CHEMICAL ENGINEERING TRANSACTIONS
VOL. 64, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Antonio Marzocchella, Tajalli Keshavarz

ISBN 978-88-95608- 56-3; **ISSN** 2283-9216
DOI: 10.3303/CET1864083

Screening of Anionic Biosurfactants Production among Fungi and Bacteria

Federica Spina[a], Giulia Spini[b], Anna Poli[a], Alice Romagnolo[a], Andrea Zanellati[a], Nicolò G. Bentivegna[a], Najoi El-Azhari[c], Tiffanie Regnier[c], Anne-Laure Blieux[c], Abdelwahad Echairi[c], Valeria Prigione[a], Edoardo Puglisi[b], Giovanna C. Varese[a*]

[a]University of Turin, Department of Life Sciences and Systems Biology, Mycotheca Univeristatis Taurinensis, Viale P.A. Mattioli 25, 10125 Turin, Italy
[b]Università Cattolica del Sacro Cuore, Department for Sustainable food process, Via Emilia Parmense 84, 29122 Piacenza, Italy
[c]Satt Grand-EST, Maison régionale de l'innovation, 64a Rue Sully, 21000 Dijon, France
cristina.varese@unito.it

Biosurfactants production is a great advantage for those microorganisms that could be involved in bioremediation processes. In the present study, 164 bacteria and 212 fungi isolated from a contaminated site were investigated for their capability to produce anionic biosurfactants. The screening was carried out by the blue agar test, providing semi-quantitative results for more than 370 strains. Bacteria response was easy to evaluate whereas fungi showed a plethora of responses that partially interfere with the evaluation of positive results. Among the tested strains, 43 bacteria and 20 fungi were identified as good producers of anionic biosurfactants, as indicated by the formation of a clear dark blue halo around the colony.

1. Introduction

Soil degradation is associated to the loss of its ecological functions that ultimately leads to the reduction of productive capacity of soil. The main causes are related to human activities, being extensive industrialization at the centre of this inauspicious scenario. The Roadmap to a Resource Efficient Europe clearly stated that soil contamination is a serious problem that is under consideration by the European Union to strategically draw the EU policies in the period 2020-2050 (SEC 2011). It has been estimated that soil pollution is affecting more than 1.5 billion people and 12.2 billion hectares of land (Tripathi et al. 2017). SEC (2011) foresees the achievement of the 'no net land take' before 2050, but in the meantime, a defined strategy for the recovery of contaminated soil is missing.
Aromatic and aliphatic hydrocarbons are among the most polluting compounds. Their presence in the soil is imputable to petroleum and coal industries as well as runoff and sediments from urban areas which contain oil and fuel residues (Alegbeleye et al. 2017). Being accumulating at different trophic levels, they could ultimately create adverse effects to the aquatic ecosystem, the soil microbiota and ultimately to human being.
Soil recovery passes through the treatment of contaminated soil. Several reviews have dealt with physical and chemical approaches, which are indeed among the most in-used techniques. Bio-based systems offer great advantages from an economic and environmental point of view (Gomes et al. 2013).
Natural attenuation of the pollutants concentration is a recurring phenomenon that involves plants and microorganisms that naturally populate the contaminated soil. Having evolved extreme adaptation skills, they could be capable of degrading contaminants into less toxic compounds. However, this process is often limited by the load of the degrading microorganisms and the availability of the pollutants that are instead in a different phase from the living microorganisms. The first issue could be overcame by the application of bioaugmentation approach, which is instead ineffective to enhance the microbial attack when the pollutants are not available. Biosurfactants are instead a cost and environmental effective technology aimed to enhance the solubility of hydrophobic compounds.

Please cite this article as: Spina F., Spini G., Poli A., Romagnolo A., Zanellati A., Bentivegna N.G., El-Azhari N., Regnier T., Blieux A.L., Echairi A., Prigione V., Puglisi E., Varese G.C., 2018, Screening of anionic biosurfactants production among fungi and bacteria, Chemical Engineering Transactions, 64, 493-498 DOI: 10.3303/CET1864083

Biosurfactants have hydrophilic (polar) and hydrophobic (non-polar) groups, helping to weaken the surface tension between water and oil or non-polar solutions. The length of the non-polar long-chain fatty acids can vary as well as the moiety of the hydrophilic residue, e.g. carbohydrate, cyclic peptide, amino acid, phosphate carboxyl acid or alcohol. According to these differences they can be classified as glycolipids, fatty acids, phospholipids, neutral lipids, polymeric and particulate biosurfactants (Santos et al. 2016).
They have a wide industrial exploitation being involved in food, cosmetics, agriculture and environmental industrial processes. For instance there is a great interest about exploiting their antimicrobial properties in oral-related health applications (Elshikh et al. 2016). Despite the research is producing the first encouraging data in the very last years, surfactants as lipopeptides, rhamnolipids and sophorolipids have already demonstrated to be able of inhibiting microorganisms (Coronel-León et al. 2016; Elshikh et al. 2017). Besides as part of pharmaceutical formulation, they could enhance the permeability of the bacterial membranes, strengthening the action of antibiotics (Joshi-Navare and Prabhune 2013; Perinelli et al. 2017). One of the major advantages of biosurfactants is that being produced by a biological production line, they should have less harmful effects than chemically-based surfactants. The use of biosurfactants in the cosmetic industry is indeed based on their potential to avoid skin irritations and allergic reactions due to their application (Vecino et al. 2017). Besides in comparison to chemically-based surfactants, biosurfactants are associated to cost-effective production, biodegradability, low toxicity and a wide range of environmental resilience (Lamichhane et al. 2017).
Most of these applications are a front-edge of the scientific research while the petroleum-related industry is the main on-going industrial exploitation. By solubilising hydrocarbons, they could be ultimately come into contact with microorganisms at the base of an effective bioremediation process (Lamichhane et al. 2017).
Microorganisms can produce biosurfactants with different molecular structures and surface activities. Since the early ’60, most of the attention has been given to bacteria. Among others, probably *Pseudomonas aeruginosa* is the most commonly used and most studied bacterium for the production of biosurfactants as rhammolipids useful for bioremediation purposes (Cheng et al. 2017; Deepika et al. 2015). Very few information could be found about the capability of fungi and yeast to produce biosurfactants (Shekhar et al. 2015).
The present study is aimed to investigate the capability of bacteria and fungi to produce biosurfactants that could represent a clear advantage for the degradation of aromatic and aliphatic hydrocarbons. Blue agar growth test is a semi-quantitative assay for the detection of extra cellular glycolipids or other anionic surfactants. It has been mostly applied to bacteria screening (Elazzazy et al. 2015; Pacwa-Płociniczak et al. 2016), lacking information about fungal response to this method. This approach have been selected because feasible for a large screening procedure providing fast responses and easy to define.

2. Material and Methods

The production of anionic biosurfactants was investigated by the Methylene Blue Agar Test. The fungal and bacterial strains were cultured in optimal conditions in order to stimulate their surfactants-producing metabolism.

2.1 Tested Microorganisms

Fungi and bacteria were isolated from a 100-years-old industrially exploited contaminated soil (data not shown), located in Fidenza (PR, Italy): 164 bacteria and 212 fungi were screened.

2.2 Preparation and Cultivation of Bacteria and Fungi

Microorganisms were pre-grown on agar plates with rich medium: Malt Extract Agar (MEA) for fungi and Trypticase Soy Agar (TSA) for bacteria. Plates were incubated at 24 °C for few days (3 and 7 day for bacteria and fungi, respectively).
The cultural media were prepared as follow:

1. MEA: 20 g/L glucose, 20 g/L malt extract, 2 g/L peptone and 18 g/L agar.
2. TSA: 15 g/L tryptone, 5 g/L enzymatic digest of soybean meal, 5 g/L sodium chloride, 15 g/L agar.

2.3 Screening for Biosurfactant-Producing Microorganisms

Blue agar test is a semi-quantitative assay for the detection of extra cellular anionic surfactants, developed by Siegmund and Wagner (1991). Methylene blue agar plates were prepared modifying a mineral salt medium (MSM). MSM contained microelements needed for an efficient bacterial and fungal growth (KH_2PO_4 0.03 % w/v, $MgSO_4$ 0.03 % w/v, $NaNO_3$ 0.3 % w/v) and glucose as source of carbon (2 % w/v). Cetyltrimethylammonium bromide (CTAB 0.5 mg/mL) and methylene blue (0.2 mg/mL) were added to the medium.

Fungi and bacteria were inoculated into the prepared blue plates, as a spot of culture for bacteria and 4 mm fungal mycelium plugs taken the margin of actively growing colonies. During the 1-2 weeks of incubation at 24 °C, the plates were observed to detect the formation of a dark blue halo around the colony. As a positive response, the halo indicated the production of anionic surfactant by the microbial strain: the anionic surfactant forms insoluble ion pair with the cationic CTAB-Methylene complex, forming the colored halo.
Negative controls were carried out evaluate the stability of the color on plates.

3. Results and Discussion

The results concerning the bacterial screening are reported in Table 1; positive colonies are shown in Figure 1. Among the 164 tested bacteria, 26 % of them (43 strains) formed dark blue halos in the methylene blue agar plate supplemented with CTAB. Among them 7 strains gave a wide dark halo outside the colony (++), highlighting their capability to produce extracellularly anionic biosurfactants.
Up to date, this is the widest screening performed on bacteria by means of blue agar test. Pacwa-Płociniczak et al. (2016) studied 42 strains, among which less than 10 % produced anionic surfactants. Elazzazy et al. (2015) investigated a smaller bacteria sample: 9 of the 23 isolates showed dark halo.
The isolation origin of these strains can be the cause of such interesting data. Being isolated from oil-polluted soil, these bacteria could have developed important adaptation skills to colonize this extreme ecological niche, including the capability of producing biosurfactants.
Most the active strains belonged to the Pseudomonas genus and in particular to *P. putida* (10 strains). This is in agreement with literature data that indicate *Pseudomonas putida* as a well-known producer of biosurfactants (Sivasankar and Kumar 2017).
Interestingly anionic biosurfactants production was species dependent but also strain dependent. The intra-specific variability is indeed a common phenomenon among microbes. For instance among the 11 *Pseudomonas chlororaphis* strains, positive responses were observed only in 1 strain. Also among *P. putida* strains, anionic biosurfactants production cannot be given for granted: only 44 % of them produced dark halo.
Similarly, only 4 out of 32 *Rhodococcus erythropolis* strains gave positive responses (Pacwa-Płociniczak et al. 2016).

Table 1: List of bacteria producing a dark blue halo.

species	response	species	response
Ochrobactrum sp.	+	Pseudomonas sp.	+
Acinetobacter calcoaceticus	++	Pseudomonas sp.	+
Acinetobacter sp.	++	Pseudomonas sp.	+
Agrobacterium sp.	+	Pseudomonas sp.	+
Bacillus idriensis	+	*Rhizobiaceae bacterium*	+
Bacillus sp.	+	*Rhizobiaceae bacterium*	+
Cupriavidus campinensis	+	*Rhizobiaceae bacterium*	+
Cupriavidus sp.	++	*Rhizobiaceae bacterium*	++
Ochrobactrum sp.	+	Rhizobium sp.	++
Ochrobactrum sp.	+	*Rhizobium radiobacter*	+
Ochrobactrum sp.	+	*Serratia marcescens*	+
Pantoea agglomerans	+	*Serratia marcescens*	+
Pantoea agglomerans	+	Sphingobacterium sp.	+
Pseudomonas chlororaphis	+	*Stenotrophomonas acidaminiphila*	+
Pseudomonas fluorescens	+	*Stenotrophomonas maltophilia*	+
Pseudomonas fluorescens	+	Unknown bacterium	++
Pseudomonas mosselii	+		
Pseudomonas putida	+		
Pseudomonas putida	+		
Pseudomonas putida	+		
Pseudomonas putida	+		
Pseudomonas putida	+		
Pseudomonas putida	+		
Pseudomonas putida	+		
Pseudomonas putida	+		
Pseudomonas putida	+		
Pseudomonas putida	+		

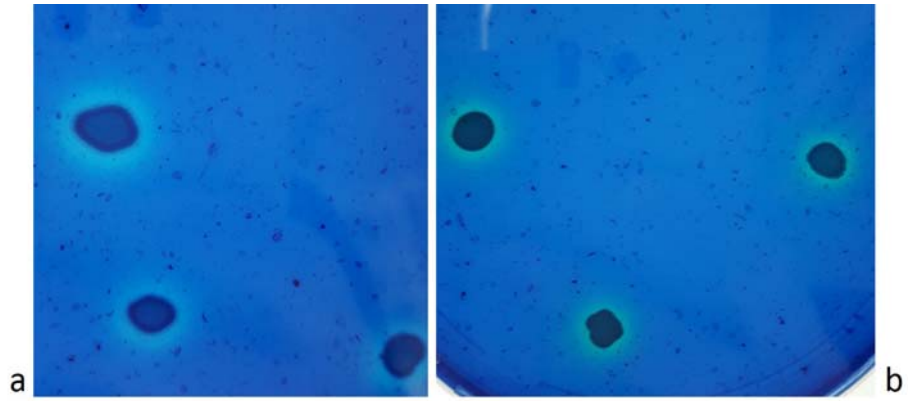

Figure 1: Dark blue halos formed around the bacterial colony on blue agar plates, indicating the production of anionic biosurfactants: a) Pseudomonas sp.; b) Pseudomonas chlororaphis.

In the present study, for the first time, the blue agar plate method was also applied to fungi. Among the 214 fungi, 20 strains displayed a clearly visible dark blue halo around the cultures (Table 2).
Noteworthy, to the best of our knowledge this is the first report of a wide screening aimed to identify anionic-biosurfactants producing fungi. Unexpectedly, about 10 % of tested fungi gave strong positive responses to the test. However according to the authors, the test underestimated the positive results since it showed some drawbacks for fungal screening. Fungi reacted to the presence of methylene blue-CTAB medium in different ways, including dye degradation and absorption. In most of cases, unclear responses were recorded due to absorption or decolourization phenomena. In detail, 39 % of fungi decolorized the blue plates, forming a clarification halo around the colony. This reaction often produced degradation metabolites as indicated by the plates turning green or red as shown in Figure 2a. This phenomenon has been already reported for other dyes, as for Acid Blue 62 breakage by white rot fungi (Vanhulle et al. 2008). Besides 27 % of fungi clearly adsorbed the colour onto the mycelium, as shown in Figure 2b.

Table 2 List of fungi producing a dark blue halo

species	response
Aspergillus nidulans	++
Aspergillus sydowii	++
Aspergillus sydowii	++
Aspergillus sydowii	++
Aspergillus terreus	++
Aspergillus terreus	++
Aspergillus terreus	++
Cladosporium pseudocladosporioides	++
Epicoccum nigrum	++
Hypocrea lixi	++
Irpex lacteus	++
Penicillum echinulatum	++
Trichoderma harzianum	++
Trichoderma harzianum	++
Trichoderma harzianum	++
Trichoderma harzianum	++
Trichoderma harzianum	++
Trichoderma harzianum	++
Trichoderma harzianum	++
Talaromyces sayulitensis	++

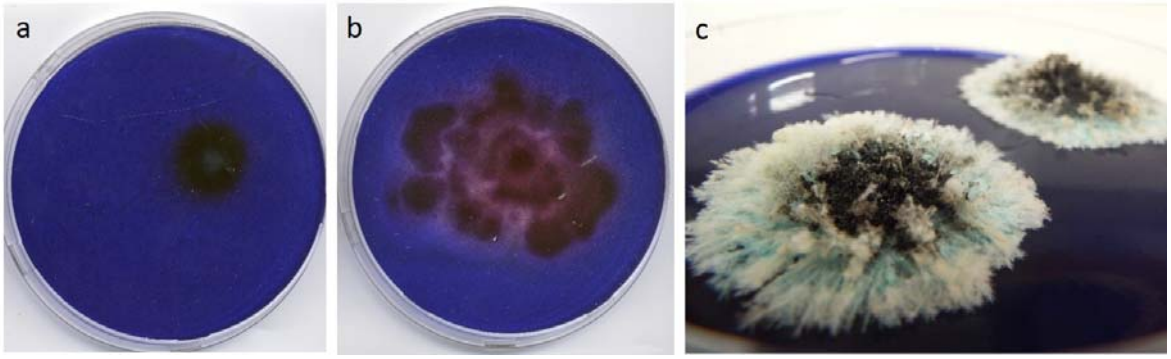

Figure 2: a) Dark halo around Aspergillus sydowii colony, indicating the production of anionic biosurfactants; b) degradation of the colour by Trametes gibbosa with formation of a red metabolite; c) absorption onto the Gliomastix murorum mycelium, showing light-blue colour in the aerial hyphae.

4. Conclusions

The present study emphasizes the need of an exhaustive screening to investigate biosurfactants producers among bacteria and fungi. Results clearly stated that this capability cannot be taken for granted even among microorganisms isolated from polluted soils. The above-describe approach is well-performing for bacteria, but not for fungi. Other tests are probable necessary to investigate more in detail fungal production of biosurfactants.

However, great interest arouses on those strains that actively produced anionic biosurfactants; in particular 43 bacteria and 20 fungi gave clear dark blue halo. Further analysis are needed to identify the anionic surfactants produced and evaluate the capability of the strains to produce also biosurfactants belonging to other chemical classes. How biosurfactants production will affect the fitness of microorganisms in contaminated environments and their outputs in bioremediation processes will be the topic of further investigations. The capability of several bacteria and fungi to produce anionic biosurfactants arouses great interest also from an applicative point of view, as the bioremediation of contaminated soils. Indeed the bioavailability of hydrocarbons is a critical issue that may weaken the microbial attack. Bacteria and fungi capable of overcoming this problem by secreting themselves surfactants can be at the base of successful technologies.

Acknowledgments

This project was funded by LIFE Program (LIFE15 ENV/IT/000396 _ LIFE-BIOREST).

Reference

Alegbeleye O.O., Opeolu B. O., Jackson V. A., 2017, Polycyclic Aromatic Hydrocarbons: A Critical Review of Environmental Occurrence and Bioremediation, Environ. Manage. 60, 758-783.

Cheng T., Liang J., He J., Hu X., Ge Z., Liu J., 2017, A novel rhamnolipid-producing *Pseudomonas aeruginosa* ZS1 isolate derived from petroleum sludge suitable for bioremediation, AMB Expr. 7, 120.

Coronel-León J., Pinazo A., Pérez L., Espuny M. J., Marqués A. M., Manres M., 2017, Lichenysin-geminal amino acid-based surfactants: Synergistic action of an unconventional antimicrobial mixture, Colloids Surf. B Biointerfaces 149, 38-47.

Deepika K. V., Sridhar P. R., Bramhachari P. V., 2015, Characterization and antifungal properties of rhamnolipids produced by mangrove sediment bacterium *Pseudomonas aeruginosa* strain KVD-HM52, Biocatal. Agric. Biotechnol. 4, 608-615.

Elazzazy A. M., Abdelmoneim T. S., Almaghrabi O. A., 2015, Isolation and characterization of biosurfactants production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia, Saudi J. Biol. Sci. 22, 466-475.

Elshikh M., Marchant R., Banat I. R., 2016, Biosurfactants: promising bioactive molecules for oral-related health applications, FEMS Microbiol. Lett. 363, 18.

Elshikh M., Funston S., Chebbi A., Ahmed S., Marchant R., Banata I. M., 2017, Rhamnolipids from non-pathogenic *Burkholderia thailandensis* E264: Physicochemical characterization, antimicrobial and antibiofilm efficacy against oral hygiene related pathogens, New Biotechnol. 36, 26-36.

Gomes H. I., Dias-Ferreira C., Ribeiro A. B., 2013, Overview of in situ and ex situ remediation technologies for PCB-contaminated soils and sediments and obstacles for full-scale application, Sci. Total Environ. 445-446, 237-260.

Joshi-Navare K., Prabhune A. A., 2013, Biosurfactant sophorolipid acts in synergy with antibiotics to enhance their efficiency. BioMed Res. Int. 7, 1-8.

Lamichhane S., Krishna K. C. B., Sarukkalige R., 2017, Surfactant-enhanced remediation of polycyclic aromatic hydrocarbons: A review, J. Environ. Manage. 199, 46-61.

Pacwa-Płociniczak M., Płociniczak T., Iwan J., Zarska M., Chorazewski M., Dzida M., Piotrowska-Seget Z., 2016, Isolation of hydrocarbon-degrading and biosurfactant-producing bacteria and assessment their plant growth-promoting traits, J. Environ. Manage. 168, 175-184.

Perinelli D. R., Vllasaliu D., Bonacucina G., Come B., Pucciarelli S., Ma R., Cespi M., Itri R., Spinozzi F., Palmieri G. F., Casettar L., 2017, Rhamnolipids as epithelial permeability enhancers for macromolecular therapeutics, Eur. J. Pharm. Biopharm. 119, 419-425.

Santos D. K., Rufino R. D., Luna J. M., Santos V. A., Sarubbo L. A., 2016, Biosurfactants: Multifunctional Biomolecules of the 21st Century, Int. J. Mol. Sci. 17, 401.

SEC, 2011, Communication from the commission to the European parliament, the council, the European economic and social committee and the committee of the regions, Roadmap to a Resource Efficient Europe, European Commission.

Shekhar S., Sundaramanickam A., Balasubramanian T., 2015, Biosurfactant Producing Microbes and their Potential Applications: A Review, Crit. Rev. Environ. Sci. Technol. 45:14, 1522-1554.

Siegmund I., Wagner F., 1991, New method for detecting rhamnolipids excreted by pseudomonas species during growth on mineral agar, Biotechnol. Tech. 5:4, 265-268.

Sivasankar P., Kumar G. S., 2017, Influence of pH on dynamics of microbial enhanced oil recovery processes using biosurfactant producing *Pseudomonas putida*: Mathematical modelling and numerical simulation, Bioresour. Technol. 224, 498-508.

Tripathi V., Edrisi S. A., Chen B., Gupta V. K., Vilu R., Gathergood N., Abhilash P. C., 2017, Biotechnological Advances for Restoring Degraded Land for Sustainable Development, Trends Biotechnol. 35, 847-859.

Vanhulle S., Enaud E., Trovaslet M., Billottet L., Kneipe L., Jiwan J-L. H., Corbisier A-M., Marchand-Brynaert J., 2008, Coupling occurs before breakdown during biotransformation of Acid Blue 62 by white rot fungi, Chemosphere 70, 1097-1107.

Vecino X., Cruz J. M., Moldes A. B., Rodrigues L. R., 2007, Biosurfactants in cosmetic formulations: trends and challenges, Crit. Rev. Biotechnol. 37, 911-923.