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

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4 Andrea Hom-Diaz [a,#], Fabiana Passos [b,c,#], Ivet Ferrer [b], Teresa Vicent [a], Paqui Blánquez
5 [a,*]

6

7 [a] Chemical, Biological and Environmental Engineering Department, Escola
8 d'Enginyeria, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Barcelona,
9 Spain

10

11 [b] GEMMA - Environmental Engineering and Microbiology Research Group,
12 Department of Civil and Environmental Engineering, Universitat Politècnica de
13 Catalunya·BarcelonaTech, c/Jordi Girona 1-3, Building D1, E-08034 Barcelona, Spain

14

15 [c] Environmental and Chemical Technology Group, Department of Chemistry,
16 Universidade Federal de Ouro Preto, 35400-000 Ouro Preto, Minas Gerais, Brazil.

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18 [#] Andrea Hom-Diaz and Fabiana Passos contributed equally to this work

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20 *Corresponding author: Paqui Blánquez
21 Chemical, Biological and Environmental Engineering Department
22 Escola d'Enginyeria
23 Universitat Autònoma de Barcelona
24 08193 Cerdanyola del Vallès. Spain
25 E-mail address: paqui.blanquez@uab.cat
26 Tel: +34 93 581 19 79; Fax: +34 93 581 20 13

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28**Abstract**

29Coupling microalgae production to wastewater treatment can reduce the costs of
30microalgae production for non-food bioproducts and energy consumption for
31wastewater treatment. Furthermore, microalgae anaerobic digestion can be enhanced by
32applying pretreatment techniques.. The aim of this study is to improve the biogas
33production from microalgal biomass grown in urban wastewater treatment systems by
34applying an enzymatic pretreatment with crude fungal broth and commercial laccase. To
35this end, the fungus *Trametes versicolor* was cultured, and the enzymatic activity of the
36culture broth analysed by measuring laccase concentration. The results showed that both
37the fungal broth and commercial laccase pretreatment (100 U/L) over an exposure time
38of 20 min increased the methane yield in batch tests. Indeed, the fungal broth
39pretreatment increased the methane yield by 74%, while commercial laccase increased
40the methane yield by 20% as compared to non-pretreated microalgal biomass. In this
41manner, laccase addition enhanced microalgal biomass anaerobic biodegradability, and
42addition of *T. versicolor* broth further improved the results. This fact may be attributed
43to the presence of other molecules excreted by the fungus.

44

45**Keywords**
46Biological pretreatment; Enzyme; Fungi; Laccase; Microalgae; Methane

47

48 **1 Introduction**

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

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

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

73used enzymes for microalgae pretreatment are commercial α-amylases,

74amyglucosidases, cellulases, xylanases, lipases or proteases [10,11]. Furthermore, it has

75been shown that using a mixture of commercial enzymes, the methane yield was higher

76than using a single enzyme specific for one substrate [10,12]. Regarding the use of

77crude fungal enzymes, those from *Aspergillus lentullus* were particularly effective at

78improving microalgae anaerobic biodegradability [13]. Ligninolytic fungi produce non-

79specific intracellular and extracellular enzymes, depending on the culture conditions

80[14]. One of the most well-known fungus that produces laccase is the white-rot fungus

81*Trametes versicolor*. Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) are a

82family of glycoproteins, classified as oxidoreductases that catalyse the monoelectronic

83oxidation of substrates at the expense of molecular oxygen. They are used for cross-

84linking of monomers, degradation of polymers and ring cleavage of aromatic

85compounds in various environmental applications (e.g. bioremediation of soils and

86wastewater, decolourization of recalcitrant dyes, kraft pulp biobleaching, biorefinery

87processes and degradation of contaminants) [15–19]. In addition, laccase can be used as

88a pretreatment step for cellulose hydrolysis [20].

89The aim of the present study is to evaluate the biogas production increase obtained by

90applying an enzymatic pretreatment to microalgal biomass in biochemical methane

91potential (BMP) tests. Two pretreatment approaches were considered, the first one using

92the commercial laccase enzyme and the second one using crude fungal enzyme from

93*Trametes versicolor*. This is the first time that the fungal broth from *T. versicolor* culture

94has been used as a microalgal biomass pretreatment for biomethanization.

95

96 ## 2 Materials and methods

97 ### *2.1 Microalgal biomass*

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

107 **Table 1** Main characteristics of microalgal biomass (substrate) and digested sludge
108 (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	-

109 ### *2.2 Fungus and chemicals*

110 *Trametes versicolor* was obtained from the American Type Culture Collection (ATCC
111 #42530). The fungus was serially subcultured on 2% malt agar slants at 25 °C until use.
112 Glucose, ammonium tartrate dibasic, malt extract and other chemicals were purchased

113from Sigma-Aldrich (Barcelona, Spain).

114*2.3 Trametes versicolor culture*

115A mycelia suspension of *T. versicolor* was obtained by inoculating four 1 cm diameter
116plugs from the growing zone of fungi on malt agar, in 250 mL malt extract medium
117(2%) in a 1 L Erlenmeyer flask. Flasks were placed on an orbital shaker (130 rpm, r =
11825 mm) at 25 ºC. After 6 days, a thick mycelial mass was formed, which was ground
119with an X10/20 (Ystral GmbH) homogenizer. This suspension was used to produce
120pellets by inoculating 1 mL of the suspension in 250 mL malt extract medium 2% in a 1
121L Erlenmeyer flask. The flasks were incubated on an orbital shaker (130 rpm, r = 25
122mm) at 25 ºC for 6 days. The pellets thus obtained were then used for fungal broth
123production.

124*2.4 Fungal broth production*

125*T. versicolor* broth was produced in 250 mL Erlenmeyer flasks containing 0.9 g cell dry
126weight of *T. versicolor* pellets in 100 mL of medium containing: 8 g L^{-1} of glucose, 3.3
127g L^{-1} of ammonium tartrate, 1.168 g L^{-1} of 2,2-dimethylsuccinate buffer, 10 and 100 mL
128L^{-1} of a micro and macronutrient solution, respectively *[23]*; adjusted to pH 4.5 with
129HCl. Pellets were cultured in six Erlenmeyer flasks, 3 of them were cultured until
130laccase production was 100 U/L (3.5 days) and the other 3 until glucose was totally
131consumed. Both parameters, laccase production and glucose consumption were daily
132monitored.

133 *2.5 Enzymatic pretreatment*

134Two enzymatic pretreatments were carried out using either the commercial enzyme
135laccase (purchased from Merck (Madrid, Spain)) enzyme or *T. versicolor* broth. In the
136first case, a stock solution of commercial laccase was prepared and added to microalgal

137biomass (31 g_{wet}) before BMP tests. The laccase concentration in BMP bottles was 100
138U L^{-1} and the contact time prior to BMP tests was 20 minutes, it was maintained at 25ºC
139and 100 rpm shaker platform (orbital shaker Kuhner, LS-X, Switzerland, r = 25 mm) .
140In the second case, broth produced by *T. versicolor* culture (sieved to remove the fungal
141pellets) containing 100 U L^{-1} of laccase enzyme was added to microalgal biomass
142following the same strategy as for commercial laccase.

143 *2.6 Biochemical methane potential tests*

144After the enzymatic pretreatment of microalgal biomass for 20 minutes, BMP tests were
145carried out in serum bottles of 160 mL, with a working liquid volume of 100 mL and a
146headspace volume of 60 mL. The inoculum was mesophilic digested sludge from an
147anaerobic digester of a municipal WWTP located in Gavà (Catalunya, Spain). Bottles
148contained a total organic matter concentration of 5 g COD/L and the substrate/inoculum
149(S/I) ratio was 0.5 g VS substrate/ g VS inoculum, based on previous studies, including
150one in which the S/I ratio was optimised for microalgal biomass grown in the same pilot
151HRAP [24,25]. Afterwards, bottles were filled with distilled water up to 100 mL,
152flushed with helium gas, sealed with butyl rubber stoppers and incubated at 35ºC until
153biogas production ceased. Biogas production was measured by the pressure increase in
154the headspace volume using an electronic manometer (Greisinger GMH 3151, error
155±0.1%). After each measurement, biogas was released from the reactor's headspace
156until atmospheric pressure in the reactor was reached; afterwards reactors were
157manually shaken.
158The following trials were carried out: (1) microalgal biomass pretreated with
159commercial laccase, (2) microalgal biomass pretreated with fungal broth, (3) non-
160pretreated microalgal biomass control, (4) commercial laccase control, (5) fungal broth
161control, and (6) blank containing only inoculum, in order to quantify the methane

162production by endogenous respiration. Blank results were subtracted from all trials to

163obtain the net biogas production. Furthermore, commercial laccase control results were

164subtracted from microalgal biomass pretreated with commercial laccase; whereas fungal

165broth control results were subtracted from microalgal biomass pretreated with fungal

166broth. All experimental trials, including pretreatments, controls and blank were

167performed in triplicate and expressed at standard temperature and pressure.

168 *2.7 Analytical methods*

169Glucose concentration was measured with an YSI 2000 enzymatic analyzer from Yellow

170Springs Instruments and Co.

171Laccase activity was measured using a modified version of the method for the

172determination of manganese peroxidase [26]: The reaction mixture used consisted of

173200 µL of 250 mM sodium malonate at pH 4.5, 50 µL of 20 mM 2,6-dimethoxyphenol

174(DMP) and 600 µL of sample. DMP is oxidized by laccase even in the absence of

175cofactor. Changes in the absorbance at 468 nm were monitored for 2 min on a Varian

176Cary 3 UV-vis spectrophotometer at 30ºC. One activity unit (U) was defined as the

177number of micromoles of DMP oxidized per minute. The DMP extinction coefficient

178was 24.8 mM^{-1} cm^{-1} [27].

179The inoculum and substrate were characterised (Table 1) by the concentration of total

180solids (TS), volatile solids (VS) and chemical oxygen demand (COD), following

181standard methods guidelines (APHA, 1999). pH was analysed with a Crison Portable

182506 pH-meter. The lipid content of biomass was determined by the Soxhlet extraction

183method [28, 29]. The total Kjeldahl nitrogen (TKN) to protein conversion factor was

1845.95, according to González López et al., [27]. Carbohydrates were determined by

185phenol– sulphuric acid method, after acid hydrolysis and measured by

186spectrophotometry (Spectronic Genesys 8) [30].

187The methane content in biogas was measured once a week with a gas chromatograph
188(GC) (Trace GC Thermo Finnigan) equipped with a Thermal Conductivity Detector, by
189injecting gas samples into a packed column (Hayesep 3m1/8 in. 100/120). The carrier
190gas was helium in splitless mode (column flow: 19 mL/min). The oven temperature was
19135 ºC with a retention time of 1.5 min. Injector and detector temperatures were 150 and
19225 ºC, respectively. The system was calibrated with methane (50% CH_4) and carbon
193dioxide (50% CO_2).
194

1953 Results and discussion

196 *3.1 Fungal broth production*

197*Trametes versicolor* cultured in Kirk's nutrient medium produces laccase enzyme and is
198appropriate for studying the ligninolytic activity of fungal cultures [31]. Laccase
199production and glucose consumption from *Trametes versicolor* culture are shown in
200Figure 1. Gradual glucose consumption along with laccase activity increase by the
201fungus *T. versicolor* can be observed.
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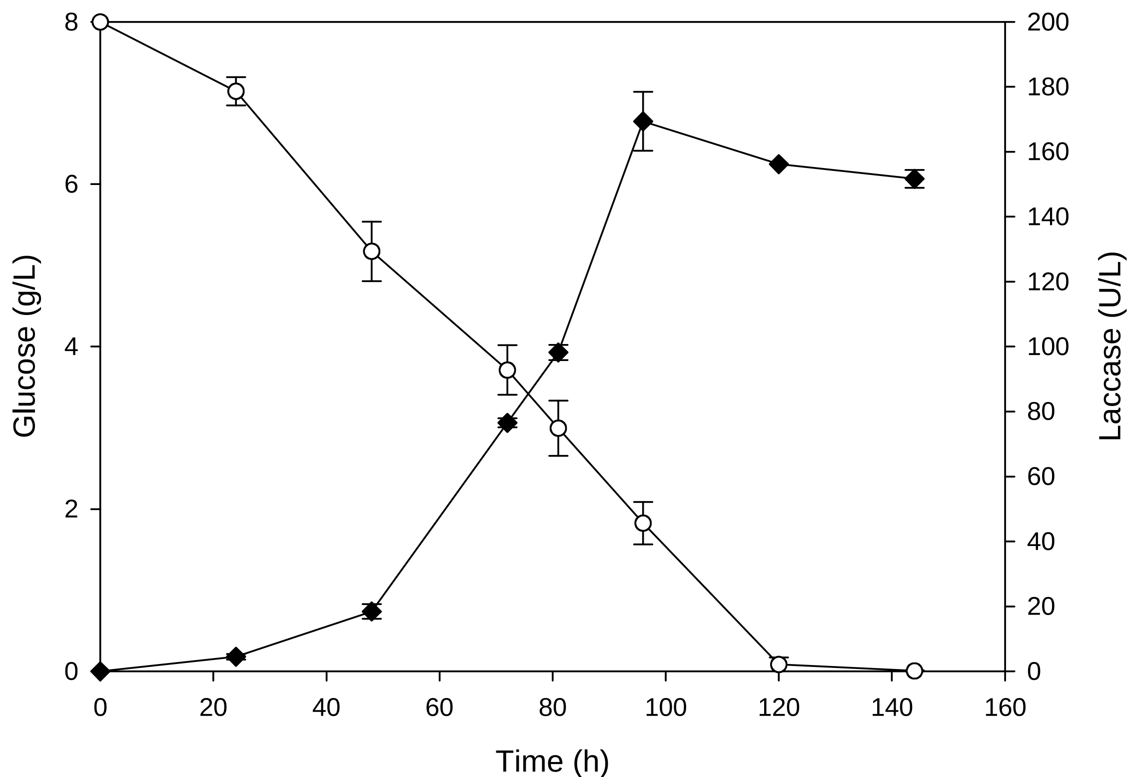


204**Figure 1.** Glucose consumption (○) and laccase production (♦) by *Trametes versicolor*

205

206Laccase enzyme is excreted by *T. versicolor* to the broth and the excretion is associated

207to both growth and glucose consumption. Enzyme production increased over the first 4

208days and, after reaching a maximum activity level (170 U L^{-1}, 4 days), it dropped, since

209the carbon source (glucose) had been consumed. The same laccase activity behaviour

210was observed by other authors [15,32]. The fungal broth obtained from *T. versicolor*

211culture in Kirk's medium is mostly rich in laccase enzyme, among other enzymes or

212mediators, and unconsumed glucose. After 3 days of cultivation, other enzymes can be

213secreted by *T. versicolor,* such as cellulases and hemicellulases [33], possibly important

214for microalgae cell wall degradation.

215 *3.2 Biogas production in BMP test*

216The fungal broth and commercial laccase were applied at a dose of 100 U $L^{-1}$of laccase

217enzyme and were used as a pretreatment for microalgal biomass solubilisation in order

218to evaluate the anaerobic biodegradability increase in BMP tests. The experiment lasted

21932 days, until accumulated biogas production reached an asymptote (Figure 2). As can

220be seen from the results, both pretreated trials increased the biogas production as

221compared to non-pretreated microalgae. Moreover, the fungal broth pretreatment

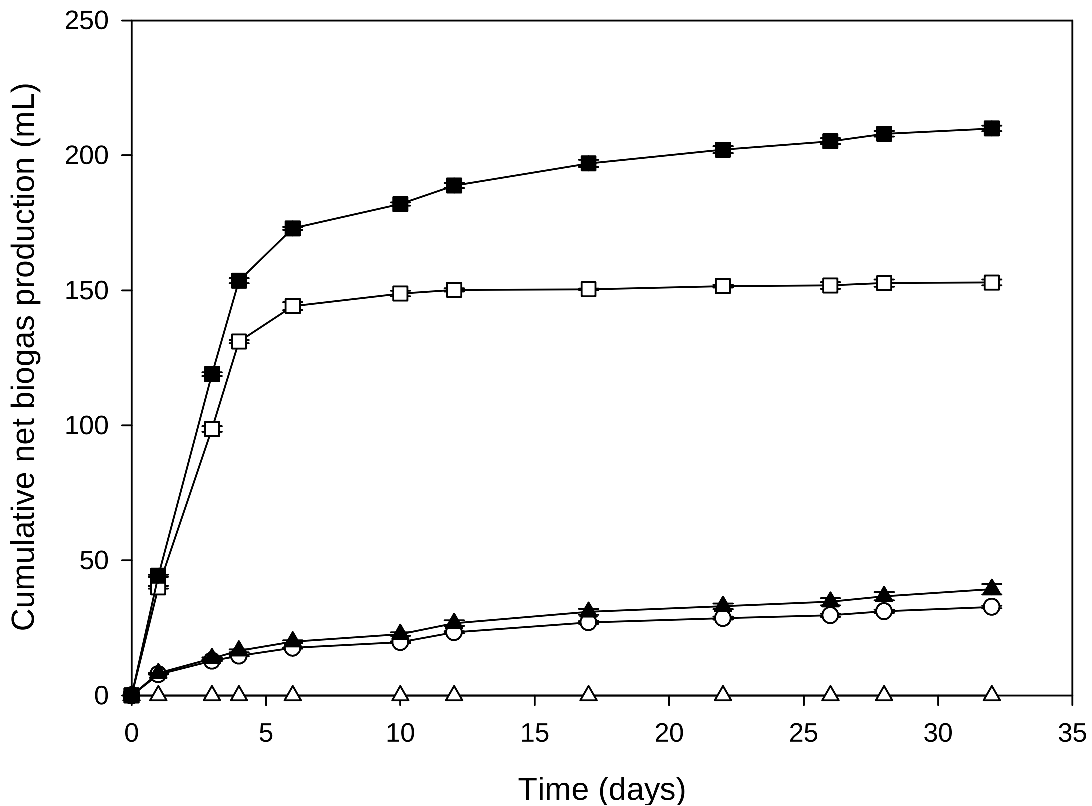
222attained the highest value. The methane content was measured along the experiment

223obtaining an average concentration of 68±4.5% CH_4. Control trials from both laccases

224(commercial and fungal broth) were subtracted from the corresponding pretreatment,

225along with the production of the inoculum, to obtain the net biogas and methane

226production along with the net methane yield (Table 2).



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

229biomass using two enzymatic pretreatments and their respective controls. Commercial

230laccase control (); Microalgal biomass control (○); Commercial laccase pretreatment

231(▲); Fungal broth control (); Fungal broth pretreatment ()

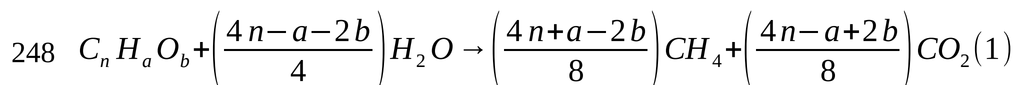
232

233**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

234

235Regarding the control trials, commercial laccase control did not produce any biogas.
236Microalgal biomass control produced little methane (22 mL CH_4), whereas the fungal
237broth control produced 104 mL CH_4. Indeed, reactors containing fungal broth produced
238more biogas than the rest, since they contained part of the nutrients (mainly glucose)
239present in the media for laccase production, which were not completely consumed by *T.*
240*versicolor.* This can be seen from Fig. 1: when 100 U L^{-1} of laccase were obtained, the
241concentration of glucose was 3 g L^{-1}. The amount of biogas produced from glucose
242remaining in the culture broth was theoretically calculated and compared with
243experimental results, using the Buswell equation [34] (equation 1 and 2). According to
244this, 112 mL CH_4 should have been theoretically produced, due to the remaining glucose
245of the medium, moreover this value does not exceed the mass-energy balance (374 mL
246CH_4/g $C_6H_{12}O_6$). This theoretical value is in accordance with the experimental one (104
247mL CH_4).

248 

$$C_n H_a O_b + \left(\frac{4n-a-2b}{4}\right) H_2O \rightarrow \left(\frac{4n+a-2b}{8}\right) CH_4 + \left(\frac{4n-a+2b}{8}\right) CO_2 \quad (1)$$

249 $$B_{o,th}\left[\frac{LCH_4}{gC_nH_aO_b}\right] = \frac{1}{8}\left(\frac{4n+a-2b}{12n+a+16b}\right) V_m \quad (2)$$

250where V_m is the molar volume of methane at standard temperature and pressure.

251The presence of glucose could also enhance the proliferation of anaerobic

252microorganisms, which may contribute to an increase of biogas production if it

253functions as a substrate to some microorganisms, which at the same time could produce

254some enzymes capable to further degrade the microalgal biomass, increasing the

255methane yield. However, since this effect cannot be measured, only the methane

256production due to glucose contribution was subtracted

257With regards to the pretreatment trials, commercial laccase pretreatment increased the

258methane yield by 20%, whereas fungal broth pretreatment increased the methane yield

259by 74% relative to non-pretreated biomass. The results suggest that laccase may

260solubilise part of the microalgal biomass substrate, enhancing its bioavailability and/or

261biodegradability by anaerobic microorganisms. However, better results were achieved

262using the fungal broth. This is probably due to the presence of other enzymes, radicals

263and other mediators produced by *T. versicolor* during its culture, which may also

264contribute to microalgal biomass solubilisation [19]. It is worth pointing out that even

265though laccase is not specifically active on glycoproteins and polysaccharides (the main

266components of microalgal cell wall), the pretreatment was effective. Therefore, results

267confirm that laccase played a role on microalgae enzymatic pretreatment, although a

268mixture of different enzymes would be preferred. This is common for complex cultures,

269such as the one of the present study, composed by several microalgae species, bacteria

270and other microorganisms with different cell wall compositions. The results are in

271accordance with previous studies, where microalgae methane yield was increased when

272non-specific enzymes were added confirming the synergistic effect [10,12,13].

273Nevertheless, a previous study using filamentous microalgae reported higher values

274than those obtained in our study. Ehimen et al. [10] obtained 115-145 mL CH_4/g TS

275after an enzymatic pretreatment over 2 days, whereas the values obtained in the present

276study were 63 and 91 mL CH_4/g TS for commercial laccase and fungal broth
277pretreatment, respectively, after 20 minutes of enzymatic pretreatment. From these
278results, contact time seems to be an important parameter that should be further
279investigated. The methane yield of *Chlorella vulgaris* was increased by 14% after
280pretreatment with the hydrolytic enzyme carbohydrolase and by 51% after pretreatment
281with protease after an exposure time of 5 h. Moreover, the same study with
282*Chlamydomonas reinhardtii* showed no increase after pretreatment with carbohydrolase
283and only 8% increase after pretreatment with protease [35]. This increase was lower
284than the ones obtained in our study (20 and 74% increase) and highlights that
285pretreatment effectiveness is species-specific and depends on the biomass complexity
286and composition.

287Finally, the results obtained in this study demonstrates that enzymatic pretreatment may
288be applied to microalgae anaerobic digestion, with better results for crude fungal
289enzymes probably due to the presence of other enzymes and other molecules produced
290by the fungus. This may be more cost-effective compared to commercial enzymes.
291Nevertheless, these results should be evaluated in continuous reactors for energy and
292economic aspects.

293

294**4 Conclusions**

295This study aimed at investigating the effect of laccase, a non-specific enzyme, on
296microalgal biomass from a pilot-scale urban wastewater treatment system as a
297pretreatment step prior to its anaerobic digestion. Comparing the effect of commercial
298laccase and the fungal broth from *Trametes versicolor*, better results were observed for
299the fungal broth, which may be due to the synergistic effect of laccase and other radicals
300or molecules produced by *T. versicolor*. The methane yield was increased by 20% for

301commercial laccase and 74% for fungal broth, as compared to non-pretreated biomass.

302Thus, these findings should be investigated in continuous anaerobic reactors for

303evaluating full-scale viability.

304

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315

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