1 **Selective carbon sources and salinities enhance enzymes and extracellular polymeric**

2 **substances extrusion of *Chlorella* sp. for potential co-metabolism**

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15

16 **Abstract**

17 This study investigated the extracellular polymeric substance (EPS) and enzyme extrusion of

18 *Chlorella* sp. using seven carbon sources and two salinities for potential pollutant co-

19 metabolism. Results indicated that the levels of biomass, EPS and enzymes of microalgae

20 cultured with glucose and saccharose outcompeted other carbon sources. For pigment

21 production, glycine received the highest chlorophyll and carotene, up to 10 mg/L. The EPS

22 reached 30 mg/L, having doubled the amount of protein than carbohydrate. For superoxide

23 dismutase and peroxidase enzymes, the highest concentrations were beyond 60 U/ml and 6

24 nmol/d.ml, respectively. This amount could be potentially used for degrading 40%

25 ciprofloxacin of concentration 2000 μg/L. When increasing salinity from 0.1% to 3.5%, the

26 concentrations of pigment, EPS and enzymes rose 3 to 30 times. These results highlighted

27 that certain carbon sources and salinities could induce *Chlorella* sp. to produce EPS and

28 enzymes for pollutant co-metabolism and also for revenue-raising potential.

29 Keywords: *Chlorella* sp., extracellular polymeric substance, superoxide dismutase,

30 peroxidase, co-metabolism.

31 1. Introduction

32 Microalgae can remediate various types of pollutants including nutrients, organic compounds,

33 heavy metals and micropollutants which differ greatly from concentration, characteristic to

34 toxicity level (Chan et al., 2014; Vadlamani et al., 2019). Microalgae use the pollutants as

35 primary substrates for cell growth and trigger enzymes production, so-called the metabolism.

36 However, most organic pollutants present in wastewater at minor concentration (e.g.

37 antibiotic, pesticide). For example, acetaminophen presents in sewage system and water

38 reservoir of many countries from 20 to 100 μg/L (Phong Vo et al., 2019). They are unable to

39 be used as sole carbon source since the obtained metabolic energy from micropollutants is

40 insufficient to microorganism's demand. Therefore, the co-metabolism for removing

41 pollutant is necessary. Cometabolism is the transformation of a non-growth substrate (e.g.,

42 organic pollutants) primed by microalgae using a growth substrate or another biodegradable

43 compound (e.g., glucose, saccharose, acetate). Microalgae can extrude extracellular

44 polymeric substances (EPS) and several types of enzymes to the culturing environment (Lv et

45 al., 2019). Positively, those EPS and enzymes catalysed the pollutant co-metabolism of

46 microalgae. Thus, microalgae can degrade long-chain organic pollutants in a more efficient

47 manner. Example enzymes are superoxide dismutase (SOD) and peroxidase (POX). The SOD

48 (EC 1.15.1.1) catalyses the dismutation (or partitioning) of the superoxide (O_2^-) radical into

49 either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). POX (EC 1.11.1.x) is a

50 large group of enzymes that can catalyse the reaction of H_2O_2 and organic compounds by

51 using them as electron donor and acceptor (Eq. 1 and 2), so-called the co-metabolism.

52 $2HO_2 \rightarrow O_2 + H_2O_2$ (Eq. 1)

53 $ROOR' + 2e- + 2H+ \xrightarrow{Peroxidase} ROH + R'OH$ (Eq. 2)

54 In practice, wastewater in the treatment plant contained various organic pollutants and carbon

55 sources. For instance, atrazine, carbamazepine, diclofenac, sulfamethoxazole are among

56 hundreds of micropollutants are detected in wastewater treatment plant (Luo et al., 2014).

57 The wastewater is discharged from domestic, pharmaceutical, alcoholic, cassava, sugar

58 refinery industries which possess high nutrient level of methanol, ethanol, acetate and sugar

59 carbon sources (Bhattacharya et al., 2017). In the latest investigations, several researchers

60 have studied the effects of different feeding carbon sources on microalgae (Lin and Wu,

61 2015; Zhang et al., 2014). Their experimented carbon sources were glucose, saccharose,

62 acetate and sodium bicarbonate (Moon et al., 2013; Tu et al., 2018; Vergnes et al., 2019;

63 Wang et al., 2016). Some important carbon sources are not fully explored, such as methanol,

64 ethanol and glycine. Also, those studies focus on the impact of carbon sources on protein,

65 carbohydrate and lipid yield in microalgae cells, but not the pollutant co-metabolism (Yew et

66 al., 2019). It is known widely the growth and pigment production of microalgae are

67 determined strongly by the type of feeding carbon source. However, still little is known with

68 insightful elaboration on the biochemical effect of the carbon source towards the pollutant co-

69 metabolism of microalgae. This is useful and applicable for using microalgae towards organic

70 pollutants cometabolism and nutrient recovery in wastewater treatment plant and industry

71 discharge. Another factor influencing microalgae is salinity. By entering microalgae cells,

72 substantial amounts of sodium and chloride ions of high salinity alter the biochemical

73 function of microalgae and interfere the remediation process (Pérez et al., 2016). Through

74　literature, only limited studies have explored the effect of different carbon sources between

75　fresh water (salinity 0.1%) and sea water (salinity 3.5%), towards pollutant co-metabolism.

76　Therefore, it was hypothesized that salinity and type of carbon source potentially alter the

77　EPS and enzyme concentration extruded by microalgae and influence the pollutant co-

78　metabolism efficiency. To address the identified gaps, this study investigated the effect of

79　different carbon sources on microalgae cultured in salinities 0.1% and 3.5%, with a focus on

80　EPS and enzymes extrusion for potential pollutant co-metabolism. Up to our knowledge, this

81　study was the first one encountered insightfully the impacts of various carbon sources and

82　salinities on the pollutant co-metabolism of microalgae.

83　In the previous work, the *Chlorella* sp. has demonstrated that it could adapt better to the

84　salinities from 0.1 to 3% than other strains (i.e., *Stichococcus* sp. and *Chlorella vulgaris*) (Vo

85　et al., 2019). In detail, the *Chlorella* sp. can accumulate 60-80% TOC while the *Stichococcus*

86　sp. only hands on less than 60%. The elemental analysis demonstrates that the C percentage

87　in the *Chlorella* sp. and cells reaches to 20%; on the other hand, the values of *Chlorella*

88　*vulgaris* and *Stichococcus* sp. stay at 9 to 15%. Hence, in this work, *Chlorella* sp. was

89　experimented in the mixotrophic condition using six organic carbon sources (i.e., methanol,

90　ethanol, glucose, saccharose, glycine, acetate) and photoautotrophic one with inorganic

91　sodium bicarbonate. Apart from the biomass and pigment, the bound and soluble EPS, SOD

92　and POX in the two selected salinities were analysed to evaluate the cometabolism potential.

93　In addition, the advanced technology such as Scanning Electron Microscope (SEM) was used

94　to explore the effect of salinity on microalgae. By clarifying those, *Chlorella* sp. would be

95　better characterised for sustainable applications in industrial wastewater to recover nutrients

96　and to increase the pollutant co-metabolism process.

97 2. Materials and methods

98 2.1 Microalgae and wastewater

99 The *Chlorella* sp. (CS-436) stock was supplied by the National Algae Supply Service

100 (Tasmania, Australia). The stock was cultured in cyanobacterial growth media MLA

101 (Ausaqua, Australia) at $20 \pm 1°C$ and 4.35 ± 0.03 klux. The cultured microalgae was

102 illuminated by a LED light bulb (11 W, 220–240 V) (Philip, Australia). The illumination

103 intensity was measured by a light meter, model QM1584 (Digitech, Australia). The

104 microalgae stock was renewed per fortnight.

105 This study used artificial wastewater for experiment which was spiked with selective carbon

106 sources. Seven carbon sources were employed separately to prepare seven wastewater types

107 including: methanol (CH_3OH), ethanol (C_2H_5OH), saccharose ($C_{12}H_{22}O_{11}$), glucose

108 ($C_6H_{12}O_6$), sodium acetate (CH_3COONa), glycine ($C_2H_5NO_2$), and sodium bicarbonate

109 ($NaHCO_3$). The MLA media was also spiked in the wastewater at dosing as advised from the

110 supplier. It served as background for microalgae growth since the MLA media contained vital

111 elements (e.g., Mg^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}). The nitrogen and phosphorus sources were from

112 $NaNO_3$ and KH_2PO_4, respectively. The concentrations of total carbon (TC), NO_3^--N and

113 $PO_4^{3-}-P$ were fixed at 300, 30 and 5 mg/L, respectively. The concentration of carbon, nitrogen

114 and phosphorus was studied and optimized in the previous work and also to maximize the

115 nutrient recovery capacity of microalgae (Vo et al., 2019). The spiked trace vitamins were

116 supplied by AusAqua Company (Australia) and the applied dose of trace vitamins followed

117 the supplier's instructions. All the chemicals were purchased from Merck (Australia) to

118 ensure the analytical grade quality.

119   2.2 Experimental design

120   Eight sealed glass bottles (1 L each) were used as the experimental photobioreactors. Seven

121   photobioreactors contained one type of carbon source each as previously described. One

122   photobioreactor served as the control, feeding MLA media only. The initial microalgae

123   concentration cultured in the photobioreactor was retained at 50 mg/L. The illumination

124   intensity and temperature for those photobioreactors were similar to the stock solution.

125   Following this the cultured solutions were gently mixed at 50 rpm. The experimental period

126   lasted for 10 d. Biomass concentration of microalgae was measured every 2 d. Other

127   parameters including pigment, EPS, enzymes and efficiencies in removing pollutants, were

128   checked on day 10th.

129   2.2.1 Biomass yield analysis

130   The biomass yield of microalgae was obtained by measuring the cell density at 680 nm using

131   a spectrophotometer (DR1900, Hach). Then, the value of cell density was converted to the

132   biomass yield by Eq (3):

133    $y = (x - 0.0222)/0.0021$ (R^2=0.95) (Eq. 3)

134   Where:

135   x: cell density

136   y: biomass yield (mg/L)

137   2.2.2 Chlorophyll and carotene analysis

138   The pigment (chlorophyll and carotene) was analysed according to methods used in the

139   previous studies (Vo et al., 2019; Xiong et al., 2017a). In brief, a 10 ml sample was

140   centrifuged at 9000 rpm for 10 min. The supernatant was disposed and the pellet was

141   suspended in 10 ml of 90% acetone. The sample was sonicated in 5 min at 4°C and incubated

142 in 24 h in darkness. It was re-centrifuged at 4°C, 4000 rpm for 15 min. The supernatant was

143 checked at various wavelengths with a spectrophotometer. The chlorophyll and carotenoid

144 concentrations were calculated as follows:

145 Chlorophyll (mg/L) = $11.64*(A_{663}-A_{750}) - 2.16*(A_{647} - A_{750}) + 0.1*(A_{630} - A_{750})$ (Eq. 4)

146 Carotene (mg/L) = $(1000 A_{470} + 243.1*A_{661} - 1267.1*A_{645})/214$ (Eq. 5)

147 Where

148 A_{λ}: cell density at wavelength λ (nm).

149 2.2.3 Extracellular polymeric substances

150 The EPS extraction procedure was modified from Deng et al. (2016). Firstly, a 30 mL sample

151 was collected and then centrifuged at 3000 rpm for 30 min. The supernatant was re-

152 centrifuged at 3000 rpm for another 30 min and filtered through a 0.45 μm Phenex-NY

153 (Nylon) syringe filter to receive soluble EPS. The pellets in the centrifuge tube were

154 suspended in phosphorus buffer solution to 30 mL, and mixed with cation exchange resin for

155 2 h at 900 rpm. By filtering the resin and liquid through a 1.2 μm Phenex-GF (Glass fiber)

156 syringe filter, the bound EPS was retained. Both the soluble and bound EPS were analysed

157 for protein and polysaccharide using the modified Lowry method (Sigma, Australia) and

158 Anthrone-sulphuric acid method, respectively (Deng et al., 2016).

159 2.2.4 Superoxide dismutase and peroxidase enzymes

160 The SOD and POX activity was analyzed by using enzyme test kits (Sigma-Aldrich,

161 Australia). At first, a 20 ml sample was collected and centrifuged at 4500 rpm for 15 min at

162 4°C. The cellular pellet and supernatant were utilised for the enzymatic activity assay in cells

163 and culturing environment, respectively. The SOD and POX analysis was conducted

164 according to the manufacturer's instructions. One SOD unit was defined as the amount of

165 enzyme exhibiting 50% dismutation of the superoxide radical. The unit U/ml (nmol/min.ml)

166 served to measure SOD activity while the unit nmol/d.ml was applied for POX activity.

167 2.2.5 Total carbon, nitrate and phosphate analysis

168 The total carbon (TC) level was analysed by Multi N/C 3100 (Analytikjena, Germany). The

169 NO_3^--N and PO_4^{3-}-P concentrations were measured by the test kits, coded 114942 and

170 100798 of Merck (Australia), respectively. The Photometer Nova 60 (Merck, Australia) was

171 employed for NO_3^--N and PO_4^{3-}-P analysis.

172 2.2.6 Scanning electron microscope

173 The surface morphology of microalgae cells were analysed using SEM (Zeiss Supra 55VP,

174 Carl Zeiss AG). The microalgae samples were filtered by glass fiber filter paper GF/C

175 (Whatman, Australia), dehydrated for 24 h at 105 °C, and coated by Au/Pd. The SEM was

176 operated at 10 kV as accelerating voltage and multiple magnifications. The SEM was used to

177 explore the effect of salinity on microalgae.

178 2.3 Model analysis of biomass yield

179 To understand the effect of different carbon sources and salinities on biomass yield, the

180 Gompertz model (Eq. 6) was applied to describe the biomass growth. Data were retrieved

181 from experimental measurement described in section 2.3.1.

182 $y = a.e^{-exp(-k(x-x_c))}$ (Eq. 6)

183 Where, y is the estimated biomass yield (mg/L), a is the maximum biomass yield (mg/L), k is

184 the biomass yield per day (mg/L.d), x is the cultured time (d), x_c is the lag time of biomass

185 yield (d). The a, k and x_c values are kinetic parameters and can be achieved using Origin 8.0

186 software.

187 2.4 Statistic analysis

188 The analysis of variance (ANOVA) was used for statistical purposes. Specifically, the

189 repeated measures of ANOVA examined the difference of biomass yield which was

190 influenced by salinities and carbon sources, according to the cultured time. For other

191 parameters, the factorial ANOVA was applied to investigate the difference of pigment, EPS,

192 enzymes and pollutants removal efficiencies impacted by salinities and carbon sources. All

193 the data were presented as mean value ± standard deviation (Mean ± SD) with duplicated

194 samples.

195 3. Results and discussion

196 3.1 Biomass yields feeding by different carbon sources and salinities

197 Both organic and inorganic carbon sources and salinity can affect the growth of *Chlorella* sp.

198 (Vo et al., 2019). The *Chlorella* sp. was cultured in seven types of carbon sources using two

199 salinities: fresh water (0.1%) and sea water (3.5%). As shown in Fig. 1, biomass

200 concentrations feeding with different carbon sources and salinities were significantly

201 different ($p<0.01$). For salinity 0.1%, the glucose and saccharose yielded the highest biomass

202 reaching beyond 600 mg/L at day 10. Other carbon sources, namely ethanol, glycine, sodium

203 acetate and sodium bicarbonate achieved smaller amounts (400 - 500 mg/L). The methanol

204 emerged the least effective carbon source for biomass growth, which was not significantly

205 different to the control, around 100 mg/L ($p>0.01$). Its biomass concentration was five-fold

206 less than those cultured in glucose and saccharose.

207 For salinity 3.5%, the biomass yield decreased by 200 mg/L for most carbon sources

208 compared to salinity 0.1%. Only the sodium acetate carbon maintained a comparable biomass

209 yield between 400 and 500 mg/L in both salinities. Similar to salinity 0.1%, both glucose and

210 saccharose presented the highest biomass yield in salinity 3.5% (400 - 500 mg/L). The

211   methanol and the control samples possessed the lowest biomass level, less than 100 mg/L.

212   Other carbon sources received biomass from 250 to 400 mg/L.

213   [Insert Fig. 1]

214   To elaborate the impact of carbon sources and salinities on biomass yield, the Gompertz

215   model was applied to validate the results of experimental parts. In this model, the three

216   kinetic parameters, including maximum biomass yield (a), biomass growth rate (k) and lag

217   time of biomass yield (x_c), were calculated. Overall, most of the models well-fitted the

218   experimental data, confirming by high R^2 values. The biomass growth rates of glucose and

219   saccharose surpassed five-fold other carbon sources. Furthermore, in salinity 3.5%, the

220   biomass growth rate reduced 20 – 30% than in salinity 0.1%. This coincided the results of

221   experimental data. It can be seen that biomass feeding by glucose and saccharose reached

222   stationary phase at day 4. Other carbon sources likely performed stationary phase since day

223   10 of the cultured period in salinity 0.1%., but not in salinity 3.5%.

224   The effects of carbon sources such as glucose, saccharose, sodium acetate and sodium

225   bicarbonate to microalgae's biomass have been studied by several researchers (Lin and Wu,

226   2015; Vo et al., 2019; Zhang et al., 2014). These particular organic carbon sources were

227   implemented in mixotrophic mode and the sodium bicarbonate was examined in

228   photoautotrophic mode. However, the effects of methanol, ethanol and glycine were not

229   investigated adequately. In this study, the biomass yields were higher than those of other

230   studies. Several technical conditions could explain this discrepancy but the most important

231   one was due to the experimented microalgae strain. The *Chlorella* sp., a highly productive

232   strain of this work, could adapt to both fresh water and sea water environments. In the

233   previous study, *Chlorella* sp. could well tolerate salinity from 0.1 to 3.5% (Vo et al., 2019).

234   For instance, the received biomass yield was 600 to 800 mg/L and the sugar-based carbon

235 sources of others achieved biomass from 350 to 450 mg/L (Moon et al., 2013; Zhang et al.,

236 2014). In details, the *Chlorella* sp. Y8-1 and *Botryococcus braunii* were fed carbon levels

237 from 500 to 12000 mg/L, that 2 to 40 times higher than in our experiment (i.e., 300 mg/L).

238 Another evidence was the *Chlamydomonas reinhardtii*. Although a comparable biomass

239 concentration (i.e., 400 mg/L) to our results has been achieved, the feeding acetate

240 concentration (i.e., 1 – 10 g/L) was 3 to 30 times higher than ours (Moon et al., 2013). This

241 further emphasised the distinct characteristics of *Chlorella* sp. For methanol, ethanol and

242 glycine carbon sources, the literature is limited for making an adequate comparison.

243 Technically, the alcohol carbon sources required microalgae more time to adapt and grow

244 (Choi et al., 2011). For example, Choi et al. (2011) cultured *Chlorella* sp. in 1% methanol

245 and eventually obtained 4.2 g biomass/L after 45 d. It can be seen from Fig. 1 that, at salinity

246 3.5%, most biomass growth curves still happened on log phase after 10 d. In practice,

247 culturing for 45 d is time-consuming and potentially reduces revenue. In contrast, *Chlorella*

248 sp. feeding ethanol and glycine carbon sources herein could grow to 250-500 mg/L within 10

249 d under both salinities. This is very comparable to the sugar-based feedstocks used in other

250 studies (Moon et al., 2013; Zhang et al., 2011; Zhang et al., 2014).

251 3.2 Pigment production under different carbon sources and salinities

252 While consuming carbon sources and necessary nutrients, *Chlorella* sp. can produce pigment

253 such as chlorophyll and carotene. High chlorophyll and carotene levels demonstrate the

254 adaptation and growth of microalgae to the culturing environment (Singh et al., 2018). The

255 concentration of chlorophyll and carotene associated to the seven carbon sources and two

256 salinities were different significantly ($p<0.01$) (Fig. 2).

257 The chlorophyll production of this microalgae strain ranged from 0.3 to 5 mg/L for all carbon

258 sources and salinities after 10 d. At salinity 0.1%, *Chlorella* sp. generated chlorophyll below

259 0.5 mg/L, the exception being glycine. The *Chlorella* sp. feeding glycine produced 4.1 mg

260 chlorophyll/L, which was 10 times more than other carbon sources. However, *Chlorella* sp.

261 performed differently in salinity 3.5%. Most carbon sources could generate chlorophyll 3 to

262 30 times more than salinity 0.1%. The *Chlorella* sp. culturing by ethanol, saccharose, glucose

263 and sodium acetate yielded chlorophyll from 2 to 5 mg/L. The saccharose one achieved

264 around 5 mg/L as the highest chlorophyll concentration. The exception was also glycine that

265 its chlorophyll level in salinity 0.1% tripled the amount in salinity 3.5%. Only the methanol

266 and bicarbonate-feeding microalgae performed insignificant change in both salinities. The

267 chlorophyll concentration of microalgae culturing in those carbon sources received less than

268 0.3 mg/L.

269 The *Chlorella* sp. yielded carotene from 0.1 to 1.5 mg/L, which was three times less than the

270 corresponding amount of chlorophyll. In salinity 0.1%, most carbon sources achieved low

271 carotene concentration, less than 0.5 mg/L, apart from glycine. In turn, at salinity 3.5%, the

272 level of carotene increased 10-fold to 1.5 mg/L, typically for the saccharose and glucose

273 carbon sources. Other carbon sources still created the carotene level below 0.5 mg/L which

274 was not significantly different from the control unit. However, *Chlorella* sp. feeding glycine

275 was again exceptional because it generated more carotene in salinity 0.1% than salinity 3.5%.

276 It can be seen that glycine was the most effective carbon source for chlorophyll and carotene

277 production. In turn, methanol and bicarbonate achieved the least pigment content. The reason

278 was elaborated in Section 3.6. In short, the photoautotrophic mode exhibited less pigment

279 production at least 10 times than the mixotrophic one.

280 [Insert Fig. 2]

281   3.3 Extracellular polymeric substances production

282   The EPS are excreted by *Chlorella* sp. to the culturing environments when they consume

283   carbon sources. They consist mostly soluble/bound protein and polysaccharide. By adding

284   different carbon sources, this impacts on the EPS extrusion of *Chlorella* sp. as shown in Fig.

285   3. For salinity 0.1%, the produced EPS differed significantly amongst carbon sources

286   ($p < 0.01$); nevertheless, the level of EPS was always below 15 mg/L. The ethanol and glycine

287   ones possessed the highest EPS concentrations, ca. 13 mg/L. The follow-up carbon sources

288   included glucose, saccharose and bicarbonate, producing around 10 mg EPS/L. Other carbon

289   sources produced less than 6 mg EPS/L. Of the EPS's components, protein concentration

290   dominated at least double the amount of polysaccharide. For example, the glycine-induced

291   protein concentration exceeded the polysaccharide concentration eight-fold. However, for the

292   saccharose and glucose carbon sources, the levels of protein and polysaccharide were close.

293   Regarding the bound and soluble forms, their concentrations were almost equal, both for

294   protein and polysaccharide.

295   Of the salinity 3.5%, *Chlorella* sp. produced excessive EPS than in salinity 0.1%. Particularly

296   for the saccharose, glucose, glycine and sodium bicarbonate, EPS value doubled the one in

297   salinity 0.1%. The amount of protein in salinity 3.5% also doubled in salinity 0.1%, while the

298   level of polysaccharide was similar. It implied that increasing salinity would enhance protein

299   production of microalgae. The largest increase in the EPS component related to the bound

300   protein. The glycine carbon source facilitated *Chlorella* sp. producing the highest EPS of 31

301   mg/L. The saccharose, glucose and sodium bicarbonate generated 20 mg EPS/L; the others

302   produced below 10 mg EPS/L. It can be seen each carbon source influenced EPS generation

303   in different ways. Unlike protein, polysaccharide was produced at a lower level at either

304   salinities. The concentration of carbohydrate did not exceed 10 mg/L in most cases and

305   preferably stayed below 5 mg/L. In salinity 0.1%, there was no significant difference between

306   the bound and soluble carbohydrate concentration. However, in salinity 3.5%, the bound

307   carbohydrate increased significantly than the soluble one, especially of the saccharose,

308   glucose, glycine and bicarbonate carbon sources.

309   [Insert Fig. 3]

310   3.4 Superoxide dismutase and peroxidase production

311   As *Chlorella* sp. was stressed by carbon sources and salinities, it started producing a number

312   of enzymes such as SOD and POX in both cells and the culturing environment. Those

313   enzymes alleviated the stress of biotic and abiotic factors and also functioned the biochemical

314   processes in microalgae. The levels of SOD and POX were affected by both carbon sources

315   and salinities as illustrated in Fig. 4. Overall, for most cases, the SOD level in microalgae

316   cells was two to three times higher than in the culturing environments. The *Chlorella* sp.

317   extruded SOD below 20 U/ml to the culturing environments while it generated 30 to 60 U/ml

318   in the cells. For all carbon sources, glucose recorded the highest value (60 U/ml) in the

319   culturing environment. Comparing the two salinities, microalgae extruded SOD to the

320   culturing liquid in salinity 3.5% double in salinity 0.1%. Nevertheless, SOD concentrations in

321   cells of microalgae were comparable in the two salinities. For instance, in the microalgae

322   cells the SOD values mostly varied from 50 to 60 U/ml.

323   POX activity in *Chlorella* sp. for most carbon sources and salinities ranged from 1 to 2

324   nmol/d.ml, which is comparable to the control samples, except for glucose and saccharose in

325   salinity 0.1%. Those produced POX in microalgae cells from 5 to 6 nmol/d.ml which was

326   double the POX activity of other carbon sources. The increase of POX in sugar carbon

327   sources rooted from the POX activity in microalgae cell. It indicated that the sugar carbon

328   sources affected the biochemical and metabolic pathways of microalgae more significantly

329   than other carbon sources. Further discussion was given in section 3.6. On the other hand,

330   other carbon sources received a consistent POX concentration in both cell and culturing

331   environment, around 1 nmol/d.ml. There was no distinct discrepancy between the two

332   salinity. In salinity 3.5%, the saccharose and glucose carbon sources received lower POX

333   concentration than in salinity 0.1%. Their POX concentration was comparable to other carbon

334   sources.

335   [Insert Fig. 4]

336   3.5 Nutrient removal influenced by different carbon sources and salinities

337   Carbon, nitrogen and phosphorus are nutritious elements and promote the growth of

338   *Chlorella* sp., and furthermore produce pigment, EPS and enzymes. As shown in Fig. 5, those

339   compounds reduced significantly after 10 d. The total carbon decreased 30 to 90%. Methanol,

340   ethanol and sodium bicarbonate carbon sources were used below 50%. The saccharose,

341   glucose and acetate ones were removed from 70 to 90%. For glycine, it was a particular case

342   given *Chlorella* sp. removed 91% in salinity 0.1% and 55% in salinity 3.5%. This result was

343   closely linked to the larger amount of chlorophyll and carotene produced in salinity 0.1%

344   when compared to salinity 3.5%. Total carbon removal of methanol in salinity 3.5% also

345   surpassed 50% in salinity 0.1%.

346   The nitrogen removal efficiencies varied from 70 to 99%, which were superior to the carbon

347   removal efficiencies. In salinity 0.1%, the nitrogen was removed from 85 to 89% for all

348   carbon sources. In salinity 3.5%, these values ranged more widely from 70 to 99%. The

349   glucose and saccharose carbon sources exhibited the highest nitrogen consumption (99%)

350   which were 20% better than others. For phosphorus, *Chlorella* sp. consumed 65 to 75% in

351   salinity 0.1%. In turn, in salinity 3.5%, it fluctuated from 30 to 75% given that the

352   phosphorus removal efficiency of the corresponding carbon sources impaired two times than

353 in salinity 0.1%. Nonetheless, the glucose and saccharose carbon sources did not follow this

354 trend as the phosphorus removal efficiencies persisted at 70%.

355 [Insert Fig. 5]

356 3.6 Effects of carbon sources and metabolic pathways on *Chlorella* sp.

357 The interesting question in this research was how and to what degree the carbon sources

358 impacted on *Chlorella* sp., since the biomass, pigment, EPS and enzyme levels related to

359 those carbon sources were different (Fig. 1-4). There were two possible explanations and they

360 originated from the chemical structure of carbon sources and the responses of microalgae.

361 Firstly, it was known that those carbon sources possessed different energy content. For

362 instance, glucose comprised more energy in its chemical structure and could produce 2.8 kJ

363 energy/mol compared to 0.8 kJ/mol of acetate (Perez-Garcia et al., 2011). In addition,

364 saccharose could create 4.2 kJ/mol. Therefore, glucose and saccharose resulted in the highest

365 biomass yield being produced. In contrast, the alcohol-based carbon sources, methanol and

366 ethanol, containing a low energy outputs of 0.1 kJ/mol and 0.5 kJ/mol, respectively, could

367 also toxify microalgae more severely than the sugar-based and inorganic carbon sources

368 (Cardol et al., 2011). Such facts are consistent with their low biomass yield (Fig. 1).

369 Secondly, regarding microalgae, those carbon sources were consumed by different metabolic

370 pathways. For instance, microalgae transformed glucose through the hexose/H^+ symport

371 system, then glucose was converted by the Embden–Meyerhof Pathway (EMP) and entered

372 the tricarboxylic acid (TCA) cycle for ATP production. Of the saccharose, microalgae

373 consumed it via two possible pathways: direct accumulation by specific carriers or

374 degradation by particular enzymes, such as sucrase, to monomers including glucose and

375 fructose (Wang et al., 2016). The monosaccharides are subsequently uptaken via the EMP

376 and TCA cycle. The EMP was an ATP economical pathway since the cells only invested one

377 ATP molecule per sugar molecule for transportation (Perez-Garcia et al., 2011). By using less

378 ATP pools, cells could yield more pigment, EPS and enzyme products for co-metabolism.

379 Microalgae also transform the supplied glucose into stored energy efficiently in the

380 phosphoanhydride bonds of ATP (12%). This explained the highest biomass, pigment, EPS

381 and enzyme levels of microalgae feeding by saccharose and glucose.

382 Of the sodium acetate, it is transported by the monocarboxylic/proton of monocarboxylic

383 molecules in the cell membrane. Inside the cells, sodium acetate is assimilated for the

384 acetylation of coenzyme A to form acetyl coenzyme A and participated in the Glyoxylate

385 Cycle. Unlike glucose, acetate encountered in the Glyoxylate Cycle, principally for lipid

386 production prior to entering the TCA cycle. This reduces the amount of acetate available for

387 biomass production. Previously, Bouarab et al. (2004) reported that glucose donates 38 moles

388 ATP and acetate donates 12 moles for microalgae growth, indicating glucose outcompeted

389 acetate for cell build-up. This explains the biomass and products of microalgae feeding by

390 acetate are lower than glucose and saccharose.

391 Glycine is the simplest amino acid which contains amino group ($-NH_2$). This makes glycine a

392 unique carbon source in this study. Glycine would be involved in the transcript encoding and

393 form a precursor of chlorophyll and carotene (Cecchin et al., 2018). By adding glycine, the

394 activity of uroporphyrinogen-III synthase, coproporphyrinogen III oxidase and magnesium

395 chelatase enzymes upregulated and resulted in the increase of chlorophyll level. This

396 confirms that by feeding glycine directly in salinity 0.1%, the *Chlorella* sp. generated more

397 chlorophyll than microalgae cultured in other carbon sources. For carotene, the carotenoid

398 biosynthetic pathway performed differently as a carotenoid oxygenase decreased in the

399 mixotrophic condition (Cecchin et al., 2018). This finding was in contrary to the one of this

400 study. However, Cecchin et al. (2018) insisted that the function of carotenoid oxygenase

401 remained unclear. In addition, the experimented microalgae strains were a case by case basis

402 (i.e., *Chlorella* sp. and *Chlorella sorokiniana).*

403 In comparison, the mechanism of methanol and ethanol accumulation were still unclear. It

404 was speculated that microalgae tend to avoid consuming them due to high toxicity of alcohols

405 as a self-defence mechanism. Consequently, the total carbon removal efficiencies of

406 methanol and ethanol were consistently poor. Also, microalgae compensate ATP energy in

407 order to repair the damage caused by alcoholic carbon sources, which accounts for 45–82%

408 of the total ATP produced (Yang et al., 2000). This explained the low products yield of

409 *Chlorella* sp. when fed by alcohol carbon sources.

410 Regarding inorganic carbon, it is common knowledge that inorganic carbon operated under

411 photoautotrophic mode resulted in less productivity than the organic carbon under

412 mixotrophic mode (Yeh and Chang, 2012). The Calvin cycle in the autotrophic mode

413 consumes 70% of the ATP stock in the cells and subsequently produces 3.11 g biomass/mmol

414 ATP, rather than 19.3 g biomass/mmol ATP of the heterotrophic and mixotrophic conditions

415 (Yang et al., 2000). By the transcript encoding, in the mixotrophic condition, 285 transcripts

416 encoding of 259 different proteins shifted, while, in the autotrophic one, 721 transcripts

417 corresponding to 620 proteins diminished. Those transcript encoding exhibited the

418 metabolisms of photosynthesis, chlorophyll, carotenoid, photorespiratory pathway,

419 carbohydrates, fatty acids and amino acids metabolism, sulphur, nitrogen, phosphate

420 assimilation (Cecchin et al., 2018). Thus, the inorganic carbon-feeding microalgae yield

421 biomass and other products less than most organic carbon sources (Fig. 1-4).

422 3.7 Effects of salinities on *Chlorella* sp.

423 Salinity is able to influence the behaviour of *Chlorella* sp. by altering biomass concentration,

424 pigment, EPS, enzyme concentrations and pollutants removal efficiencies (Fig. 1-4). The

425   mechanisms and reasons for these changes can be elaborated as follows. Firstly, increasing

426   salinity causes stress in microalgae cells with surplus sodium and chloride ions penetrating

427   into the cells. It will induce the hydroxyl radical groups substantially and interfere the PSI,

428   PSII and other metabolic pathways of *Chlorella* sp. (Vo et al., 2019). To alleviate the stress,

429   *Chlorella* sp. respond in several ways. For example, to balance the osmotic regulation,

430   microalgae accumulate compounds such as soluble carbohydrates to tolerate salinity. Sugars

431   reduce the stress conditions and trigger the functions of osmoprotection, osmotic adjustment,

432   carbon storage, and radical scavenging (Khosh Kholgh Sima et al., 2012). Also, microalgae

433   yield more chlorophyll and carotene. Those pigments are antioxidant compounds and they

434   can cope the stress conditions. Microalgae concurrently produce more enzymes and EPS in

435   the cells, extruding to the culturing environment in order to neutralise the hydroxyl radical

436   groups. Thus, it was observed that concentration of SOD enzyme and EPS was shifted to the

437   higher level in salinity 3.5% (Fig. 3 and 4). However, less phosphorus was consumed in high

438   salinity due to the decline in $H_2PO_4^-$ activity (Khosh Kholgh Sima et al., 2012). By saving

439   material pool to produce more EPS and enzymes, the biomass in higher salinity reduced

440   accordingly. The phosphorus consumption of sugar-feeding microalgae (i.e., glucose and

441   saccharose) in salinity 3.5% were still comparable to the one in salinity 0.1%. As explained,

442   the sugar-based carbon sources could alleviate the salinity stress to microalgae better than

443   other carbon sources; possibly, they consume phosphorus in the same manner in both

444   salinities.

445   For salt stress, as previously mentioned, twice the amount of protein in EPS was produced

446   compared to the carbohydrate. The reason for this could be due to the particular *Chlorella* sp.

447   strain. For instance, Xiao and Zheng (2016) reported the protein level extruded by microalgae

448   would vary case by case. In their review, the green microalgae produced protein from 0.5 to

449   10 mg/L, which was significantly less than the *Chlorella* sp. in this study (10-30 mg/L).

450   Regarding enzyme, similarly, Singh et al. (2018) found that *Chlorococcum humicola* and

451   *Chlorella vulgaris* augmented 55% SOD enzyme activity in salinity 3.5%. This outcome

452   agreed with our findings that SOD concentration rose with salinity for most cases (Fig. 4).

453   Regarding the POX enzyme, its activities were stable in most carbon sources and salinities,

454   except for saccharose and glucose. POX depended less on carbon sources and salinities, and

455   was low compared to SOD. The reason was still inconclusive but it was interpreted that the

456   POX-producing system within microalgae was less developed than that of SOD and it reacted

457   insignificantly against salinity. The POX system was more sophisticated in plants rather than

458   microalgae. For example, Soltani Nezhad and Mansouri (2019) found that POX in the plant

459   *Robinia pseudoacacia* increased to 40 U/mg at salinity 4M. Very few studies have reported

460   POX produced by microalgae so far.

461   In salinity 3.5%, although microalgae extruded more EPS and enzyme, it did not imply that

462   the pollutants were removed in a better manner. It might get worse. For example, salinity

463   diminished the $H_2PO_4^-$ activity and reduced phosphorus accumulation efficiency of

464   microalgae as previously mentioned (Khosh Kholgh Sima et al., 2012). Another evidence

465   was organic pollutants. In salinity 1%, the *Scenedesmus obliquus* removed 93.4%

466   levofloxacin of concentration 1 mg/L (Xiong et al., 2017b). Increasing salinity to 3% and 5%

467   impaired the levofloxacin removal efficiency to below 10%. However, the *Scenedesmus*

468   *obliquus* only removed 4% and 80% at salinity 0% and 0.3%, respectively, that less than the

469   value in salinity 1%. This indicated each strain preferred a particular salinity. Choosing an

470   appropriate salinity range for microalgae was critical as excessive salinity could face several

471   challenges. One of the problem was salt layer forming on microalgae cells which captured by

472   the SEM. This layer could hinder the pollutant assimilation process and reverse pollutant to

473   culturing environment. Apart from the salt layer, EPS could deposit on microalgae cell as

474 well. The thickness of EPS layer ranged up to 4 μm which further impaired the pollutant

475 assimilation (Vergnes et al., 2019).

476 3.8 Implications for green pollutant co-metabolism process

477 Overall, the results of this study delivered a basis for further research and practical

478 applications. Industries such as molasses, vinegar, pharmaceuticals, aquaculture and cattle

479 discharge large amounts of wastewater into sewerage systems. Such waste streams contain

480 sufficient carbon sources (i.e., sugar, acetate, alcohol) for microalgae cultivation

481 (Bhattacharya et al., 2017). According to our results, wastewater containing glucose and

482 saccharose would receive the highest amount of removed pollutants, EPS and enzyme levels.

483 Hence, nutrients and valuable pigments such as chlorophyll and carotene could be recovered,

484 up to 10 mg/L. It indicated a sustainable revenue-raising potential. Also, enzymes and EPS in

485 the cultured effluent encountered significant amounts of organic pollutants as a co-

486 metabolism process (Bilal et al., 2019; Ufarté et al., 2015). After harvesting microalgae, the

487 cultured effluent could be reused for removing organic pollutants. Containing up to 30 mg

488 EPS/L, the cultured effluent possessed strong antioxidant properties and could catalyse the

489 degradation of pesticides, plastics and antibiotics (Xiao et al., 2019; Xiong et al., 2017a).

490 Several studies have reported the feasibility of EPS in the cometabolism of organic

491 pollutants, such as 2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl) acetamide, toluene

492 and trichloroethylene (Ahmad, 2020; Yang et al., 2019). Apart from the organic pollutants,

493 EPS could absorb heavy metals, such as Cr (VI) (Johnson et al., 2020). By binding the heavy

494 metal in the EPS, cells exposed less to heavy metal and, consequently, it reduced toxicity

495 causing to the cells. It increased the biodegradation of organic pollutants. This conditions the

496 cometabolism of mg 1,4-Dioxane/L up to 100%. Based on the sustainable criteria, the

497 application of cultured liquid for pollutant co-metabolism was proposed as following (Fig. 6):

498   [Insert Fig. 6]

499   EPS from microalgae has been applied to agricultural crops to alleviate salt stress (Arroussi et

500   al., 2018). The EPS-incubated crops increased their protein levels by 31.5% compared to

501   non-incubated ones. In addition, the SOD and POX levels decreased by 30%, thus indicating

502   salt stress was less severe when implementing EPS-incubation. Apart from glucose and

503   saccharose, glycine yields significant amounts of chlorophyll, carotene and EPS. Also,

504   acetate can increase the carotene yield in salinity 3.5% (1.5 mg/L). In addition, the sodium

505   acetate could stimulate microalgae degrading ciprofloxacin three times higher to 40%, using

506   initial ciprofloxacin 2000 μg/L and those microalgae produced enzyme level similarly to our

507   result (Xiong et al., 2017a). The algae-producing industry can reuse wastewater containing

508   glycine for mass production purposes. Glycine and acetate are part of the amine-rich

509   wastewater that is released from the degradation of amine-based adsorbents (Dong et al.,

510   2019). By culturing microalgae in amine-rich wastewater, the wastewater is detoxified,

511   nutrients are recovered and pigments are harvested. It can be seen that this work has solved to

512   the two major issues for microalgae-based sustainability: gaining revenue and remediating

513   pollutant by co-metabolism.

514   In practice, this work implied potential improvement. Bacteria in wastewater might compete

515   with microalgae and consume nutrients at the same time. Knowing how to harmonize and

516   combine microalgae and bacteria when using those carbon sources was important. For

517   example, purple bacterium was a prominent candidate because it functioned as a notable

518   pollutant remover in saline wastewater (Hülsen et al., 2019). Another issue was that sugar-

519   based carbon sources likely favoured microalgae rather than any others. However, in

520   wastewater, various carbon sources co-existed and it was anticipated that the competition

521   amongst carbon sources might occur. This is a problem that needs to be addressed.

522   4. Conclusion

523   Overall, the effects of seven carbon sources under two salinities on the cultivation of

524   *Chlorella* sp. were comprehensively studied. The sugar-based carbon sources achieved the

525   highest EPS and enzymes concentration. The produced EPS mainly comprised protein rather

526   than carbohydrate. When assessing the influence of salinity, EPS and enzymes concentrations

527   in salinity 3.5% rose by 3 to 30 times. The increasing EPS and enzymes concentration

528   suggested that the EPS and enzyme increased co-metabolism for degrading pollutants. In

529   EPS, there would be a number of enzyme types involved in the cometabolism which required

530   further address.

531   E-supplementary data of this work can be found in online version of the paper.

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659

660   Figure captions

661   Fig. 1. Biomass concentrations of *Chlorella* sp. feeding with different carbon sources under

662   two salinities, fitted Gompertz model. The symbols demonstrate experimental measurements

663   and dash lines demonstrate model fit. All measures showed significant differences ($p<0.01$)

664   amongst carbon sources and between the two salinities.

665   Fig. 2. Chlorophyll (A) and carotene (B) of *Chlorella* sp. at day 10th feeding with different

666   carbon sources under two salinities. All measures showed significant differences ($p<0.01$)

667   amongst carbon sources and between the two salinities

668   Fig. 3. EPS of *Chlorella* sp. at day 10th feeding with different carbon sources under two

669   salinities. All measures showed significant differences ($p<0.01$) amongst carbon sources and

670   between the two salinities.

671   Fig. 4. SOD (top) and POX (bottom) concentration of *Chlorella* sp. at day 10th feeding with

672   different carbon sources. All measures of SOD showed significant differences ($p<0.01$)

673   between the two salinities. The POX concentration of the two salinities were insignificant

674   difference ($p>0.01$).

675   Fig. 5. Total carbon, nitrate and phosphate removal efficiencies of *Chlorella* sp. at day 10th

676   feeding with different carbon sources under two salinities. All measures showed significant

677   differences ($p<0.01$) amongst phosphorus removal between the two salinities. The removal

678   efficiencies of total carbon and nitrate were insignificant difference ($p>0.01$).

679   Fig. 6. Proposed application of culturing liquid for pollutant co-metabolism.

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682   List of figures
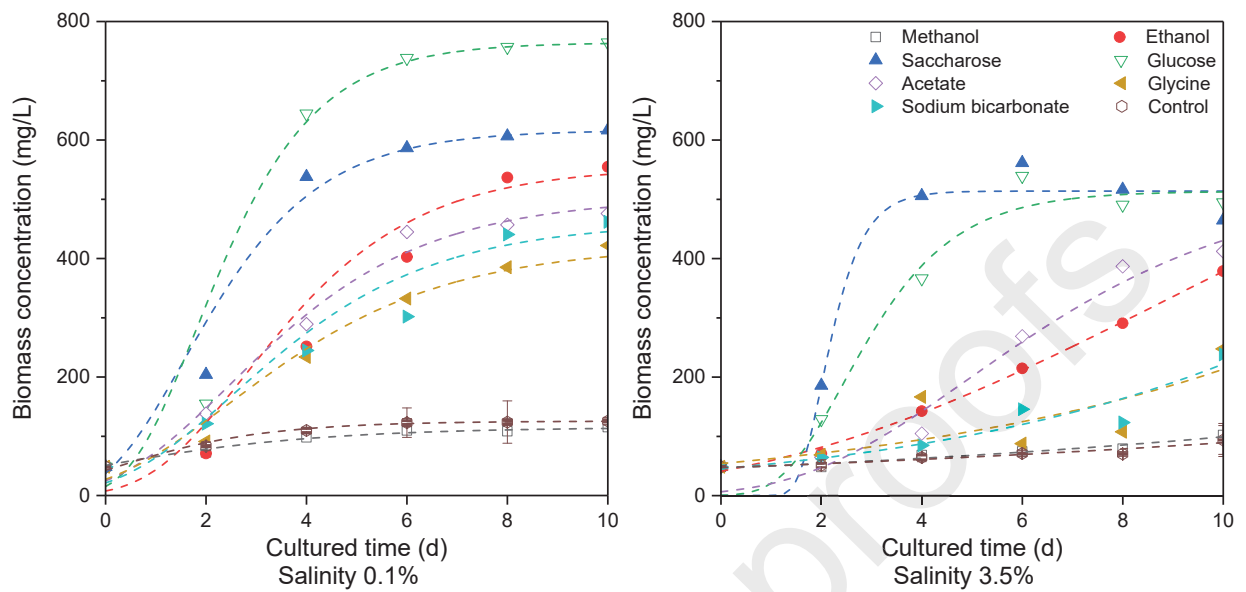
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684   Fig. 1. Biomass concentrations of *Chlorella* sp. feeding with different carbon sources under

685   two salinities, fitted Gompertz model. The symbols demonstrate experimental measurements

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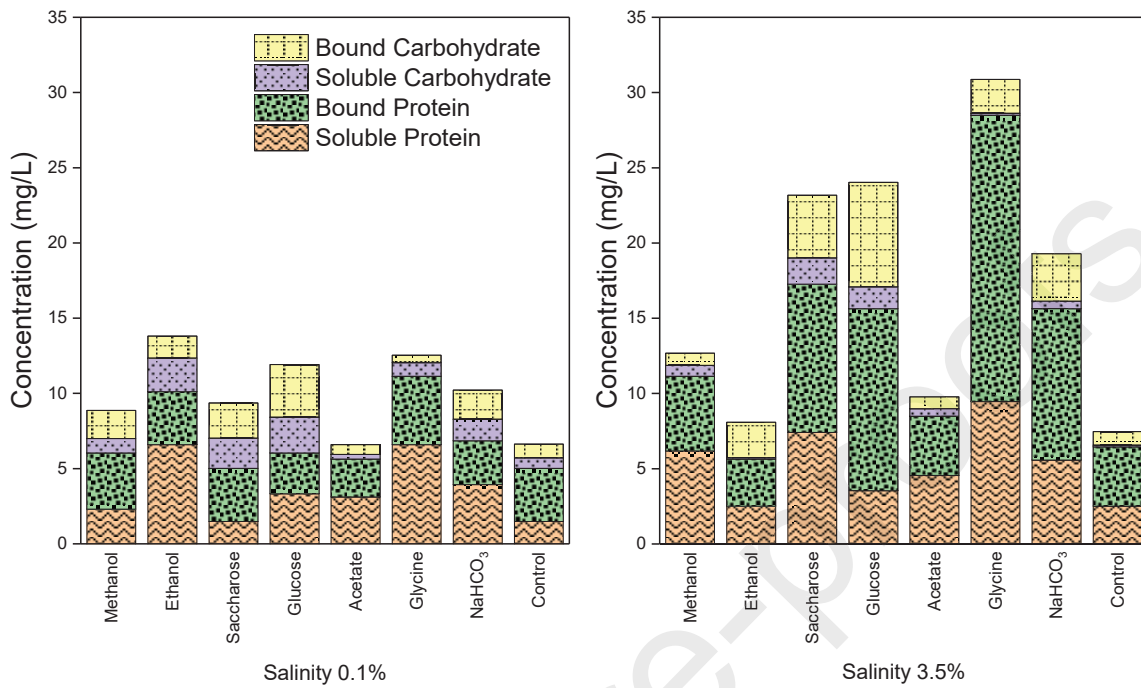687   amongst carbon sources and between the two salinities.

688
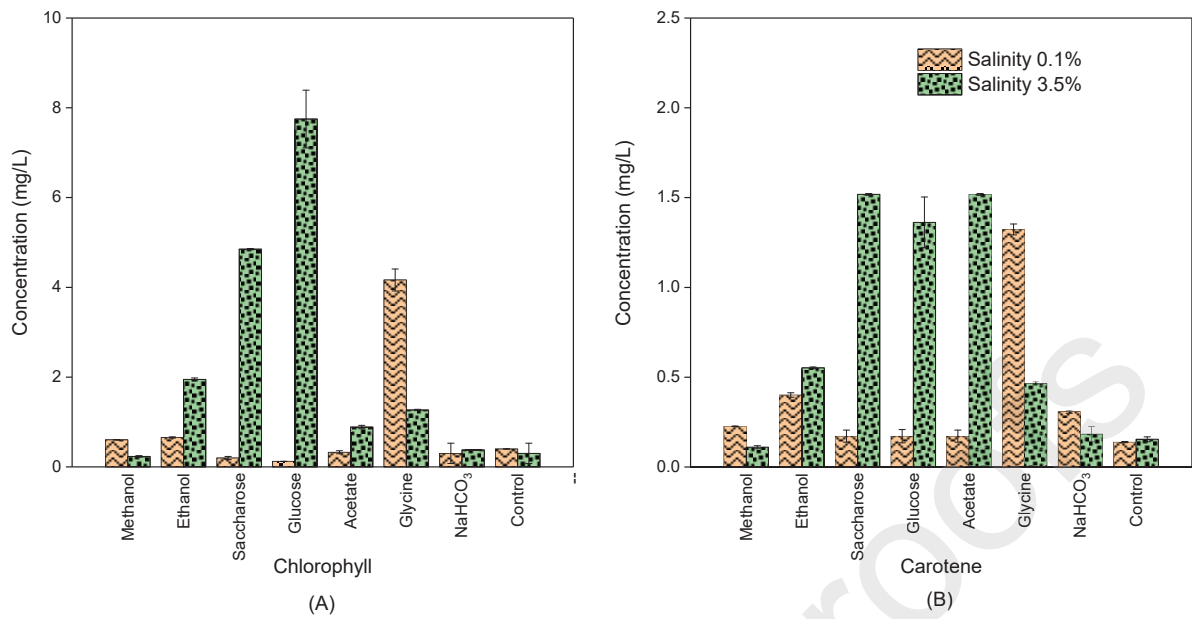
689

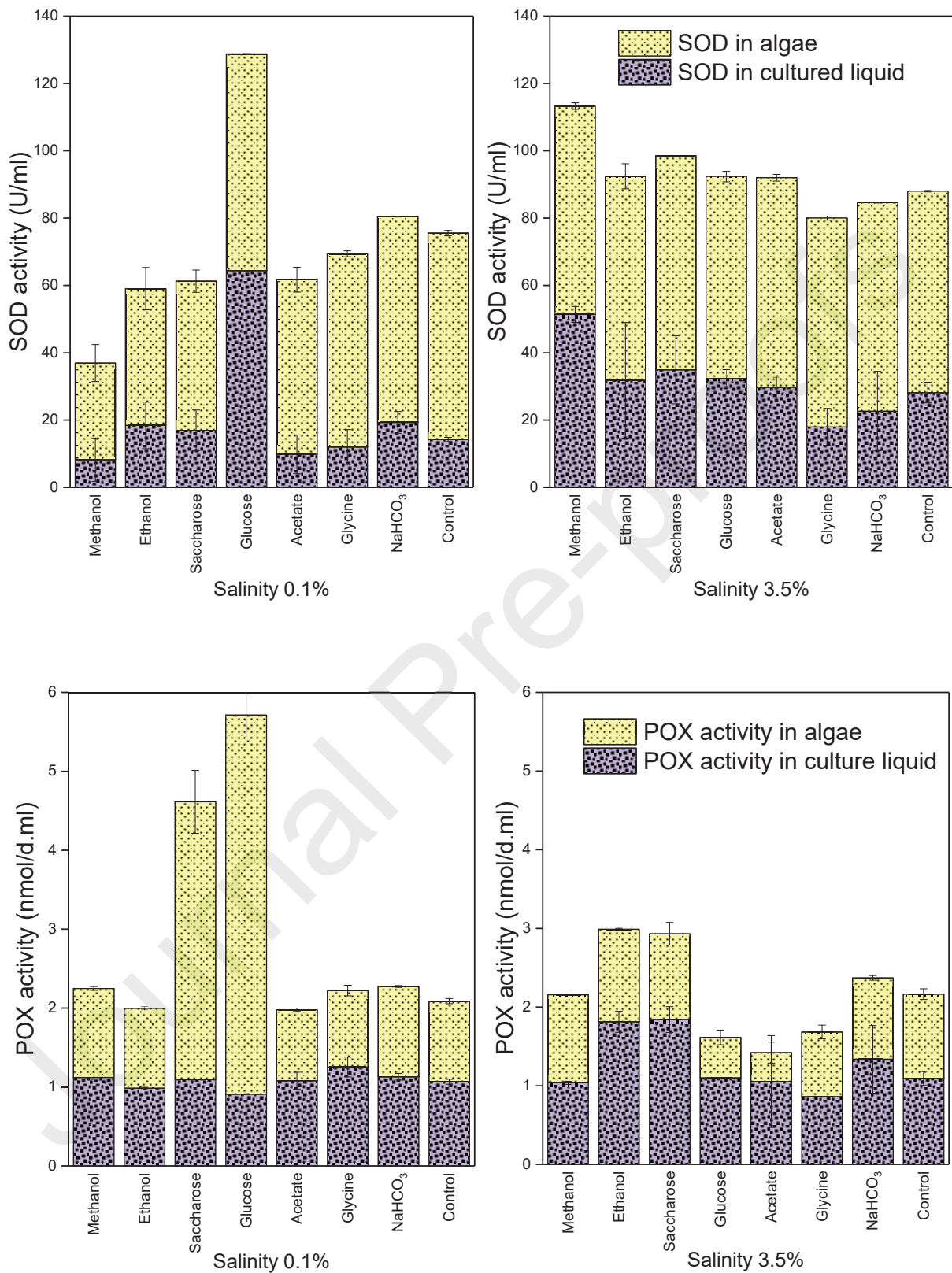Fig 2. Chlorophyll (A) and carotene (B) of *Chlorella* sp. at day 10th feeding with different

carbon sources under two salinities. All measures showed significant differences ($p < 0.01$)

amongst carbon sources and between the two salinities.

693

694

Salinity 0.1%   Salinity 3.5%

695
696 Fig. 3. EPS of *Chlorella* sp. at day 10th feeding with different carbon sources under two

697 salinities. All measures showed significant differences (p<0.01) amongst carbon sources and
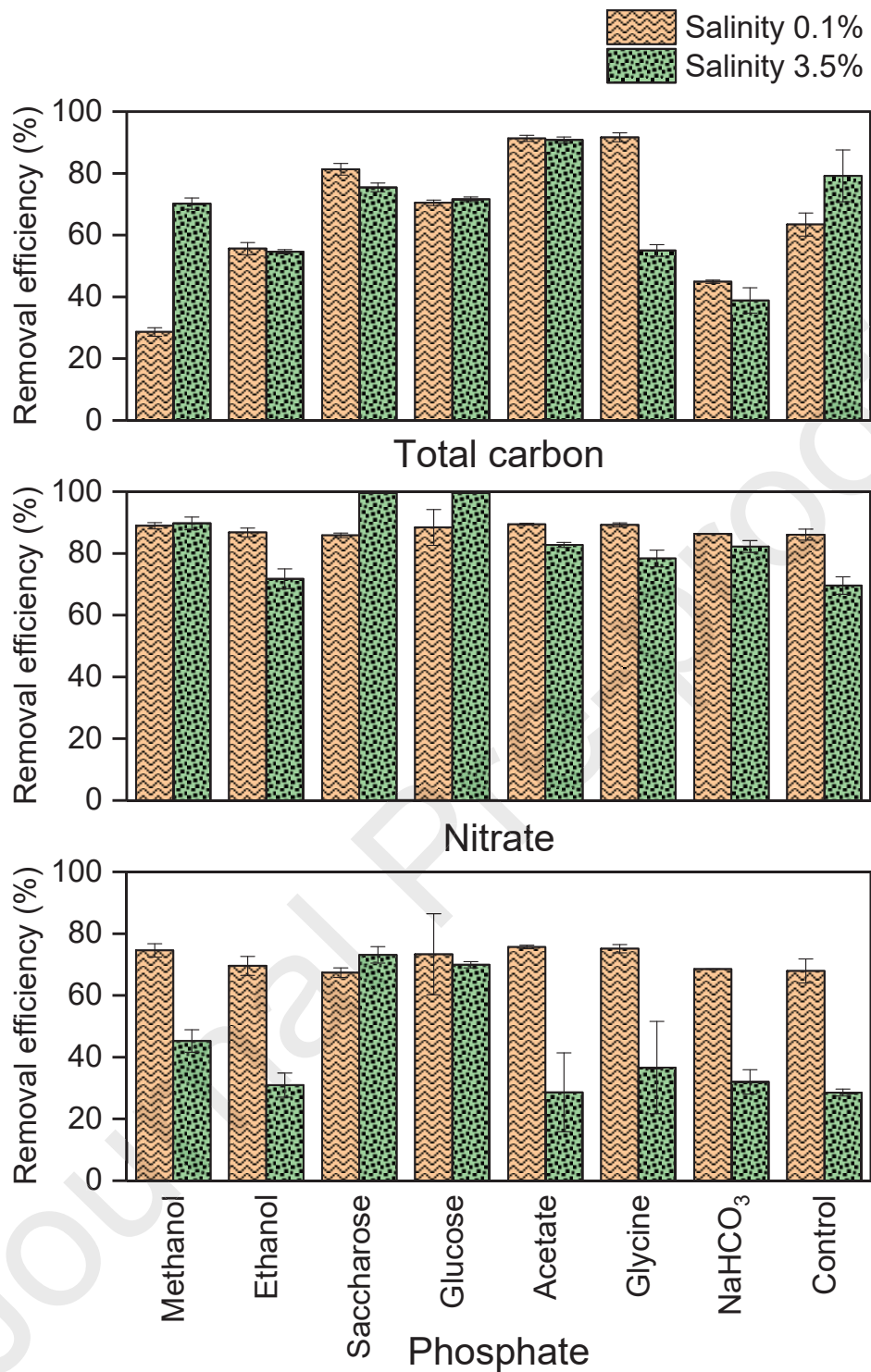
698 between the two salinities.

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701
702   Fig. 4. SOD (top) and POX (bottom) concentration of *Chlorella* sp. at day 10th feeding with

703   different carbon sources. All measures of SOD showed significant differences (p<0.01)

704   between the two salinities. The POX concentration of the two salinities were insignificant

705   difference ($p > 0.01$).

706

Fig. 5. Total carbon, nitrate and phosphate removal efficiencies of *Chlorella* sp. at day 10th

feeding with different carbon sources under two salinities. All measures showed significant

709   differences ($p<0.01$) amongst phosphorus removal between the two salinities. The removal

710   efficiencies of total carbon and nitrate were insignificant difference ($p>0.01$).

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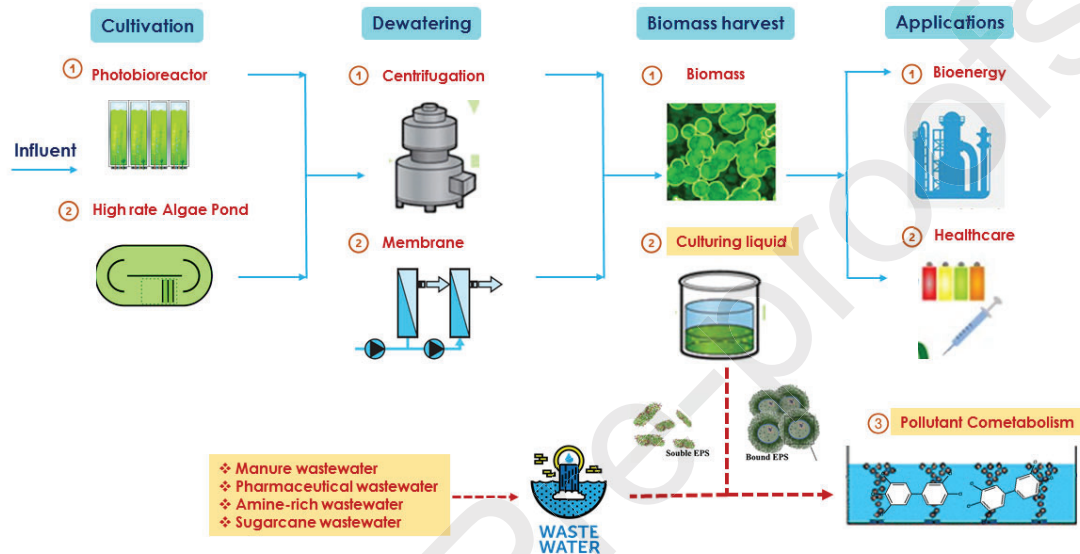
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714   Fig. 6. Proposed application of culturing liquid for pollutant co-metabolism.

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Author Contribution Statement

Hoang Nhat Phong Vo: investigation, writing - original draft, methodology, formal analysis, data curation
Huu Hao Ngo: supervision, investigation, project administration, conceptualization, review & editing
Wenshan Guo: supervision, investigation, review & editing
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Soon Wang Chang: investigation, project administration, review & editing