Mechanisms for rhamnolipids-mediated biodegradation of hydrophobic organic compounds

Zhuotong Zeng [a, 1], Yang Liu [b, 1], Hua Zhong [b, c, 1] Rong Xiao[a,1], Guangming Zeng [a,b,*], Zhifeng Liu [b], Min Cheng [b], Cui Lai [b], Chen Zhang [b], Guansheng Liu [b], Lei Qin [b]

[a] *Department of Dermatology, Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, P. R. China*

[b] *College of Environmental Science and Engineering, Hunan University and Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, P.R. China*

[c] *State Key Laboratory of Water Resources and Hydropower Engineering Science, Wuhan University, Wuhan 430070, China*

∗ Corresponding authors: Tel.: +86–731–88822754; fax: +86–731–88823701. Email address: zgming@hnu.edu.cn (Guangming Zeng) and xiaorong65@csu.edu.cn (Rong Xiao).

ABSTRACT

The widespread existence of hydrophobic organic compounds (HOCs) in soil and water poses a potential health hazard to human, such as skin diseases, heart diseases, carcinogenesis, etc. Surfactant-enhanced bioremediation has been regarded as one of the most viable technologies to treat HOCs contaminated soil and groundwater. As a biosurfactant that has been intensively studied, rhamnolipids has shown to enhance biodegradation of HOCs in soils, however, the underlying mechanisms are not fully disclosed. In this paper, properties and production of rhamnolipids are summarized. Then effects of rhamnolipids on the biodegradation of HOCs, including solubilization, altering cell affinity to HOCs, and facilitating microbial uptake are reviewed in detail. Special attention is paid to how rhamnolipids changes the bioavailability of HOCs, which are crucial for understanding the mechanism of rhamnolipids-mediated biodegradation. The biodegradation and toxicity of rhamnolipids are also discussed. Finally, perspectives and future research directions are proposed. This review adds insight to rhamnolipids-enhanced biodegradation process, and helps in application of rhamnolipids in bioremediation.

Keywords: Rhamnolipids; Hydrophobic organic compounds; Microorganism; Biodegradation; Bioremediation

Contents

1. Introduction

The anthropogenic environmental pollution by hydrophobic organic compounds (HOCs) is well documented (Luo et al., 2014; Wang et al., 2010). The widespread existence of HOCs in soil and water causes serious problems to ecosystem and human health, and thus has drawn increasing attentions (Cheng et al., 2016; Lee et al., 2014; Lin and Gan, 2011). It is reported that these compounds are carcinogenetic and teratogenetic, and could cause allergy, skin diseases, heart diseases, etc., after long-term exposure (Cheng et al., 2018; Xiong et al., 2018; Daifullah and Girgis, 2003; Štandeker et al., 2007). The sound and effective techniques to treat HOCs contaminated sites have been proposed, and bioremediation is considered to have higher ecological significance and greater promise (Budd et al., 2009; Cheng et al., 2017b; Zhu et al., 2010). However, due to the hydrophobicity, most of HOCs either exist as non-aqueous phase liquids (NAPLs) or strongly adsorb onto soil matrix, which greatly decrease the bioremediation efficiency (de la Cueva et al., 2016; Ren et al., 2018).

Various studies have shown that the addition of surfactants facilitates removal of HOCs from contaminated soil and water (Mao et al., 2015; Trellu et al., 2016; Zhong et al., 2017). They are able to decrease the surface/interfacial tension of immiscible phase, increase the apparent solubility of HOCs, and thereby enhance the bioremediation (Cheng et al., 2017b; Zhang et al., 2015). Compared to chemical surfactants, biosurfactants have higher solubilizing ability towards hydrophobic pollutants (Barnadas-Rodríguez and Cladera, 2015; Yu et al., 2015). In addition, biosurfactants are more eco-friendly than most chemical synthetic surfactants (De et al., 2015; Yadav et al., 2016). As a result, biosurfactants have been promising alternatives in surfactant-based bioremediation (Zhong et al., 2017). Rhamnolipids, as a class of

anionic glycolipid biosurfactant, have attracted particular interest. They present the maximum number of patents and publications among biosurfactants. According to Müller et al. (2012), more than 200 patents were registered for biosurfactants until 2012, and 50% of them are related to rhamnolipids. At the end of 2017, the numbers of publications on rhamnolipids and biosurfactants have reached 2100 and 4500, respectively.

Rhamnolipids are the most extensively studied and used biosurfactant in bioremediation area (De et al., 2015; Kim et al., 2015). They are biodegradable, less toxic, and can be produced from renewable resources (Gudiña et al., 2015; Ramírez et al., 2015). Studies also suggested that rhamnolipids are as good or better than synthetic surfactants (e.g., Tween 80 and Triton X-100) in enhancing aqueous solubility of HOCs, such as alkanes (Kiran et al., 2016), polycyclic aromatic hydrocarbons (PAHs) (Mahanty et al., 2017), polychlorinated biphenyls (PCBs) (Chakraborty and Das, 2016), and pesticides (Singh et al., 2016). Moreover, it has been demonstrated that the presence of rhamnolipids could decrease the energy consumption of biodesulfurization by resting cells in biphasic O/W systems with hydrocarbon as the oil phase (Raheb et al., 2012). Due to these advantages, many studies have been performed on rhamnolipids-enhanced bioremediation in recent years (Lladó et al., 2012; Tahseen et al., 2016).

Some review papers (Bai et al., 2017; Hošková et al., 2013; Lamichhane et al., 2017; Shao et al., 2017) and few book chapters (Galabova et al., 2014; Leitermann et al., 2010) have summarized data on the application of rhamnolipids in bioremediation. To the best of our knowledge, however, these articles are mainly focused on biosynthesis and characteristics of rhamnolipids (Bai et al., 2017; Hošková et al., 2013), influence of rhamnolipids on microbial metabolism process (Shao et al., 2017),

or simply the remediation efficiency (Lamichhane et al., 2017). To date, a comprehensive overview on mechanisms for rhamnolipids to enhance biodegradation of HOCs from a microscopic view point of interactions between rhamnolipids, HOCs, and microorganisms, are still in scarce. However, such an overview is important to fill the knowledge gap and definitely required, and thus is the focus of this article.

2. Rhamnolipids

As a biosurfactant produced by *Pseudomonas aeruginosa,* rhamnolipids were first reported in 1949 (Jarvis and Johnson, 1949). They are composed of L-rhamnose and β-hydroxy fatty acids moieties (Kiran et al., 2016). Up to date, over 60 congeners and homologues of rhamnolipids have been reported in literatures (Kourmentza et al., 2018). They are different in the number of rhamnose rings, chain length, and the saturability of fatty acid moiety (Lovaglio et al., 2015). Four common rhamnolipid homologues are Rha-C_{10}-C_{10}, Rha-C_{10}, Rha_2-C_{10}-C_{10} and Rha_2-C_{10}, respectively (Liu et al., 2017).

It was reported that rhamnolipids can lower the interfacial tension of hexadecane/water from 43 to below 1 mN/m, decrease the surface tension of water from 72 to < 30 mN/m, and have critical micelle concentration (CMC) value in the range of 10 to 200 mg/L (Dubeau et al., 2009; Hörmann et al., 2010; Müller et al., 2012). CMC is an important characteristic for surfactants, defined as the concentration of surfactants at which micelles begin to form and corresponds to the point at which the surfactant achieves the lowest stable surface/interfacial tension (Santos et al., 2016). Surface activity of rhamnolipids can be maintained even under extreme conditions of temperature (able to withstand 90 °C up to 120 min, and even 120°C for 15 min) and pH (range from 3 to 11) (Hošková et al., 2015; Jackson et al.,

2015; Pornsunthorntawee et al., 2008). Patel et al. (1997a, 1997b) reported that the hydrophilic/lipophilic balance (HLB) is 13 for monorhamnolipid and 21 for dirhamnolipid, which are indicative of the strong emulsifying capacity. According to Lebrón-Paler et al. (2006), pKa values of Rha-C_{10}-C_{10} are 4.28 and 5.50, respectively, with the concentration below and above the CMC, suggesting rhamnolipids belong to weak acid, which is due to their terminal carboxylic group. In compare with the synthetic surfactants, rhamnolipids are more biocompatible, which enable them to be used as a carbon source supporting microbial growth (Galabova et al., 2014; Leitermann et al., 2010). In addition, rhamnolipids have a minimal toxic influence on aquatic microorganisms, plants, and indigenous microbial communities (Johann et al., 2016). Due to these properties, rhamnolipids are suitable for various industrial applications, such as wetting, solubilization, foaming, emulsification, detergents, phase dispersion, and lubrication (Lovaglio et al., 2015).

Bacteria of *Pseudomonas* genus are the main rhamnolipids-producing strains (De et al., 2015); however, many other species also have been found to produce rhamnolipids, e.g., *Pseudoxanthomonas sp.* (Nayak et al., 2009), *Acinetobacter calcoaceticus* (Hošková et al., 2013), *Burkholderia sp.* (Tavares et al., 2013) and *Streptomyces sp.* (Hošková et al., 2015). An overview of rhamnolipids producing bacteria is shown in Table 1.

Please insert Table 1

It has been reported that many microorganisms can utilize renewable resources to produce rhamnolipids (De et al., 2015; Prabu et al., 2015; Radzuan et al., 2017), for example, a *P. aeruginosa* can produce as much as 0.43 g/L of rhamnolipids when they grow on agro-industrial by-products (Radzuan et al., 2017). This is conducive to producing various homologs (Lovaglio et al., 2015; Ramírez et al., 2015).

152 The carbon, nitrogen and phosphorus source types have significant impacts on
153 the production of rhamnolipids (Hošková et al., 2015; Varjani and Upasani, 2016).
154 Rhamnolipids are generally produced under growth-limiting conditions, but
155 C-limitation was not included (Müller et al., 2012). P-limitation and N-limitation have
156 been mostly described for rhamnolipids production (De et al., 2015; Varjani and
157 Upasani, 2017). Interestingly, the replacement of nitrogen source, for example $NaNO_3$
158 instead of $(NH_4)_2SO_4$, could significantly enhance the total rhamnolipids
159 concentration (Hošková et al., 2013). In addition to N-limitation and P-limitation,
160 limitation of trace element salts and multivalent ions, such as Mg, Ca, K, and Na can
161 also improve the yield of rhamnolipids (Arora et al., 2016; Giraldiño et al., 2015). The
162 most important conditions influencing production of rhamnolipids by *P. aeruginosa*
163 have been discussed in detail by Müller et al. (2011). Several possible strategies are
164 proposed to optimize the production of rhamnolipids, including (a) process
165 optimization (Long et al., 2017); (b) screening for new non-pathogenic
166 rhamnolipids-producing strains (Zhao et al., 2015); (c) recombinant production of
167 rhamnolipids (Tiso et al., 2015); and (d) biocatalysis for customized rhamnolipids
168 glycolipids (Müller et al., 2012).
169
170 **3. Solubilization of HOCs**
171 *3.1 Solubilization mechanism*
172 Comparing with the bulk phase, the intermolecular forces of an interface are not
173 balanced because of excessive free energy, which is measured as interfacial tension
174 (Özdemir and Malayoglu, 2004; Prosser and Franses, 2001). Rhamnolipids are
175 composed of a hydrophilic head (one or two rhamnose molecules) and a hydrophobic
176 tail (one or two 3-hydroxy fatty acid chains) (Galabova et al., 2014). The addition of

177 rhamnolipids to a given solution will reduce the interfacial tension due to the
178 adsorption of rhamnolipids at liquid-air or liquid-liquid interface (Pacwa-Płociniczak
179 et al., 2011). Based on the classic surfactant aggregation theories, at concentrations
180 lower than CMC, surfactant molecules exist alone as monomers in aqueous phase, and
181 accumulate at the liquid-liquid or air-liquid interface (Ansari et al., 2013; Guo et al.,
182 2016). Once the surface adsorption of rhamnolipids reaches its threshold, the
183 monomers in the bulk phase start to form aggregates as the Gibbs energy required for
184 establishing non-polar chains in contact with water is higher than that of the repulsive
185 head group interactions (Rodrigues, 2015). Manko et al. (2014) systematically studied
186 the thermodynamic properties of rhamnolipid micellization and adsorption. The
187 maximal surface excess concentration of rhamnolipids at water–air interface was
188 determined as 2.01×10^{-6} mol/m^2 by using the Gibbs adsorption equation. The
189 corresponding minimal area occupied by one rhamnolipid molecule at the water–air
190 interface was measured as 82.6 Å^2. Physical rhamnolipids interactions with HOCs will
191 enhance their aqueous dispersion, which arises from hydrophobic interactions between
192 HOCs and rhamnolipid monomers below the CMC, or rhamnolipids encapsulation of
193 HOCs into micelle cores above the CMC (Hua et al., 2003; Zhang and Miller, 1994).
194 The process of partitioning HOCs into a micellar core is called solubilization.
195 For HOCs contaminated soil environment, the addition of rhamnolipids can be
196 expected to enhance bioremediation by desorption and solubilization of HOCs (Cheng
197 et al., 2017a). In generally, the hydrophilic head of rhamnolipids tends to enter into
198 the water and the hydrophobic tail is apt to combine with HOCs. At low
199 concentrations, the accumulation of rhamnolipid monomers at the soil-oil interface
200 would cause the repulsive force between solid phase and rhamnolipid hydrophilic
201 head, resulting in desorption of HOCs from soil (Cheng et al., 2017a; He et al., 2015).

As concentration increasing, the interfical tension would be decreased due to rhamnolipid molecules gradually occupying interficial sites (Santos et al., 2016). When rhamnolipid concentrations in the aqueous phase are above CMC, HOCs would be incorporated into hydrophobic cores of micelles through strong competetion between rhamnolipid micelles and soil particles (Lamichhane et al., 2017; Pacwa-Płociniczak et al., 2011). This solubilization facilitates the mobilization and availability of HOCs, which assists in the subsequent treatments.

It is generally accepted that solubilization is mainly caused by the formation of micelles when surfactant concentrations are above the CMC. However, several reports have suggested that solubilization activity of rhamnolipids to HOCs is excellent even at very low concentration. For example, in a recent study by Zhong et al. (2016) it showed that rhamnolipids could enhance the solubility of octadecane and hexadecane with concentrations both below and above the CMC, and the solubilization was more efficient at sub-CMC concentrations. Similarly, Singh et al. (2016) reported rhamnolipids could effectively enhance the aqueous phase solubility of chlorpyrifos at very low concentrations (below CMC).

It was hypothesized that the solubilization activity of rhamnolipids to HOCs is related to the aggregation behavior at low concentrations (Zhong et al., 2016). Studies have shown the concentrations at which rhamnolipids form aggregates, namely critical aggregation concentration (CAC), can be lower than CMC. Using dynamic lighter scattering method, Abbasi et al. (2013) observed the signs of aggregate formation in multi-component rhamnoliplids system with the concentrations below CMC. Recently, the results of cryo-transmission electron microscopy (cryo-TEM) and dynamic light scattering (DLS) further demonstrated the occurrence of dirhamnolipid aggregates when below CMC (Zhong et al., 2015).

227 The mechanism for rhamnolipids to enhance aqueous dispersion of HOCs can be
228 summarized as follows (Fig. 1). (i) Rhamnolipids monomers accumulate at the
229 interface between HOCs and aqueous phase. By reducing the interfacial tension they
230 decrease the repulsive forces between these two phases, and allow formation of micro
231 droplets. (ii) Rhamnolipids molecules form co-aggregates with some HOCs or form
232 micelles to incorporate HOCs, which are responsible for HOC solubilization at
233 rhamnolipids concentrations below CMC and above CMC, respectively.

234 Please insert Figure 1

235 *3.2 Biodegradation of solubilized HOCs*

236 Bioavailable is a key term in biodegradation, which is defined as "substrates are
237 freely available to cross microbial cell membrane from the medium the
238 microorganism inhabits at a given time" (Semple et al., 2004). For HOCs, the
239 biodegradation involves degrading sorbed or NAPL-state HOCs at the interface,
240 aqueous HOCs (dissolution as a molecular state), and micellar HOCs
241 (pseudo-solubilization). It is known that the rhamnolipids/HOCs co-aggregates, as
242 tiny HOCs reservoirs, can enhance the mass transfer to microbial cells (Bordoloi and
243 Konwar, 2009; Sponza and Gök, 2010). Therefore, the addition of rhamnolipids can
244 enhance bioavailability of the sorbed or NAPL-state HOCs (Brown, 2007; Guha and
245 Jaffé, 1996a, 1996b; Lanzon and Brown, 2013). However, it has been observed that
246 the increasing apparent solubility of HOCs due to rhamnolipids solubilization does
247 not always result in enhancement of bioavailability (Liu et al., 2017; Zhong et al.,
248 2014). Potential mechanism regarding these contradictory results is whether to form
249 hemispherical micelle or not (Brown, 2007; Brown and Al Nuaimi, 2005). When
250 surfactants adsorb onto a surface, they will form hemi-micelles on it, which is similar
251 to the formation of micelles in the aqueous phase. These hemi-micelles have

252 hydrophobic cores and can provide additional partitioning sites for HOCs (Lanzon
253 and Brown, 2013; Zhou and Zhu, 2005). According to a model developed by Guha
254 and Jaffe´ (1996a, 1996b), aqueous HOCs can be transported into cells, and the
255 pathway is described as (A) in Fig. 2. For the mass transfer from micellar cores into
256 microbial cells, it was assumed to have three steps (pathway (B)). The first step is
257 transporting surfactant/HOCs aggregates from the bulk fluid to cells. Then micellar
258 HOCs will be transported into hemi-micelles adsorbed on the cell surface under the
259 condition of micelle breakdown due to micellar relaxation kinetics. Finally, HOCs
260 will be transferred from hemi-micelles into cells. The later research found that the
261 formation of hemi-micelles on the cell surface is necessary for surfactant-enhanced
262 biodegradation of HOCs (Brown, 2007; Brown and Al Nuaimi, 2005). And thus a
263 limiting case was supplemented in the process of mass transfer (pathway (C)): if there
264 is no hemi-micelles formation on cell surface, the direct transport of micellar HOCs
265 into cells will not occur.

266 Please insert Figure 2

267 Based on above revised model, Lanzon and Brown (2013) made a series of
268 experiments and the results demonstrated that the effect of surfactant solubilization on
269 the biodegradation of HOCs is related to following aspects. (1) The formation of
270 hemi-micelles on cell surface. Specifically, when hemi-micelles adsorbed on the cell
271 surface are dominant in the system, micellar HOCs are directly available to cells;
272 while surfactant monomers are dominant in the system, micellar HOCs can't be
273 directly available to bacterial cells. (2) The impact of partitioning and mass transfer
274 on bioavailable HOCs concentration. A system is at equilibrium in which has a
275 sufficiently small mass of HOCs. After adding surfactant, if solid-phase HOCs aren't
276 residual due to partitioning into micelles, the bioavailable HOCs concentration will be

277 decreased, and thus depress biodegradation rate. (3) The ability of microbe utilizing
278 the enhanced available HOCs. For example, when microbial growth is already at
279 maximum specific growth rate, the addition of surfactant will not affect the total
280 biodegradation rate.
281
282 **4. Effect of rhamnolipids on affinity between cells and HOCs**
283 Rhamnolipids not only have the ability to increase the solubility of HOCs, but
284 also have biological effects of modifying cell surface properties (De et al., 2015). Cell
285 surface hydrophobicity (CSH) is an important parameter for microorganisms. It has
286 been known that CSH can affect the efficiency of many bioprocesses, including cell
287 adherence to HOCs and cell-to-cell interactions (Habimana et al., 2014). It has been
288 well reported that bacterial CSH can be affected by surfactants (Owsianiak et al., 2009;
289 Sun et al., 2016). For example, Owsianiak et al. (2009) found that rhamnolipids could
290 increase the CSH of microbial consortia with low hydrophobicity, while reduce the
291 CSH microbial consortia with high hydrophobicity. Knowledge of how rhamnolipids
292 affect CSH is important for evaluation on the affinity between cells and HOCs and
293 thus biodegradation of HOCs.
294 *4.1 Rhamnolipids-induced removal of outer membrane components*
295 CSH depends on the proportion of hydrophilic and hydrophobic regions on the
296 cell envelope. For most of Gram-negative microorganisms, the hydrophobicity is
297 attributed to certain lipids and proteins presented in the outer membrane of the cells
298 (Zimmermann et al., 2016). For example, outer membrane (OM) of Gram-negative
299 bacteria comprises an inner leaflet of phospholipids, an outer leaflet of LPS, and
300 proteins inserted in the lipid bilayer (Whitfield et al., 1997). From inside to outside,
301 lipid A tail, core oligosaccharide including 2-keto-3-deoxyoctonic (KDO), and

O-antigen together constitute the typical structure of LPS (Kastowsky et al., 1992). One way for rhamnolipids to change CSH is to induce the removal of LPS from bacterial cell envelope, which has been firstly reported by Al-Tahhan et al. (2000). The possible mechanisms for rhamnolipids-induced LPS release have been proposed (Figure 3), which are: 1) rhamnolipids could directly remove LPS or the O-antigen part of LPS through micellar capture, resulting in the exposure of hydrophobic LPS lipid A (Bhattacharjee et al., 2016; Zhao et al., 2011); 2) rhamnolipids form complex with Mg^{2+}, which is crucial for bridging LPS molecules and maintaining stability of LPS-LPS interactions, leading to direct release of LPS; 3) rhamnolipids can affect the structure of OM proteins which are responsible for the synthesis of LPS (Andersen and Otzen, 2014), and this has been evidenced by Fourier Transform Infrared Spectroscopy (FTIR) spectra (Zeng et al., 2011). It should be noted that the replacement or denaturation of components in OM would result in the irreversible alteration of CSH (Zhang and Zhu, 2014).

Please insert Figure 3

4.2 Adsorption of rhamnolipids

Another way to modify the CSH can be attributed to adsorption of rhamnolipids on the cell surface driven by polar interactions between rhamnolipid molecules and functional groups on bacterial outer envelope serving as adsorption sites (Hou et al., 2017). The bacterial cell surface contains hydrophilic and hydrophobic sites. The orientation of rhamnolipids adsorbed onto the cell surface determines the effect of rhamnolipids on CSH. Figure 4 illustrates the relationship between the orientation and the change of CSH. On the one hand, rhamnolipid may adsorb to the cell surface through the interactions between carboxyl or rhamnosyl groups and polar structures of cell surface by hydrogen bonding, dipolar, electrostatic, or short-term forces (e.g.,

O-antigen of LPS), turning cell surface more hydrophobic (Liu et al., 2014). On the other hand, the adsorption could also be driven by van der Waals and hydrophobic forces between nonpolar structures of cell surface (e.g., lipids and some proteins) and hydrophobic tails of rhamnolipids, causing the decrease of CSH (Górna et al., 2011; Zhong et al., 2008). Overall, adsorption of rhamnolipids on cell surface may result in the exposure of the group with an opposite polarity into the environment (Mańko et al., 2014). Such a way of orientation of rhamnolipids is always inclined to change CSH from hydrophilic to hydrophobic, or from hydrophobic to hydrophilic (Zhong et al., 2007). The CSH of *Bacillus subtilis* BUM (with 71.5% of initial CSH) significantly decreased with the adsorption of rhamnolipids (Zhao et al., 2011). For relatively hydrophilic *P. aeruginosa*, the adsorption of rhamnolipids at low concentration resulted in a significant increase of CSH (Zhong et al., 2008). However, authors found that CSH could be slightly reduced at high rhamnolipids concentration. This is probably due to the double layer adsorption of rhamnolipids, or the accumulation of micelles on the hydrophilic sites of cell surface (Mohanty and Mukherji, 2013).

Please insert Figure 4

4.3 Effect of rhamnolipids concentration on CSH

It is shown by many studies that the change of CSH is highly related to rhamnolipids concentration (Domingues et al., 2014; Sun et al., 2016). Sun et al. (2016) reported the addition of rhamnolipids significantly enhanced the CSH of *P. stutzier* KS0013, and CSH was increased from 14.9% to 24.1, 27.0, 29.2, 30.1 and 33.5% with 0.005, 0.010, 0.015, 0.020 and 0.025% of rhamnolipids concentrations, respectively. The control of CSH through rhamnolipids concentration could be an important strategy to improve the efficiency of bioremediation.

352 The orientation of rhamnolipid monomers and micelle deposition on cell surface
353 are the basic means for altering CSH when rhamnolipids concentrations are low and
354 high, respectively (Zhong et al., 2007). The effect of monomer adsorption is even
355 more significant than that of micelle deposition (İkizler et al., 2017; Liu et al., 2014).
356 When rhamnolipids are at low concentration, the adsorption is the presence of
357 tight-binding of one moiety of rhamnolipid molecules to the chemical groups on cell
358 surface (İkizler et al., 2017), and the orientation always tends to alternate the CSH
359 (Liu et al., 2014). While at high concentration level, the change of CSH is less
360 sensitive to micelle deposition since it is a simple accumulation of rhamnolipid
361 micelles on originally hydrophilic sites of cell surface or pre-adsorbed rhamnolipids
362 layer (Zhong et al., 2007). At these points, using low-concentration of rhamnolipids
363 can be a way for controlling CSH (Liu et al., 2014).
364 The native hydrophobicity of microorganisms is related to the proteins and lipids
365 on cell surface (Yoneda et al., 2016). Al-Tahhan et al. (2000) showed that
366 rhamnolipids at concentrations much less than the CMC caused the removal of LPS,
367 leading to an increase in CSH. In contrast, the study by Sotirova et al. (2009)
368 demonstrated when the concentration was above CMC, rhamnolipids caused the
369 decrease of total LPS content of 22%, associated with an increase in CSH to 31%
370 adherence. When the concentration of rhamnolipid was decrease to below CMC,
371 however, rhamnolipids did not influence the LPS component of OM but caused
372 significant changes in outer membrane protein (OMP) composition of *P. aeruginosa*
373 (Galabova et al., 2014; Sotirova et al., 2009). According to above results, the removal
374 of proteins and lipids from cell surface is related to the concentration of rhamnolipids,
375 but no obvious relationship was found among them.

5. Rhamnolipids-induced enhancement of cell membrane permeability and uptake of HOCs

It is reported that the permeability barriers imposed by cell envelopes lower whole-cell catalyzed reactions about 10 to 100 folds comparing with free enzymes catalyzed reactions (Sotirova et al., 2008). The permeability of OM is an important parameter for substrate uptake for Gram-negative bacteria. Solutes and metabolites less than 5 kDa are able to freely permeate OM, mainly owing to the presence of a plentiful protein (Schmidt et al., 2016). The induced permeability enhancement for microbial cells will probably enhance the enzyme reaction (Nesin et al., 2011).

One of the theoretical bases for the application of rhamnolipids in bioremediation processes is the enhancement in cell permeability (Jadhav et al., 2011; Magalhães and Nitschke, 2013). The permeabilization can facilitate the mass transfer and reduce the toxic effect of prolonged incubation with HOCs, thus leading to the increase of mineralization rate (Tecon and van der Meer, 2010). Jadhav et al. (2011) investigated the potential of mono-rhamnolipid to permeabilize *Bacillus sp* VUS NCIM 5342. It was shown that mono-rhamnolipid had excellent performance in *Bacillus* cell permeabilization, and the efficiency of textile dye Brown 3REL decolorization was enhanced by 50%. On the other hand, permeabilized cells can be as a source of proteins and insoluble enzymes with analogous effects as those immobilized by conventional methods, allowing them to be tested under the identical conditions as those observed in vivo (Oliveira et al., 2016). Rhamnolipids can partition into microbial membrane because of the amphiphilicity, which causes the alteration of membrane in physicochemical properties and function (e.g., transport and energy generation) (Bai and McClements, 2016). Recently, many studies focus on the membrane actions of rhamnolipids, especially the induction of membrane

401 permeabilization in liposome system (Diaz De Rienzo et al., 2016; Inès and Dhouha,
402 2015). The mechanism underlying rhamnolipids-induced leakage of liposomes might
403 be that rhamnolipids adsorb onto the outer leaflet of microbial membrane, flip the
404 inner leaflet, and then properly intercalate the phospholipid molecules, leading to
405 destabilization of the membrane (Sánchez et al., 2010; Zhang and Zhu, 2014). Some
406 researchers suggested rhamnolipids could induce the release of cell surface materials,
407 such as LPS and outer membrane protein (OMP) (Kim et al., 2015; Sotirova et al.,
408 2009; Galabova et al., 2014) which are not only responsible for cell surface
409 hydrophobicity, but also responsible for cell permeability characteristics (Amro et al.,
410 2000). The removal of cellular LPS is probably due to solubilization of OM through
411 binding the aggregated rhamnolipids to the membrane, followed by the reduction of
412 LPS (Sotirova et al., 2009). This usually occurs when the concentration of
413 rhamnolipids is above CMC. When its concentration is below CMC, rhamnolipids
414 could cause a marked reduction in the amount of proteins. This is probably because
415 rhamnolipids monomers can cause alterations in membrane organization (Galabova et
416 al., 2014). Fig. 5 shows the rhamnolipids-induced membrane permeabilization.
417 Please insert Figure 5
418 In the studies by Magalhães and Nitschke (2013), they observed an increase in
419 cell permeability with the presence of rhamnolipids, and the hypothetical action site is
420 the phospholipids in cell membrane, although the mechanism was not completely
421 understood. In order to confirm the permeabilizing effect of rhamnolipids,
422 Scanning-Electron Microscopy (SEM) was used to observe the morphologic changes
423 of strain cells by Sotirova et al. (2008). The results showed strain cells in 0.5%
424 rhamnolipids solution had significant changes in cell shapes and membranes fold, and
425 formed various cavities with different shapes and sizes as compared to the untreated

426 cells. Result of several other studies showed that the addition of rhamnolipids can
427 eliminate cyclopropane fatty acids of 17:0 cyclo and 19:0 cyclo which have been
428 recognized can assist in tolerance of disturbance and stabilize membrane lipids
429 (Denich et al., 2003; Mrozik et al., 2007). Moreover, Sánchez et al. (2010) studied the
430 action of direhamnolipid on biological membrane through determining the release of
431 carboxyfluorescein, and the results showed that permeabilization of dirhamnolipid
432 induced leakage in liposomes with concentrations below the CMC, at which the
433 solubilization of membrane was not observed.

434 The permeabilization can facilitate the mass transfer of HOCs through cell
435 membrane, and thus lead to an increase in HOC uptake rate (Teron and van der Meer,
436 2010). Jadhav et al. (2011) investigated the potential of mono-rhamnolipid to
437 permeabilize *Bacillus sp* VUS NCIM 5342. It was shown that mono-rhamnolipid had
438 excellent performance in *Bacillus* cell permeabilization, and the efficiency of textile
439 dye Brown 3REL decolorization was enhanced by 50%.

440

441 **6. The biodegradation of rhamnolipids**

442 Mohan et al. (2006) investigated the biodegradation of rhamnolipids and the
443 results showed that rhamnolipids could be rapidly degraded under aerobic conditions,
444 while the degradation was remarkably slower under anaerobic conditions. In another
445 study it was shown that microorganisms can degrade rhamnolipids after the
446 biodegradation of solubilized HOCs (Oberbremer et al., 1990). Maslin and Maier
447 (2000) proposed that rhamnolipids by themselves may serve as a carbon source. This
448 observation caused increasing attentions because preferred utilization of rhamnolipids,
449 as an alternative carbon source, may affect biodegradation efficiency of primary
450 contaminants (Ławniczak et al., 2013). Ghosh and Mukherji (2016) carried out the

biodegradation experiment of pyrene by *P. aeruginosa* with the presence of rhamnolipids JBR 515, and they found that rhamnolipids were preferentially degraded as compared to pyrene. According to observations concerning the preferential use of rhamnolipids over HOCs, a negative impact of rhamnolipids supplementation may well exist in environmental biodegradation trials. Moreover, it is also plausible that rhamnolipids may be co-degraded with substrates, which means their effect on biodegradation of substrates will be slowly diminished. Lin et al. (2011) observed a significant increase in the biodegradation rate of diesel oil in the initial stage, while the process efficiency was similar to that of the control group (without rhamnolipids) in the latter stages.

However, biodegradability can be an advantage of rhamnolipids for HOC degradation. It has been reported that the biodegradation of surfactants may cause the release of HOCs from the micellar cores into the aqueous phase, eliminating the blocking effect of surfactants (Liu et al., 2017; Pęziak et al., 2013). Under such conditions, the biodegradability of rhamnolipids is beneficial for the degradation of the solubilized hydrocarbon. Zeng et al. (2011) found that the metabolism of rhamnolipids as carbon and energy source contributed to the growth of *Candida tropicalis*, which further enhanced the degradation of hexadecane. However, contradictive results were obtained by Ghosh and Mukherji (2016), who confirmed that the preferred utilization of rhamnolipids decreased the specific growth rate during the biodegradation of pyrene. These results indicate that unintended effects of rhamnolipids on HOCs biodegradation efficiency will occur when rhamnolipids are available to microorganisms in the system. Therefore, in practical applications, it is necessary to find the balance between the biodegradability of rhamnolipids and their effects on the HOCs biodegradation (Kumar et al., 2017; Maire and Fatin-Rouge,

476 2017). Parameters to be considered include the physical properties of rhamnolipids
477 (stability, etc.), solubilization capacity of rhamnolipids for HOCs, and a suitable
478 degree of biodegradability (Brycki et al., 2014).
479
480 **7. Toxicity of rhamnolipids**
481 Surfactants can be toxic to functionally important bacteria or may change
482 bacterial community composition (Álvarez-Paino et al., 2015). Therefore, it is
483 necessary to know their potential toxic effect to microorganisms when considering the
484 environmental impacts of rhamnolipids.
485 One opinion is that rhamnolipids have no toxic effect on the microbial cells
486 cultured in medium (Banat et al., 2010; Hadibarata and Kristanti, 2014; Solaiman et
487 al., 2016). Hadibarata and Kristanti (2014) investigated the effects of diverse
488 surfactants on the growth of *Armilaria* sp. F022, and they observed that the system
489 with rhamnolipids (10 mg/L) obtained the highest biomass. Solaiman et al. (2016)
490 found that the lag phase of bacteria could be shortened by the presence of
491 rhamnolipids. Several studies also have shown that addition of rhamnolipids can
492 enhance the activity of indigenous microbes in the soil and sediment (Guo et al., 2016;
493 Liao et al., 2015; Mathurasa et al., 2012). Liao et al. (2015) reported that the
494 microbial number significantly increased with increasing concentrations of
495 rhamnolipids. Besides, it was also found that rhamnolipids can promote microbial
496 growth in solid-state fermentation systems (Liu et al., 2010; Zhou et al., 2011). For
497 instance, Zhou et al. (2011) reported that rhamnolipids caused a significant increase of
498 *P. simplicissimum* biomass. The promoting effect might be directly due to
499 rhamnolipids, or the greater levels of dissolved organic matter released by the
500 surfactants, serving as carbon sources for additional microbial growth.

The second opinion is that rhamnolipid has toxic effect on the growth of microorganisms during the HOCs biodegradation but it depends on the concentration of rhamnolipids. Sotirova et al. (2008) found that the application of low concentration of rhamnolipids has no effect on the growth of Gram-negative *P. aeruginosa* and Gram-positive *B. subtilis*, but high concentration (above CMC) of rhamnolipids showed toxic effects to *B. subtilis*. Fuchedzhieva et al. (2008) reported that the presence of rhamnolipids suppressed *B. cereus* growth on fluoranthene solution, and the inhibitory effect of rhamnolipids was better expressed when rhamnolipids concentrations are above 100 mg/L. This phenomenon was also shown by Mukherjee et al., (2006), and they suggested that the toxicity of rhamnolipids towards the microorganisms at high concentrations could be an issue hindering their applicability. It was suggested that with the increase of surfactant concentration, the formed surfactant micelles may solubilize cell membranes by forming mixed micelles with cell membrane lipids, leading to the necrosis of cells (Kim et al., 2013). In all, these results demonstrated thatconcentration is an important factor that should be seriously considered for successful application of rhamnolipids in bioremediation.

8. Conclusions and perspectives

Rhamnolipids have been frequently employed to enhance the bioremediation of HOCs polluted soil and water environment due to their high solubilizing ability, environmental friendly, etc. This paper provides a comprehensive review on the interaction mechanisms of rhamnolipids with HOCs and microorganism including solubilization, changing affinity through rhamnolipids adsorption or LPS release, permeabilization, with the aim of a better understanding and controlling of rhamnolipids-mediated HOCs biodegradation. In addition, effects from

biodegradation and toxicity of rhamnolipids should be considered since the factors are also important for the successful application of rhamnolipids in bioremediation of HOCs pollution.

Rhamnolipids-mediated biodegradation provides a promising way to remediate HOCs contaminated environment. The following main areas need to be considered for subsequent work in research and practical application:

(1) The commercial application of rhamnolipids is limited due to the high cost of production. Some measures could be taken to make the production of rhamnolipids more profitable and economically feasible, for example, using cheaper renewable substrates, optimizing growth/production conditions and employing original and effective multi-step downstream processing methods. Moreover, it is also necessary to find recombinant and mutant microorganisms that could utilize a wide range of cheap substrates to grow or produce rhamnolipids in high yield, bringing a real breakthrough for their economic production.

(2) Currently, the data on the formation of rhamnolipid/HOCs aggregates below CMC concentration is even less clear. The research is needed to describe the morphology and stability of formed aggregates, as well as the sub-CMC solubilization ability for different HOCs. Moreover, it is necessary to verify whether the conclusions on rhamnolipid micelles are still suitable for sub-CMC aggregates, for example, the mechanism for micellar bioavailability based on hemi-micelles formation on cell surface.

(3) The mechanisms of rhamnolipid-induced release of LPS and rhamnolipid adsorbed on cell surface to change CSH have been recognized. However, how to regulate rhamnolipids achieving the optimal microbial CSH remains

551 rarely discussed. In addition, the studies about rhamnolipid-induced release
552 of LPS and rhamnolipid adsorption changing CSH are carried out
553 independently. The question is how rhamnolipids perform in the actual
554 application system. It is of importance to solve these problems in the near
555 future.
556 (4) The study of rhamnolipids permeabilization is built mainly on indirect
557 evidence, such as the measure of released cell surface materials. The direct
558 analysis and determination are needed to further investigate the
559 permeabilization mechanism through advanced instruments and inspection
560 methods.
561 (5) In some cases, the preferential biodegradation of rhamnolipids might result in
562 the less effectiveness in the contaminant bioremediation process. Therefore,
563 it is of importance to solve these problems in the further, for example, the
564 investigation of suitable strain and environmental conditions.
565 (6) Future researches should not only focus on exploring how to enhance the
566 efficiency, but also on extending this challenging problem through
567 illuminating the complex mechanisms underlying the whole system based on
568 the extensive data of other surfactants, e.g., interactions among rhamnolipids,
569 microorganisms and HOCs.
570 (7) A great deal of research efforts have been devoted to enhance the
571 biodegradation of HOCs by means of rhamnolipids. However, most of the
572 attempts are limited to the laboratory or theory study, and larger scale
573 experiments are needed to demonstrate the feasibility of field application of
574 this technique.
575 (8) Another important consideration is that most studies have been conducted

with simulated wastewater or single HOCs in growth media, which means that few studies are executed on actual polluted water. The wide differences could be obtained between contaminants removal efficiencies in simulated and actual polluted wastewater due to the fact that the compositions of real wastewater are more complex. Hence a massive effort is required to assess these application technologies of rhamnolipids for use with actual contaminated wastewater.

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Figure legends:

Fig. 1 Schematic representation of rhamnolipids-enhaned the aqueous dispersion of HOCs

Fig. 2 Schematic diagram describing the uptake of HOCs by the bacterial cell Pathway (A): the transfer of aqueous HOCs into the cell; Pathway (B): the direct mass transfer of micellar HOCs into the cell when the formation of hemi-micelles on the cell surface occurs; Pathway (C): the micellar HOCs are not directly bioavailable when no hemi-micelles are formed on the cell surface. Adapted with permission from refs (Brown, 2007; Lanzon and Brown, 2013).

Fig. 3 Schematic diagram for removal of LPS by rhamnolipids (I) Direct removal of LPS or the O-antigen part of LPS by rhamnolipids micellar capture as previously described (Zhao et al., 2011); (II) Complex formation between rhamnolipids and Mg^{2+} (Al-Tahhan et al., 2000); (III) Inhibition of synthesis and transport of LPS caused by the effect of rhamnolipids on protein described by Andersen and Otzen (2014).

Fig. 4 Orientation of rhamnolipids at cell surface of microorganisms. The possible adhesion of microbial cells to hydrophobic or hydrophilic interface is indicated. The hydrophobic (hydrophilic) moiety of rhamnolipids will contact microbial cells with relative high (low) CSH (cell surface hydrophobicity), and the hydrophilic (hydrophobic) moiety of rhamnolipids exposed to environment reduces (increase) the CSH (Górna et al., 2011; Liu et al., 2014).

Fig. 5 Schematic diagram of rhamnolipids-induced the permeabilization of cell membrane: The intercalation of rhamnolipids monomers into phospholipid molecules cause the destabilization of the membrane (Zhang and Zhu, 2014); the release of several cell surface materials induced by rhamnolipids increase the permeability of the membrane (Amro et al., 2000; Kim et al., 2015).

1084 **Table 1**

1085 An overview of recent studies on rhamnolipid producing bacteria

Strain	Carbon source	Main composition	Reference
Burkholderia thailandensis	glycerol	di-RLs	Funston et al. (2016)
Burkholderia kururiensis	glycerol	di-RLs	Tavares et al. (2013)
Pseudomonas aeruginosa	sunflower oil	Rha-C_{10}-C_{10} Rha_2-C_{10}-C_{10}	Amani et al. (2013)
Acinetobacter calcoaceticus	sunflower oil/ sodium citrate	di-RLs	Hošková et al. (2013)
Enterobacter asburiae	sunflower oil/ sodium citrate	mono-RLs	Hošková et al. (2013)
Pseudomonas chlororaphis	waste cooking oil	di-RLs	Lan et al. (2015)
Pseudomonas nitroreducens	glucose	a mixture of rhamnolipid	Onwosi and Odibo (2012)
Pseudomonas stutzeri	lignite coal	a mixture of rhamnolipid	Singh and Tripathi (2013)

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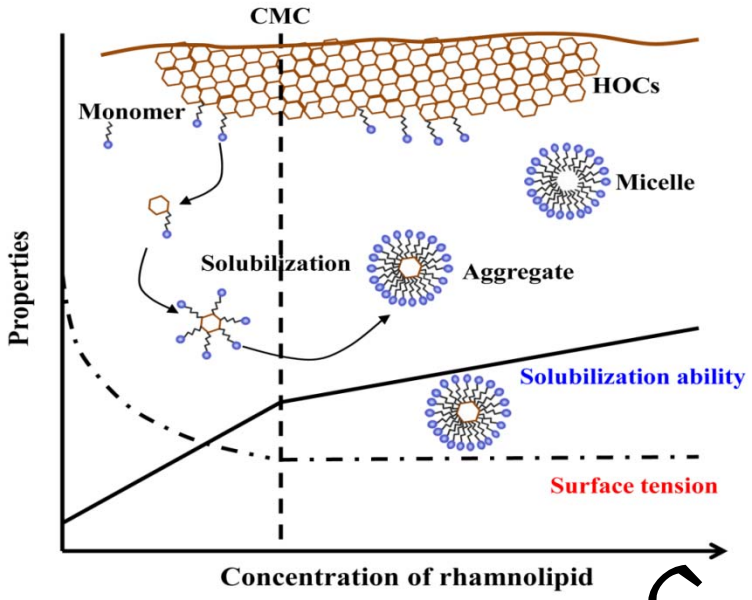

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1089 Figure 1

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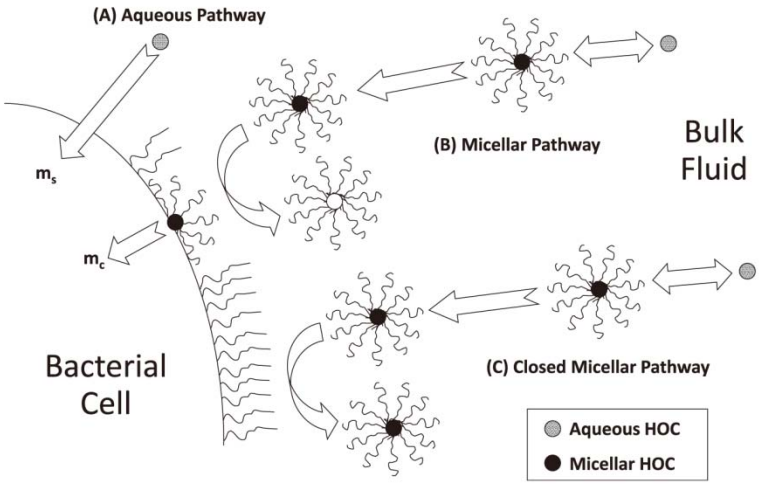

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1092 Figure 2

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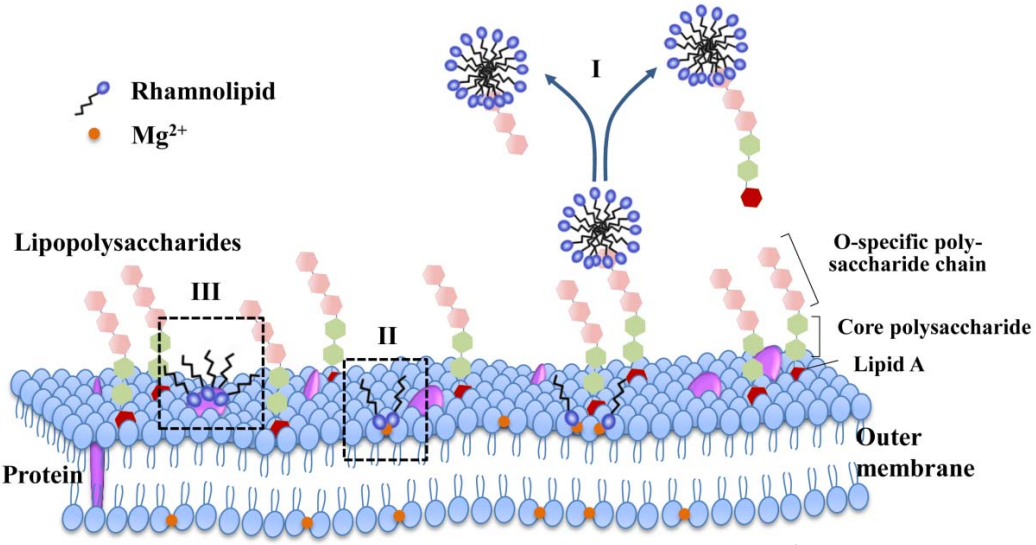

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1095 Figure 3

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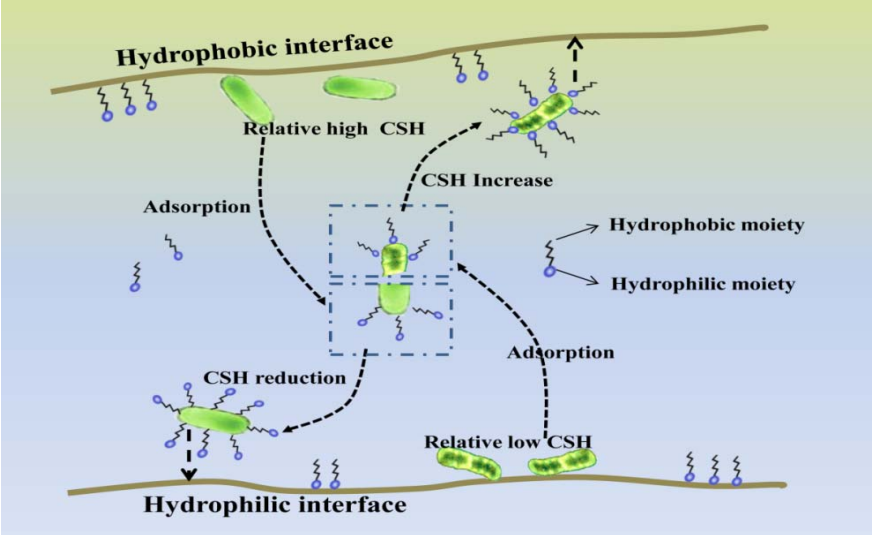

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1098 Figure 4

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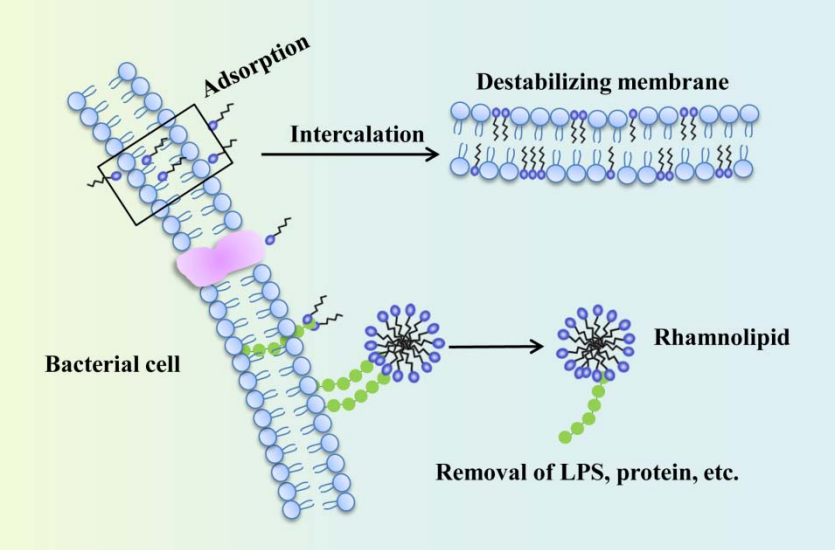

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1101 Figure 5