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# Demonstrating the viability of halolipase production at a mechanically stirred tank biological reactor

Sergio Colangiuli, Ana Rodríguez, María Ángeles Sanromán, Francisco J. Deive*

*Department of Chemical Engineering, University of Vigo, 36310 Vigo, Spain*

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ABSTRACT

The definition of halophiles as "the coming stars of industrial biotechnology" in a recent review demands new research efforts for their efficient production at bioreactor scale. In this sense, the scarcity of information about halolipases production has furthered the research on the viability of *Halomonas* sp. LM1C culture in a mechanically stirred bioreactor. The operating conditions have been optimized by means of a Central Composite Face-Centered (CCFC) design. The operation at low aerations (0.25 vvm) and moderate agitation rates (583 rpm) led to activity levels near 8000 U/L, which clearly surpasses the typical values detected for other extremophilic enzymes. The process at optimum conditions has been kinetically characterized and the oxygen volumetric mass transfer coefficient ($K_La$) has been determined.

## 1. Introduction

Currently, the fact that fossil fuels constitute 80% of the primary energy consumption has favored a booming research interest in the biocatalytic synthesis of biofuels. More specifically, the use of biocatalysts is one of the cornerstones to alter the biofuel reaction yield and sustainability (Mardhiah et al., 2017). The reason is that they entail advantages over conventional catalysts such as their lower energy requirements and costs due to the simplification of the process and the operation at mild conditions (Aransiola et al., 2014). In this case, one of the promising alternatives for the production of biodiesel can be the use of triacylglycerol acylhydrolases or lipases (E.C. 3.1.1.3), enzymes that typically act on the cleavage or formation of ester bonds (in the presence or absence of water, respectively). Additionally, their ability to catalyze both esterification and transesterification is useful because it simultaneously allows transforming triacylglycerols and free fatty acids in biodiesel. Actually, the assumption that biocatalytic production of biodiesel can be only viable from useless or waste fats makes it interesting to employ these enzymes, as they have been demonstrated to enable the conversion of inedible substrates containing more than 80% of free fatty acids (Lai et al., 2005).

The new research trends in this field are focused on the introduction of neoteric solvents as replacement of conventional organic compounds in order to improve the process efficiency and sustainability (Gutiérrez-Arnillas et al., 2016a). However, the obverse side of the coin refers to the deactivating phenomena recorded in mainstream enzymes in the presence of these chemicals. Thus, physical properties like polarity, hydrophobicity or hydrogen bonding capacity have been described as crucial aspects that must be taken into account to avoid the existence of deleterious effects both at a structural and biocatalytic level (Gutiérrez-Arnillas et al., 2016a; Shah and Gupta, 2007; Rodrigues et al., 2014).

To avoid this, extremophilic microorganisms may be envisaged as an ideal platform for obtaining robust enzymes able to optimally exert their biocatalytic potential. Depending on the habitat where they are adapted to thrive, these organisms are classified as halophiles, thermophiles, psychrophiles, alkalophiles, acidophiles, piezophiles, xerophiles, metallophiles and radiophiles (Deive et al., 2012a). Among them, halophiles have been underscored as resilient contenders to withstand high concentrations of some neoteric solvents employed in the production of biodiesel like ionic liquids (Gutiérrez-Arnillas et al., 2016b; Deive et al., 2011). Therefore, these microorganisms could be a suitable source of enzymes maintaining their lipolytic activity in the presence of these molten salts. Beside this, they are envisaged to acquire a great predominance in future (Yin et al., 2015) by making biotechnological processes more competitive regarding conventional chemical alternatives. The main reasons are the possibility of saving fresh water (using marine water) or the avoidance of costly sterilization stages (Yin et al., 2015; Chen, 2012).

However, despite the promising potential of halophilic enzymes, they are still not commercially available probably due to the scarcity of knowledge in this field. Efforts in the design of optimum medium components and operating conditions are thus needed to achieve economically viable production processes. In this vein, the operation at bioreactor and the scale-up is usually the bottleneck for successfully

* Corresponding author.
*E-mail address:* deive@uvigo.es (F.J. Deive).

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implementing the enzyme production at an industrial scale (Wolf-Márquez et al., 2017). Most of the work in this field has been focused on the isolation, identification and characterization of different strains (Moreno et al., 2009; Pérez et al., 2011; Li et al., 2014), while the production at bioreactor scale and the optimization of the operating conditions is comparatively scarce.

As a continuation of our previous research works with a novel lipase producer, *Halomonas* sp. LM1C (Gutiérrez-Arnillas et al., 2016b, 2017), in this study we have researched how the operating conditions in a stirred tank bioreactor affected the extracellular lipase biosynthesis. Once the optimum was identified, the experimental kinetic data from the biological process were fitted to logistic equations. Finally, the oxygen volumetric mass transfer coefficient ($K_La$) was ascertained in order to elucidate the role of oxygen supply in the development of the biological process.

## 2. Materials and methods

### 2.1. Microorganism

The bacterium *Halomonas* sp. LM1C was isolated and genetically identified from samples obtained in saltworks from Parque Natural de las Lagunas de La Mata y Torrevieja (Alicante, Spain) as demonstrated previously (Gutiérrez-Arnillas et al., 2016b).

### 2.2. Culture conditions

The medium employed, adapted from the Halophile I medium (Spanish Culture Type Collection – CECT 17), has the following composition: casein peptone (7.5 g/L), yeast extract (10 g/L), $Na_3C_6H_5O_7{\cdot}2H_2O$ (3 g/L), KCl (2 g/L), $MgSO_4{\cdot}7H_2O$ (20 g/L), $MnSO_4{\cdot}H_2O$ (0.25 g/L), $FeSO_4{\cdot}7H_2O$ (0.05 g/L). In addition, the culture was supplemented with NaCl (15% w/v) and Triton X-100 (1 g/L). All the components of the culture medium were provided by Sigma-Aldrich. The pH of the culture was adjusted to 6.9 and this medium was sterilized at 121 °C for 20 min. All the cultures were carried out at 21.6 °C, in line with the conclusions reported elsewhere (Gutiérrez-Arnillas et al., 2017). Inocula were preserved following the instructions provided in a previous research work (Gutiérrez-Arnillas et al., 2016b).

### 2.3. Bioreactor culture

A mechanically stirred tank bioreactor (1.5 L working volume) equipped with a dual Rushton-type impeller and connected to a control unit (Biostat B, Braun, Melsungen, Germany) was used. The medium was inoculated (3% v/v) with actively growing cells, cultured in Erlenmeyer flasks at the abovementioned pH and temperature. An experimental design Central Composite Face-Centered (CCFC) was the tool employed to optimize the lipolytic enzyme production when the aeration rate and agitation speed were varied.

### 2.4. Sampling treatment

5 mL-samples were taken and centrifuged at 10,000 rpm for 10 min at 4 °C. While the supernatant was set aside for determining the extracellular lipolytic activity, the precipitate was re-suspended in 2 mL of Tris/HCl buffer for biomass determination.

### 2.5. Biomass determination

UV spectrophotometry at 600 nm was used to quantify the cell dry weight after a previous calibration step (Biomass (g/L) = $0.515{\cdot}Abs_{600}$: $R^2 = 0.972$). This calibration was carried out as follows: a known volume (20 mL) of culture at the stationary phase was centrifuged at 10,000 rpm for 5 min. The supernatant was discarded and the cells were resuspended in 10 mL of distilled water. This operation was repeated twice and the obtained cell suspension was then vacuum-filtered (pore size 0.44 μm) and introduced in an oven at 120 °C for 24 h and the weight was recorded. 8 dilutions of the original culture sample were carried out in order to obtain the relationship between biomass and absorbance at 600 nm.

**Table 1**
Summary of experiments used for operating conditions optimization.

| Experiment | Agitation speed (rpm) | Aeration rate (vvm) | Maximum Biomass (g/L) | Maximum Lipolytic activity (U/L) |
|---|---|---|---|---|
| 1 | 300 | 0.25 | 3.34 | 3550 |
| 2 | 300 | 0.50 | 3.27 | 2390 |
| 3 | 300 | 0.75 | 3.44 | 1988 |
| 4 | 500 | 0.25 | 3.37 | 6901 |
| 5 | 500 | 0.50 | 3.41 | 4626 |
| 6 | 500 | 0.75 | 3.16 | 2905 |
| 7 | 700 | 0.25 | 3.34 | 6124 |
| 8 | 700 | 0.50 | 3.03 | 4188 |
| 9 | 700 | 0.75 | 3.00 | 3502 |

### 2.6. Quantification of lipolytic activity

UV spectrophotometry at 400 nm was employed to determine the lipolytic activity. Thus, the hydrolysis of *p*-nitrophenyl laurate (2.5 mM) (Sigma-Aldrich) was monitored and one activity unit was defined as the amount of enzyme leading to the release of 1 μmol of *p*-nitrophenol per minute at the assay conditions (Fuciños et al., 2005).

### 2.7. Statistical analysis and validation of the experimental model

Response Surface Methodology (RSM) was used to maximize lipase production varying the aeration rate (0.25 vvm–0.75 vvm) and agitation speed (300 rpm–700 rpm), as detailed in Table 1. The lipolytic activity was described by means of a second order polynomial equation:

$$Y = a_0 + a_1X + a_2Z + a_{12}X{\cdot}Z + a_{11}X^2 + a_{22}Z^2 \quad (1)$$

being $X$ and $Z$ the independent variables, aeration rate and agitation speed, respectively, $Y$ the dependent variable (lipolytic activity) and $a_0$, $a_1$, $a_2$, $a_{12}$, $a_{11}$, and $a_{22}$ the optimized parameters of linear, quadratic and interaction effects. The analysis of variance (ANOVA) was obtained by means of the software Design Expert® 9.0.0 (Stat-Ease Inc., Minneapolis, USA).

## 3. Results and discussion

### 3.1. Maximizing lipolytic biosynthesis in a mechanically stirred tank bioreactor

In previous research works of our group, we have isolated a promising halophilic bacterium, genetically identified as *Halomonas* sp. LM1C, able to produce high levels of extracellular lipases in hypersaline environments (about 250 U/L) (Gutiérrez-Arnillas et al., 2016b). After having optimized the culture medium, pH and temperature, a drastic increase (more than 1 order of magnitude) of lipolytic activity was recorded during the stationary phase of cultivation (3000 U/L) (Gutiérrez-Arnillas et al., 2017). This enzyme displays an optimum activity at high NaCl concentrations (even at 30%), and which does not suffer from deactivation phenomena when concentrated in an AMICON cell (YM-10 membrane). However, a lack of knowledge is detected on how the operating conditions (agitation speed and aeration rate) at a mechanically stirred bioreactor would affect the performance of a halophilic biotechnological process for lipase production, as there are no previous research works tackling this topic. In this case, the bioreactor is equipped with a sparge ring made of stainless steel placed bellow a 6-flat-bladed Rushton disc turbine, as it is the most commonly employed