Hom-Diaz, A., Passos, F., Ferrer, I., Vicent, T., Blanquez, P. (2016) Enzymatic pretreatment of microalgae using fungal broth from Trametes versicolor and commercial laccase for improved biogas production. Algal Research 19 (2016) 184–188.

Enzymatic pretreatment of microalgae using fungal broth from *Trametes versicolor* and commercial laccase for improved biogas production

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Abstract

Coupling microalgae production to wastewater treatment can reduce the costs of microalgae production for non-food bioproducts and energy consumption for wastewater treatment. Furthermore, microalgae anaerobic digestion can be enhanced by applying pretreatment techniques.. The aim of this study is to improve the biogas production from microalgal biomass grown in urban wastewater treatment systems by applying an enzymatic pretreatment with crude fungal broth and commercial laccase. To this end, the fungus *Trametes versicolor* was cultured, and the enzymatic activity of the culture broth analysed by measuring laccase concentration. The results showed that both the fungal broth and commercial laccase pretreatment (100 U/L) over an exposure time of 20 min increased the methane yield in batch tests. Indeed, the fungal broth pretreatment increased the methane yield by 74%, while commercial laccase increased the methane yield by 20% as compared to non-pretreated microalgal biomass. In this manner, laccase addition enhanced microalgal biomass anaerobic biodegradability, and addition of *T. versicolor* broth further improved the results. This fact may be attributed to the presence of other molecules excreted by the fungus.

Keywords

Biological pretreatment; Enzyme; Fungi; Laccase; Microalgae; Methane

1 Introduction

Microalgae have long been studied for wastewater treatment because of their high capacity for nutrient and organic matter removal in symbiosis with heterotrophic bacteria, resulting in a much lower energy requirement compared to conventional activated sludge systems which demand mechanical aeration [1]. Furthermore, the produced microalgal biomass may be converted into biofuels, including biodiesel, biohydrogen, bioethanol, biomethane, or non-food bioproducts, such as biofertilizers and biomaterials.

Biogas production from microalgal biomass through anaerobic digestion has raised interest due to the low complexity, minimal processing requirements and availability of a technology that has long been used for sludge treatment in wastewater treatment plants (WWTP) [2]. Despite the potential of anaerobic digestion, most microalgae species growing in WWTP have a complex cell wall composed of resistant structural carbohydrates, limiting the hydrolysis step [3]. Thus, pretreatment techniques have been studied to increase microalgae solubilisation and methane yield [4]. Thermal processes at low and high temperatures and mechanical methods like ultrasound and microwave enhance microalgae biodegradation and biogas production [5], although the energy consumed during the pretreatments may be too high for full scale application, especially in the case of mechanical techniques.

Recently, biological methods like the use of enzymes has been tested. They are regarded as a low-cost, eco-friendly pretreatments for enhancing microalgal biomass anaerobic biodegradability [6,7]. Enzymes are selected according to the main microalgal cell wall compoundss namely cellulose, hemicelluloses, pectin, glycoproteins, and even lignin [8,9]. Indeedthe specific composition depends on the strain, age of the culture, nutrient concentration and ambient conditions, among others [6]. The most commonly used

enzymes for microalgae pretreatment are commercial α-amylases, amyglucosidases, cellulases, xylanases, lipases or proteases [10,11]. Furthermore, it has been shown that using a mixture of commercial enzymes, the methane yield was higher than using a single enzyme specific for one substrate [10,12]. Regarding the use of crude fungal enzymes, from those *Aspergillus lentullus* were particularly effective at improving microalgae anaerobic biodegradability [13]. Ligninolytic fungi produce non-specific intra and extracellular enzymes, depending on the culture conditions [14]. One of the most well-known fungus that produces laccase is the white-rot fungus *Trametes versicolor*. Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) are a family of glycoproteins, classified as oxidoreductases that catalyse the monoelectronic oxidation of substrates at the expense of molecular oxygen. They are used for cross-linking of monomers, degradation of polymers and ring cleavage of aromatic compounds in various environmental applications (e.g. bioremediation of soils and wastewater, decolourization of recalcitrant dyes, kraft pulp biobleaching, biorefinery processes and degradation of contaminants) [15–19]. In addition, laccase can be used as a pretreatment step for cellulose hydrolysis [20].

The aim of the present study is to evaluate the biogas production increase obtained by applying an enzymatic pretreatment to microalgal biomass in biochemical methane potential (BMP) tests. Two pretreatment approaches were considered, the first one using the commercial laccase enzyme and the second one using crude fungal enzyme from *Trametes versicolor*. This is the first time that the fungal broth from *T. versicolor* culture has been used as a microalgal biomass pretreatment for biomethanization.

2 Materials and methods

2.1 Microalgal biomass

In this article, the term microalgal biomass refers to the mixed culture of green microalgae, mainly *Oocystis* sp., diatoms, bacteria and other microorganisms such as protozoa, grown spontaneously in experimental raceway ponds treating urban wastewater [21]. This microalgal biomass was harvested from pilot raceway ponds used for secondary treatment of real urban wastewater, located outdoors at the Department of Civil and Environmental Engineering of the Universitat Politècnica de Catalunya·BarcelonaTech (Barcelona, Spain). A full description of the system operation may be found elsewhere [22]. Average characteristics of harvested biomass are summarised in Table 1.

Table 1 Main characteristics of microalgal biomass (substrate) and digested sludge (inoculum) used for BMP tests.

Parameter	Microalgal biomass	Inoculum
pH	7.8	7.4
TS [%(w/w)]	3.28	3.63
VS [%(w/w)]	2.07	2.57
VS/TS (%)	63%	71%
COD (g/L)	31.3	31.2
Proteins (% VS)	58	-
Carbohydrates (% VS)	22	-
Lipids (% VS)	20	-

2.2 Fungus and chemicals

Trametes versicolor was obtained from the American Type Culture Collection (ATCC #42530). The fungus was serially subcultured on 2% malt agar slants at 25 °C until use. Glucose, ammonium tartrate dibasic, malt extract and other chemicals were purchased from Sigma-Aldrich (Barcelona, Spain).

2.3 Trametes versicolor culture

A mycelia suspension of *T. versicolor* was obtained by inoculating four 1 cm diameter plugs from the growing zone of fungi on malt agar, in 250 mL malt extract medium (2%) in a 1 L Erlenmeyer flask. Flasks were placed on an orbital shaker (130 rpm, r = 25 mm) at 25 ºC. After 6 days, a thick mycelial mass was formed, which was ground with an X10/20 (Ystral GmbH) homogenizer. This suspension was used to produce pellets by inoculating 1 mL of the suspension in 250 mL malt extract medium 2% in a 1 L Erlenmeyer flask. The flasks were incubated on an orbital shaker (130 rpm, r = 25 mm) at 25 ºC for 6 days. The pellets thus obtained were then used for fungal broth production.

2.4 Fungal broth production

2.5 T. versicolor broth was produced in 250 mL Erlenmeyer flasks containing 0.9 g cell dry weight of T. versicolor pellets in 100 mL of medium containing: 8 g L^{-1} of glucose, 3.3 g L^{-1} of ammonium tartrate, 1.168 g L^{-1} of 2,2-dimethylsuccinate buffer, 10 and 100 mL L^{-1} of a micro and macronutrient solution, respectively [23]; adjusted to pH 4.5 with HCl. Pellets were cultured in six Erlenmeyer flasks, 3 of them were cultured until laccase production was 100 U/L (3.5 days) and the other 3 until glucose was totally consumed. Both parameters, laccase production and glucose consumption were daily monitored.Enzymatic pretreatment

Two enzymatic pretreatments were carried out using either the commercial enzyme laccase (purchased from Merck (Madrid, Spain)) enzyme or *T. versicolor* broth. In the first case, a stock solution of commercial laccase was prepared and added to microalgal biomass (31 g_{wet}) before BMP tests. The laccase concentration in BMP bottles was 100 U L^{-1} and the contact time prior to BMP tests was 20 minutes, it was maintained at 25ºC

143 and 100 rpm shaker platform (orbital shaker Kuhner, LS-X, Switzerland, r = 25 mm) .
144 In the second case, broth produced by *T. versicolor* culture (sieved to remove the fungal
145 pellets) containing 100 U L^{-1} of laccase enzyme was added to microalgal biomass
146 following the same strategy as for commercial laccase.

147 *2.6 Biochemical methane potential tests*

148 After the enzymatic pretreatment of microalgal biomass for 20 minutes, BMP tests were
149 carried out in serum bottles of 160 mL, with a useful volume of 100 mL and a
150 headspace volume of 60 mL. The inoculum was mesophilic digested sludge from an
151 anaerobic digester of a municipal WWTP located in Gavà (Catalunya, Spain). Bottles
152 contained a total organic matter concentration of 5 g COD/L and the substrate/inoculum
153 (S/I) ratio was 0.5 g VS substrate/ g VS inoculum, based on previous studies, including
154 one in which the S/I ratio was optimised for microalgal biomass grown in the same pilot
155 HRAP [24,25]. Afterwards, bottles were filled with distilled water up to 100 mL,
156 flushed with helium gas, sealed with butyl rubber stoppers and incubated at 35ºC until
157 biogas production ceased. Biogas production was measured by the pressure increase in
158 the headspace volume using an electronic manometer (Greisinger GMH 3151, error
159 ±0.1%). After each measurement, biogas was purged from the reactor's headspace until
160 atmospheric pressure; afterwards reactors were manually shaken.
161 The following trials were carried out: (1) microalgal biomass pretreated with
162 commercial laccase, (2) microalgal biomass pretreated with fungal broth, (3) non-
163 pretreated microalgal biomass control, (4) commercial laccase control, (5) fungal broth
164 control, and (6) blank containing only inoculum, in order to quantify the methane
165 production by endogenous respiration. Blank results were subtracted from all trials to
166 obtain the net biogas production. Furthermore, commercial laccase control results were
167 subtracted from microalgal biomass pretreated with commercial laccase; whereas fungal

broth control results were subtracted from microalgal biomass pretreated with fungal broth. All experimental trials, including pretreatments, controls and blank were performed in triplicate and expressed at standard temperature and pressure.

2.7 Analytical methods

Glucose concentration was measured with an YSI 2000 enzymatic analyzer from Yellow Springs Instruments and Co.

Laccase activity was measured using a modified version of the method for the determination of manganese peroxidase [26]: The reaction mixture used consisted of 200 µL of 250 mM sodium malonate at pH 4.5, 50 µL of 20 mM 2,6-dimetoxiphenol (DMP) and 600 µL of sample. DMP is oxidized by laccase even in the absence of cofactor. Changes in the absorbance at 468 nm were monitored for 2 min on a Varian Cary 3 UV-vis spectrophotometer at 30ºC. One activity unit (U) was defined as the number of micromoles of DMP oxidized per minute. The DMP extinction coefficient was 24.8 mM^{-1} cm^{-1} [27].

The inoculum and substrate were characterised (Table 1) by the concentration of total solids (TS), volatile solids (VS) and chemical oxygen demand (COD), following standard methods guidelines (APHA, 1999). pH was analysed with a Crison Portable 506 pH-meter. The lipid content of biomass was determined by the Soxhlet extraction method [28]. The total Kjeldahl nitrogen (TKN) to protein conversion factor was 5.95, according to González López et al., [27]. Carbohydrates were determined by phenol–sulphuric acid method, after acid hydrolysis and measured by spectrophotometry (Spectronic Genesys 8) [30].

The methane content in biogas was measured once a week with a gas chromatograph (GC) (Trace GC Thermo Finnigan) equipped with a Thermal Conductivity Detector, by injecting gas samples into a packed column (Hayesep 3m1/8 in. 100/120). The carrier

gas was Helium in split less mode (column flow: 19 mL/min). The oven temperature was 35 ºC with a retention time of 1.5 min. Injector and detector temperatures were 150 and 25 ºC, respectively. The system was calibrated with methane (50% CH_4) and carbon dioxide (50% CO_2).

3 Results and discussion

3.1 Fungal broth production

Trametes versicolor cultured in Kirk's nutrient medium produces laccase enzyme and is appropriate for studying the ligninolytic activity of fungal cultures [31]. Laccase production and glucose consumption from *Trametes versicolor* culture are shown in Figure 1. Gradual glucose consumption along with laccase activity increase by the fungus *T. versicolor* can be observed.

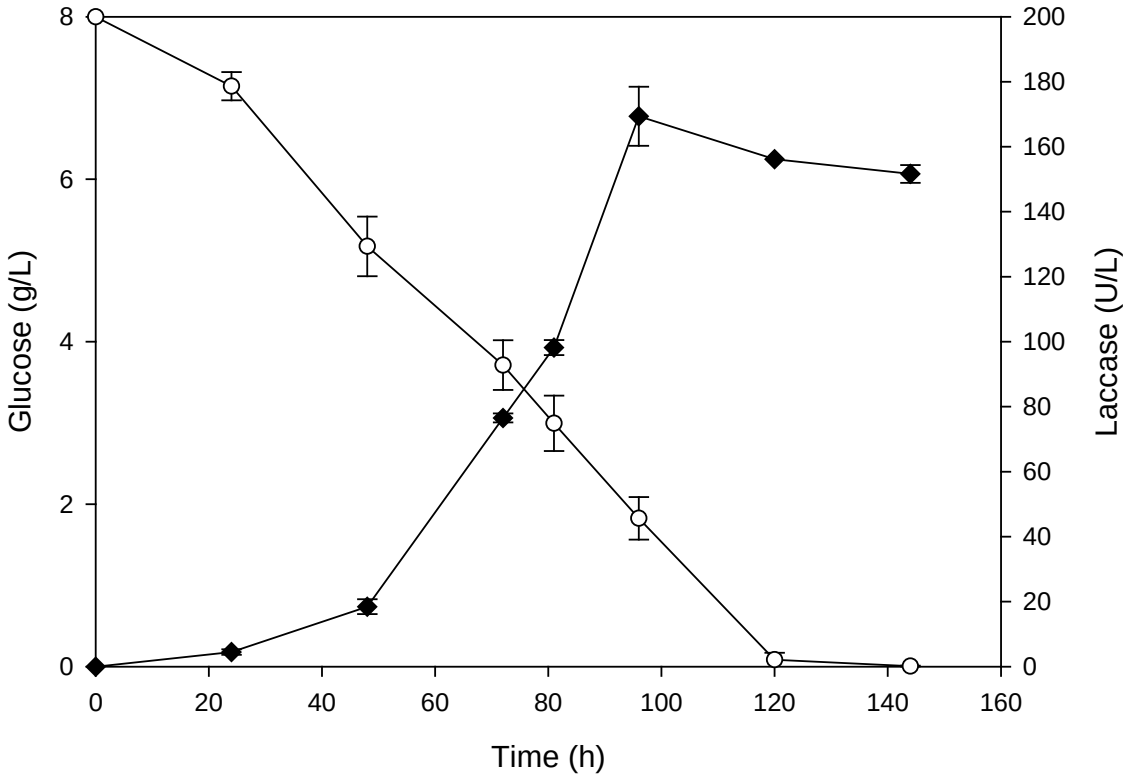
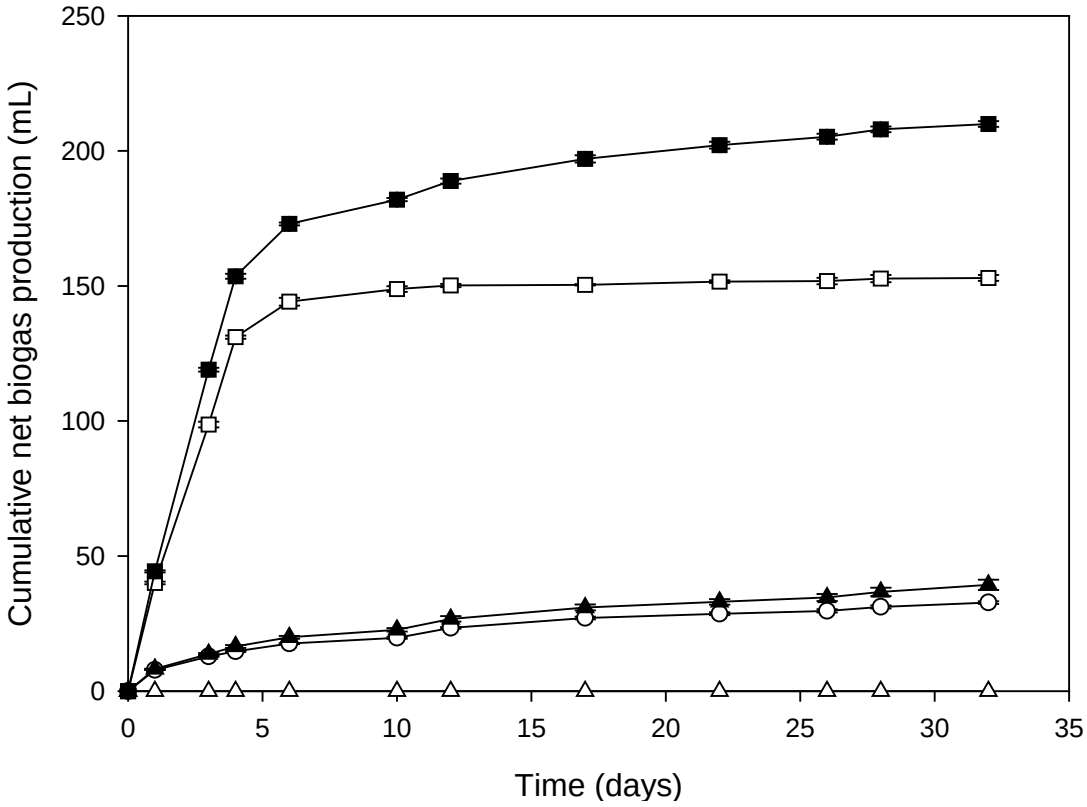


Figure 1. Glucose consumption (○) and laccase production (♦) by *Trametes versicolor*

Laccase enzyme is excreted by *T. versicolor* to the broth, which is associated to both growth and glucose consumption. Enzyme production increased over the first 4 days and, after reaching a maximum activity level (170 U L^{-1}, 4 days), it dropped, since the carbon source (glucose) had been consumed. The same laccase activity behaviour was observed by other authors [15,32]. The fungal broth obtained from *T. versicolor* culture in Kirk's medium is mostly rich in laccase enzyme, among other enzymes or mediators, and unconsumed glucose. After 3 days of cultivation, other enzymes can be secreted by *T. versicolor*, such as cellulases and hemicellulases [33], possibly important for microalgae cell wall degradation.

3.2 Biogas production in BMP test

The fungal broth and commercial laccase were applied at a dose of 100 U $L^{-1}$of laccase enzyme and were used as a pretreatment for microalgal biomass solubilisation in order to evaluate the anaerobic biodegradability increase in BMP tests. The experiment lasted 32 days, until accumulated biogas production reached an asymptote (Figure 2). As can be seen from the results, both pretreated trials increased the biogas production as compared to non-pretreated microalgae. Moreover, the fungal broth pretreatment attained the highest value. The methane content was measured along the experiment obtaining an average concentration of 68±4.5% CH_4. Control trials from both laccases (commercial and fungal broth) were subtracted from the corresponding pretreatment, along with the production of the inoculum, to obtain the net biogas and methane production along with the net methane yield (Table 2).



230

231 **Figure 2** Cumulative net biogas production for the anaerobic digestion of microalgal

232 biomass using two enzymatic pretreatments and their respective controls. Commercial

233 laccase control (△); Microalgal biomass control (○); Commercial laccase pretreatment

234 (▲); Fungal broth control (□); Fungal broth pretreatment (■)

235

236 **Table 2 Net methane production and yield for the different trials of the BMP test**

Trial	**Biogas production (mL)**	**Methane production (mL CH_4)**	**Methane yield (mL CH_4 g VS^{-1})**
Microalgal biomass control	33±0.5	22±0.5	83±1
Commercial laccase control	0.0	0.0	-
Fungal broth control	153±1.1	104±1.1	-
Commercial laccase pretreatment	40±1.3	27±1.3	100±7
Fungal broth pretreatment	210±0.3	143±0.3	144±2

237

Regarding the control trials, commercial laccase control did not produce any biogas. Microalgal biomass control produced little methane (22 mL CH_4), whereas the fungal broth control produced 104 mL CH_4. Indeed, reactors containing fungal broth produced more biogas than the rest, since they contained part of the nutrients (mainly glucose) present in the media for laccase production, which were not completely consumed by *T. versicolor*. This can be seen from Fig. 1: when 100 U L^{-1} of laccase were obtained, the concentration of glucose was 3 g L^{-1}. The amount of biogas produced from glucose remaining in the culture broth, was theoretically calculated and compared with experimental results, using the Buswell equation [34] (equation 1 and 2). According to this, 108 mL CH_4 were theoretically produced, due to the remaining glucose of the medium. This theoretical value is in accordance with the experimental one (104 mL CH_4).

$$C_nH_aO_b + \left(\frac{4n-a-2b}{4}\right)H_2O \rightarrow \left(\frac{4n+a-2b}{8}\right)CH_4 + \left(\frac{4n-a+2b}{8}\right)CO_2 \qquad (1)$$

$$B_{o,th}\left[\frac{L\ CH_4}{g\ C_nH_aO_b}\right] = \frac{1}{8}\left(\frac{4n+a-2b}{12n+a+16b}\right)V_m \qquad (2)$$

Where V_m is the molar volume of methane at standard temperature and pressure.

The presence of glucose could also enhance the proliferation of anaerobic microorganisms, which may contribute to an increase of biogas production. However, since this effect cannot be measured, only the methane production due to glucose contribution was subtracted.

With regards to the pretreatment trials, commercial laccase pretreatment increased the methane yield by 20%, whereas fungal broth pretreatment increased the methane yield by 74% relative to non-pretreated biomass. The results suggest that laccase may solubilise part of the microalgal biomass substrate, enhancing its bioavailability and/or biodegradability by anaerobic microorganisms. However, better results were achieved using the fungal broth. This is probably due to the presence of other enzymes, radicals

and other mediators produced by *T. versicolor* during its culture, which may also contribute to microalgal biomass solubilisation [19]. It is worth pointing out that even though laccase is not specificly on active glycoproteins and polysaccharides (the main components of microalgal cell wall), the pretreatment was effective. Therefore, results confirm that laccase played a role on microalgae enzymatic pretreatment, although a mixture of different enzymes would be preferred. This is common for complex cultures, such as the one of the present study, composed by several microalgae species, bacteria and other microorganisms with different cell wall compositions. The results are in accordance with previous studies, where microalgae methane yield was increased when non-specific enzymes were added confirming the synergistic effect [10,12,13]. Nevertheless, a previous study using filamentous microalgae reported higher values than those obtained in our study. Ehimen et al. [10] obtained 115-145 mL CH_4/g TS after an enzymatic pretreatment over 2 days, whereas the values obtained in the present study were 63 and 91 mL CH_4/g TS for commercial laccase and fungal broth pretreatment, respectively, after 20 minutes of enzymatic pretreatment. From these results, contact time seems to be an important parameter that should be further investigated. The methane yield of *Chlorella vulgaris* was increased by 14% after pretreatment with the hydrolytic enzyme carbohydrolase and by 51% after pretreatment with protease after an exposure time of 5 h. Moreover, the same study with *Chlamydomonas reinhardtii* showed no increase after pretreatment with carbohydrolase and only 8% increase after pretreatment with protease [35]. This increase was lower than the ones obtained in our study (20 and 74% increase) and highlights that pretreatment effectiveness is species-specific and depends on the biomass complexity and composition.

Finally, the results obtained in this study demonstrates that enzymatic pretreatment may be applied to microalgae anaerobic digestion, with better results for crude fungal enzymes probably due to the presence of other enzymes and other molecules produced by the fungus. This may be more cost-effective compared to commercial enzymes. Nevertheless, these results should be evaluated in continuous reactors for energy and economic aspects.

4 Conclusions

This study aimed at investigating the effect of laccase, a non-specific enzyme, on microalgal biomass from a pilot-scale urban wastewater treatment system as a pretreatment step prior to its anaerobic digestion. Comparing the effect of commercial laccase and the fungal broth from *Trametes versicolor*, better results were observed for the fungal broth, which may be due to the synergistic effect of laccase and other radicals or molecules produced by *T. versicolor*. The methane yield was increased by 20% for commercial laccase and 74% for fungal broth, as compared to non-pretreated biomass. Thus, these findings should be investigated in continuous anaerobic reactors for evaluating full-scale viability.

Acknowledgements

This work was funded by the Spanish Ministry of Economy and Competitiveness and FEDER (CTM2013-48545-C2-1-R and CTQ2014-57293-C3-3-R) and supported by the Generalitat de Catalunya (Consolidated Research Groups 2014SGR476 and 2014SGR116). The Department of Chemical Engineering of the Universitat Autònoma de Barcelona is member of the Xarxa de Referència en Biotecnologia de la Generalitat

de Catalunya. Andrea Hom-Diaz acknowledges her PhD scholarship from AGAUR (2013FI_B 00302). Fabiana Passos appreciates her Post-Doctorate scholarship funded by the National Council for Scientific and Technological Development (CNPq) from the Brazilian Ministry of Science, Technology and Innovation.

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