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Rhamnolipid CMC prediction

Kłosowska-Chomiczewska I. E.[a], Mędrzycka K.[a], Hallmann E.[a], Karpenko E.[b], Pokynbroda T.[b], Macierzanka A.[a], Jungnickel C.[a*]

[a] Department of Colloid and Lipid Science, Faculty of Chemistry, Gdansk University of Technology, Narutowicza St. 11/12, 80-233 Gdansk, Poland

[b] Department of Physical Chemistry of Fossil Fuels InPOCC, National Academy of Sciences of Ukraine, 3a Naukova St., Lviv, 79053, Ukraine

*Corresponding author: christian.jungnickel@pg.gda.pl, tel: +4858 347 2469

Abstract

Relationships between the purity, pH, hydrophobicity ($log K_{ow}$) of the carbon substrate, and the critical micelle concentration (CMC) of rhamnolipid type biosurfactants (RL) were investigated using a quantitative structure–property relationship (QSPR) approach and are presented here for the first time. Measured and literature CMC values of 97 RLs, representing biosurfactants at different stages of purification, were considered. An arbitrary scale for RLs purity was proposed and used in the modelling. A modified evolutionary algorithm was used to create clusters of equations to optimally describe the relationship between CMC and $log K_{ow}$, pH and purity (the optimal equation had an R^2 of 0.8366). It was found that hydrophobicity of the carbon substrate used for the biosynthesis of the RL had the most significant influence on the final CMC of the RL. Purity of the RLs was also found to have a significant impact, where generally the less pure the RL the higher the CMC. These results were in accordance with our experimental data. Therefore, our model equation may be used for controlling the biosynthesis of biosurfactants with properties targeted for specific applications.

Keywords

biosurfactant, rhamnolipid, CMC, purity, prediction, QSPR

Abbreviations list

CMC	critical micelle concentration
di-RL	dirhamnolipid
JBR	JBR 425, purified rhamnolipid produced by Jeneil Biosurfactant Co.
mono-RL	monorhamnolipid
RBC	rhamnolipid biocomplex
RL	rhamnolipid

1. Introduction

Rhamnolipids (RLs) are the product of biosynthesis of a variety of microorganisms such as *Pseudomonas* [1, 2], but also *Burkholderia [3], Streptomyces* [4, 5], *Acinetobacter* [6], and *Enterobacter* [6], and are usually comprised of a mixture of mono-RLs and di-RLs as shown in Figure 1. RLs have found application in a wide variety of processes, in which they display marked advantages over other surfactants, in that they are biodegradable, active in a wide range of conditions, and no additional cost is required for their disposal. For example, RLs may be applied in the cleaning of oil storage tanks and recovering hydrocarbons from the removed sediments [7]. RLs are efficient demulsifiers, and thereby recover over 98% of crude oil from refractory wastes, both in laboratory scale and pilot plants [8]. RLs are also efficient in washing out up to 95% of synthetic oil from sand [9]. Additionally, RLs have been approved by the Environmental Protection Agency to be applied on

horticultural and agricultural crops as an effective biofungicide [10]. They also find use in improving germination and seedling development; where by treating seeds with RL solutions the rate of germination increased by up to 75% [11].

However, their application is often limited by their availability and cost, since the purification of the RL is extensive [12]. This is due to the fact that RLs are synthesized by microorganisms, as a mixture of lipids and other metabolites such as polysaccharides [13], diketopiperazines, phenazine alkaloid antibiotics [14], and 3-(3-hydroxyalkanoyloxy)-alkanoic acids [15], which need to be removed from the mixture. Purification, therefore, and its exact methodology is an important factor influencing the quality and properties of the final product.

The purification of RLs is done either by *in situ* product removal or batch-wise, after cultivation. For the *in situ* product removal, membrane techniques and foam fractionation are often applied [16]. The batch-wise purification usually consists of the precipitation of a cell-free broth under acidic conditions (pH 2-3) and consequent solvent extraction with ethyl-acetate [17, 18] or mixtures of chloroform and methanol [19-22] for example. For further purification, preparative chromatography in silica gel-filled columns is often used with a variety of organic solvents [17, 18, 21-23]. Thus, RL use is heavily reliant on various solvent extraction techniques. To this end, a number of ideas were proposed to reduce the use of solvents and limit the number of the purification steps. One such idea is the use of GMO. In this case, bacteria or fungi are modified to yield a higher quantity of RL, and thus require less solvent per given amount of RL produced. An example of this is the mutagenesis of *Pseudomonas aeruginosa* MR01 which increased biosurfactant production by more than 1.5-fold [24].

The production of RLs (as opposed to traditional surfactants) has the added benefit, that cheap or waste materials may be used as carbon substrates for the growth of RL producing bacteria. The application of RLs therefore has a doubly positive effect, since the biosurfactant biosynthesis results in the production of a valuable product, as well as the utilization of waste such as cheese whey waste, used canola oil, and corn steep liquor [17, 25].

It is worth pointing out that literature has not reached a consensus on terms such as crude, unpurified and purified biosurfactants. In fact, a number of papers [26, 27] discuss the use of "crude" RLs, which have been purified by solvent extraction and chromatographic separation. Therefore, for the sake of consistency we have created a simple purity scale, which will be discussed later.

The exact nature of the RL produced depends on a number of factors including strain type, substrate, and purification technique used. When the RL is applied to a specific scenario the physicochemical properties of the product should be well characterized [28]. Since the properties are largely dependent on the above mentioned factors, knowledge of these properties is only possible if they are measured specifically for each scenario. To this end, hundreds of papers exist in which researchers usually synthesize, purify and then measure the properties of RLs [6, 20, 21, 29, 30]. We want to show here a method of simplifying this issue, by presenting a method to predict one of these physicochemical properties, in this case the CMC. A number of successful attempts of quantitative CMC modelling was described in literature. However, they concern mostly synthetic surfactants alone [31-35] or in mixtures [36-38], and there are no models for predicting CMC of biosurfactants described. Understanding the different factors influencing the CMC is crucial for predicting properties and designing biosynthesis of biosurfactants targeted for specified applications. Understanding the different factors influencing the CMC is crucial for predicting properties and designing biosynthesis of biosurfactants targeted for specified

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applications. Generally, CMC is dependent on pH, temperature, ionic strength and surfactant structure. As the RLs are synthesized in an ionic medium, pH will have most significant influence on CMC among the above mentioned parameters.

Therefore, for the purpose of this paper, we have analyzed a number of RLs of different purity and produced from different substrates. We have attempted to quantify and justify these differences. We have also proposed an arbitrary scale for the RLs purity classification since it is a very cloudy issue, and descriptions found in many publications are inconsistent. Finally, we have attempted to predict the CMC of RLs based on their purity, the pH conditions and the character of the carbon substrate used for their production.

2. Materials and Methods

2.1 Rhamnolipids

Microbial synthesis of the biosurfactant was conducted using the *Pseudomonas* sp. PS-17 strain (kindly provided by the L.M. Litvinenko Institute of Physical Organic and Coal Chemistry, National Academy of Sciences of Ukraine). The strain synthesizes homologous extracellular rhamnolipids and extracellular biopolymer (acidic polysaccharide such as alginic acid) with an optimized liquid medium using glycerol (30 g/L) as a carbon source. RLs and biopolymer form a surface-active complex known as a rhamnolipid biocomplex (RBC) [39, 40]. The RBC was isolated from the cell-free cultural broth via acid precipitation (10% HCl to pH 3) and purified by re-precipitation. The content of RLs in RBC was determined spectrophotometrically (UVmini – 1240, Shimadzu, Japan) using the orcinol method [41]. The RLs were then isolated by extraction with the Folch mixture (chloroform-methanol 2:1) from the RBC which was further separated and analyzed using TLC [41].

The 25% neutral aqueous solution of di-RL:mono-RL ratio was 0.97:1 (JBR 425 – lot. No. 040714) which was kindly provided by the Jeneil Biosurfactant Company (Saukwille, WI, USA). The structural formulas of the pure components mono-RL [α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydrohydecanoate] and di-RL [2-O-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydrohydecanoate] are shown in Figure 1. The JBR molecular weight of mono-RL is 504 g mol^{-1} and that of di-RL is 650 g mol^{-1}. The RBC and JBR solutions were prepared by serial dilution.

Figure 1 about here

2.2 *Pendant drop shape analysis*

Equilibrium interfacial tension measurements were performed in buffered biosurfactant solutions, as RLs have good tolerance to ionic strength [42, 43] and the effect of pH is more significant (thus influences the adsorption parameters CMC) [43, 44]. The buffer, boric acid (> 99.5%, Sigma Aldrich, Germany), phosphoric acid (85% p.a., Chempur, Poland) and acetic acid (99.5-99.9% p.a., POCH, Poland) each at concentrations of 0.04 M, had a pH which was adjusted by titration with 0.2M NaOH (POCH, Poland) solution. Biosurfactant solutions were analyzed for equilibrium surface and interfacial tension using the pendant drop shape technique (DSA 10 analyzer, Krüss GmbH, Germany).The pendant drop shape analysis of RBC and JBR 425 solutions at different pH yielded plots of surface tension versus biosurfactant concentration. From the plots we calculated parameters such as CMC, interfacial tension at CMC (σ_{cmc}), Gibbs surface excess Γ_{max} and minimal area of

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interface per surfactant molecule A_{min}. The position of the breakpoint indicating CMC was determined using Phillips method [45]. According to Phillips the CMC is defined as a concentration corresponding to the maximum change in a gradient of the measured property versus concentration plot [46] as shown in the equation (1):

$$\left(\frac{d^3\phi}{dC_T^3}\right)_{C_T=CMC} = 0 \qquad \text{Eq. 1}$$

where C_T is the total concentration of the biosurfactant, ϕ is a parameter quantifying the property used to determine CMC and is represented as follows:

$$\phi = a[C_s] + b[C_m] \qquad \text{Eq. 2}$$

where a and b are constants, C_s and C_m are concentrations of the monomeric biosurfactant or micelle, respectively. CMC was determined by computing the second derivative of the local polynomial fit, and finding the maximum. The calculations of the local polynomial fit and its derivatives were done in R statistical software [47] package with the locpoly function from the KernSmooth library [48]. All analyses were performed in quadruplicate at 25°C. Doubly-distilled water (ROpureST/NANOpure system, Barnstead, USA) was used for instrument calibration. The purity of water was additionally controlled by the surface tension measurements before solution preparation.

2.3 Computational Modelling

For the computational modelling Eureqa Pro v1.24.0 (build 9367) [49] was used. Eureqa uses a modified evolutionary algorithm to generate a cluster of equations for a given target expression y = f(x) (in our case *e.g.* logCMC = $f(log K_{OW})$) [50].The software was allowed to run for each target expression for a minimum of $5x10^9$ generations. In each generation, the software would attempt to develop an equation to optimize the coefficient of determination R^2. For training and validation 100% of the data was used. Only basic mathematical operators were chosen for the correlation, and the correlation had to be as accurate as possible with minimal complexity. The optimal equation was chosen as the one with the lowest complexity, and highest coefficient of determination R^2, which can otherwise be described as a breakpoint in a graph depicting R^2 and complexity, as shown in Figure 2.

Figure 2 about here

The complexity of solution is a measure of complexity of mathematical building blocks found in the equation, and it is determined as the sum of complexities of operators that comprise the equation [50]. Within every model equation presented here a relative impact (sensitivity, S) that each variable has on the CMC was calculated with Eureqa Pro using equation (3):

$$S = \overline{\left|\frac{\partial CMC}{\partial x}\right|} \cdot \frac{SD(x)}{SD(CMC)} \qquad \text{Eq. 3}$$

where S is the sensitivity (evaluated at all input data points), x is any variable taken into account ($log K_{OW}$, pH or purity), $\overline{\left|\frac{\partial CMC}{\partial x}\right|}$ is the mean, absolute value of the partial derivative of CMC with respect to x, and $SD(x)$ is the standard deviation of the variable x. The sensitivity provides an insight into the impact of the input variables on the output variable. Additionally, the percentage of data points which provide a positive or negative contribution

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to the output CMC may be determined. These are the percent of data points where $\frac{\partial CMC}{\partial x} > 0$ for positive contribution, and $\frac{\partial CMC}{\partial x} < 0$ for negative contribution. Magnitude provides information of the sensitivity of the positive and negative contribution separately.

The octanol-water partition coefficient ($log K_{ow}$) of the substrate for biosynthesis was determined by molinspiration [51]. Since many of the oils used as substrates consist of a mixture of compounds, the three major components were used for the calculation (Table S1).

3. Results and Discussion

3.1 Biosurfactant composition

The composition of the RBC synthesized for this paper is presented in Table 1. The biosurfactant contained 79-80% RLs, 20-21% alginic acid, and traces of protein. The analysis of the lipid fraction gave an di-RL:mono-RL ratio of 10:1.

Table 1 about here

When analyzing literature of RLs, a vast plethora of terms are used to describe their purity. For example RL crude extract [52], RL supernatant [52], crude RLs solution [53], RL organic crude extract [43], crude biosurfactant [26, 53], or standard RL [54] are often used expression. None of these terms provide any reliable way of assessing purity. In addition, these terms are not even consistently used. For example, the term ”crude biosurfactant” sometimes refers to a culture-free broth containing 35 g/L RLs [53], and another time it refers to RLs purified by acidic precipitation and extracted with ethyl acetate, composed of 50% mono-RL and 50% di-RL [27] or even a mixture of 11 different RLs [26]. The inconsistency occurs not only between different publications but may also be found within the same publication. For example, one paper described a method to obtain “purified biosurfactant”, and later the results for “crude biosurfactant extract solutions” were given with no further explanation [55]. To avoid and solve these inconsistencies we decided to systematize the nomenclature, and thus we prepared a classification of RL purity, which is presented in Table 2. Such an arbitrary scale describes purity of biosurfactant in a more sophisticated way than just giving the content of RLs (% or g/L), since the yield of RL depends on a number of other factors, such as carbon substrate, microorganism strain and so on, and moreover can be given at every purification step, either for supernatant, or for pure RL solution.

Table 2 about here

The RBC analyzed for the purpose of this publication is a representative of the purity 3, of which only a few instances were described in the literature [56-60].

3.2 Equilibrium interfacial tensions and pH dependence

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The results of the RBC (purity 3) and JBR 425 RLs (purity 5) surface tension measurements are shown in Figure S1, and the numerical values are given in Table 3.

Table 3 about here

As shown in Table 3 from our own measurements (RBC and JBR) and literature sources, the CMC is dependent on pH. As the carboxylic acid dissociates the charge on the RL increases, thereby increasing repulsion between the molecules, and thus inhibiting micelle formation. At low pH the RL is fully protonated, and thus behaves like a non-ionic surfactant, with a resulting low CMC. This very general trend may only be observed for the purity 5 RLs (Figure 3A). However, these pH dependent changes disappear as the purity decreases (as shown in Figure 3B). As one may expect with decreasing purity the number of "junk" molecules such as polysaccharides (including in our example, alginic acid), increases. These junk molecules, offer alternate interaction sites for the RLs, therefore decreasing their effective concentration in solution. Thus, it would be expected that JBR should have a lower CMC than RBC, which at pH 7 is the case, with CMCs of 41.5 and 62.1 mg/L respectively. At pH 9 the CMC for both was almost the same due to dissociation of the acidic polysaccharides as these also develop a negative charge, and the RLs no longer interact with them.

Figure 3 about here

The Gibbs surface excess concentration, Γ_{max}, and the surface area per surfactant molecule, A_{min}, were also calculated for the RBC and JBR biosurfactants, using the Gibbs equation (4):

$$\Gamma_{max} = \frac{-1}{RT \cdot 2.303n}\left(\frac{\partial \gamma}{\partial \log C}\right) = \frac{1}{A_{min} \cdot N_A} \qquad \text{Eq. 4}$$

where R is the gas constant, T is temperature, n is a constant which depends on the number of species constituting the surfactant and which are adsorbed at the interface, A_{min} is the head group area per surfactant molecule and N_A is the Avogadro's number.

The A_{min} values have been calculated using $n = 2$ for both, RBC and JBR biosurfactants [61, 99]. The values of about 75Å^2 have been obtained for RBC biosurfactant at both pH values and 70 and 104 Å^2 for JBR at pH 7 and 9, respectively, as shown in Table 3. The surface area A_{min} for RBC was a little larger than the corresponding value obtained for JBR RLs at pH 7. At the same time it was very similar to the values reported for RLs previously, which were in the range of 57-135 Å^2 [44, 61-63, 100, 101]. This suggests that the RBC RLs (purity 3) surface monolayer is as closely packed as a monolayer of JBR RLs (purity 5) at pH 7. However, at pH 9 JBR RLs formed a monolayer which was rather loosely packed since A_{min} was higher (104 Å^2 as in Table 3). This might be due to a stronger repulsive interaction between the anionic head groups of the RLs. This repulsion seemed to be weaker in RBC since no changes in monolayer packing was observed, which is probably due to the presence of alginic acid and other compounds adjacent to the RLs.

3.4 Octanol-water partition coefficients of substrate

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When analyzing Table 3, we can see that the CMCs of the RL are in fact vastly different, ranging from 6.5 mg/L to 400 mg/L. This range of CMCs of the same RLs is due to two aspects. First, it is due to the type and quantity of impurities which affect the CMC, and secondly to the composition of the RLs, which in turn depend on the different carbon substrates that the various bacteria metabolize. Rikalovic *et al.* stated that the number of unsaturated bonds, branching and length of the alkyl chain of the substrate can affect the CMC values of RLs [75]. RLs with more hydrophobic (*e.g* .saturated) fatty acids form micelles at a lower concentrations [6]. Can we expect therefore that a more hydrophobic substrate will result in a more hydrophobic RL? Yes we can; Nitschke *et al.* showed that when *P. aeruginosa* LBI grows on hydrophilic substrates such as glycerol and glucose, the di-RL predominates (higher CMC), whereas for the hydrophobic carbon sources the mono-RL (lower CMC) is the predominant homologue present in the RLs mixture [65].

To verify this hypothesis, a relationship of $log K_{ow,}$ of the feedstock and CMC was expected. However, a clear relationship was not observed at any purities, as shown in Figure 4. The absence of a clear trend however may not necessarily indicate that one does not exist, but simply that the magnitude of the effect of the $log K_{OW}$ of the carbon substrate alone is not enough. For that reason other variables need to be considered.

Figure 4 about here

3.5 Prediction of the CMC of RLs

Given the number of variables that influence the CMC, successful prediction has significant scientific importance. In addition, the prediction would also enable us to calculate the magnitude of effect that each variable has. Our aim was to create a technologically relevant correlation, in which we assumed that many bacteria utilize similar metabolic pathways, and thus similar metabolites may be expected using the same feed material, thus allowing us for the purpose of this paper to ignore the microorganism type.

The data of Table 3 was used for the creation of the correlation. The carbon substrate, pH, CMC, surface tension at CMC, Gibbs surface excess, and minimal area per RL headgroup are given when provided by the authors. The discontinuous data sets were ignored. The purity classification was used as given in Table 2.

If we exclude the purity as a variable, the first equation clusters generated by Eureqa were for purity 2 and 5. For purity 1, 3 and 4 no separate equation clusters could be generated because not enough data points were available. The target expressions for those clusters are given in Table 4.

Table 4 about here

As can be seen from Table 4, both purity 2 and 5 equation clusters provided very satisfying fits (equations and graphs of complexity vs R^2 for the equation clusters are shown in the Supplementary Information in Figure S2 and Table S2, and Figure S3 and Table S3 for purity 2 and 5 respectively), with R^2 close to unity. If all the available data was included, over all purities, surprisingly the R^2 dropped significantly to 0.3420. The equations and graphs of complexity vs R^2 are shown in Figure S4 and Table S4. To explain this phenomenon, we concluded that the effect of the purity is on each variable independently. That is, each variable has a different

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magnitude of effect on the CMC at various purities. This is visible when the sensitivity of each variable at different purities is compared, as shown in Table 5.

Table 5 about here

For purity 2, the pH did not even appear in the equation with highest parsimony. This is due to the large number of junk molecules in the system and their influence being greater than the state of association/dissociation of the RL molecules. The $log K_{ow}$ is the deciding factor for predicting the CMC of the RL, as it influences the number of rhamnose units on the RL (mono-RL or di-RL), and the length of the lipid chain, which are influenced by the carbon substrate. At purity 5 this effect is even more pronounced as the number of junk molecules is removed. Since the RL is almost pure, the subtle effects of pH becomes visible, as reflected by the small magnitude of the effect of pH. Thus the effect that each variable has on the target value must depend on the purity.

To improve the accuracy of the predictions, the purity variable was included in the target expression. This resulted in an optimal equation with an R^2 of 0.7326. It represented a definitive improvement, however logically inconsistent since the purity, as an arbitrary magnitude, has differing effects on $log K_{ow}$ and pH as explained above. Therefore, we proposed a target expression that used nested functions within the target expression to imply that the effect of purity on each variable pH and $log K_{ow}$ is independent and orthogonal. In this case, the R^2 resulting from the optimal equation was 0.8366, and the relation is shown in Figure 5.

Figure 5 about here

The sensitivity of the variable of this final expression (Table 6) paints a clear picture, as to the processes and factors governing the micelle formation phenomenon of RLs at different purities.

Table 6 about here

The $log K_{ow}$ has a strongly positive effect, just as before, indicating that the feed material is one of the main variables to influence the CMC. The rhamnose units generate steric hindrance in the micelle formation process. The longer lipid units on the other hand encourage the micellization phenomenon. The effect of the pH is as previously explained, the higher the pH the more dissociated the RL becomes, and thus the more negative, which in turn would inhibit micellization. Purity of the RL has a clear effect on the CMC of the RL, that is, the less pure the RL the higher the CMC. Our experimental results, as shown in Table 3, are in agreement with these findings.

4. Conclusions

The interfacial properties of non-purified RLs from *Pseudomonas* sp. PS-17 were described here for the first time. When we compared our data to literature we noticed that a wide spectrum of purities of RLs is reported [26, 43, 52-54]. Even though a lot of research exists that create and measure the properties of RLs [6, 21, 30], it is difficult to draw concrete conclusions or comparisons from the data due to a lack of consistency. We

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therefore, would like to highlight the need for more stringent characterization and reporting of the biosurfactants before publication. In order to quantify the purity we assigned and presented here for the first time an arbitrary scale of RLs purity, which may facilitate this.

Moreover, we have presented a method to quantitatively predict the CMC of RLs. A number of models exist to predict CMC in literature however, they concern mostly synthetic surfactants [31-35] or their mixtures [36-38]. Apart from our attempt, no models for predicting CMC of biosurfactants are described. In our case the modelling was conducted using a modified evolutionary algorithm. We attempted to use the $log K_{ow}$ of the substrate, pH of solution, and purity of biosurfactant as variables. The use of the purity, in the modelling and subsequent prediction, revealed that the purity has a significant and independent effect on pH and $log K_{ow}$, and these in turn influence the CMC. To separate the purity effects on both variables, we applied nested target equations. The final equation provided new insights into the influence of these parameters on the CMC formation process in biosurfactant solutions. This approach enables the improvement of the biosurfactant technologies by allowing to design target-specific RLs by controlling their biosynthesis. This paper presents a pioneering study, on predicting CMC of RLs, and is expected to play significant role in biosurfactant property modelling.

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