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A fast and effective analytical method to quantify the emulsifying activity: design and validation

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Introduction

Biosurfactants (BS) and bioemulsifiers (BE) are amphiphilic molecules that are produced by a wide range of microorganisms. According to Willumsen and Karlson [1], BS/BE are both surface active biomolecules, but while the surfactants play the role of surface tension reduction, emulsifiers are involved in formation and stabilization of emulsions. However, some biomolecules possess both surfactant and emulsifying properties, which contributes to their unique features, including high biodegradability, low toxicity, effectiveness at extremes of temperature, pH and salinity, and special biological activities (e.g., antimicrobial, antiviral, anticancer, etc). These attributes make them an alternative to their chemical counterparts and allows them to have key roles in several fields [2, 3].

In fact, the chemical composition of BS and BE is different, and this may contribute to their specific roles in nature and biotechnological applications; however, both BS/BE have recognized emulsifying properties, which are the focus of this study. The idea of quantifying the emulsifying activity rather than quantifying the emulsifiers and/or surfactants themselves has been studied before [4]. The concept that stands out is the emulsification index (E24: % emulsification after 24 h) proposed by Cooper and Goldenberg [4], which is still applied, and some of its adaptations such as the one proposed by Trebbau de Acevedo and McInerney [5]. These authors have defined one unit of emulsifying activity as the amount of emulsifier that results in an emulsification (E24) of 20%. Although theoretically simple, these approaches require considerable sample volume, have a long wait (24 h) and are lengthy. Moreover, they can be subjective, since two substances might induce complete emulsion at 24 h, with one resulting in a much denser emulsion. This may indicate more BS/BE activity; however, it is not easily comparable, or demonstratable. Furthermore, due to nature of emulsions and the factors that influence them, small differences in test conditions, such as shape or size of the tubes, or nature of the hydrophobic layer, can generate drastic differences, which hinders reproducibility between authors.

In this context, it is crucial to have a reliable method that not only allows for an accurate evaluation of the BS/BE emulsifying activity during its production conditions, but also that constitutes a valid parameter, which can be consistently comparable for different BS/BE. Therefore, the main goal of this study was the development and validation of a simple, expeditious and inexpensive analytical method to detect, quantify and compare the emulsifying activity of microbial culture broths or BS/BE solutions. Hence, a quantitative assay was developed to measure the emulsifying activity (EA) of any water-soluble compound, based on its ability to form and stabilize emulsion with n-heptane, in a fast, sensitive and reproducible manner.

Materials and methods

Microorganism and BS/BE production - The microorganism used in this work was the bacterium *Gordonia alkanivorans* strain 1B, isolated on our laboratory [6] and kept at a culture collection of microorganisms (CCM at LNEG, Portugal, Lisbon). The BS/BE production by strain 1B was performed as described by Silva et al. [7].

Emulsifying activity (EA) test design and its validation - For the method development and validation, several synthetic surfactants (Tween 80, Tween 20, Triton X-100, SDS and STC), and industrial/domestic dishwashing detergents (Magilar[®] S100 and Fitipol[®], Ultra Pro[®], Super Pop[®], and Fairy[®]) were tested in parallel with the crude BS/BE produced by strain 1B. The preparation of aqueous solutions for synthetic surfactants, industrial/domestic detergents and for crude BS/BE was made by dissolving the different products in ultrapure water to obtain a set of specific known increasing concentrations for each one. After, for each test product concentration, an aliquot (e.g. 10 µL) was diluted in water, up to 1 mL aqueous fraction



in 4 mL screw cap glass tubes (10x75 mm, ND10 caps with PTFE septum), and then 1 mL of n-heptane was added and mixed by vortexing at high speed for 2 min. Lastly, the tubes were left resting vertically for 10 minutes for emulsion detection. A set of emulsification tests with increasing volumes of test product solutions were carried out until 100% emulsion was obtained in the organic phase. One emulsification unit (1 U) consists of the minimum volume of product (volume of emulsifier/surfactant = Vol_{min}) needed to form and maintain 100% emulsion in 1ml of n-heptane. Thus, the corresponding EA value is presented in U/mL and calculated as: $EA(\text{product}) = 1 U/Vol_{min}(\text{mL})$.

Results and conclusions

In this study, a new and simple method to assess the emulsifying activity (EA) was developed and further validated both for BS/BE and for synthetic surfactants/commercial detergents. It is an easy test that enables the obtention of a quantitative value within 10 minutes, by mixing 1 mL of an aqueous solution, containing the product with emulsifying properties (e.g., BS/BE or synthetic surfactants), with 1 mL of n-heptane as the organic phase. In this analytical approach, the EA can be evaluated through the quantification of emulsification units per mL (U/mL), with one emulsification unit (1 U) defined as the minimum amount of test product (volume, up to 1 mL) needed to form and maintain 100% emulsion of 1 mL of n-heptane. So, for the crude BS/BE (0.352 g/L solution), 1 U = 350 μ L and the corresponding EA = $1/0.350 = 2.86$ U/mL, while for Tween 80 (0.289 g/L solution), 1 U = 30 μ L and the correspondent EA = $1/0.065 = 15.38$ U/mL.

In this test method a rapid and stable emulsion is obtained, and, moreover, the sample amount required is usually very small. These features are an advantage towards a quick quantification of the EA for a great number of samples, primarily for screen/monitoring assays of products with emulsifying properties (BS/BE).

Furthermore, the specific emulsifying activity (SEA in U/g) for each product tested can be estimated through correlation analysis (linear regression) between volumetric emulsifying activity (U/mL) and product concentration (g/L) allowing actual data comparison between different products with emulsifying properties (BS/BE or synthetic surfactants/detergents).

In overall, the novel analytical method proposed seems to be a fundamental tool for at-line quantification/estimation of emulsifying activity required for the prospecting of new microbial sources of BS/BE and monitoring the production of these natural compounds within microbial cultivation process.

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