study the effect of medium composition on rhamnolipid production and for strain selection or screening studies [70, 117]. Being interested in selecting rhamnolipids-productive strains and in evaluating the effects of different culture conditions, the above method was employed in the early phase of this study. As described in more detail later, it was found that many *P. aeruginosa* strains produced pigments that also surrounded the colonies and made it very difficult to correlate quantitatively the halo areas with the rhamnolipid concentrations.
When the method was evaluated for use in this laboratory, it was also found that the edges of the blue halos were not always clearly distinguishable and could shift depending on the intensity and location of the light source as well as the background color. In this part of the project, the methylene blue (MB)/CTAB agar plate method was revisited for improved understanding and application using a set-up including fixed underneath illumination such as that of a dissecting microscope or a UV transilluminator and a common image analysis software. Some potential limitations of this semi-quantitative method were identified using the modified set-up on different *P. aeruginosa* strains. The complexation between anionic rhamnolipids and MB/CTAB as well as the effect of pH on the SW agar plates were also investigated.

4.2. Materials and methods

The microorganisms and medium used in this part of the study were described in section 3.1. After preparing the agar plates containing MB and CTAB, the improved procedure of Gunther et al. [70] was used to cut shallow wells on the agar plate as described in subsection 3.2.1. The plates were incubated after inoculation for 48 h at 34 °C and then stored at 4 °C for at least 24 h (typically 48 h). The cold storage tended to darken the blue color, making visible recognition less difficult [70]. In this study the halo edges (and multilayered structures) were found to be more discernible when the plates were lighted on a fixed light source such as that of a dissecting microscope (Olympus SZX12 ILLB100) or a UV transilluminator (at 312 nm, Fisher Scientific). An image analysis software was used to determine the halo diameters after taking pictures of the plates with a digital camera (Olympus C-4000 Zoom) connected to the UV
transilluminator or a digital camera (Olympus DP71) connected to the dissecting microscope.

The same procedure was also used to observe the halo formation when standard solutions of different rhamnolipid concentrations (e.g., 11, 23, 57 and 114 g/L) were added to the wells on the agar plates. In some experiments the CTAB concentration was varied (at 0, 0.05, 0.1, 0.2, 0.3, and 0.5 g/L) to investigate the role and effect of CTAB in the agar plates. Because the rhamnolipids were added all at once in these systems (instead of being produced by the cells over the duration of experiment), these plates were incubated for a shorter period of 24 h (instead of 48 h).

MB and CTAB were purchased from Fisher Scientific (Waltham, MA). The rhamnolipid standard was provided by Ecochem Canada Ltd. (Delia, AB, Canada).

4.3. Results and discussion

The blue halos formed on MB/CTAB agar plates were found to change with the intensity and incidence angle of the light source, affecting the accurate determination of the halo areas. In this study, different ways of viewing the plates were explored. Additionally, the effects of CTAB and pH on the method as well as the remaining difficulties of its application were also discussed.

4.3.1. Use of dissecting microscope or UV transilluminator for clear observation of SW plates

The clarity was found to be improved by observing the plates using either a dissecting microscope or a UV transilluminator. In both cases, the plate was illuminated
from underneath and with a fixed light source. For comparison, pictures of SW plates taken with the use of a UV transilluminator and a dissecting microscope, respectively, are shown in Figure 4.1. Each plate had 4 wells, added with 10 µL of standard solutions containing different rhamnolipid concentrations.

Figure 4.1. Pictures of a SW agar plate with 4 wells, each added with 10 µL of a standard solution: (a) picture taken with the plate lighted on a dissecting microscope, and (b) picture taken with the plate lighted on a UV-Transilluminator.

Using a dissecting microscope the circles developed surrounding the wells had up to 4 distinguishable layers (Figure 4.1-a): an innermost light area, a bluest halo, a lighter blue ring outside the darker blue halo, and an outermost brown ring. Similar 4-layered structures were observed with the UV transilluminator but the colors were different (Figure 4.1-b). (The outermost layer was light and not always clearly shown on pictures.) Without the help of fixed underneath illumination, these layers were not always evident and would vary depending on the background and lighting condition. The areas shown on
Figure 4.1-a appear larger than in the UV transilluminator (Figure 4.1-b) due to the magnifying lens connected to the dissecting microscope.

In Figure 4.2 both the total areas (including all 4 layers) surrounding the wells and the blue areas, i.e., the innermost light area and the bluest halo area, which together correspond to the blue halo area used originally by Siegmund and Wagner [30]), determined with the dissecting microscope, were plotted against the rhamnolipid concentrations in the 10-µL solutions added to the wells (The results shown in Figure 4.2 were the averages and standard deviations obtained from three agar plates).

![Empirical linear correlation of the affected areas (surrounding the well added with 10 µL of a rhamnolipid solution) with the employed rhamnolipid concentration. The total areas are for all 4 layers; the blue layer areas are for the combined innermost light layer and the bluest halo, which were originally used by Siegmund and Wagner [30].](image)

Figure 4.2. Empirical linear correlation of the affected areas (surrounding the well added with 10 µL of a rhamnolipid solution) with the employed rhamnolipid concentration. The total areas are for all 4 layers; the blue layer areas are for the combined innermost light layer and the bluest halo, which were originally used by Siegmund and Wagner [30].
Good linear correlations, with $R^2 > 0.99$, were obtained with either of the two types of areas. In addition, either the dissecting microscope or the UV transilluminator set-up allowed the determination of the areas with the use of image analysis software (As a result, the standard deviations at each rhamnolipid concentration were smaller as compared to those determined not using the modified set-up (Figure 4.2)).

4.3.2. Effect of CTAB concentration

The phenomena observed on SW agar plates were associated with the ion-pairing complexation between anionic rhamnolipids and cationic MB and CTAB [30]. Nonetheless, the individual roles of MB and CTAB were not clearly established to allow adjustment of their concentrations for attaining better results for different applications. With aqueous solutions, instead of the complex agar plates, the following observations were obtained (described in more detail in Chapter V, section 5.2.1): (1) Aqueous MB solutions showed clear absorbance bands near 292, 615, and 664 nm. (2) CTAB had no detectable absorbance and the mineral salts medium used in SW agar plates had only one very weak absorbance band near 300 nm. (3) Rhamnolipid solutions had no absorbance at concentrations below 100 mg/L. At higher concentrations, the “absorbance” appeared and expanded to increasingly longer wavelengths with increasing concentration. The “absorbance” was attributed to light scattering by the micelles formed. (4) With solutions of 5 mg/L MB (the same concentration as in SW plates) and varying rhamnolipid or CTAB concentrations (to get different molar ratios of rhamnolipids-to-combined MB and CTAB), the characteristic absorbance at 664 nm increased only within a narrow range of molar ratio, i.e., 1:1 to 2:1, supporting the occurrence of ion-pairing complexation.
Clearly, MB was included not only for being a cationic compound but also because of its characteristic blue color. However, only very low MB concentrations could be used; at higher concentrations, the intense blue color would mask the color change due to the ion-pairing complexation. Using MB alone at the very low concentrations would limit the applicability of the method to detection of very low rhamnolipid levels. The addition of absorbance-free CTAB enabled the detection of rhamnolipids at the levels produced by *P. aeruginosa* colonies.

The effect of CTAB concentration was next investigated in a study using agar plates containing 5 mg/L MB and different CTAB concentrations (0, 0.05, 0.1, 0.2, 0.3, and 0.5 g/L). Rhamnolipid solutions of 10, 25, 50, and 125 g/L were added to the wells of these plates. The total areas of the 4-layered circles surrounding these wells, revealed on the UV transilluminator, are summarized in Table 4.1. Pictures of some of these plates, i.e., those with 0, 0.1, and 0.5 g/L CTAB, are shown in Figure 4.3. It was found that, at the same rhamnolipid concentration, the area affected by the complexation decreased with increasing CTAB concentrations. On the plates of high CTAB concentrations, 0.3 and 0.5 g/L, the only circles detectable were those surrounding the wells added with solutions of at least 50 g/L rhamnolipids. With lower CTAB concentrations, the circles from lower rhamnolipid concentrations became more recognizable but the circles from high rhamnolipid concentrations became very diffused and harder to distinguish the edges. On the plate of no CTAB, no circles could be detected; instead, the whole plate looked darker than the other plates. Totally 3.64 µmol of rhamnolipids were added to the 4 wells of each plate. Without CTAB, the plate contained only 0.27 µmol of MB. The
added rhamnolipids must have spread over the entire plate and complexed with all the MB.

Table 4.1. Total affected areas surrounding the wells added with 10 µL of rhamnolipid (RhL) solutions of different concentrations, on agar plates with 5 mg/L methylene blue and various concentrations of cetyl trimethylammonium bromide (CTAB).

<table>
<thead>
<tr>
<th>RhL (g/L)</th>
<th>CTAB (g/L)</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>125</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.05</td>
<td>10.32</td>
<td>34.96</td>
<td>145.06</td>
<td>489.16</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>5.80</td>
<td>29.97</td>
<td>119.88</td>
<td>306.90</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>11.27</td>
<td>32.42</td>
<td>180.39</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>0</td>
<td>0</td>
<td>24.64</td>
<td>153.98</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>12.28</td>
<td>116.71</td>
<td></td>
</tr>
</tbody>
</table>

Note: (1) Areas are reported in mm². (2) ND – Not detectable.

According to the above understanding, it is recommended to adjust the CTAB concentration in the SW plates (without changing the MB concentration) for different applications or purposes. With the much improved detection sensitivity and clarity provided by the use of dissecting microscope and UV transilluminator, lower CTAB concentrations than that in the original formula can be used. For example, if the purpose is to ensure complete deactivation of genes responsible for rhamnolipid synthesis, the
CTAB concentration should be reduced significantly so that even small amounts of rhamnolipids produced can be revealed with larger complexation circles.

Figure 4.3. UV-transilluminated pictures of plates containing 5 mg/L methylene blue and different CTAB concentrations: (a) 0 g/L, (b) 0.1 g/L, and (c) 0.5 g/L. Rhamnolipid solutions of 10, 25, 50, and 125 g/L were added to the wells of these plates.

4.3.3. Effect of pH

The effect of pH on circle formation on SW plates was examined at 5 pH values: 3, 5, 7, 9, and 11. Circles were clearly identifiable at pH 7 and 9. At pH 3 and 5, the circles were not apparent even when inspected with a dissecting microscope or a UV
transilluminator. This finding is reasonable because ion-pairing requires the rhamnolipids to be present as anionic ions. Rhamnolipids were reported to have pKa in the range of 4.8-5.5 at concentrations higher than the critical micelle concentration (CMC, ranging from 5 to 200 mg/L, depending on the rhamnolipid structure) [48]. (pKa was lower, 4.3-4.4, at concentrations lower than CMC.) When pH was lowered to near or below the pKa, increasingly more of the rhamnolipids became protonated and the neutral molecules were incapable of forming complex with the cationic MB and CTAB. At pH 11, the circles were formed with comparable sizes to those at pH 7 and 9. The agar plates, however, had a much paler blue color than the plates at lower pH values. The reason of the color change at this high pH is not clear but it can be attributed in part to the light reflection by the larger amount of precipitates of medium components formed when the pH was adjusted to 11, and to the known property of MB to become colorless in its reduced form [141]. The circles are expected to have similar areas at pH 7, 9 and 11 (all significantly higher than pKa of rhamnolipids) because MB and CTAB are quaternary ammonium cations that are permanently charged independent of the pH.

The above findings indicated the need for considering the potential change of local pH by cell metabolism in the colonies when using SW plates for strain selection. If significant acid production is expected, a stronger buffer should be used in the SW plates to avoid the problem of unsuccessful complexation caused by low local pH.

4.3.4. Molar complexation ratios

An attempt was made to determine the molar ratios of rhamnolipids-to-combined MB and CTAB, i.e., (MB + CTAB), involved in the complexation. The total area
(including all 4 layers) of the circle developed around each well was used to determine the number of moles of (MB + CTAB) participated in the complexation with the number of moles of rhamnolipids added to the well. The resultant molar complexation ratios are given in Table 4.2. The molar ratios of rhamnolipids-to-(MB + CTAB) turned out to be not 1:1 but to increase with increasing rhamnolipid concentration in the solution added. The ratio was as high as $(3.18 \pm 0.04):1$ when the 10 µL solution added to the well contained 114 g/L of rhamnolipids (Table 4.2).

Table 4.2. Molar complexation ratios of rhamnolipids-to-(MB + CTAB) and SDS-to-(MB + CTAB).

<table>
<thead>
<tr>
<th>Surfactant concentration* (g/L)</th>
<th>Molar complexation ratio</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rhamnolipids:(MB + CTAB)</td>
<td>SDS:(MB + CTAB)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.68 ± 0.11</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>1.99 ± 0.08</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>2.57 ± 0.07</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>114</td>
<td>3.18 ± 0.04</td>
<td>1.39</td>
<td></td>
</tr>
</tbody>
</table>

* Concentration of either rhamnolipids or sodium dodecyl sulfate (SDS) in the 10 µL solution added to the well in an agar plate

\( \text{a} \) Molar ratios determined from 3 agar plates

\( \text{b} \) Molar ratios determined from 1 agar plate

To check if the observed deviation from 1:1 complexation was caused by erroneous experimental or computational procedures, the same procedures were applied to another set of SW plates whose wells were added with solutions of different concentrations of sodium dodecyl sulfate (SDS), a common synthetic anionic surfactant.
Clear 4-layered complexation circles were also observed on these plates (but the circle colors were considerably darker than in the rhamnolipids case) (Figure 4.4). For these SDS-complexed plates, the determined molar complexation ratios of SDS:(MB + CTAB) were much closer to a 1:1, ranging from 1.2:1 to 1.4:1, and had no apparent dependency on the SDS concentration employed (Table 4.2). The large, concentration-dependent molar complexation ratios observed with the rhamnolipids-complexed SW plates were therefore not caused by experimental or computational errors; instead, they reflected the more complicated nature of the complexation between rhamnolipids and MB and CTAB in agar plates.

The anionic SDS has a simple structure – a straight C12 hydrocarbon with a sulfate end group. The SDS-MB/CTAB complexes are expected to be rather water insoluble and the ion-pairing stable. On the other hand, each rhamnolipid molecule has

Figure 4.4. Picture of a MB/CTAB agar plate with 4 wells, each containing SDS solutions of different concentrations (picture taken with the plate lighted on a dissecting microscope).
the sugar moiety of one or two rhamnose linked to a chain of one or two $\beta$-
hydroxyalkanoic acid (mainly C10). Even after being complexed with MB/CTAB, the
sugar moiety of rhamnolipids continues to provide hydrophilicity, possibly resulting in
incorporation of the formed complexes as part of micelles as the added rhamnolipid
solutions diffused outwards from the wells. The complexation may therefore be of a loose
association nature, with the cations dynamically interacting with several anionic
rhamnolipids. This association nature would also explain why higher rhamnolipids-to-
(MB + CTAB) ratios occurred with the wells added with more concentrated rhamnolipid
solutions.

Nevertheless, the form of complexation between rhamnolipids and MB/CTAB
hypothesized above is yet to be verified. The mechanisms responsible for formation of
the unique 4-layered circles are also unknown. Furthermore, unlike in liquid solutions,
the complexation behaviors used for rhamnolipid detection in SW plates were in a
transitional state, instead of being in equilibrium. Together with the changing molar
complexation ratios observed, these factors make the agar plate-based method unsuitable
for quantitative analysis of rhamnolipid concentration or amount. The plate-based method
is recommended only for strain screening and other qualitative purposes.

4.3.5. Pigment formation

For demonstration the SW plates were used to evaluate the rhamnolipid producing
ability of 10 $P.\ aeruginosa$ strains. In general, all the tested strains showed better growth
on plates containing glycerol as the carbon source than on the glucose-based plates. The
only exception was the ATCC 9027 strain, which grew similarly well on both substrates.
Also, the complexation circle formation was easier to detect on the glycerol-based plates, at least partially due to better growth.

The total complexation areas due to rhamnolipid production in 48-h incubation of the culture are summarized in Table 4.3. Using the average molar complexation ratio of 2.3:1 for rhamnolipids-to-combined MB and CTAB (from Table 4.2), the amounts of rhamnolipids produced during the 48-h cultivation can be roughly estimated. The estimates are also given in Table 4.3. Further included in Table 4.3 (last column) are qualitative descriptions for the extents of pigment formation by different strains.

While the use of fixed illumination from below and the image analysis software allowed for a better determination of the different layers, one possible complication remains with the use of methylene blue/CTAB plates. Many *P. aeruginosa* strains produced fluorescent and non-fluorescent pigments that deposited around the colonies. The pigment formation and the problems that this causes for rhamnolipid quantification have been also reported by others [117]. These pigments could affect recognition of the boundary of complexation circle and, potentially, the accuracy of complexation area determination [117]. Two extreme examples are shown in Figure 4.5: one had high rhamnolipid productivity and minimal pigment formation; the other produced an excessive amount of pigments and prevented the detection of complexation circles, if any, formed by rhamnolipids. As shown in Table 4.3, four of the tested strains, PAO1, E03-40, E03-36, and H05-25, were found to be good rhamnolipid producers without excretion of considerable amounts of pigments.
Table 4.3. Affected areas and estimated amounts of rhamnolipids produced by different *P. aeruginosa* strains on SW agar plates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Area (mm²)</th>
<th>Rhamnolipids (µmol)†</th>
<th>Rhamnolipids (mg)†</th>
<th>Extent of pigment formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E03-36</td>
<td>85.8</td>
<td>0.39</td>
<td>0.23</td>
<td>+</td>
</tr>
<tr>
<td>E03-40</td>
<td>128.6</td>
<td>0.59</td>
<td>0.34</td>
<td>+</td>
</tr>
<tr>
<td>H05-25</td>
<td>108.3</td>
<td>0.49</td>
<td>0.28</td>
<td>+</td>
</tr>
<tr>
<td>H05-45</td>
<td>99.6</td>
<td>0.45</td>
<td>0.26</td>
<td>++</td>
</tr>
<tr>
<td>H05-03</td>
<td>101.8</td>
<td>0.46</td>
<td>0.27</td>
<td>++</td>
</tr>
<tr>
<td>F04-63</td>
<td>97.8</td>
<td>0.45</td>
<td>0.26</td>
<td>++</td>
</tr>
<tr>
<td>F04-67</td>
<td>49.9</td>
<td>0.23</td>
<td>0.13</td>
<td>+</td>
</tr>
<tr>
<td>ST55</td>
<td>58.9</td>
<td>0.27</td>
<td>0.15</td>
<td>++</td>
</tr>
<tr>
<td>PAO1</td>
<td>101.0</td>
<td>0.46</td>
<td>0.27</td>
<td>+</td>
</tr>
<tr>
<td>ATCC 9027</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+++</td>
</tr>
</tbody>
</table>

† Calculated using a molar complexation ratio of 2.3:1 rhamnolipids:(MB + CTAB), which is the average ratio obtained from those reported in Table 4.2.

‡ Estimated with an average rhamnolipid MW of 576, assuming similar composition as the rhamnolipid standard.

+ Pigments visible only inside the well of initial inoculation

++ Pigments visible in the area immediately surrounding the well

+++ Pigments visible in an extensive area outside the well, preventing detection of complexation circles.
4.4. Conclusions

In summary, the use of a set-up including fixed underneath illumination, such as that of a dissecting microscope or a UV transilluminator, and an image analysis software is an alternative for improved determination of the areas corresponding to the rhamnolipid complexation with MB/CTAB. For different applications and with this modified set-up, the CTAB concentration could be adjusted, particularly to lower concentrations according to the amounts of rhamnolipids to be detected. Low local pH conditions should be avoided when using methylene blue/CTAB agar plates as the complexation did not occur at pH 5 or lower. Additionally, an accurate detection of rhamnolipid production using the plates would also be difficult for strains that synthesize large amounts of pigments.
This improved semi-quantitative method was effectively applied for selecting the strains used for further experiments to advance the denitrifying rhamnolipid production strategy (described in Chapter VI).
CHAPTER V

QUANTITATIVE ANALYSIS OF RHAMNOLIPIDS BIOSURFACTANTS IN LIQUID FERMENTATION SAMPLES BY METHYLENE BLUE COMPLEXATION

5.1. Introduction

The new approaches for an economical rhamnolipid production have increased the interest on fast and reliable rhamnolipid identification and analysis. Commonly used methods for rhamnolipid quantification are either indirect measuring only the sugar moiety of the rhamnolipid molecule (i.e. rhamnose) or rely on the availability of expensive equipment (HPLC-MS). Even though analytical methods provide detailed structural information of the rhamnolipids, the equipment involved is expensive and preliminary purification steps are required. Among the colorimetric methods, anthrone, phenol-sulfuric acid, and orcinol are commonly used. However, as described in section 2.5.3, these methods are tedious, laborious, and operationally hazardous because of the strong acid involved. They are also indirect: rhamnose, instead of the rhamnolipids, is actually detected and the accurate determination of rhamnolipid concentration requires additional steps for establishing the ratio between the sugar and the lipid moieties in the mixture.
As described in Chapter IV, for fast identification of active rhamnolipid-producing strains, Siegmund and Wagner [30] proposed a simple method based on agar plates containing methylene blue (MB) and CTAB. Nevertheless, this method has some disadvantages and even though the alternative set-up that was developed in the previous chapter significantly improved the rhamnolipid detection on the plates, the method could not be used for quantitative determination of rhamnolipids in liquid samples.

The principle behind the agar plates consisting on the formation of an insoluble ion-pair between anionic rhamnolipids and cationic MB and CTAB was further studied in this part of the project in order to develop a quantitative method for rhamnolipid determination directly applicable to liquid fermentation samples.

Therefore, the complexation of rhamnolipids with MB and CTAB was investigated and a simple quantitative method based on whole rhamnolipid molecules was developed. The potential interferences from compounds present in culture broths were examined. The procedure was modified accordingly and verified by comparing the results with those obtained using the anthrone assay. The developed method was successfully applied to analysis of samples taken from batch *P. aeruginosa* fermentations.

5.2. Materials and methods

The materials and methods involved in the development of the new method are described in the following subsections.
5.2.1. Reagents

Methylene blue, CTAB, chloroform, and decahydrated sodium tetraborate (Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7}•10H\textsubscript{2}O) were purchased from Fisher Scientific. The borax buffer and methylene blue solution were prepared in similar manner to those previously reported by Jurado et al. [142]. The borax buffer reagent was prepared at 50 mM in distilled water, with pH adjusted to 10.5. A 1 g/L methylene blue solution in 10 mM borax buffer was prepared, stabilized by adjusting the pH to 5.5 (± 0.2), and then stored refrigerated in an amber bottle.

Standard rhamnolipid solutions were prepared using a purified rhamnolipid sample from Ecochem Canada Ltd. This sample was analyzed as described in subsection 3.3.6 to determine the types of rhamnolipids present in the standard mixture.

5.2.2. Rhamnolipid analysis on liquid samples

A description of both the methylene blue method for rhamnolipid analysis developed in this part of the project and the common anthrone technique is provided below.

5.2.2.1. Methylene blue analysis procedure

The principle of this method developed in this study is similar to that for determining the concentrations of anionic surfactants in water samples [101, 142, 143] but the procedure had to be modified throughout the study to minimize the interferences from rich fermentation broths. The finalized procedure is described below: The sample pH was first adjusted to 2.3 ± 0.2 using 1 N HCl. The acidified sample was then extracted
with 5-fold volume of chloroform. 4 mL of the chloroform extract were carefully removed and put in contact with a freshly prepared methylene blue solution containing 200 μL of the 1 g/L methylene blue reagent, and 4.9 mL of distilled water. The pH of this aqueous solution of methylene blue was adjusted to 8.6 ± 0.2 by adding the 50 mM borax buffer (~15 μL). After being vigorously mixed for 4 minutes, the samples were left to stand for 15 minutes. (Varying the contact time in the range of 10 to 30 minutes did not give statistically different results.) The chloroform phase was transferred into a cuvette and the absorbance was measured at 638 nm with a Shimadzu UV/Vis spectrophotometer (Model 1601) against a chloroform blank (The reading tended to slowly decrease. The initial value was used in this study). The absorbance values were converted to rhamnolipid concentrations using a calibration curve established by applying the same procedure to standard rhamnolipid solutions of different concentrations.

5.2.2.2. Anthrone analysis

The sample was acidified and extracted with 4-fold volume of ethylacetate. The ethylacetate extract was air dried to remove the solvent and the remaining lipids were re-dissolved in 0.05 M NaHCO₃. The standard anthrone analysis (as described in subsection 3.3.4.2) was then applied to determine the final rhamnose concentration in the samples.

5.2.3. Microorganism, medium, and fermentation

The microorganism used in this study was *P. aeruginosa* E03-40, obtained from the ARS (NRRL) Culture Collection. The medium used for the fermentations was described in section 3.1 and the set-up in subsection 3.2.2. These batch experiments were
carried out in a 2-L glass fermentor containing 0.8 L of medium. The temperature was maintained at 34°C and an automatic pH control, with acid/base addition, was used to keep the pH at 7.0 ± 0.1.

5.3. Results and discussion

The complexation of rhamnolipids with MB and CTAB in different systems was investigated and subsequently used to develop a simple quantitative method for rhamnolipid determination. The discussion of these results as well as the successful application of the method is presented in the next subsections.

5.3.1. Absorbance spectra of individual components

The original report by Siegmund and Wagner [30] did not contain enough information for more fundamental understanding on the phenomena occurring on the MB agar plates. Nonetheless, the complexation by ion-pairing between anionic rhamnolipids and cationic MB and CTAB must play a critical role in the phenomena.

A series of experiments were first carried out to examine how the complexation affected the absorbance spectra. Aqueous solutions of CTAB had no detectable absorbance in the entire range examined, 200-800 nm. The mineral salts medium used for *P. aeruginosa* culture had a weak absorbance band near 300 nm (Figure 5.1). On the other hand, aqueous solutions of methylene blue had clear absorbance bands near 292, 615, and 664 nm (Figure 5.1); the absorption in the red-light range gave it the characteristic blue color. Solutions of the rhamnolipid standard showed no absorbance at low rhamnolipid concentrations (< 100 mg/L). At higher concentrations, the absorbance
started to appear at the shortest wavelengths examined and the absorbance expanded to increasingly longer wavelengths with increasing rhamnolipid concentrations (Figure 5.2, where the rhamnolipid solutions were prepared in the presence of methylene blue and CTAB at the same concentrations as those used in the SW agar plates). The “absorbance” observed in solutions of rhamnolipids alone is therefore believed to be the result of micelle formation at higher concentrations that would increase the light scattering of the samples.

Figure 5.1. Absorbance spectra of methylene blue (MB) and mineral salts medium.
5.3.2. Complexation of rhamnolipids with methylene blue and/or CTAB in aqueous samples

A series of experiments was first carried out to examine how the complexation, by ion-pairing between anionic rhamnolipids and cationic methylene blue and CTAB, affected the absorbance spectra in aqueous samples. The concentrations of methylene blue and CTAB used were the same as those in the originally reported agar plates, i.e., 0.013 mM and 0.549 mM, respectively [30]. Different rhamnolipid concentrations were used, i.e., 0.032, 0.16, 0.33, 0.49, 0.65, and 1 g/L, which corresponded to the following molar ratios of rhamnolipids-to-combined methylene blue and CTAB: 0.1:1, 0.5:1, 1:1, 1.5:1, 2:1, and 3.15:1, respectively. As shown in Figure 5.3-a, the presence of
rhamnolipids at these low concentrations did not substantially alter the basic pattern of absorbance spectra of methylene blue; thus, all of the solutions remained blue in color. While the extent of absorbance indeed changed with different molar ratios, as summarized in Figure 5.3-b, the changes were too small and did not correlate with the rhamnolipid concentrations monotonically. Instead, the changes of absorbance occurred only in a narrow range of molar ratio, as shown in Figure 5.3-b.

The systems with molar ratios of 1:1 to 2:1 showed higher absorbance, with almost a constant step increase, throughout the wavelength range of 200-800 nm (Figures 5.3-a and 5.3b). Presumably, this step increase in absorbance reflected the colloidal (not truly soluble) nature of the ion-paired complexes. This increased absorbance due to complexation would have caused the formation of darker blue halos on the agar plates as originally reported by Siegmund and Wagner [30].

It is interesting to note that the step increase in absorbance did not appear at the higher molar ratio of 3.15:1 (Figure 5.3). In even higher rhamnolipid concentrations (i.e. 10, 25, and 50 g/L, corresponding to the molar ratios of 31:1, 77:1, and 153:1), the absorbance peak originally at 664 nm shifted to shorter wavelengths (and the solutions shifted toward green color as shown in Figure 5.4)) and the absorbance decreased with increasing rhamnolipid concentrations (Figure 5.2). Together, these observations suggested that the hydrophobic complexes became solubilized in the rhamnolipid micelles at high rhamnolipid concentrations. The decrease and shift of the absorbance peak in the systems with very high rhamnolipid concentrations might be the result of interference to methylene blue absorbance by the increasingly stronger light scattering of rhamnolipid micelles.
Figure 5.3. Complexation of rhamnolipids with MB and CTAB in aqueous systems. (a) Absorbance spectra of aqueous samples with 0.005 g/L MB, 0.2 g/L of CTAB, and different rhamnolipid concentrations (0.032, 0.16, 0.33, 0.49, 0.65, and 1 g/L) corresponding to the different molar ratios (0.1:1, 0.5:1, 1:1, 1.5:1, 2:1, and 3.15:1) of rhamnolipids-to-combined MB and CTAB, labeled as RL:(MB+CTAB). (b) Absorbance at 664 nm in aqueous solutions with different RL:(MB+CTAB).
Figure 5.4. Aqueous solutions with 0.005 g/L MB, 0.2 g/L of CTAB, and high rhamnolipid concentrations (0, 10, 25, and 50 g/L). As the rhamnolipid concentration increased, the solutions shifted toward green color.

These results led us to study the formation of the complex in aqueous-organic two-phase systems and to use the complex partitioned into the organic phase (chloroform) as the basis for analysis of rhamnolipids in liquid samples.

5.3.3. Complexation of rhamnolipids and methylene blue in aqueous-organic two-phase systems

It was previously demonstrated that in a water-organic phase system, anionic surfactants or methylene blue alone did not partition into chloroform [143] but when both were present, the complex formed would transfer into the organic phase [142]. It was hypothesized that the anionic rhamnolipids would show a similar behavior and the absorbance of the partitioned rhamnolipids-methylene blue complex in the organic phase would be useful for rhamnolipid analysis.
In the early phase of this study, both CTAB and methylene blue were used, as in the original formulation for agar plates. The high CTAB concentration used (0.549 mM) caused the formation of rather stable emulsions and prevented the easy separation of the two phases, making it difficult to obtain reliable measurements of the absorbance of the organic phase. Subsequently, CTAB was removed and different methylene blue concentrations, 0.013, 0.06, 0.12, and 0.18 mM, were evaluated. 0.12 mM was found to give the best results: it allowed higher rhamnolipid concentrations to be analyzed while not giving an absorbance that was so high as to extend into the optical nonlinear range, a problem that was encountered with the 0.18 mM methylene blue solution.

The absorbance spectra of the complex in the chloroform phase are shown in Figure 5.5. The maximum peak of methylene blue seen at 664 nm in the aqueous solutions was found to shift to 638 nm in the chloroform phase. In addition, the complex showed a new broad band around 525 nm. When establishing the calibration curves using rhamnolipid standards, it was found that the absorbance at 525 nm did not show good linear correlation with rhamnolipid concentration while the absorbance at 638 nm did. The latter absorbance was therefore chosen as the basis for measuring the rhamnolipids in different samples.

5.3.4. Interferences avoided or minimized by the modified procedure

Some fermentation medium components could interfere with the rhamnolipid analysis if the aqueous samples, after addition of methylene blue, were directly contacted with chloroform. Among the common medium compounds used for *P. aeruginosa* fermentation, nitrate caused the most significant interferences: higher absorbance values
were found in the nitrate-containing samples. When the medium containing nitrate was
diluted, the interfering absorbance decreased considerably. Table 5.1 summarizes the
absorbance values of the chloroform phase after being contacted with the nitrate-free
medium and the nitrate-containing media at different dilution factors. Another potential
source of interferences occurring with the original procedure was the non-rhamnolipid
organic materials produced by the cells. Some of these materials were observed to
partition into chloroform, particularly when facilitated by complexation with methylene
blue. By first extracting rhamnolipids from the aqueous sample into chloroform without
the addition of methylene blue, the modified procedure completely avoided the nitrate
interference and minimized the interferences from other organic materials.

Figure 5.5. Absorbance spectra of the chloroform phase after being contacted with
aqueous solutions of different rhamnolipid concentrations. The absorbance spectra of MB
in aqueous and organic (chloroform) phases after extraction are also included.
Table 5.1. Absorbance of chloroform phase contacted with nitrate-containing and nitrate-free media diluted at different dilution factors in the absence of rhamnolipids. The concentration of NaNO$_3$ in the fresh medium was 2 g/L.

<table>
<thead>
<tr>
<th>Dilution factor of fresh medium before extraction</th>
<th>Absorbance (650 nm) of samples with NO$_3^-$</th>
<th>Absorbance (650 nm) of samples without NO$_3^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.401</td>
<td>0.1</td>
</tr>
<tr>
<td>25</td>
<td>0.307</td>
<td>0.101</td>
</tr>
<tr>
<td>50</td>
<td>0.129</td>
<td>0.11</td>
</tr>
<tr>
<td>80</td>
<td>0.107</td>
<td>0.082</td>
</tr>
<tr>
<td>100</td>
<td>0.096</td>
<td>0.089</td>
</tr>
</tbody>
</table>

5.3.5. Calibration for rhamnolipid analysis in liquid samples

The previously reported method for analysis of anionic surfactants in water samples involved the direct contact of the water samples, after addition of methylene blue, with chloroform [142, 143]. It was found that this method could not be readily adopted for fermentation samples because of potential interferences, as described in the previous section. The procedure was therefore modified to extract the rhamnolipids from the aqueous sample into chloroform. An aqueous methylene blue solution was then added to the collected chloroform extract, to induce the complex formation in the chloroform phase.

The rhamnolipid standard was found to have a limited solubility (~ 10 mg/L) in chloroform. For complete rhamnolipid extraction, chloroform had to be used in a high volume ratio (5:1) to the aqueous sample. Four (4) ml of the chloroform phase were
carefully removed without taking any liquid from the top aqueous layer. This extract was brought into contact with the methylene blue solution prepared with distilled water and borax buffer, in order to ensure a pH of about 8.5. The pH of this solution must not be higher than 9.5 in order to prevent oxidation of methylene blue to dimethylthionoline, a phenomenon which would result in the formation of pink colors in the chloroform phase [144]. The finalized procedure for analysis of rhamnolipids in fermentation samples using the methylene blue analysis is summarized in Figure 5.6. As shown in Figure 5.7, the calibration curve developed using this procedure resulted in a linear relation in the range from 5 to 250 mg/L of rhamnolipids in the original aqueous sample. At higher rhamnolipid concentrations, stable emulsions were formed, causing difficult phase separation and interfering with absorbance measurements.

Figure 5.6. Methylene blue analysis procedure.
5.3.6. Methylene blue rhamnolipid analysis of fermentation samples

The developed method was further tested with samples taken from *P. aeruginosa* fermentations carried out in 2-L fermentors using glycerol as the carbon source. For comparison, the profiles of rhamnolipid concentrations measured, respectively, with the common anthrone colorimetric method and the methylene blue method developed in this study are shown in Figure 5.8 for a typical fermentation batch. Clearly, the profiles obtained with the two analysis methods were consistent. Applicability of the methylene blue method to real fermentation samples was demonstrated.
Figure 5.8. Rhamnolipid production profiles determined by the anthrone method and the methylene blue-based method, respectively, for a *P. aeruginosa* batch fermentation experiment.

It is important to mention that there is a fundamental difference in the analysis principles of the two methods. The methylene blue-based analysis measures the molar rhamnolipid concentration. The analysis is presumably insensitive to the rhamnolipid structures because all known major rhamnolipids have single carboxyl end group [36] which, upon deprotonation, forms the basis of molar complexation with methylene blue. On the other hand, anthrone analysis measures the concentration of rhamnose in the rhamnolipids. Conversion of the measured rhamnose concentration to rhamnolipid concentration requires the knowledge of actual mixture composition and structures of the rhamnolipids involved. In Figure 5.8 this conversion was done by assuming a 2.3:1 rhamnolipid-to-rhamnose mass ratio, similar to the ratio for the rhamnolipid standard.
(estimated according to the structures and relative peak areas of total ion intensity determined by the HPLC-MS analysis, as described earlier).

Additionally, the anthrone analysis is significantly more complicated, lengthy, and hazardous to perform than the developed method. No drying of extract or redissolution of rhamnolipids in NaHCO₃ is required in the methylene blue-based analysis. In addition, the anthrone analysis requires careful preparation of the anthrone reagent in concentrated sulfuric acid. The addition of anthrone reagent to the NaHCO₃ solution (containing redissolved lipids) needs to be slow (drop-wise) and cautious while the sample tube is gently shaken in an ice bath to remove the rapidly generated reaction heat. The anthrone added sample then needs to be heated at 95°C for an additional 20 minutes to complete the reaction part of the anthrone method.

5.4. Conclusions

The methylene blue method developed in this work is a rapid and simple procedure that allows the quantification of rhamnolipids in liquid cultures throughout the cultivation period to study the changes in the production with time. Additionally, this method is easier than the other colorimetric methods. It is carried out at room temperature and does not involve lengthy drying, redissolution and reaction. More importantly, it avoids the use of hazardous concentrated sulfuric acid in the procedure. Compared with the original agar plate method, this procedure allows easier and more accurate quantification of rhamnolipids in liquid samples. These characteristics are very helpful for the development of an improved fermentation process for industrial rhamnolipid production.
CHAPTER VI

ADVANCED MICROAEROBIC DENITRIFYING TECHNOLOGY FOR RHAMNOLIPID PRODUCTION USING GLYCEROL AS CARBON SOURCE

6.1. Introduction

The productivity of processes based on aerobic fermentation strategies is often limited by the oxygen availability in the bioreactor. Vigorous agitation and high aeration rates are commonly provided in microbial fermentations to supply the oxygen necessary for adequate culture performance. As a result, foaming is usually present in aerobic fermentations. To control the foaming, mechanical foam breakers and chemical antifoaming agents are used. However, the highly foaming nature of the broth in rhamnolipid fermentations makes it very difficult to maintain the required oxygen concentration in the bioreactor. The foaming in aerobic rhamnolipid fermentation appears extremely fast and is too stable to be handled by common foaming control methods [28]. Moreover, chemical antifoams can have adverse effects on the downstream recovery and purification processes besides the considerable extra costs associated with the large amounts of antifoam required to match the rhamnolipids produced along the fermentation process. Even though different strategies have been attempted to break the foams [28, 99], the foaming was still difficult to control and the aeration rate had to be significantly
lowered. This caused oxygen limitation compromising cell viability as well as rhamnolipid productivity.

An alternative fermentation technology based on the ability of *P. aeruginosa* to perform anaerobic respiration based on nitrate (denitrification) was previously studied by Chayabutra et al. [13]. In that study, *P. aeruginosa* ATCC 10145 was able to produce rhamnolipids under denitrifying conditions, using a phosphorus limited medium with palmitic acid as the carbon source [13]. While the rhamnolipid concentration obtained (0.4 g/L as rhamnose) under the denitrifying conditions was lower than it was usually reported by aerobic conditions, this approach was found to completely eliminate the problems associated with the severe foaming nature of the rhamnolipid broth.

In this chapter, the denitrifying fermentation technology was further investigated using different *P. aeruginosa* strains with the main goal of improving the cell and rhamnolipid concentrations and therefore the productivity of the process. The NAD(P)H fluorescence monitoring was incorporated to the process and allowed for a more accurate nitrate supplementation according to the needs of the culture. Successful cell growth and rhamnolipid production in liquid culture and under denitrifying conditions was obtained using glycerol as substrate. However, the cells were found to stop growing even when there was no limitation from the medium components. The possible reasons for this behavior were further investigated.

Glycerol is a water-soluble substrate that has been previously used for rhamnolipid production. The rhamnolipid concentrations and yield that had been attained with this carbon source varied depending upon the strain, nitrogen source, and fermentation approach [27, 89, 91] (Table 2.2). As described in section 2.8, the price of
glycerol has decreased considerably because it is being generated in large quantities as a major byproduct of the biodiesel industry. Currently there are not enough uses of glycerol which created an urgent need to find innovative ways for its economic conversion to more value-added products and prevent it from becoming an environmental problem. Hence, this part of the study also evaluated the feasibility of using glycerol as energy and carbon source for the production of high-value rhamnolipid biosurfactants via the denitrifying fermentation strategy. Even though glycerol was found to support the growth of the cells as well as the rhamnolipid production, it was also found to promote the production of other compounds under anaerobic denitrifying conditions.

6.2. Materials and methods

The following subsections provide a description of the materials and methods employed in the investigation and application of the denitrifying fermentation technique for rhamnolipid production using glycerol in free cell systems.

6.2.1. Biodiesel glycerol and pure glycerol as carbon source

The strain used in this part of the study was P. aeruginosa PAO1. The media comparing the biodiesel glycerol (88 % pure, obtained from a biodiesel production plant) with pure glycerol and glucose contained 6 g/L NH₄Cl, 9.3 g/L NaNO₃, 6 g/L KH₂PO₄, 0.9 g/L MgSO₄•7H₂O, 0.03 g/L CaCl₂•2H₂O, 0.004 g/L FeSO₄•7H₂O, 1.5 g/L NaCl, 0.03 g/L MnCl₂•4H₂O. The initial volume in each system was 50 mL and the initial carbon source concentration was 10 g/L. The pH was adjusted to 7.3 ± 0.1 prior sterilization of the media and the temperature was maintained at 34°C during the
experiment. Each system was inoculated with 2% of the inoculum (prepared in TSB as explained in Chapter III) and then placed in the rotatory shaker incubator at 280 rpm. Samples were taken periodically to follow the cell concentration in the systems.

6.2.2. Osmotic stress effect on rhamnolipid production

The methylene blue/CTAB agar plate method was used to test the effect of high osmotic pressures on the growth and rhamnolipid production of *P. aeruginosa* E03-40. The medium used for the assays under high salt concentration and containing an osmoprotectant (betaine) was similar to the one described in section 3.1: 20 g/L of glycerol, 0.7 g/L KH$_2$PO$_4$, 0.9 g/L Na$_2$PO$_4$, 2 g/L NaNO$_3$, 0.4 g/L Mg$_2$SO$_4$•H$_2$O, 0.1 g/L CaCl$_2$•2H$_2$O, and 2 mL of a trace element solution containing 2 g/L FeSO$_4$•7H$_2$O, 1.5 g/L MnSO$_4$•H$_2$O and 0.6 g/L (NH$_4$)$_6$Mo$_7$O$_2$$_4$•4H$_2$O. The agar plates were prepared by adding 0.2 g of CTAB and 0.005 g of methylene blue to 1 L of the medium described above. The plates were also added with 0.5 M of NaCl to ensure conditions of high osmolality. Additionally, the plates prepared to test the effect of the osmoprotectant betaine on the rhamnolipid production of *P. aeruginosa* E03-40 were added with 0.5 mM betaine (from a 10 g/L solution that was previously filter sterilized).

6.2.3. Batch denitrifying fermentations for rhamnolipid production

*P. aeruginosa* E03-40 and *P. aeruginosa* PAO1 were selected to conduct the batch fermentation experiments in the 2 and 3-L glass fermentors. The medium used for batch fermentations typically contained glycerol as carbon source, 3 g/L NaNO$_3$, 2 g/L KH$_2$PO$_4$, 0.3 g/L Mg$_2$SO$_4$•7H$_2$O, 0.03 g/L CaCl$_2$•2H$_2$O, 0.1 g/L FeSO$_4$•7H$_2$O, 1.5 g/L
NaCl, 0.03 g/L MnCl₂•4H₂O and 2 mL/L of a trace element solution containing 0.08 g/L FeCl₃•6H₂O, 0.75 g/L ZnSO₄•7H₂O, 0.08 g/L CoCl₂•6H₂O, 0.075 g/L CuSO₄•5H₂O, 0.75 g/L MnSO₄•H₂O, 0.15 g/L H₃BO₃, and 0.05 g/L Na₂MoO₄. The amounts of NH₄Cl and NaNO₃ varied according to the experiment. NH₄Cl was usually added in excess to ensure that sodium nitrate was only consumed for denitrification and not as nitrogen source for growth. Additionally, a sterile 10 g/L betaine solution was used as an osmoprotectant to provide a final concentration of 0.5 mM in the fermentors.

The inoculum was typically 5% of the total volume of the culture and the pH of the medium was adjusted before sterilization to 7.1 ± 0.1. A diagram of the experimental setup typically used for the batch fermentations in glass fermentors was presented in Figure 3.1 and the details of the operation of the fermentors were given in section 3.2.2.

6.2.4. Effect of rich media and iron on the growth of *P. aeruginosa* E03-40

These experiments were carried out in shaker flasks as described in section 3.2. A layer of cheesecloth-wrapped cotton was used to cover the flasks while allowing gas transfer. Gas exchange between the liquid broth and headspace took place through the medium surface. The medium used to test the effect of rich media contained 10 g/L glycerol, 6 g/L NH₄Cl, 6 g/L NaNO₃, 2 g/L KH₂PO₄, 0.3 g/L Mg₂SO₄•7H₂O, 0.03 g/L CaCl₂•2H₂O, 0.1 g/L FeSO₄•7H₂O, 1.5 g/L NaCl, 0.03 g/L MnCl₂•4H₂O and 2 mL/L of the trace element solution described above. The first system was used as a control and only contained glycerol and the mineral salts medium, the second system was supplemented with 2.5 g/L of yeast extract, and the third system was added with 2.5 g/L of yeast extract and 2.5 g/L of peptone. The inoculum was 5% of the total volume of the
culture and the pH of the medium was adjusted before autoclaving to 7.1 ± 0.1. After inoculation the systems were put in a rotatory shaker incubator at 34 °C and 280 rpm.

Similarly, the effect of different iron concentrations on *P. aeruginosa* E03-40 was studied using shaker flasks containing 25 g/L glycerol, 6 g/L NH₄Cl, 3 g/L NaNO₃, 2 g/L KH₂PO₄, 0.3 g/L Mg₂SO₄•7H₂O, 0.01 g/L CaCl₂•2H₂O, 0.5 g/L NaCl, 0.01 g/L MnCl₂•4H₂O and 2 mL/L of the trace element solution described above in section 6.2.3. Four different systems were included in this experiment having different iron concentrations as FeSO₄•7H₂O: 0.001, 0.01, 0.04, 0.1 g/L, respectively. The pH of the medium was adjusted before autoclaving to 7.1 ± 0.1. The inoculum was 5% of the total volume of the culture. The experiment was carried out in a rotatory shaker incubator at 34 °C and 280 rpm.

6.2.5. Inhibitory compound(s) produced during denitrifying fermentation conditions

250-mL shaker flasks containing a working volume of 80 mL were used for this part of the study. A layer of cheesecloth-wrapped cotton was used to cover the flasks while allowing gas transfer. For the first system, the broth (80 mL) from a *P. aeruginosa* E03-40 denitrifying fermentation carried out in a fermentor was collected and centrifuged under sterile conditions. The spent medium (supernatant) was separated from the old cells, and was later inoculated with 4 mL of fresh cells prepared by the normal inoculum preparation procedures. The glycerol and ammonia concentrations in the spent medium were measured and adjusted accordingly so that it had the same starting concentration as the fresh medium systems. For the second system, the old cells separated from the spent medium were resuspended in fresh medium and used as inoculum in a fresh medium.
A third system containing fresh medium and inoculated with the same volume of fresh cells was used as a control. The fresh medium contained 30 g/L glycerol, 6 g/L NH₄Cl, 6 g/L NaNO₃, 2 g/L KH₂PO₄, 0.3 g/L Mg₂SO₄•7H₂O, 0.03 g/L CaCl₂•2H₂O, 0.1 g/L FeSO₄•7H₂O, 1 g/L NaCl, 0.03 g/L MnCl₂•4H₂O, 1 g/L yeast extract, 1 g/L peptone, and 2 mL/L of the trace element solution described above in section 6.2.3. These three systems were then grown in a rotatory shaker incubator at 34 ºC and 280 rpm for 50 hours.

6.2.6. Denitrifying fermentation of wild type PAO1 and its ΔrhlI, ΔlasI, and ΔlasIΔrhlI mutants

*P. aeruginosa* PAO1 and three mutant strains, namely ΔrhlI, ΔlasI, and a ΔlasIΔrhlI double mutant (provided by Dr. Sang-Jin Suh from the Department of Biological Sciences at Auburn University) were used in this experiment. The fermentations were carried out in four 500-mL glass fermentors (with 300 mL working volume) provided with automatic pH control, surface aeration, and glycerol and sodium nitrate addition with pumps connected to timer controls. The initial medium contained 25 g/L glycerol, 6 g/L NH₄Cl, 6 g/L NaNO₃, 2 g/L KH₂PO₄, 0.3 g/L Mg₂SO₄•7H₂O, 0.01 g/L CaCl₂•2H₂O, 0.05 g/L FeSO₄•7H₂O, 0.5 g/L NaCl, 0.03 g/L MnCl₂•4H₂O, 2 g/L yeast extract, 2 g/L peptone, and 2 mL/L of the trace element solution described in section 6.2.3. The pH of the medium was adjusted before autoclaving to 7.1 ± 0.1 and was maintained at that pH with automatic addition of 2N HNO₃. Additionally, a sterile 10 g/L betaine solution was used as an osmoprotectant to provide a final concentration of 0.5 mM in the fermentors. Each system was inoculated with 15 mL of the respective strain.
which was grown overnight in TSB at 34 °C and 280 rpm. The fermentors were agitated by stirring bars using a magnetic multi-stirrer (Bellstir Multistir 4, Bellco Glass Inc.) for direct comparison of their growth under denitrifying conditions.

6.2.7. Fermentation analytical methods

Samples taken from the fermentor (or shaker culture flasks when applicable) were periodically taken and first centrifuged to separate the cells from the medium (supernatant). The pelletized cells were kept to measure the cell concentration and the supernatant was used to measure the concentrations of carbon source, NO₃⁻-N (and NO₂⁻-N), rhamnolipid, extracellular protein, alginate, and carbohydrates. The procedure for each one of these analyses was described in section 3.3.

6.2.8. Thermal gravimetric analysis (TGA)

After the cells were separated by centrifugation, 3 mL of the denitrifying fermentation supernatant samples were completely air dried. TGA was carried out on the dried samples using a TGA Q500 Thermogravimetric Analyzer (TA Instruments) under nitrogen flux (balance gas 40 mL/min and sample gas 60 mL/min), over a temperature range from 25°C to 600°C. The material of the pan was platinum and two different scan rates were used: (1) 10 °C/min until 600 °C and (2) a variable scan rate of 10 °C/min to 120 °C, 1 °C/min to 550 °C and 10 °C/min to 600 °C.
6.2.9. Size exclusion chromatography analysis (SEC)

The broth obtained from denitrifying fermentations was first centrifuged to separate the cells and then the supernatant was recovered and diluted with HPLC water. The samples and standards were analyzed using a HPLC (HP 1100 series) equipped with a diode array detector (DAD) (HP 1100 series) set at a wavelength of 230 ± 50 nm and a refractive index (RI) detector (model HP 1047A). The mobile phase used to estimate the molecular weight of the compounds in the supernatant was HPLC water and the column was a PL aquagel-OH MIXED 8 μm (300 mm × 7.5 mm, Polymer Laboratories). The flow rate and injection volume were 0.7 mL/min and 10 μL, respectively. Polyethylene glycol (PEG) of different molecular weights was used as the standard to generate the calibration curve.

6.3. Results and discussion

In the next subsections the results of the investigation and application of the denitrifying fermentation technique for rhamnolipid production using glycerol in free cell systems are presented and discussed accordingly.

6.3.1. Selection of *P. aeruginosa* strains using glycerol as carbon source

The improved method proposed in Chapter IV for the screening of high rhamnolipid producer strains was used to evaluate the rhamnolipid producing ability of 10 *P. aeruginosa* strains. The procedure used for this evaluation was described in section 4.3.5. Four of the tested strains, *P. aeruginosa* PAO1, E03-40, E03-36, and H05-25, were selected as the best rhamnolipid producers for the experiments carried out in this chapter.
This selection was made taking into account not only that the areas surrounding the wells were large (which represented high rhamnolipid production) but also that these strains did not excrete a considerable amount of pigments. This last consideration was also important because the amount of pigments secreted by different *P. aeruginosa* strains has been found to vary depending on the substrate [116]. Keeping the production of these virulence factors to a minimum under glycerol conditions could be beneficial to the rhamnolipid productivity and further purification.

6.3.2. Biodiesel glycerol and pure glycerol as carbon source

Crude glycerol (88% pure) obtained from a biodiesel production plant was evaluated as the carbon source using *P. aeruginosa* PAO1. For comparison, pure glycerol (99%) as well as glucose was also included as sole carbon sources to determine the degree of toxicity or inhibition of biodiesel glycerol. As shown in Figure 6.1, the systems containing glucose and crude glycerol had a very similar growth profile reaching a maximum cell concentration of approximately 2 g/L. Biodiesel glycerol was clearly shown to support the growth of *P. aeruginosa* PAO1. The system containing pure glycerol showed a slower growth rate in the initial hours of the experiment and did not reach stationary phase until later in the run. However, the maximum cell concentration attained in this system resulted in a slightly higher cell concentration of 2.9 g/L. This result suggested that glycerol was a better carbon source than glucose for growth of PAO1 under the conditions of the experiment. The reason for the differences in cell concentration when pure glycerol was compared to biodiesel glycerol is unknown. It is difficult to conclude that biodiesel glycerol had a negative effect on the cells because the
growth was still comparable to the growth using glucose. Furthermore, shaker flask studies had the disadvantage of not having an automatic pH control. The changes in pH could also have affected the performance of the culture. Nevertheless, all three systems grew similarly suggesting that biodiesel glycerol did not have any toxic or inhibitory effects on the growth of *P. aeruginosa* PAO1.

![Graph](image)

**Figure 6.1.** Growth profiles of *P. aeruginosa* PAO1 using 10 g/L of crude glycerol, glucose, and pure glycerol as sole carbon sources.

Additionally, the ability of all four high rhamnolipid producer strains (selected in section 6.4.1) to withstand and grow with considerably higher glycerol concentrations was evaluated. All four strains, PAO1, E03-40, E03-36, and H05-25, grew well under these conditions with no significant differences among them.
Figure 6.2. Growth profiles of the four high rhamnolipid producer *P. aeruginosa* strains under high pure glycerol concentration of 100 g/L.

The growth of PAO1 under high glycerol concentrations appeared to be slower during the first 24 hours when compared to its growth under lower glycerol concentrations of approximately 10 g/L (Figure 6.1). However, the growth continued at a similar rate for the remaining of the experiment and the cells grew to similar cell concentrations. It is also important to note, that the cell concentration attained in the high glycerol concentration systems was not as high as expected considering that the glycerol concentration was ten times higher than in the experiment comparing pure and crude biodiesel glycerol with *P. aeruginosa* PAO1 (see Figure 6.1 above). The medium in both experiments was provided with excess of all components which would assure no limitation (except for that of carbon source in the experiment comparing biodiesel glycerol to other carbon sources). In the case of the comparison of pure and crude
glycerol (and glucose) as sole substrates, the limitation was in fact the carbon source. On the other hand, in the experiment with high glycerol concentration the limitation could either be the result of a drastic change in the pH or from the insufficiency of electron acceptors for respiration. Even though there was plenty of potassium phosphate in the medium serving as a buffer, the pH could have increased to higher values due to the consumption of nitrate. Additionally, under the conditions of the experiment without submerged aeration, the dissolved oxygen was probably extremely low after the first hours of the experiment. In addition, only 500 mg/L NO₃⁻-N were initially added to the shaker flasks, which was probably not enough to sustain the growth of the cells via denitrification.

Among all four high rhamnolipid producer strains, *P. aeruginosa* E03-40 and *P. aeruginosa* PAO1 were selected to conduct the batch denitrifying experiments since they produced the largest amount of rhamnolipids on the methylene/blue CTAB agar plates (Table 4.3).

6.3.3. Denitrifying fermentation of *P. aeruginosa* E03-40

*P. aeruginosa* E03-40 batch denitrifying fermentation studies were conducted using glycerol as substrate. The effect of different factors on the cell growth and rhamnolipid concentration was also evaluated in order to improve the process for better rhamnolipid production. These results are presented and discussed in the next subsections.
6.3.3.1. Rhamnolipid production under denitrifying condition using glycerol as substrate

One of the main purposes of using the denitrifying fermentation technology in this study was to avoid the problems caused by the highly foaming nature of rhamnolipid-producing fermentation. Accordingly, the 2-L fermentor, with 800 ml of culture broth, was only magnetically stirred and surface-aerated (i.e., without submerged bubbling). Under such conditions, the DO results clearly showed that the fermentor turned into dissolved oxygen-limiting state only a few hours after the normal 5% inoculation as demonstrated in Figure 6.3. NO$_3^-$-N, provided only by nitric acid, was then consumed by the cells as the electron acceptor for respiration.

![Figure 6.3. Oxygen concentration in the initial hours of denitrifying fermentation.](image)

Figure 6.3. Oxygen concentration in the initial hours of denitrifying fermentation.
The profiles of cell growth, glycerol, ammonia, and nitrate concentrations, as well as rhamnolipid production during this denitrifying fermentation run are shown in Figure 6.4. The cells reached stationary phase, presumably due to nitrogen limitation, at around 73 h of the experiment with a maximum cell concentration of 2 g/L. This concentration was similar to the cell concentration that can be supported under most aerobic rhamnolipid fermentations [85, 91, 145, 146].

Glycerol was consumed almost completely during the first 48 hours of growth.

Considering that a cell yield from carbohydrate under anaerobic conditions could be
around 30% [147], only 6 g/L of glycerol would be necessary to grow the approximately 2 g/L maximum cell concentration. However, the cells consumed large amounts of glycerol even during the initial hours of growth when they were not producing rhamnolipids. After 48 hours, glycerol was added into the fermentor using a pump connected to a timer control to provide enough glycerol to avoid carbon limitation. The glycerol needs of the culture were not as high as the rate provided causing glycerol accumulation as shown in Figure 6.4. The glycerol addition per day was also shown in this figure and was illustrated by vertical arrows. The consumption was shown as the discontinuous lines.

The foaming in the process was completely avoided allowing for a more continuous production of rhamnolipids. Rhamnolipid production started after NH$_4^+$-N was consumed and increased linearly during the rest of the experiment. Rhamnolipid accumulation coincided with the end of the exponential growth phase which agrees with the fact that rhamnolipids are secondary metabolites and their production is at least partially regulated by the RpoS sigma factor [74]. The rhamnolipid concentration obtained at 312 h was 3.8 g/L. This rhamnolipid concentration was higher than the concentration reported by Rahman et al. [27] of 1.77 g/L under aerobic conditions using glycerol as carbon source as one of the potential low cost raw materials for rhamnolipid production. Additionally, it was higher than that reported by Anna et al. [89] and Arino et al. [91] also using glycerol as carbon source. Moreover, the rhamnolipid production obtained with this alternative denitrifying fermentation set-up was considerably higher than that obtained by Chayabutra et al. [13] under denitrifying conditions. In that report, using palmitic acid as carbon source, approximately 0.9 g/L rhamnolipids (reported as
0.35 g/L rhamnose) were produced at a much later time on the experiment (i.e. around 450 h).

The obtained product yield \( Y_{PS} \) with glycerol was 8.7%, which was higher than that previously reported for rhamnolipid production under denitrifying conditions [13], and even higher than some reports under aerobic conditions using different carbon sources (summarized in Table 2.2). However, it was still lower than some estimated yields obtained using different vegetable oils under aerobic conditions (also shown in Table 2.2). Even though the glycerol was found to be accumulating by the end of the run, the low yield was mostly due to the high glycerol consumption rate and low cell concentrations obtained during the fermentation. The possible causes of these results were described in subsection 6.3.3.3 and investigated later in this chapter.

The cell concentration in the fermentor decreased to approximately 1.1 g/L at around 140 hours. Since there was plenty of glycerol in the fermentor, this behavior probably showed that the nitrate addition was not enough for the respiration needs of the culture. Nitrate was supplied by nitric acid addition every 3 hours starting after 48 hours, and at 191 hours the addition was increased by adding the acid every 2 hours. The nitrate analysis showed that nitrogen was depleted in the fermentor during the entirety of the run even with faster nitric acid addition. The addition was not increased further because the pH decreased due to the strong acid involved and did not recover completely by the denitrification mechanism which caused the addition of large amounts of base to maintain the pH within an acceptable range.
6.3.3.2 Osmotic stress effect on rhamnolipid production

An osmoprotectant, betaine (0.5 mM), was included in all the glycerol
denitrifying fermentations because high glycerol concentrations and large amounts of
base (NaOH) added to the fermentor (such as in the experiment described above) could
result in a high osmotic pressure environment. Hyperosmotic conditions had been
reported to negatively affect not only the growth but the rhamnolipid production of *P.
aeruginosa* PAO1 [148, 149]. By adding an osmoprotectant such as glycine betaine to the
medium, this strain was shown to adapt to high osmolality conditions and was able to
produce rhamnolipids under circumstances in which otherwise the rhamnolipid synthesis
was repressed [148, 150].

Using the improved method based on methylene blue/CTAB agar plates described
in Chapter IV, the rhamnolipid production of *P. aeruginosa* E03-40 was found to be
affected by high salt concentrations. Figure 6.5 shows the rhamnolipid production under
conditions of low salt concentration, high salt concentration, and high salt concentration
with betaine as osmoprotectant.

![Figure 6.5](image1)

Figure 6.5. Rhamnolipid production of *P. aeruginosa* E03-40 on methylene blue/CTAB
agar plates with (a) low salt concentration, (b) high salt concentration – 0.5 M NaCl, and
(c) high salt concentration – 0.5 M NaCl and osmoprotectant betaine (0.5mM).
Rhamnolipid production was found to be repressed when high salt concentrations were used and betaine was found to help relieve the osmotic stress of the cells by partially restoring the rhamnolipid production. Under high salt concentrations of 0.5 M, the betaine-added (0.5 mM) plates showed better growth and rhamnolipid production.

6.3.3.3.Effect of ammonia as nitrogen source under denitrifying fermentation conditions

Denitrifying fermentation experiments were also conducted without NH₄⁺-N using only nitrate as nitrogen source for growth and respiration. In these cases, 500 mg/L NO₃⁻-N were included in the initial medium and during the fermentation experiment both nitric acid (triggered by pH control) and sodium nitrate (100 mg/L NO₃⁻-N per day added using a timer control every 4 hours) were added. However, as shown in Figure 6.6a, the cell concentration attained was very low. At 167.5 h, the maximum cell concentration was only 1.2 g/L even though 4 g/L per day of glycerol was added to the fermentor to ensure the cells had plenty of carbon source (Figure 6.6b). After glycerol started to accumulate, the addition was decreased to 2 g/L per day. When the stationary growth phase was reached (after 100 hours of the experiment) the glycerol consumption decreased from approximately 4.3 g/L to 1.2 g/L per day. Interestingly, after 200 hours the glycerol was consumed faster. The consumption increased from 1.2 at 192 hours to 9.7 g/L per day at 336 hours of the experiment, when there were no apparent increases in the cell and rhamnolipid concentrations (Figure 6.6b). The finding suggested that glycerol was used for other purposes besides growth and rhamnolipid production.

The nitrate analysis showed that the cells were consuming the entirety of the nitrate that was supplied (Figure 6.6b) probably causing poor conditions in the
fermentation since the nitrogen from nitrate was used not only for respiration but also for growth. Additionally, these conditions were not optimal for rhamnolipid production, and in contrast with the systems initially containing NH$_4^+$-N (Figure 6.4 and 6.7), the rhamnolipid concentration in this experiment was very low (Figure 6.6a).

In Figure 6.7 the profiles of a fermentation containing 600 mg/L NH$_4^+$-N in the initial medium are shown (note that this concentration was two times higher than the concentration used in the fermentation described in Figure 6.4 in which 2 g/L of cells was obtained). Under denitrifying conditions, the initial addition of NH$_4^+$-N was proven to stimulate the growth of *P. aeruginosa* E03-40, reaching higher cell concentration in a considerably shorter amount of time (when compared to those in which NH$_4^+$-N was not included in the medium).

However, the cell concentration was not as high as it was expected from doubling the NH$_4^+$-N concentration in the initial medium. In an attempt to obtain even higher cell concentrations for better rhamnolipid productivity, ammonium chloride was added to the fermentation after it had been completely consumed. From a sterile 100 g/L solution of ammonium chloride, 500 mg/L NH$_4^+$-N were provided to the fermentor but the cell concentration did not increase further (Figure 6.7). Even more, the NH$_4^+$-N was consumed and completely depleted after 120 hours without any change on the cell concentration. Additionally, the rhamnolipid production was not affected by the addition and continued increasing until the end of the run.
Figure 6.6. *P. aeruginosa* E03-40 denitrifying fermentation without NH$_4^+$-N and only NO$_3^-$-N as nitrogen source for growth and respiration. (a) Cell and rhamnolipid concentration; and (b) NO$_3^-$-N concentration and glycerol consumption and concentration profiles.
Figure 6.7. *P. aeruginosa* E03-40 denitrifying fermentation with 600 mg/L NH$_4^+$-N (×) as nitrogen source for growth in the initial medium and supplemented with additional 500 mg/L NH$_4^+$-N in the middle of the run. Cell (◇), rhamnolipid (△), and NO$_3^-$-N (□) concentration profiles are also shown in the figure.

The osmoprotectant betaine was included in these experiments and other components of the medium were initially provided in excess to avoid other limitation. Also, the cells stopped growing before the initial ammonia was depleted which confirmed that the limitation was not associated with nitrogen, or to other component limitation, or to high osmotic pressure. Then, other possible reasons for the difficulty to obtain even higher cell concentrations under denitrifying conditions were investigated and could be described as follows: (1) the mineral salts medium is not enough to support the growth of *P. aeruginosa* E03-40 and possibly some co-factors are needed, (2) the nitrate provided during the fermentations was not enough to support the respiration needs of a culture with
higher concentration, (3) there was an inhibitory compound produced during the
denitrifying fermentation conditions, or (4) there was a control of density population by
the quorum-sensing systems (section 2.4). These reasons were investigated and described
in the next sections by (1) studying the effect of rich media on the growth, (2) following
the NAD(P)H fluorescence response of the culture, (3) studying the inhibitory effect of
the spent medium from a common denitrifying fermentation, and (4) comparing a wild
type strain to its ΔrhlI, ΔlasI, and ΔlasIΔrhlI mutants under denitrifying conditions. By
identifying and understanding the reasons related to the inhibition behavior, higher cell
concentrations can possibly be attained in order to increase the rhamnolipid volumetric
productivity of this process.

6.3.4. Effect of rich media and iron on the growth of P. aeruginosa E03-40

Shaker flask studies were carried out to test the effect of yeast extract and peptone
supplementation and the effect of different iron concentrations on the growth of P.
aeruginosa E03-40. In the first study, three different systems with and without yeast
extract and peptone containing the original mineral salts medium and glycerol as carbon
source were tested: (1) a control with only glycerol as carbon source, (2) a system
supplemented with peptone, and (3) a system supplemented with both yeast extract and
peptone. The cell concentration attained after 40 hours of the experiment was not
significantly different among all three systems (Table 6.1). Rich media did not seem to
have an effect on the growth of P. aeruginosa E03-40 under these conditions. However,
these systems contained nitrate as well as yeast extract and peptone which caused the pH
to increase due to denitrification and to the consumption of these components. The final
pH of the systems is included in Table 6.1 and was found to be 8 or higher. The cell growth and concentration could have also been affected by the high pH conditions.

Table 6.1. Cell concentration attained after 40 hours of growth in shaker flask systems containing mineral salts medium with and without yeast extract and peptone.

<table>
<thead>
<tr>
<th>Mineral salts medium supplements</th>
<th>Cell concentration after 40 hours (g/L)</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>2.11</td>
<td>8.5</td>
</tr>
<tr>
<td>Glycerol + yeast extract + peptone</td>
<td>2.01</td>
<td>8</td>
</tr>
<tr>
<td>Glycerol + yeast extract</td>
<td>2.06</td>
<td>8</td>
</tr>
</tbody>
</table>

Denitrifying fermentation experiments with automatic pH control and with yeast extract and peptone in the initial media showed that the addition of these components resulted in a faster cell growth. Nevertheless, the final concentration was not much higher than the concentration obtained when a more frequent nitrate addition was incorporated in the fermentation as described below in section 6.2.5. To illustrate better this comparison, Figure 6.8 shows a comparison of the cell growth obtained in separate denitrifying fermentations controlling the pH around 7.1 ± 0.1, with and without yeast extract and peptone, respectively.
Figure 6.8. Cell growth profile of *P. aeruginosa* E03-40 under denitrifying fermentation conditions with and without yeast extract and peptone. The experiments were carried out in fermentors with automatic pH control set to 7.1 ± 0.1.

In another shaker flask study, the iron concentration effect on the cell growth and the maximum cell concentration attainable was investigated with *P. aeruginosa* E03-40. Iron has not only been reported as a poor growth-limiting factor [13], but also, at different concentrations, as an inhibitory compound affecting the cell growth and rhamnolipid production of several *P. aeruginosa* strains [151]. The cell growth of *P. aeruginosa* E03-40 was followed in systems that had different iron concentrations (as FeSO₄·7H₂O): 0.001, 0.01, 0.04, and 0.1 g/L. Figure 6.9 shows the cell concentration and glycerol consumption profiles obtained with the different iron concentrations in the media. The increasing iron concentration did not have a negative effect on the growth of this strain confirming that iron was not the cause of the inhibition behavior.
6.3.5. Denitrifying fermentation monitored by online NAD(P)H fluorescence

NAD(P)H fluorescence monitoring was used to follow the change of cellular electron-accepting state during denitrification of *P. aeruginosa* E03-40. The results of these studies and their implications on the rhamnolipid production are described below.

6.3.5.1. Denitrifying fermentation with frequent nitrate addition as nitric acid

A denitrifying fermentation was carried out using 6 g/L of NH₄Cl and 1000 mg/L of NO₃⁻-N in the initial medium. Throughout the experiment and as shown in Figure 6.10, ammonia was not exhausted. Its concentration increased during the first hours because of the consumption of yeast extract and peptone (1 g/L each) that were added in the initial medium and that would cause production of ammonia.

Nitrate was subsequently provided to the culture as nitric acid using different time intervals to follow the NAD(P)H fluorescence response. Initially, nitric acid was added every hour to ensure the culture would have enough nitrate to respire during exponential growth (Figure 6.10). Doses of 40 mg-N/L of nitrate were added using a timer controlled pump. Even though the cells were doing denitrification causing the pH to increase when the nitrate was consumed, the percentage recovery of the pH was not 100%. During that time, base was added once a day to increase the pH back to 6.8 ± 0.1. This method caused large pH swings that could have affected the cells.
Figure 6.9. *P. aeruginosa* E03-40 grown under different iron concentration (0.001, 0.01, 0.04, and 0.1 g/L): (a) Cell growth and (b) glycerol consumption profiles.
When the cells stopped growing and the nitrate started accumulating (at around 51 h of the experiment), the nitrate addition was changed to every 2 hours to stabilize the system and directed it to nitrate depletion conditions to follow the fluorescence changes.

Once nitrate was depleted, the time intervals of nitrate addition were modified according to the information obtained by the fluorescence profile in terms of how fast the nitrate was being consumed after each addition. Figure 6.10 indicates the time intervals when the nitric acid addition was decreased from 2 h to 1 h, then to 40, 30, and 20 minutes and its effect on the cell growth. Cell concentration increments were clearly the result of a more frequent nitrate supplementation which was guided by the NAD(P)H fluorescence signal response.

Figure 6.10. Nitrate addition intervals (illustrated by arrows) used along the denitrifying fermentation experiment guided by the NAD(P)H fluorescence signal. Cell (○), NH₄⁺-N (△), and NO₃⁻-N (□) concentration profiles are also shown in the figure. The initial medium contained 1 g/L of yeast extract and 1 g/L of peptone.
The fluorescence signal had the expected changes corresponding to the shifting electron-accepting mechanism of the culture (Figure 6.11a when addition was made every 40 minutes): After each nitrate addition the fluorescence showed a rapid drop and then a fast increase that corresponded to the consumption of the nitrate. It also clearly showed how fast the metabolism of the culture adapts to the changes produced by a more frequent nitrate addition (Figure 6.11b points the change of the nitrate addition from 1 h to 40 minutes). Figure 6.11b includes the offline measurement of nitrate corresponding to different points in the NFU signal cycle. The offline analysis confirmed that the nitrate concentration was zero when the signal stopped decreasing. These findings allowed shortening the time of addition from 2 hours to 1 hour, then to 40, 30, and 20 minutes, accordingly. More frequent nitrate addition had a positive effect on the cell concentration. Nonetheless, when the nitric acid was finally added every 20 minutes, the cell concentration did not increase further and started decreasing even though the nitrate and glycerol concentration was high in the fermentor (glycerol concentration was about 32 g/L by the end of the run). The frequent addition of the concentrated acid was thought to have a negative effect on the cells causing the cell concentration to drop.

Figure 6.11 also contains the changes of the pH signal when nitric acid was added to the fermentor. These changes were found to correlate to the changes in the fluorescence signal due to denitrification. When nitric acid was added, the pH would obviously decrease, and as soon as the addition stopped the pH started to recover due to the consumption of nitrate by the cells. The pH increased with a steep slope up to a certain value at which the recovery would slow down until it would become almost flat.
Figure 6.11. NAD(P)H fluorescence signal at two different times in the fermentation (a) from 150 to 155 h, showing the response of the signal to the addition every 40 minutes, and (b) 118 to 122 h, showing the corresponding offline nitrate measurement and the change of the signal when addition was decreased from 1 hour to 40 minutes.
By comparing the pH profile to the fluorescence profile, the slope change on the pH profile was found to be responding to the complete exhaustion of nitrate. When the fluorescence signal started to increase sharply (after nitrate was consumed), the slope of the pH decreased considerably. This finding was initially considered to set-up a more accurate nitrate addition based on the changes of pH when the nitrate levels are low. However, there is the possibility that the acid could have damaged the cells if added as the only nitrate source since concentrated acid had to be used to avoid large volume changes (shown in Figure 6.10 as the drop in cell concentration when the acid was added too frequently). For that reason, nitrate was afterwards set to be provided with both nitric acid and other nitrate source (i.e. sodium nitrate) to fulfill the respiration needs of the culture and attain higher cell concentrations for higher rhamnolipid productivity.

6.3.5.2. Higher cell concentrations attained with both nitric acid and sodium nitrate under denitrifying conditions

The most serious challenges to the denitrifying fermentation approach resulted from the need of a well controlled addition of nitrate. Although nitrate is highly water soluble and is largely not harmful to the bacterium under aerobic conditions [12], it cannot be added in batches of high concentrations for denitrification-based fermentations. Under anaerobic, denitrifying conditions, nitrate and particularly nitrite, which is an intermediate formed in the denitrification process, are inhibitory and/or toxic to *P. aeruginosa* depending on the concentrations added. Nitrite could also accumulate rapidly to toxic levels in starved or stressed denitrifying cultures [12].
With the findings from the NAD(P)H fluorescence signal monitoring, it was clear that the cells did not have enough nitrate to support the respiration needs of the culture and when the frequency of addition was increased the cell concentration also increased. Nitric acid was then set to be added in the fermentor only to control the pH and sodium nitrate was provided frequently (every hour) in batches of 30 mg/L NO$_3^{-}$-N to maintain a base concentration of ~ 400 mg/L NO$_3^{-}$-N. Figure 6.12 shows that the nitrate concentration was difficult to maintain at a certain range because it still relied on offline sampling and varied with the culture metabolism. Nevertheless, the above system allowed the achievement of a higher cell concentration of 6 g/L, which was higher than the concentration obtained when only nitric acid was added. At the end of the run the cells stopped growing even when there was still nitrate available in the fermentor which again indicated that there was other possible cause(s) preventing the cells from further growing.

Although the nitrogen provided from ammonium chloride was totally consumed, there was always some nitrogen in the fermentor from the nitrate, which as expected repressed the rhamnolipid production. Most likely the rhamnolipid production would resume as normal as soon as the cells reach other limitation from the medium.

6.3.6. Inhibitory compound(s) produced during denitrifying fermentation conditions

The broth from a denitrifying fermentation was collected to examine the possible existence of certain metabolite(s) that were produced during the fermentation and that could inhibit the cells from further growing. The broth was centrifuged under sterile conditions. The spent medium (supernatant) was separated from the old cells, and was
later inoculated with fresh cells prepared by the normal inoculum preparation procedures. The glycerol and ammonia concentrations in the spent medium were measured and adjusted accordingly so that it had the same starting concentration as the fresh medium systems.

Figure 6.12. Higher cell concentration (▲) under denitrifying conditions using nitric acid for automatic pH control and frequent sodium nitrate addition keeping nitrate concentration above zero. Rhamnolipid (△), glycerol (●), NO₃⁻-N (──), and NH₄⁺-N (···) concentration obtained under these conditions are also shown in the figure.

Another system was included in the experiment to examine if the old cells themselves could have been affected by the inhibition or by the way the nitrate was being delivered into the fermentor (as strong nitric acid). For this system, the old cells separated from the spent medium (which had been exposed to denitrifying conditions for long time) were resuspended in fresh medium and used as inoculum in a fresh medium system. A
third system containing fresh medium and inoculated with the same volume of fresh cells was used as a control. These three systems were then grown in shaker flasks at 34 °C and 280 rpm for 50 hours.

As shown in Figure 6.13 the three systems grew similarly initially but by 21 hours the cell growth in the spent medium slowed down considerably. At that moment there were still 250 mg/L of NO$_3^-$-N indicating that the cells had enough nitrate for respiration and that it was not the cause of the limitation. Even at 32 hours of the experiment the cells had about 40 mg/L of NO$_3^-$-N in the flask.

![Graph showing cell concentration over time for three systems: fresh medium - fresh cells (control), fresh medium - old cells, and old medium - fresh cells.](image)

Figure 6.13. Comparison of the growth of *P. aeruginosa* E03-40 in spent denitrification medium inoculated with fresh cells and in fresh medium inoculated with old cells.
On the other hand, the old cells growing in fresh medium grew similarly to the control. The cells in both new medium systems completely consumed the nitrate (1000 mg/L NO$_3^-$-N) within the first 21 hours. These results confirmed that the attained cell concentration in the fermentors under denitrifying conditions was not because the cells were in some way damaged by the nitrate delivery but because there was some inhibitory compound(s) in the medium. The inhibitory compound(s) was probably produced during the denitrifying fermentation and affected the cells after anaerobic conditions were reached. This would explain the normal initial growth while the surface aeration occurring in the shaker flask was still sufficient to meet the respiration rate of the cells at low concentrations.

6.3.7. Denitrifying fermentation of wild type PAO1 and its $\Delta$rhII, $\Delta$lasI, and $\Delta$lasI$\Delta$rhII mutants

As described in section 2.4, \textit{P. aeruginosa} contains at least two quorum-sensing systems that interact with each other, the \textit{las} (\textit{lasR} and \textit{lasI}) and \textit{rhl} (\textit{rhlR} and \textit{rhlI}) systems. As shown in Figure 2.3, these are directly involved in the synthesis of autoinducer molecules $N$-(3-oxododeN-(3-oxododecanoyl) homoserine lactone (PAI-1) and $N$-butyryl homoserine lactone (PAI-2). These molecules participate in the cell-to-cell signal mechanism that can alter the individual bacterium's metabolism based on the population density of its own species and the surrounding environment [152]. More recently, a study on the roles of quorum sensing on the denitrification of \textit{P. aeruginosa} PAO1 concluded that the quorum sensing mechanism was involved in controlling bacterial respiration [153]. In order to establish if the quorum sensing was regulating the
cell growth under the conditions tested on this project, the denitrifying growth of the wild type PAO1 was compared to the growth of \(\Delta rhlI, \Delta lasI,\) and \(\Delta lasI\Delta rhlI\) mutants. The \(\Delta\) symbol represented a deletion of the respective gene. These mutants were then expected not to produce the autoinducer molecules directly affecting the quorum sensing mechanisms.

As described in the materials and methods section 6.2.6, four 500-mL Erlenmeyer flasks were used as the fermentors with a 300-mL working volume and automatic pH control. Sodium nitrate was added according to the needs of each culture and to maintain a concentration close to 500 mg/L NO\(_3\)^\(-\). Glycerol was measured often and added accordingly to have enough carbon source in each system.

Figure 6.14a illustrates the growth profiles of PAO1 and the quorum sensing mutant strains under denitrifying conditions. \(\Delta rhlI\) and \(\Delta lasI\) mutants, which were deficient in PAI-2 and PAI-1, respectively, were found to grow similarly to the wild type PAO1. These findings were in agreement to the growth profiles obtained by Toyofuku et al. [153] under denitrifying conditions. Additionally, PAO1 consumed a lot more glycerol than all the other strains and probably stopped growing due to carbon limitation (Figure 6.14b). The glycerol additions were illustrated in the figure by vertical discontinuous arrows. Glycerol was added to all the systems in the first two additions (5 g/L each) but the final addition (4 g/L) was only provided to the PAO1 system. The frequent nitrate addition ensured nitrate was available for respiration in the fermentors throughout the run.
Figure 6.14. Growth profiles of PAO1 (□) and its ΔrhlI (▲), ΔlasI (●), and ΔlasIΔrhlI (×) mutant strains under denitrifying conditions: (a) Cell and (b) glycerol concentration.
In agreement with the findings of Toyofuku et al. [153], the ΔlasI mutant consumed more nitrate than the wild type PAO1 and the ΔrhlI mutant strains during the first 19 hours of the experiment. In that report, ΔlasI mutant was found to have the highest NO$_3^-$-respiring activity when compared to the wild type PAO1 and the ΔrhlI mutant during a cultivation period of 12 hours. This activity was repressed by 70% for ΔlasI mutant when the respective autoinducer molecule (PAI-1) was added to the system (resulting in similar activity as that of the wild type). Toyofuku et al. [153] hypothesized that even though the growth of the wild type and the mutant strains was similar, the quorum sensing appeared to repress the denitrification process. The lack of further growth in the quorum-sensing mutant systems was explained by the accumulation of toxic levels of nitric oxide (NO), which could have affected the growth. However, nitric oxide accumulation to higher levels was not likely in the present study because surface aeration was continuously provided into the flasks. As an alternative of the nitric oxide accumulation assumption, the obtained similar initial growth could have been the result of the quorum sensing mechanism becoming significant in the later stage of the cultures and the study by Toyofuku et al. [153] was only done for 12 hours.

ΔrhlI and ΔlasIΔrhlI double mutant was found to have higher respiring activity than PAO1 in the report by Toyofuku et al. [153] which did not agree with the nitrate consumption results obtained in this study. Additionally, considering both the nitrate provided by the acid addition as well as the sodium nitrate addition rates, PAO1 consumed the largest amount of nitrate for the remainder of the run. (An average estimation of the nitrate consumption rate for each strain was included in Table 6.2.). Additionally, at the end of the run the cell concentration of the ΔlasIΔrhlI mutant
increased sharply and the glycerol and nitrate were consumed fast. The other two mutant strains also showed this behavior and grew to higher concentrations. The reason for this behavior was established not to be due to contamination. A more thorough study of the mutant strains profiles under denitrifying conditions is still required to conclude on the effect of the quorum sensing on the cell concentration attained under denitrifying conditions.

Table 6.2. NO$_3^-$-N consumption during the first 19 hours of growth and estimated average NO$_3^-$-N consumption rate of wild type PAO1 and its $\Delta$rhlI, $\Delta$lasI, and $\Delta$lasI$\Delta$rhlI mutants considering the nitrate from nitric acid and sodium nitrate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NO$_3^-$-N consumption during first 19 h (mg/L)</th>
<th>Average NO$_3^-$-N consumption rate (mg/L•h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>142</td>
<td>175.5</td>
</tr>
<tr>
<td>$\Delta$lasI</td>
<td>842</td>
<td>101.2</td>
</tr>
<tr>
<td>$\Delta$rhlI</td>
<td>42</td>
<td>81.9</td>
</tr>
<tr>
<td>$\Delta$lasI $\Delta$rhlI</td>
<td>42</td>
<td>37.5</td>
</tr>
</tbody>
</table>

6.3.8. Identification of byproducts and possible inhibitory compounds

One of the major challenges associated with the anaerobic denitrifying process for rhamnolipid production was the high glycerol (and nitrogen) consumption rate that considerably affected the overall yield of the fermentations. The possible production of considerable quantities of compounds different from rhamnolipids was examined and described in the next subsections.
6.3.8.1 Glycerol and nitrogen sources consumption during fermentation

Throughout this chapter it was apparent that under anaerobic denitrifying conditions the cells consumed more glycerol than that required to maintain the cell concentration that was attained, especially when glycerol was accumulating in the fermentors. For example, in the fermentation described in section 6.3.5.2 almost 20 g/L of glycerol were consumed in 24 hours during the stationary phase to maintain a cell concentration of approximately 4 g/L. Considering that a typical bacterial cell contains about 50% of carbon and 14% of nitrogen [154], about 8 g/L of glycerol would be necessary to maintain that cell concentration. Additionally, only an average of 0.3 (± 0.08) g/L rhamnolipids was produced per day. The culture consumed more than 50% of glycerol for either maintenance energy or synthesis of other products.

Maintenance energy can be described as the energy that cells spend for essential metabolic functions: motility, transport of nutrients and products in and out of cells, repair damaged cellular structures, etc. The consumption of carbon source for maintenance energy may vary within a wide range depending on the strain and the culture conditions. For instance, in penicillin fermentation up to 70% of the substrate was used for maintenance and in industrial riboflavin production approximately 45% of the glucose was expended in maintenance with a maintenance coefficient of 0.67 g/(g (CDW)·h) [155].

On the other hand, during the denitrifying fermentations not only glycerol was consumed in amounts larger than expected but also nitrate and ammonia. As described in section 6.2.5.2, 30 mg/L of NO₃⁻-N (or more) were immediately consumed even when the addition was as short as 30 minutes. Ammonia was also consumed in large amounts. For example, out of the 600 mg/L of NH₄⁺-N added to the fermentation described in
section 6.2.3.3, only 280 mg/L of NH$_4^+$-N would be needed for cell growth (assuming 14% N in the dry cells at a 2 g/L concentration). However, the ammonia was completely consumed within the first days of the fermentation. Moreover, 500 mg/L of NH$_4^+$-N were added in the middle of the run to test if the addition would promote further cell growth. The cell concentration did not increase and the ammonia was again rapidly and completely consumed. These results and the changes in the fermentation broth in terms of color and turbidity suggested that the nitrogen and carbon sources were not only consumed for cell growth and maintenance but for the production of other organic compounds.

6.3.8.2. SEC and TGA for identification of side products

In an attempt to confirm the presence of other byproducts, size exclusion chromatography (SEC) and thermal gravimetric analysis (TGA) were carried out on the fermentation broth. Refractive index (RI) detector was used for the SEC analysis. The calibration curve for the SEC analysis obtained with polyethylene glycol (PEG) standards of different molecular weights is shown in Figure 6.15. A 10 g/L glycerol standard was also included as a reference. Table 6.3 was included to specify the molecular weight of each standard as well as the average retention time.

Broth samples from the fermentation in which higher cell concentrations were obtained with a more frequent nitrate addition (described in section 6.2.5.2) were diluted and injected to obtain the molecular weight range of the different compounds. However, the column did not allow a good separation and only one broad peak was obtained even when the flow rate was lowered. In any case, the average estimated molecular weight
(from the linear range of the calibration curve above) was 1,484 (± 420), with an average retention time of 15.658 minutes (± 0.16).

![Calibration Curve](image)

Figure 6.15. SEC calibration curve with PEG and glycerol as standards.

In addition, different dilutions of the sample shifted the retention time of the peak suggesting a possible agglomeration of the compounds (or micelle formation) caused by local pH changes along the column. Phosphate buffer (100 mM in HPLC water) to maintain the pH around 7.0 in the column was introduced in the mobile phase. With this change, the peak did not shift dramatically with different dilutions and a shoulder was obtained suggesting a better separation but the shape of the peak still had some differences with varying dilutions. The separation was still not complete. The chromatograms obtained with and without phosphate buffer are shown in Figure 6.16.
Table 6.3. Molecular weight of different standards and respective retention time for SEC.

<table>
<thead>
<tr>
<th>Standard - MW</th>
<th>Retention time (min)</th>
<th>Log MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol - 92</td>
<td>16.53</td>
<td>1.96</td>
</tr>
<tr>
<td>PEG - 600</td>
<td>16.27</td>
<td>2.78</td>
</tr>
<tr>
<td>PEG - 3400</td>
<td>15.16</td>
<td>3.53</td>
</tr>
<tr>
<td>PEG - 5000</td>
<td>14.93</td>
<td>3.70</td>
</tr>
<tr>
<td>PEG - 10000</td>
<td>14.56</td>
<td>4.00</td>
</tr>
<tr>
<td>PEG - 20000</td>
<td>14.34</td>
<td>4.30</td>
</tr>
<tr>
<td>PEG - 35000</td>
<td>14.15</td>
<td>4.54</td>
</tr>
</tbody>
</table>

These results showed that the byproducts were not polymers of high molecular weights and if the agglomeration was not totally prevented it was possible that the compounds could be of even lower molecular weights. A more systematic separation with a narrower range column is still needed to find out the molecular weight details of each compound in the broth.

Pure glycerol was used as a reference for the TGA carried out on the dried samples from the denitrifying fermentations since glycerol was present in the fermentor with a known concentration. The TG mass loss curve of pure glycerol obtained with the two scan rates tested is shown in Figure 6.17a. The decomposition of glycerol was rapid and complete at 215 °C under a constant scan heating rate of 10 °C/min and at 162 °C under a much slower rate of 1 °C/min. (The latter was actually operated with variable scan rates: 10 °C/min to 120 °C, 1 °C/min to 550 °C and 10 °C/min to 600 °C. But the
rate was essentially 1 °C/min during the temperature range of interest, i.e., 120-550 °C.) Differences in the initial and final mass loss temperature depending on the heating scan rate were expected since decomposition is a chemical reaction that is time dependent. Consequently, TGA results are strongly affected by the heating rate selected. Fast heating rates usually shift the onset temperature of the samples to higher values spreading the weight change over a wider time span and temperature range. The profiles for pure glycerol obtained in this study at a rate of 10 °C/min were in agreement with those reported by Dou et al. at the same rate [156].

Figure 6.16. Size exclusion chromatogram of a sample from a denitrifying fermentation broth. (a) HPLC water as mobile phase without pH adjustment. (b) Mobile phase with phosphate buffer (100 mM in HPLC water) pH 7.0 ± 0.1.
Figure 6.17. TGA results of pure glycerol (as a reference) and of a denitrifying fermentation sample at two different heating scan rates: (1) a constant rate of 10 °C/min and (2) a variable rate of 10 °C/min to 120 °C, 1 °C/min to 550 °C and 10 °C/min to 600 °C: (a) mass loss curves and (b) derivative thermogravimetric profile.
The fermentation sample also showed differences when the scan rate was varied from 10 °C/min to 1 °C/min (Figure 6.17a). The water loss due to vaporization was clearly seen at 100 °C using both heating rates but the decomposition temperature profile also shifted to lower temperatures at the slower scan rate. Additionally, two inflection points beyond the decomposition temperature of glycerol were evident at 170 °C and 200 °C when using the slower rate. This distinction was not as obvious at the faster rate but it became apparent in the derivative thermogravimetric profile (Figure 6.17b). After ~ 500 °C the mass loss slowed down considerably resulting in a final mass (at 600 °C) of 15.2 % of the initial weight. This remaining mass was most likely due to inorganic compounds coming from the medium components that typically have a decomposition temperature above 600 °C.

The slower heating rate certainly showed clearer details of the composition of the sample. Using the TGA profile obtained at this rate the amount of water was estimated to be 6 % of the initial weight of the sample. Considering the percentage of water and that of the inorganic compounds (15.24 %), the organic content of the sample was estimated to be approximately 78.6 %. One of the main organic compounds in the sample was the glycerol used as the carbon source in the fermentations. The glycerol content was therefore estimated from the decomposition temperature at 162 °C, resulting in approximately 36 % of the sample. The rest of the organic compounds accounted for about 42.6 % of the sample. The concentration of organics in the sample could then be calculated taking into account the estimated percentage of glycerol and organics and that the measured glycerol concentration in that specific sample was 38 g/L. The concentration of organics (without glycerol) was 45 g/L, which was approximately 35 %
of the total glycerol consumed by the cells up to the day when that specific sample was taken from the fermentor (total glycerol consumed was 128 g/L). This high organic content could have also contributed to the viscosity of the fermentation samples that considerably increased during the duration of the runs.

The TGA results as well as the calculated organic content suggested that the large amount of glycerol (and probably of nitrogen sources) consumed by the cells was not only being used for maintenance energy but also to produce other side compounds. Some of these products could also be the inhibitory compounds affecting the growth of the cells during rhamnolipid fermentation.

6.3.8.3. Further identification of the organic content of the fermentation products

Further characterization of the organic content of the sample was carried out by analyzing the extracellular protein as well as the carbohydrate content in the sample. The protein concentration of the denitrifying fermentation sample was analyzed using the common Lowry assay for proteins and resulted in an average of only 2.45 (± 0.3) g/L.

As illustrated in Figure 2.2, alginate is another common byproduct of the *P. aeruginosa* metabolism from glycerol. Alginate is a negatively charged exopolysaccharide considered to be one of the virulence factors produced by the bacterium to protect itself from certain antibiotics and other environmental threats [157, 158]. In order to determine if alginate was being produced under the fermentation conditions, a precipitation step was first carried out with three volumes of ethanol and drop-wise addition of concentrated KCl, as explained in the methods section. The precipitate was separated and washed by centrifugation and the alginate assay was
performed on the precipitated sample. A pink color of the sample after the heating step of
the alginate assay would indicate the presence of alginate (clearly seen in the sodium
alginate standards used to establish the calibration curve). However, when the assay was
performed on the sample, the color was green instead of pink, and the concentration was
just 1.42 g/L (± 0.5) suggesting that alginate was not the main carbohydrate product.

The total carbohydrate content was estimated using the anthrone assay with
glucose as a standard to obtain the calibration curve. The linear range of the calibration
(up to 0.08 g/L of glucose) was used to calculate the concentration of the samples from
denitrifying fermentations. The average concentration of carbohydrate was 0.9 g/L (±
0.25) which was very similar to the concentration obtained with the alginate analysis
above. These results and the green color obtained with the alginate method again implied
that the contribution from carbohydrates was not from alginate. The results of
extracellular protein and carbohydrates did not account for more than 10 % of the
glycerol consumed by the cells, suggesting the presence of a large quantity of other
organic compounds.

An attempt to determine the fatty acid content was also carried out by acidifying
the sample (to a pH of 2.5 ± 0.1) and collecting the precipitate obtained after the
acidification. The precipitate was then dried in an aluminum pan at 90 ºC for 24 hours.
The estimated concentration of the acid precipitate was approximately 1.96 g/L, from
which only 0.1 g/L were rhamnolipids (during this particular fermentation). The acid
precipitate was considerably smaller than that obtained with the ethanol precipitation
carried out in the first step of the alginate assay.
Considering this information as well as the molecular weight range, and the characteristics of the precipitation, it was established that the majority of the organic compounds produced from glycerol were not extracellular proteins, alginate, or other carbohydrates. The presence of other fatty acids could not be ruled out as there could be some with hydrophilic moieties that would not precipitate at low pH. Other organic compounds that could be inhibiting the growth of the cells could be pigments that are characteristic of *P. aeruginosa* (and that have been recognized as virulence factors [116]) and other lipids. Further investigation of the remaining 30 % (estimated from the non-glycerol organics calculated from TGA) of the compounds produced from glycerol is required to identify and eliminate the inhibiting compound affecting the cell growth.

6.4. Conclusions

In summary of this chapter, biodiesel glycerol was shown to support the growth of *P. aeruginosa* E03-40 in a similar manner as pure glycerol. Additionally, successful cell growth and rhamnolipid production were obtained using glycerol as substrate under denitrifying fermentation conditions. The foaming problems associated with commonly used aerobic rhamnolipid fermentations were avoided using the denitrifying approach in which nitrate was employed as electron acceptor for respiration instead of oxygen. The cell concentrations attained were similar to those generally obtained under aerobic conditions for rhamnolipid production. Additionally, the rhamnolipid concentrations were also comparable or higher to previous reports relying on oxygen as electron acceptor and glycerol as carbon source. By following the NAD(P)H fluorescence signal of the culture and investigating the nitrate consumption, a more accurate nitrate supplementation was
implemented and as a result, higher cell concentrations were achieved. In addition, ammonium was found to be a better nitrogen source for growth and rhamnolipid production as compared to nitrate.

When trying to increase the cell concentration to attain higher rhamnolipid productivity, further cell growth was found to be inhibited by a compound produced under denitrifying conditions. Another difficulty associated with the process was the high glycerol consumption rate that considerably affected the overall yield of the denitrifying fermentations. Using SEC and TGA, glycerol was found to promote the production of a large amount of organic compounds besides rhamnolipids (some of these compounds could also be causing the inhibitory behavior). Preliminary identification of these compounds suggested that the majority of the byproducts were not extracellular proteins, alginate, or carbohydrates. Further characterization on the denitrifying samples is still needed to determine the byproducts and their potential inhibition. By removing the inhibitory compounds, much higher cell concentrations can be attained using denitrifying conditions. This will increase the volumetric productivity and allow the use of the denitrifying fermentation technology for large scale rhamnolipid production.
CHAPTER VII
IMMOBILIZED SYSTEM FOR RHAMNOLIPID PRODUCTION UNDER DENITRIFYING CONDITIONS USING GLYCEROL AS CARBON SOURCE

7.1. Introduction

One of the main challenges for the production of rhamnolipids under the normally used aerobic conditions with free-cell systems is the uncontrollable foam due to the highly foaming nature of the fermentation broth and the biosurfactant produced. In an aerated reactor with magnetic agitation, foaming has been reported to appear at rhamnolipid concentrations as low as 0.15 g/L and once the concentration is higher than 2 g/L the foaming completely fills the fermentor [28]. In these cases, it is very difficult to retain the cells in the fermentor once they start to be removed with the foam. Additionally and to reduce the foaming, the aeration needs to be reduced. The reduced aeration led to lower oxygen supply rates, which in turn compromised cell viability due to poor respiration conditions and ultimately the rhamnolipid productivity.

An alternative to free cell systems is the immobilized cell systems in which the cells are fixed in or on an inert support. The immobilized cell systems have a tremendous advantage over free cell systems because most of the cells remain trapped and the products can be easily recovered since the biomass is already separated from the medium.
The cell immobilization can be carried out by binding to a solid support, by physical separation from the medium using membranes or fiber systems, and by entrapment into a porous matrix [28]. Rhamnolipid production using immobilized cell systems has been previously studied using various supports such as polyvinyl alcohol beads [31], kaolinite, loose polypropylene fibers [25], calcium alginate beads, and polyacrylamide [28]. However, in these studies the immobilized cells still relied on oxygen as electron acceptor for respiration. Foaming problems occurred also in these systems. Large amounts of antifoam agents had to be used to control the foam. Additionally, poor fixing ability onto the support as well as diffusion limitation especially when using non-polar substrates were reported. The oxygen transfer limitation also affected the cell viability presenting difficulties for continuous rhamnolipid production.

Immobilized cell systems using nitrate as electron acceptor for respiration were explored in this part of the study. The immobilization approach was to physically separate the cells from the medium using either a dialysis tube or a hollow fiber cartridge. This denitrification-based approach completely eliminated the foaming difficulties related to aerobic systems. In addition, the hollow fiber set-up did not show carbon or nitrogen source transfer limitation and was found suitable for continuous rhamnolipid production using glycerol as carbon source. The recovery of rhamnolipids was also easier since there was no need for a preliminary separation from the cells.
7.2. Materials and methods

A description of the materials and methods used in the evaluation of different immobilized cell systems under denitrifying conditions for rhamnolipid production is provided in the next subsections.

7.2.1. Microorganisms and medium

The microorganism used for immobilized systems was *P. aeruginosa* E03-40, isolated from the soil sample of a biodiesel production plant (West Central Cooperative) in Ralston, Iowa. With glycerol as the carbon substrate, this bacterium was found to be a high rhamnolipid producer as described in Chapter IV.

The inoculum was prepared in Tryptic Soy Broth and grown for 24 hours at 34 °C in a shaker incubator. After that it was transferred into a 250-mL flask that served as the pre-culture for the immobilized systems. The pre-culture flask held 100 mL of the medium containing 20 g/L glycerol as carbon source, 2 g/L NH₄Cl, 7.3 g/L NaNO₃, 2 g/L KH₂PO₄, 0.3 g/L Mg₂SO₄•7H₂O, 0.01 g/L CaCl₂•2H₂O, 0.03 g/L FeSO₄•7H₂O, 0.5 g/L NaCl, 0.01 g/L MnCl₂•4H₂O, 2 g/L yeast extract, 2 g/L peptone and 2 mL/L of a trace element solution containing 0.08 g/L FeCl₃•6H₂O, 0.75 g/L ZnSO₄•7H₂O, 0.08 g/L CoCl₂•6H₂O, 0.075 g/L CuSO₄•5H₂O, 0.75 g/L MnSO₄•H₂O, 0.15 g/L H₂BO₃, and 0.05 g/L Na₂MoO₄. The pH of the medium was adjusted before autoclaving to 7.1 ± 0.1.

The medium used to initiate the immobilized systems was the same as the one described for the pre-culture. Additionally, a sterile 10 g/L betaine solution was used as an osmoprotectant to provide a final concentration of 0.5 mM in the reactors.
7.2.2. Dialysis membrane tubing set-up as immobilized system for rhamnolipid production

A diagram of the experimental set-up for the dialysis membrane immobilized system is presented in Figure 7.1.

![Figure 7.1. Denitrifying immobilized cell system based on physical separation by dialysis membrane tubing.](image)

The experiment was carried out in a 600-mL beaker with 400 mL of fresh medium and fitted with a rubber stopper to maintain sterility. The stopper also held the pH probe and other ports required for the fermentation operation such as nitrate, glycerol, and nitric acid feeding lines as well as a sampling port. The pre-culture was grown for 20 hours at 34 °C in a shaker incubator and after that the cells were centrifuged down under sterile conditions in order to concentrate the cells and start the experiment with high cell
density. The concentrated cells were then transferred into a sterile dialysis tube with a nominal molecular weight cut off of 12,000 – 14,000 (wall thickness of 20 μm and flat width of 45 mm, Fisherbrand, Fisher Scientific). The tube containing the cells was then put into the beaker and immersed in the fresh medium. An automatic pH control set up, with acid/base (HNO₃/NaOH) addition, was used to keep the pH at 7.1 ± 0.1. The pH profile was monitored with a LabView program to follow the pH changes in the cell-free medium area of the fermentor due to denitrification of the cells inside of the membrane. During the fermentation, samples were taken periodically from the cell-free medium and typically used to monitor glycerol, nitrate (and nitrite), ammonia, and rhamnolipid concentrations.

7.2.3. Hollow fiber cartridge set-up as immobilized system for rhamnolipid production

The second immobilized system was a polysulfone hollow fiber bioreactor (C2025, FiberCell® Systems) with a pore size of 0.1 μm. Twenty (20) hollow fibers were sealed in the cartridge case. The medium was circulated from a 500-mL flask containing 300 mL of medium, through the inside of the fibers, and back to the original flask. The cells were grown on the outside of the fiber in the extra-capillary space (ECS). The pH was automatically controlled to pH 7.1 ± 0.1 with acid (HNO₃) addition in the cell-free medium flask. The pH changes were monitored using a LabView program that also controlled the addition of NaNO₃ with adjustable time intervals. Glycerol was added using a timer control connected to a digital pump and surface aeration was provided with an air pump connected to a rotameter (0.1 L/min) and a 0.2 μm sterile filter. In order to prevent the accumulation of inhibitory gases generated from the denitrification process in
the ECS, the side ports were connected to a 250-mL flask. This flask was used to recirculate the cells as well as to eliminate the gases, and was provided with surface aeration with filter-sterilized air passing through the flask headspace at 0.1 L/min. Both flasks, containing the cell-free medium and the cells, were magnetically stirred at 200 rpm and 800 rpm, respectively. A diagram of the experimental set-up using the hollow fiber cartridge is presented in Figure 7.2.

Figure 7.2. Denitrifying immobilized cell system based on physical separation using a hollow fiber cartridge in which the cells were in the extra-capillary space (ECS) and the medium was passed through the inside of the fibers.

Similarly as in the dialysis-tube immobilized system, samples were taken periodically from the cell-free side of the set-up and were used to monitor glycerol,
nitrate (and nitrite), ammonia, and rhamnolipid concentrations. Additionally, a sampling port was also included to the cell recirculation line to analyze the samples and check for mass transfer limitation problems.

7.2.4. Analytical methods

Samples taken from the cell-free medium flask were used to measure the glycerol, NO$_3^-$-N (and NO$_2^-$-N), and rhamnolipids concentrations. The samples taken from the cell recirculation line were first centrifuged to separate the cells from the medium (supernatant) and then the pelletized cells were used to measure the cell concentration in the system. Again, the carbon source, NO$_3^-$-N (and NO$_2^-$-N), and rhamnolipids concentrations were measured using the supernatant according to the analyses procedures described in section 3.3.

7.3. Results and discussion

The results obtained from the evaluation of immobilized systems for rhamnolipid production using a dialysis tube and a hollow fiber cartridge are described and discussed in the following subsections.

7.3.1. Dialysis tube as immobilized system

Before inoculation into the dialysis membrane, the cells from the pre-culture were concentrated four times after centrifugation to have a concentration of approximately 4 g/L. To allow the cells to grow to even higher cell concentrations within the dialysis tubing, the medium in the system outside the membrane initially contained all the
nutrients as well as glycerol. Nitrate was provided into the system using both nitric acid and sodium nitrate to maintain a concentration above 500 mg/L NO₃⁻-N. Nitric acid served also for the purpose of controlling the pH of the system.

The dialysis tubing had a molecular weight cut-off that allowed glycerol, nitrate, and other nutrients to diffuse from the outside medium into the membrane due to a concentration gradient created when the cells inside consumed these components. Additionally, rhamnolipids and other products could diffuse out to the medium and be easily recovered. This mechanism is illustrated in Figure 7.3.

![Figure 7.3. Dialysis membrane mechanism for immobilized cell system.](image-url)

Even though the pH was measured in the medium outside the tube, the pH profile reflected the cell metabolism changes and was found to be very similar to that on the free-cell systems described in the previous chapter. Responding to denitrification, the pH increased up to the set point at which the acid pump was activated and nitric acid was added causing the pH to drop. As soon as the addition stopped, it started to increase back
to the set point (see the profile in Appendix A). The pH profile suggested that the mass transfer through the membrane was not slow since the pH changes outside the membrane reflected the variations coming from the cells inside of the dialysis tube as they started consuming nitrate.

The main difficulty that was found with the membrane immobilized system was that after around 48 hours there were cells growing outside the membrane. The medium became turbid and observation under the microscope as well as centrifugation of the sample and cell concentration measurements confirmed the growth. This behavior was most likely due to the penetration of some cells originally inside the dialysis tube through the very thin and stretchable membrane. The high pressure inside the membrane, due to osmotic pressure and the pressure created by the denitrification-generated gas bubbles, could have stretched the membrane, enlarged a few pores, and pushed out a very small number of cells. Additionally, the medium was rich in nutrients which caused this few cells to reproduce rapidly outside the membrane. After that it was not possible to follow the state of the culture inside of the membrane since the cells outside were also doing denitrification and affected the pH of the system. To minimize this difficulty, the medium outside of the membrane was passed through a set of glass tubing before being recirculated back to the beaker. The glass set-up was put inside a chamber (Raytech ultraviolet equipment, Model VB-8, Raytech Industries, Inc.) in which a UV lamp (Raytech ultraviolet equipment, Model LS-88, Raytech Industries, Inc.) was used with a shortwave ultraviolet light directed to the tubing that contained the cells to control the growth. With this change the cell growth outside of the membrane could be controlled for a while but still was not eliminated completely. A sterile glass fiber filter was also
introduced in the system to trap the cells but it was quickly clogged because of the particles coming from the nutrients that were not completely soluble in water.

The cell growth outside of the dialysis tubing remained as a challenge when the medium did not contain any limitation and the system wanted to be maintained as a growing system. Therefore, this set-up had to be used as a resting cell system (i.e. introducing a nutrient limitation from the beginning of the experiment) with initially high cell densities inside the membrane for better efficiency and rhamnolipid productivity.

7.3.2. Rhamnolipid production using the immobilized dialysis membrane system

In order to introduce a limitation into the immobilized membrane system, the medium was changed several times to ensure that the contents inside of the membrane were free of the limiting nutrient. A phosphorus (P) limiting medium was used for the rest of the experiment (removing potassium phosphate, yeast extract, and peptone from the fresh medium solution before putting it in contact with the membrane). The changes of nitrate concentration as well as glycerol and nitrite before and after each change of medium are shown in Figure 7.4a. Additionally, the rhamnolipid concentration outside the membrane is shown in Figure 7.4b. Considering that the rhamnolipids outside the dialysis membrane (in approximately 380 mL) were obtained from the production by the cells inside of the membrane (that started with 20 mL), an estimation of the real rhamnolipid production within the dialysis tubing was made and shown in that same figure.

As soon as the P limitation was introduced in the system, the cells started producing rhamnolipids. However, from the first to the fourth change of medium there
was apparently some P left in the process since there were some cells still growing outside of the membrane. Only after the fourth change, the medium was clear and no outside cell growth was observed. During this last period bubble formation was seen inside of the dialysis membrane confirming that the cells were doing active denitrification. Nevertheless, the bubbles coming from the gases generated during the denitrification process (i.e. nitric and nitrous oxides as well as nitrogen) could not diffuse out fast enough and were accumulating in the membrane. This probably caused inhibition to the enzymes involved in the reduction of nitrite and the oxides as well as toxicity to the cells. That could be one of the reasons why the nitrite levels in the system were increasing (Figure 7.4a). The other possible reason for the nitrite accumulation could be oxygen repression of nitrite reductase which is the enzyme catalyzing nitrite reduction. However, the levels of oxygen were possibly very low inside the membrane since the cell concentration was high from the beginning of the experiment. Any oxygen present in the system would have been consumed at that point.

The rhamnolipid production during that last period was not as active as after the second medium change. Even though P limitation had been previously reported as a good limiting substrate for rhamnolipid production under denitrifying conditions [13], it was difficult to conclude if this limitation was effective using the immobilized set-up since after the last medium change the production slowed down considerably. This behavior could have also suggested that the cell metabolism was indeed being affected by the high gas concentration inside the membrane which was also influencing the rhamnolipid production.
Figure 7.4. Concentration profiles under denitrifying conditions using a dialysis membrane immobilized system: (a) Nitrate (□), nitrite (×), and glycerol (◇) profiles. (b) Rhamnolipid concentration outside the membrane and rhamnolipid production estimation from the cells inside the membrane considering the differences in volume (outside vs. inside).
Yet, the rhamnolipid concentration attained with this set-up was more than 6 g/L confirming the feasibility of an immobilized system for rhamnolipid production based on denitrifying conditions. This concentration was comparable to the concentration attained in the free-cell denitrifying processes described in Chapter VI and also to other reports on rhamnolipid production using immobilized system based on oxygen as electron acceptor [31].

Interestingly and similarly to the free-cell fermentations, glycerol was consumed in large quantities even when rhamnolipids were not being produced. This behavior was clearly depicted in Figure 7.4a during the last stage of the process in which 15 g/L of glycerol were rapidly consumed suggesting the production of other materials.

Even though this set-up allowed the production of rhamnolipids, the accumulation of gases within the membrane and the leakage of the cells to the outer medium prevented its use for continuous production.

7.3.3. Hollow fiber cartridge as immobilized system for *P. aeruginosa* E03-40

The cell concentration of the culture inoculated into the hollow fiber cartridge was 3.5 g/L. Immediately after the cells were inoculated the recirculation of the medium from the reservoir flask was started at a low flow rate (1.4 mL/min) and slowly increased up to (14 mL/min). By using hollow fibers of 0.1 μm pore size, a relatively small volume of cell-containing broth was separated from a larger reservoir containing the cell-free medium. The fiber pore size was large enough for the products (and inhibitory compounds) to transfer to the circulating cell-free medium but small enough to retain the cells. Additionally, the fibers also allowed the exchange of nutrients, glycerol, and
nitrate. Figure 7.5 illustrates the mechanism of the hollow fiber cartridge used for bacterial immobilization under denitrifying conditions.

![Diagram of hollow fiber cartridge](image)

**Figure 7.5.** Cross section of a hollow fiber cartridge showing the transfer mechanism of nutrients and products.

The hollow fiber cartridge system was set up to avoid nutrient limitation by initially adding all medium components in excess as well as monitoring the glycerol concentrations to ensure that the cells would have enough carbon and energy source. This would allow the cells to grow to higher cell concentrations within the ECS of the cartridge. Nitrate was also monitored and provided into the medium reservoir using both nitric acid and sodium nitrate to maintain a concentration of around 500 mg/L NO₃⁻-N. Nitric acid served also for the purpose of controlling the pH of the system. As soon as the medium started flowing through the fibers, the pH profile of the system followed the denitrification profile of the cells that were growing in the ECS showing that there was not serious initial mass transfer limitation. As in the dialysis membrane immobilized configuration, the pH started increasing up to the set-point and immediately decreased.
when the acid addition was automatically triggered. When the acid addition stopped, the pH gradually climbed back up to the set-point.

As expected, the active denitrification activity of the cells caused the formation of bubbles from the gases generated by the reduction of nitrate. To get rid of the bubbles a recirculation system of the ECS contents was included (as illustrated in Figure 7.2). The cell-containing broth was circulated between the cartridge ECS and a flask that contained a small volume of surface-aerated broth. This arrangement eliminated the bubbles in the cartridge and probably any inhibition from these to the cells. Additionally, it allowed taking samples from the cell side in the ECS. Figure 7.6 illustrates the time when the cell recirculation was started as well as each medium change to completely replace the medium in the cell-free medium flask. The first medium change was made using the same initial composition to decrease the nitrite in the system. The other changes used a phosphorus (P) free medium to investigate its effect on rhamnolipid production. These later medium replacements were made several times, gradually diluting out the P left in the cell-containing broth and eventually leading to a P-free system.

As soon as the P-free medium was brought in the system, the pH profile responded by having larger pH variations when the acid was added. This variation was expected because the phosphate that also acted as a buffer in the system was no longer present in large quantities (see Appendix B).
Figure 7.6. Concentration profiles in the reservoir and in the ECS of the hollow fiber immobilized set-up under denitrifying conditions: (a) Glycerol and (b) nitrate and nitrite concentrations. The vertical discontinuous arrows show the glycerol addition and the discontinuous vertical lines represent the time when the medium was changed.
As shown in Figure 7.6a, the difference in glycerol concentration (before and after each medium change) between both sides (reservoir and ECS) was very small. This profile as well as the nitrate (and nitrite) concentration profiles (Figure 7.6b) showed that there was no mass transfer limitation between both sides of the system even until the end of the run (which was more than 1500 hours). In this figure, it could also be seen that glycerol was consumed in large quantities especially before the last medium change. Glycerol (10 g/L) had to be added at some points during the experiment (depicted with arrows in the figure) to ensure that the cell side would have enough carbon source.

Before the last medium change (see Figure 7.6b), nitrite was again accumulating to even higher concentrations than in the dialysis membrane system. Using the pH profile to monitor the changes in the denitrification mechanism of the cells, the high nitrite concentrations were found not to slow down the denitrification rate. Even more, nitrite did not affect the cells which maintained around similar cell concentrations of 2.9 (± 0.17) g/L when the nitrite concentrations increased from 1000 to 1500 mg/L NO₂⁻-N. This finding was unexpected since nitrite has been reported to be toxic to cells when it accumulates to high concentrations [125]. The cell concentration profile obtained after starting the cell recirculation is included in Figure 7.7.

It is important to note that after the final medium change, the surface aeration in the cell side was stopped and the medium level was increased (causing the lower cell concentration shown in Figure 7.7). This change was made with the purpose of decreasing the dissolved oxygen concentration that could have been present in the system. Interestingly, the nitrite concentration started to decrease and the denitrification rate slowed down. These results suggested that, prior to the above operation changes, the
dissolved oxygen concentration in the cell side might have indeed been high enough to repress the nitrite reductases and cause the nitrite to accumulate. It has been reported that nitrite reductases are more sensitive than nitrate reductases to oxygen inhibition [125].

Figure 7.7. Cell concentration profile in the ECS of the hollow fiber immobilized set-up. The discontinuous vertical lines represent the time when the medium was changed.

Additionally, nitrate consumption also slowed down when nitrite concentrations decreased and the pigments produced by the cells had a dramatic change at this same time of the experiment. The initial culture produced a large amount of a red pigment that got darker with time. The culture changed to produce a green pigment coinciding with the last medium change and the decrease in nitrite concentration (Figure 7.8). However, it cannot be concluded that the color change was related to the nitrite variation since it could also have been related to the complete exhaustion of P. Nevertheless, the
production of large amounts of pigments during the run was evident. This could have been the cause of the consumption of the large amounts of glycerol as well as nitrate.

Figure 7.8. Pictures of the hollow fiber cartridge with the different pigments obtained: (a) red pigment observed during the first 1400 hours and (b) green pigment observed afterwards.

7.3.4. Rhamnolipid production with hollow fiber cartridge as immobilized system

The rhamnolipid concentration profile in both sides (ECS and reservoir) is shown in Figure 7.9. The P-free medium introduced in the system appeared to have a positive effect on the rhamnolipid production by the cells. In addition, the changes of medium in the reservoir side possibly diluted the concentration of other products that with time might have caused inhibition to the cells and to the rhamnolipid production. The rhamnolipid production was enhanced after each medium change. The concentration increased in both sides but the concentration was noticeably higher in the ECS. This difference probably suggested that the concentrations in the ECS reached the CMC of the rhamnolipids causing the formation of micelles. The mass transfer of rhamnolipids was
slower with increasing concentrations because micelles could not be easily transferred through the fibers.

Figure 7.9. Rhamnolipid concentration measured in both the reservoir and the ECS of the hollow fiber immobilized set-up under denitrifying conditions.

The measured rhamnolipid concentration was close to 5 g/L. Considering the rhamnolipids that were taken out from the system with each sample (which was a significant amount considering that the ECS had a small volume) and adding those to the measured concentration, the total rhamnolipid production could be estimated. This profile is shown in Figure 7.10 in which the rhamnolipid concentration was estimated to be close to 8 g/L. Additionally, from the profile, high nitrite levels did not appear to have a
negative effect on the rhamnolipid production (at 1000 hours, nitrite concentration was as high as 1000 mg/L NO₂⁻-N).

Figure 7.10. Total rhamnolipid production obtained in the ECS of the hollow fiber immobilized system considering the rhamnolipids harvested with each sample.

7.4. Conclusions

In contrast to the dialysis tube, the hollow fiber immobilized set-up effectively separated the cells from the medium. For the duration of the run no cell growth was observed in the medium reservoir side of the system even when all the nutrients were present in the initial 600 hours. Additionally, the high nitrite levels did not affect the cells or the rhamnolipid production. It was also found that the nitrite accumulation was most likely due to oxygen presence in the cell side because of the low volume in this part of
the system. Once the volume was increased and the surface aeration stopped, the nitrite levels decreased to zero. The P-free medium as well as the medium changes that probably removed other inhibitory products allowed a continuous rhamnolipid production using this immobilized system.

Pigments were clearly produced in large quantities during the experiment. Their production (or the production of other products) was probably responsible for the large amounts of nitrate and glycerol consumed during the process. With this set-up these products were not only diluted from the ECS into the larger volume of medium in the reservoir but also removed when the medium was replaced preventing any inhibitory effects. In addition, rhamnolipids also diffusing through the fibers into the circulating medium could be harvested without disturbing the hollow fiber cartridge. The cartridge can be potentially reused many times for continuous rhamnolipid production.
8.1. Summary and conclusions

The large scale production of the biosurfactant rhamnolipids still involves many challenges. In this study two main obstacles were identified and addressed. The first was the lack of a simple and rapid method to quantify rhamnolipids in liquid fermentation samples. The second was related to the foaming difficulties that eventually cause high production costs and low product yields in the typical aerobic fermentation approach. Accordingly, this research was focused on the development of a new method for the quantitative determination of rhamnolipids in liquid fermentation samples and on the improved production of rhamnolipids under denitrifying conditions. Additionally, the use of glycerol as carbon source was investigated for the production of this high-valued biosurfactant using this denitrification strategy considering that glycerol is nowadays an inexpensive and abundant substrate.

A semi-quantitative method using agar plates containing methylene blue (MB) and CTAB was proposed earlier by Siegmund and Wagner [30]. On such agar plates, the colonies producing rhamnolipids were surrounded by dark blue halos and, ideally, the areas of the halos could be correlated with the rhamnolipid concentrations [30]. Being
interested in selecting rhamnolipids-productive strains and in evaluating the effects of
different culture conditions, the above method was employed in the early phase of this
study. It was found that many *P. aeruginosa* strains produced pigments that also
surrounded the colonies and made it very difficult to correlate quantitatively the halo
areas with the rhamnolipid concentrations. When the method was evaluated for use in this
laboratory, it was also found that the edges of the blue halos were not always clearly
distinguishable and shifted depending on the intensity and location of the light source as
well as the background color. In the first part of the project, the methylene blue/CTAB
agar plate method was revisited for improved understanding and application. A set-up
including fixed underneath illumination (such as that of a dissecting microscope or a UV
transilluminator and a common image analysis software) was proposed as an alternative
for improved determination of the areas corresponding to the rhamnolipid complexation
with MB/CTAB. Additionally, for different applications and with this modified set-up,
the CTAB concentration could be adjusted, particularly to lower concentrations
according to the amounts of rhamnolipids to be detected. Low local pH conditions were
also found not suitable when using methylene blue/CTAB agar plates as the
complexation did not occur at pH 5 or lower. This improved semi-quantitative method
was effectively applied for selecting the strains used for further fermentation experiments
to advance the denitrifying rhamnolipid production strategy. Four (i.e. *P. aeruginosa*
PAO1, E03-40, E03-36, and H05-25) of the 10 different glycerol-utilizing *P. aeruginosa*
strains isolated from soil samples of a biodiesel production plant were found to be good
rhamnolipid producers without excretion of considerable amounts of pigments.
The procedure with agar plates was found to be useful to differentiate between producers and non-producers strains. However, when comparing among rhamnolipid producer strains, it was very difficult to accurately compare and select among strains producing similar amounts of rhamnolipid. In view of this fact, the principle behind the complexation by ion-pairing between anionic rhamnolipids and cationic methylene blue and CTAB on agar plates was further studied in order to develop a simple method directly applicable to liquid cultures. The methylene blue method developed in this work is a rapid and simple procedure that allows the quantification of rhamnolipids throughout the cultivation period to study the changes in the production with time. Additionally, this method is easier to apply than the other colorimetric methods commonly used to determine the sugar moiety of the rhamnolipid molecule. It is carried out at room temperature and does not involve lengthy drying, redissolution, or reaction. More importantly, it avoids the use of hazardous concentrated sulfuric acid in the procedure. The methylene blue method was verified with the results obtained from the commonly used anthrone reaction technique and was effectively applied to the analysis of batch denitrifying fermentation samples. Compared with the original agar plate method, this procedure allows easier and more accurate quantification of rhamnolipids in liquid samples. These characteristics are considered to be very helpful for the development of an improved fermentation process for industrial rhamnolipid production.

The alternative fermentation approach for rhamnolipid production investigated in this study was based on the ability of *P. aeruginosa* to perform denitrification. This approach was found to completely eliminate the problems associated with the severe foaming nature of the rhamnolipid broth by using nitrate as an electron acceptor instead
of oxygen, commonly used in the aerobic approach. Successful cell growth and rhamnolipid production were obtained using glycerol as substrate under denitrifying fermentation conditions with different nitrate addition approaches. The cell concentrations attained were similar to those generally obtained under aerobic conditions for rhamnolipid production. Additionally, the rhamnolipid concentrations were also comparable or higher to previous reports relying on oxygen as electron acceptor and glycerol as carbon source. The online NAD(P)H fluorescence monitoring allowed a more accurate supply of the nitrate required by the cells for respiration via denitrification. As a result, higher cell concentrations were achieved.

When trying to increase the cell concentration to reach higher rhamnolipid productivity, further cell growth was found to be inhibited by a compound produced under denitrifying conditions. Another difficulty associated with the process was the high glycerol consumption rate that considerably affected the overall yield of the denitrifying fermentations. Using SEC and TGA, glycerol was found to promote the production of a large amount of organic compounds besides rhamnolipids that could also be responsible for the inhibitory behavior. Preliminary identification of these compounds suggested that the majority of the byproducts were not extracellular proteins, alginate, or carbohydrates.

Other findings from the denitrifying fermentations were the detrimental effect of high osmotic pressures on the culture that was solved by adding the osmoprotectant betaine to the denitrifying fermentations. Also, the effectiveness of ammonium as nitrogen source for growth was also identified. However, rhamnolipid production was found not to initiate until the concentration of ammonium was very low. Finally, the use
of rich media, containing yeast extract and peptone, did not have a significant effect on the cell concentrations attained.

As a comparison to the free cell fermentations, immobilized systems were also explored in this study using nitrate as electron acceptor. Two different approaches were investigated: a dialysis membrane and a hollow fiber immobilized systems. Even though the former set-up allowed the production of rhamnolipids, the accumulation of gases within the membrane and the leakage of the cells to the outer medium prevented its use for continuous production. In contrast, the hollow fiber immobilized set-up effectively separated the cells from the medium. For the duration of the run no cell growth was observed in the medium reservoir side of the system even when all the nutrients where present. In spite of nitrite accumulation, the high levels did not affect the cells or the rhamnolipid production. Nitrite accumulation was most likely due to oxygen presence in the cell recirculation side. Once the volume was increased and the surface aeration stopped, the nitrite levels decreased to zero.

Rhamnolipids were found to be continuously produced when the phosphorus limitation was introduced into the immobilized system. Pigments were also produced in large quantities during the experiment. Their production (or the production of other products) was probably responsible for the large amounts of nitrate and glycerol consumed during the process. With this set-up these products were not only diluted from the ECS into the larger volume of medium in the reservoir but also removed when the medium was replaced preventing any inhibitory effects. In addition, rhamnolipids also diffusing through the fibers into the circulating medium could be harvested without
disturbing the hollow fiber cartridge. The cartridge can be potentially reused many times for continuous rhamnolipid production.

The immobilized hollow fiber set-up approach based on denitrification eliminated the common oxygen transfer limitation problems associated with immobilized rhamnolipid systems and was found suitable for a continuous rhamnolipid production using glycerol as carbon source.

8.2. Recommendations for future work

Although the denitrifying fermentation approach was substantially improved in this study, the cell concentrations required for large scale rhamnolipid production still need to be increased. Further characterization on the denitrifying samples must be carried out to determine the possible byproducts and their potential inhibition to cell growth and/or rhamnolipid production. By removing any inhibitory compounds accumulating in the batch fermentations, much higher cell concentrations can be attained using denitrifying conditions. This would increase the volumetric productivity and allow the use of the denitrifying fermentation technology for more economical rhamnolipid production.

Additionally, the product yield could also be increased by finding ways to reduce the amount of glycerol consumed by the cells. Considering that the cells grew faster during the first hours of the fermentations in which microaerobic conditions were likely present, a microaerobic-denitrifying condition, instead of totally anaerobic condition, could be investigated to improve the yield of the process.
Finally, the immobilized hollow fiber set-up can be used to optimize the medium composition for improved rhamnolipid production under denitrifying conditions. At the same time, different methods for continuously harvesting the rhamnolipids from the medium reservoir can be tested in the immobilized system taking advantage of the physical separation of the cells from the medium that this set-up provides.
REFERENCES


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APPENDIX A

pH PROFILE OBSERVED IN AN EXPERIMENT MADE WITH THE DIALYSIS TUBE IMMOBILIZED SYSTEM

Figure A.1. pH profile obtained with the dialysis membrane immobilized system set-up under denitrifying conditions after the last medium change.
APPENDIX B

pH Profiles Observed in the Experiment Made with the Hollow Fiber Immobilized System

Figure B.1. pH profile of hollow fiber immobilized system before P limitation was introduced.
Figure B.2. pH profile of hollow fiber immobilized system during change to P limitation medium.

Figure B.3. pH profile hollow fiber immobilized system during final change to P limitation medium.