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6 **Evaluation of microalgae production coupled with wastewater**

7 **treatment**

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Evaluation of microalgae production coupled with wastewater treatment

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In the present study the feasibility of microalgae production coupled with wastewater treatment was assessed. Continuous cultivation of *Chlorella sorokiniana* with wastewater was tested in lab-scale flat panel photobioreactors. Biomass productivity was determined for four dilution rates ($4.32\ d^{-1}$, $3.6\ d^{-1}$, $1.8\ d^{-1}$ and $0.72\ d^{-1}$). The productivity peak was $1.524\ g\ l^{-1}d^{-1}$ at the dilution rate of $2.41\ d^{-1}$. Nitrogen and phosphorus removals were found to be inversely proportional to dilution rates, while COD removal was found to be 50% at all the tested conditions. The biomass obtained at the highest dilution rate was characterized for its content of lipids, proteins and pigments. The average yields of fatty acid methyl esters (FAME), protein, lutein, chlorophylls and β-carotene was 62.4 mg, 388.2 mg, 1.03 mg, 11.82 mg and 0.44 mg per gram dry biomass, respectively. Economic analysis revealed that potentially more than 70 % of revenue was from the production of pigments, i.e. chlorophyllin (59.6%), lutein (8.9%) and β-carotene (5.0%) while reduction in discharging costs of the treated wastewaters could account for 19.6% of the revenue. Due to the low yield of FAME and the low market price of biodiesel, the revenue from the above was found to be the least profitable (1.4%). Even when taking into account all these different revenues combined, this cultivation strategy was found with the current prices to be uneconomical. Power consumption for artificial light was responsible for the 94.5% of the production costs.

Keywords: *Chlorella sorokiniana*, biorefinery, wastewaters, photobioreactors, economic analysis

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Introduction

Increasing concerns about climate change and sustainability of fossil fuels based economies have brought interest to microalgae for potential to establish bio-based economy, mainly due to their higher areal productivity over traditional biomasses [1]. Nevertheless, algal biomass production cost is still one major obstacle for commercialization of algae-derived products, especially for the low-value ones such as biofuels. As a consequence, current application of algal biomass is centered on high-value products (i.e. health, cosmetics, nutraceutical and food) [2]. In order to make the production of algal biomass profitable, efforts can be made on process integration, algal biology and cultivation system design [1, 3]. First, it is strongly recommended to produce biofuel simultaneously with value-added co-products, following a biorefinery strategy [4]. Furthermore, the combination of microalgae production with wastewater treatment for removal of nutrients and hazardous compounds can lead to a further step towards a cost-effective process, by saving the costs for N and P fertilizers when using nutrient rich streams [5, 6]. Moreover, revenue from wastewater treatment would help the overall process economy.

In this context, selection of appropriate algal species is pivotal: the ability of the species to grow in specific wastewaters and then generate biomass suitable for further transformation to high value products has a direct impact on the potential revenues. Furthermore, the use of wastewater as the culturing media adds stricter requirements for robustness of microalgae against adverse conditions, such as contamination with possible toxic compounds and competition with undesired microorganisms [7, 8]. Zhou et al. [9] isolated multiple species from natural environments and screened five potential high lipid producers in concentrated municipal wastewater by DNA sequencing: *Auxenochlorella prototrophocoides*, *Hindakia* sp., *Scenedesmus* sp. and two *Chlorella* sp.

77 A similar work found two *Chlorella* species, *C. protothecoides* and *C. kessleri* were

78 growing better in wastewater compared to 14 other algal strains [10]. Additionally,

79 several studies dealing with algal consortia suggested *Chlorella* sp. and *Scenedesmus*

80 sp. as relatively robust species that can grow in wastewater [11-13].

81   Apart from the selected species, biomass production coupled with wastewater

82 treatment depends on a variety of operation parameters such as type of wastewater, light

83 intensity and cycle, pH, temperature, dilution rate, etc. [14]. Flow rate of medium, that

84 determines the rate of nutrient supply, largely impacts the growth rates of the

85 microorganisms. Biomass concentration at steady state depends on the equilibrium

86 between specific growth rate and the imposed dilution rate [15]. Dilution rate is

87 following the growth rate of algae up to maximum growth rate whereafter at higher

88 dilution rates wash out would happen. As a consequence, the maximum biomass

89 productivity would be reached at a specific dilution rate which is close (but lower) to

90 the maximum growth rate of the algae at that specific condition. Previous studies

91 investigated the effect of dilution rates on the overall productivity and observed that the

92 optimal productivity corresponds to medium values of the dilution rates. This is

93 probably due to less optimal growth conditions which not support maximum rates of the

94 algae, such nutrients deficiency or content of potential inhibitors [16, 17].

95   Reducing production cost and/or increasing productivity are possible ways to

96 improve the economics of algal biomass production. The present study aims to further

97 investigate and assess the biomass productivity and the biomass composition of selected

98 microalgae species grown in wastewater, instead of widely used synthetic media for

99 supply of nutrients. Use of wastewater would reduce cost for nutrients (necessary for

100 the cultivation) into revenue deriving from the removal of the same nutrients as

101 environmental service. In this context, the algal biomass was used as a source for high

4

102   added value products and biofuels to offset the production costs. Additionally, attempts

103   to improve the productivity via strain selection and optimization of cultivation-

104   operation were made. Based on the data generated, the economics of algal biomass

105   production was assessed in four scenarios considering an annual production of 330

106   days.

107

108   **Materials and methods**

109   ***Algal strains, medium and wastewater***

110   Microalgal species *Chlorella sorokiniana* and *Scenedesmus obliquus* were chosen for

111   the initial screening because they are frequently found in different wastewaters [11-13]

112   and thus are expected to show robust growth in such environments. The strains were

113   obtained from SCAAP (Scandinavian Culture Collection of Algae & Protozoa,

114   Denmark) and cultivated in sterilized Woods Hole medium (MWC) [18] containing

115   selenium.

116   Mixed influent industrial/municipal wastewater from Kohtla-Järve, Estonia was

117   selected for testing with algae based on the assumption that it represents typical

118   conditions in larger municipalities where industrial and municipal wastewaters as well

119   as storm water are mixed and then treated together. The mixed industrial/municipal

120   probe represented time–adjusted average water sample collected over 24 hours. The

121   water sample has been analysed by the Estonian Environment Research Centre and the

122   list of substances for the analyses involved COD_{Cr}, TOC, BOD_7, NO_2-N, NO_3-N, NH_4-

123   N, N_{tot}, PO_4-P and P_{tot}. A number of hazardous compounds were present in the

124   wastewater and were analysed by Kohtla-Järve WWTP using standard procedures

125   (Table S1 in Supplementary Material). Part of the collected water sample was frozen (-

126   20°C) and transported to Danish Technical University for further tests with microalgae.

127 For all the cultivation experiments, wastewater underwent sedimentation to remove the

128 majority of solid particles. Sedimentation is considered an economic method in large

129 scale applications for gross separation of larger particles and therefore it was chosen as

130 separation methodology. Analysis of nutrients and organic compounds of the

131 supernatant after sedimentation was performed at the Technical University of Denmark.

132 Due to storage and sedimentation of the wastewater samples, some changes in

133 the water quality occurred, resulting in lower COD, N_{tot}, and P_{tot} concentrations and

134 higher NH_4-N content (Table 1).

135

136 *Microplate screening*

137 Screening for the best performing algal strain in the wastewater was carried out in 24-

138 well microplates (PE VISIPLATE, 24 well black-walled, clear bottomed). The

139 microplates were incubated at room temperature, illuminated by LED at 400 ± 50 µmol

140 photons m^{-2} s^{-1} and shaken at 140 rpm with a 50 mm throw. Growth was monitored by

141 fluorescence (440 nm emission, 690 nm detection) using a Synergy Mx microplate

142 reader (BioTek Instruments, Inc., USA).

143 Cultivation procedures, well-top membranes, growth rate calculations, and

144 detection limits were as described in recent study [19]. Each of the strains was

145 inoculated in triplicates in 100% wastewater or mixtures of wastewater and MWC + se

146 medium with varying percentages of wastewater (75%, 50% and 25%). Culture volume

147 in each well was 2 ml. The screening was repeated for two generations for both species.

148

149 *Photobioreactor cultivation*

150 A flat-panel photobioreactor (Algaemist reactor, Wageningen University) was used to

151 cultivate *C. sorokiniana* with the wastewater pretreated by sedimentation. Undiluted

152 wastewater was used for this set of cultivation experiments due to the positive results

153 obtained from the microplate screening where cultivation in undiluted wastewater

154 supported algal growth (see Results and discussion: Microplate screening).

155   The cultivation was initiated in batch mode. Parameter settings in this

156 experiment are listed in Table 2, and were chosen according to the optimal growth

157 condition for this species [20-22]. When the growth reached early stationary phase, the

158 cultivation was switched to continuous mode. The dilution rate was set to 4.32 d^{-1},

159 which was close to the maximum specific growth rate observed during the exponential

160 phase in batch mode. Thereafter, the dilution rate was stepwise decreased to 3.6 d^{-1}, 1.8

161 d^{-1} and 0.72 d^{-1}. Optical density (OD_{750}) throughout the cultivation was monitored.

162 Moreover, biomass was collected for each dilution rate when the OD value was stable.

163 The temperature of the effluent was maintained at 4°C to inhibit algae metabolism and

164 growth after harvest.

165

166 ***Analytical methods***

167 The samples obtained from the highest dilution rate was subject to lipid, protein and

168 pigment quantification.

169

170 *Cell growth and dry cell weight*

171 Cell growth of algae was monitored by measuring optical density at 680 and 750 nm

172 using a Hach Lange DR2800 spectrophotometer. The correlation between optical

173 density (OD) and dry weight (DW) concentration of samples (C_x) was determined as

174 described in Van Wagenen et al. [17]. The correlation curve between OD_{750} of cell

175 suspensions and dry weight of the biomass resulted to be linear, $C_X = 0.31 OD_{750} - 0.04$

176 with a $R^2 > 0.95$.

177

Lipid determination

179 The procedure for the quantification of fatty acid methyl esters (FAMEs) was based on

180 the modified Folch method [23]. 10 mg of freeze-dried and powdered biomass was

181 mixed to a solvent mixture of chloroform: methanol (2 mL, 2:1, v/v) in duplicate. After

182 vortexing for 20 minutes, FAMEs were formed by addition 1 mL of methanol and 300

183 µL of H_2SO_4 and incubation at 100°C for 20 minutes. After cooling down, 1 mL of

184 distilled water was added to the sample, which was then vortexed for 5 minutes and

185 centrifuged at 4,000 rpm for 10 minutes. The lower layer including the organic solvent

186 was analysed with gas chromatography (HP 5890, Agilent, USA) with a flame ionized

187 detector (FID) and INNOWAX capillary column (Agilent, USA). The GC column

188 temperature was programmed as follows: (1) initial column temperature at 50 °C, hold

189 for 1 min, (2) increase to 200 °C at a rate of 15 °C min^{-1}, hold for 9 min, and (3)

190 increase to 250 °C at a rate of 2 °C min^{-1}, maintain for 2 min. Individual FAME

191 component was identified and quantified by comparing the retention times and peak

192 areas with those of the FAMEs standard solutions, respectively. The internal standard

193 was Supelco 37 Component FAME Mix, item no. 47885- U, Sigma−Aldrich.

194

Protein determination

196 For protein hydrolysis, duplicates of 50 mg biomass were suspended in 6 ml of 6N HCl

197 and transferred in close vessels. The vessels were flashed with nitrogen to prevent

198 oxidative degradation of some oxygen/sensitive amino acids. The vessels were then

199 microwaved for 30 min at 150 and 500W (Multiwave 3000, Anton Paar). Samples were

200 then freeze-dried to remove HCl. The residues were resuspended in 400 milliQ H_2O

201 and filtered through 0.22 syringe filters before the protein quantification by in-needle

202   derivatization HPLC-FLD (Dionex UltiMate 3000, Thermo Scientific). Amino-acids

203   were separated in a c18 reversed phase column (Eclipse Plus C18, Agilent

204   Technologies, USA) with an in-line guard column (EC 4/2 Universal RP, Macherey-

205   Nagel, Germany) and mobile phases A (10mM Na_2HPO_4, 10 mM $Na_2B_4O_7$) and B

206   (methanol: acetonitrile: water, 45:45:10). The flow rate was 0.420 mL min^{-1}.

207   Quantitative analyses were performed by means of calibration curves using a

208   commercial amino-acid mix standard (AAS18 Fluka).

209

210   *Pigments determination*

211   Two milligrams of freeze-dried biomass were mixed with 3 ml of 90% acetone in

212   duplicates. Well mixed samples were sonicated in ice bath for 10 min (Branson

213   3510MT). The supernatant was separated from the residual biomass by centrifugation at

214   13,000 rpm for 10 min. A Zorbax Eclipse plus C8 RRHD 1.8 μm 3.0×150 mm column

215   was used for UHPLC separation at 60 °C with a 75 min separation time. Detection

216   utilized UV−VIS at 450 nm. Quantification was done relative to individual pigment

217   standards obtained from DHI, Hørsholm, diluted from 15 to 1500 μg L^{-1}.

218

219   *Nutrient measurements*

220   Samples corresponding to each dilution rates were centrifuged in order to harvest

221   biomass. The supernatants were collected for nutrient composition analysis. Contents of

222   COD, total nitrogen (N_{tot}), total phosphorus (P_{tot}) and ammonium were determined for

223   the supernatant using Hach Lange Cuvette Kits. (LCK314, LCK238 and LCK348, while

224   Spectroquant® ammonium test (Merck Millipore) was used for the measurement of

225   ammonium.

226

227 *Estimation of biomass market value*

228 Evaluation of economic potential of algae biomass was performed by calculating the

229 gross profit, taking into account only the difference between revenue and the operating

230 cost, without deducting costs for overhead, payroll, taxation and interest.

231 Specifically, a value of unit biomass was calculated as sum of revenues from all

232 products of interest, including biodiesel, proteins and pigments (e.g. lutein, chlorophylls

233 and β-carotene) as well as benefit for removing COD, N and P from the wastewater.

234 Market value for each bioproduct obtained per unit biomass can be calculated from the

235 experimentally obtained yields, i.e. FAME (C_f), amino acid (C_{aa}) and pigments (C_p).

236 Prices of desirable products (Table 3) were obtained from an e-commerce website:

237 www.alibaba.com. Specifications of the benchmark products can be found on the

238 company pages. The revenue from bio-products is the sum of production of each

239 product (P_i) multiplied with its price, shown in the following equation.

$$Revenue_b = \sum_i C_i \cdot Price_i$$

240 Estimation of production cost was based on data from literature. Aim with this

241 preliminary economic assessment was to estimate which costs – revenues are more

242 important for the operational cost balance. The estimation only includes operation costs

243 and not initial investment costs. The rationale behind this was to generate a dataset that

244 could serve as a preliminary assessment of the profitability of this specific concept. In

245 case the process resulted to be not economically feasible based on operational costs and

246 revenues, it would be logical to assume investments for facilities construction would

247 make the economic prospects even more difficult. CO_2 supply was the only input

248 needed cost, while nitrogen and phosphorus were considered free as present in the

249 wastewater. Power consumptions for light, CO_2 sparging and harvesting were

250 considered main items of production cost for algal biomass. Additionally, cationic

10

251 coagulant was chosen for the estimation of the harvesting costs due to its effectiveness

252 and low cost compared to others [24]. Detailed calculation can be found in

253 supplementary material.

254

255 *Scenarios for potential cost reduction*

256 A basic economic analysis was conducted to evaluate potential cost reduction

257 opportunities. In addition to the base case (where costs for CO_2 and LED were both

258 taken into account), three alternative scenarios were proposed. Case (1) assumed

259 industrial flue gas containing CO_2 was provided freely e.g. from a nearby power plant

260 without significant influence on cell growth and composition. In case (2), the cost for

261 power of lighting was eliminated by substituting artificial light with natural light source

262 (i.e. sunlight). Because of the unstable supply as a consequence of day-night cycle and

263 seasonal variation, specific growth rate and cell density was assumed to decrease by

264 14% and 31%, respectively [25]. In the third scenario, assumptions in case (1) and (2)

265 were combined.

266

267 *Statistics analysis*

268 IBM SPSS Statistics (Version 22) was used for statistical analysis. Data comparison

269 was performed using one way ANOVA test and unpaired t-test with 95% confidential

270 intervals.

271

272 **Results and discussion**

273 *Microplate screening*

274 Based on specific growth rate (Figure 1), *C. sorokiniana* shows higher robustness in this

275 wastewater over *S. obliquus* at all conditions. The highest specific growth rates are 2.40

11

276   d^{-1} and 2.04 d^{-1} for *C. sorokiniana* and *S. obliquus*, respectively, which are obtained in a

277   mixture with 50% wastewater in the second generation. Acclimation in the second

278   generation was observed for both species. Furthermore, when wastewater concentration

279   was higher than 50%, growth rates were inversely proportional to wastewater

280   concentration for both species, which suggests possible inhibitory effects of wastewater

281   on the algal growth.

282   This could be due to presence of hazardous compounds from the oil-shale

283   industry in the KJ wastewater, which can potentially be harmful to microalgae species.

284   At the same time, undiluted wastewater contains the highest concentration of nutrients

285   and therefore leads to the highest cell density of *C. sorokiniana* (Figure 2), even with a

286   lower growth rate. The same tendency was observed in a previous study, where 100%

287   wastewater resulted in initial inhibition to algae, but eventually it resulted in the highest

288   algae density compared to diluted concentrate [26]. Based on these results and on

289   considerations that dilution of wastewater would be more technical complex and costly,

290   undiluted wastewater was used for the photobioreactor (PBR) experiments.

291

292   ***Algae productivity***

293   Average biomass productivities and biomass concentration measured at steady states of

294   four dilution rates are shown in Figure 3. The cultivation was initiated with the dilution

295   rate (4.32 d^{-1}) close to the maximal specific growth rate (4.56 d^{-1}) observed in a batch

296   cultivation in the same wastewater. This dilution rate led to the lowest biomass

297   concentration (0.18 g l^{-1}) and, as a consequence, to the lowest productivity (0.8 g $l^{-1}d^{-1}$).

298   With the decrease of dilution rates, biomass concentration rose to 1.44 g l^{-1}, (dilution

299   rate of 0.72 d^{-1}) corresponding to low productivity (0.95 g $l^{-1}d^{-1}$). The highest biomass

300   productivity (1.46 g $l^{-1}d^{-1}$) was exhibited at a dilution rate of 1.8 d^{-1}. The curve

301 describing the correlation between dilution rate and biomass productivity was fitted to a

302 binomial equation, and the highest productivity was estimated to be 1.524 g $l^{-1}d^{-1}$ at a

303 dilution rate of 2.41 d^{-1}, corresponding to a cell density of 0.63 g l^{-1}.

304 The trend seen with decrease of cell concentration with increasing dilution rates

305 is contradictory to the theoretical expected. The expected trend would be that the cell

306 concentration was stable with increasing dilution rate, until initiation of wash out which

307 would correspond to a sharp decrease the cell concentration.

308 The explanation to the observed relationship could be due to the spontaneous

309 flocculation and wall attachment occurred during the cultivation (Figure 4). The

310 calibration curve (section Analytical methods) used to calculate cell concentration was

311 generated using homogeneously suspended cells, and therefore OD measurements do

312 not reflect cell concentrations of flocculant cell associations. High flow rates (high

313 dilution rates) in upflow reactor systems are causing selection pressure to the cells. Only

314 cells managing to create flocs are resisting wash out, by creating flocs presenting larger

315 diameter than the single cells and thereby having a higher sedimentation rate, while the

316 suspended cells are washed out of the reactor. Therefore high dilution rates are

317 promoting flocculation and thereby OD measurements at these high rates are giving an

318 underestimation of the cell concentration.

319 Previous studies employed the same photobioreactor system (flat plate) used in

320 the current one [16, 17] and have found similar trends. The operation conditions and

321 growth data achieved in these previous publications listed in Table 4 for comparison. In

322 Van Wagenen et al. [17] parallel experiments were conducted with a high light intensity

323 (2100 μmol m^{-2} s^{-1}) and a low light intensity (200 μmol m^{-2} s^{-1}). The operating

324 conditions of the present study (wastewater instead of synthetic media and low light

325 intensity) are very similar.

326   However, even if the light intensity in the present work was twice as much as the

327 low light experiment in Van Wagenen et al. [17], lower biomass density and

328 productivity were obtained. A reason for this difference could be the different nutrient

329 supplements in the media used. The nutrient content, especially nitrogen in Kohtla-Järve

330 influent wastewater was considerably lower compared to the aforementioned study

331 (Table 5). It has been proven that biomass concentration and NO_3-N supply are

332 positively correlated, up to a saturation level of about 30 mg NO_3-N l^{-1} (further increase

333 of cell density was limited, which may be caused by the limitation of other nutrients)

334 [27]. The positive effect of increasing nitrogen and phosphorus concentration on algal

335 growth was also reported, demonstrating that the highest level of algal biomass

336 corresponded to the highest initial N_{tot} of 25 mg l^{-1} [28].

337

338 ***Nutrient removal***

339 Nitrogen and phosphorus concentrations were determined for the treated wastewater

340 and for the resulting biomass after harvesting. Nutrient contents of the treated

341 wastewater were compared with the composition of untreated wastewater.

342   Removal efficiencies at different dilution rates are shown in Figure 5. Overall,

343 the highest removal efficiencies (> 90%) were observed at the lowest dilution rate (0.72

344 d^{-1}). With the decrease of dilution rate, the removals of total nitrogen, total phosphorus

345 and ammonium were steadily increased. However, the removal of COD for all dilution

346 rates remained around 50%. Limited COD reduction was also previously reported [29,

347 30]. This indicates that the residual ~50% of COD consisted by organics not degradable

348 by microalgae. This also shows that organic carbons were consumed very quickly in

349 these experiments and therefore were the preferred carbon source by *C. sorokiniana*

350 over CO_2 (heterotrophy/mixotrophy). This is in agreement with a previous study, in

14

351 which batch cultivations of *C. sorokiniana* were conducted at increasing concentration

352 of organic carbon, with the highest growth rate corresponding to the highest

353 concentration [31].

354 Van Wagenen et al. [17] observed very high removal efficiencies for PO_4-P in

355 all the tested dilution rates. In the present work phosphorus removal rate was instead

356 increased with dilution rate. An explanation for this could be the fact that phosphorus

357 was in excess in the wastewater used in this previous study (N/P ratio was 36.5:1 in Van

358 Wagenen et al. [17] while it was only 14.9:1 in the Kohtla-Järve influent wastewater

359 which we used in this study).

360 Finally, average concentrations of mineral elements present in the algal biomass

361 are 8.87 % N and 1.04 % P, which partly represent the nutrients transferred from

362 wastewater to biomass. Similar N and P contents were also reported when microalgae

363 were grown in dairy manure and obtained biomass consisting of 7 % N and 1% P [32].

364

365 *Biomass characterization*

366 Compositional analysis of the algal biomass grown in wastewater is listed in Table 6.

367 Palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:1) and linolenic acid (18:3)

368 were found to be the most abundant fatty acids present in the algal biomass (Table 7).

369 This is in agreement with typical fatty acid composition of *C. sorokiniana* found in

370 literature [33-36].

371 Fatty acid content in *C. sorokiniana* can vary from 0.6% to 47.51% depending

372 mainly on the growth conditions (Table 8). FAME yield of current study is relatively

373 low compared to fatty acid contents of *C. sorokiniana* reported in literature.Nitrogen

374 starvation has been widely recognized as a stress condition which stimulates the

375 accumulation of lipids. Li et al. [46] showed that the initial nitrogen concentration in the

15

376   medium was positively correlated with the growth of *C. sorokiniana,* but reversely

377   correlated with the lipid content. Lipid accumulation is believed to be a consequence of

378   the inhibition of proteins and starch biosynthesis which usually occurs in stationary

379   phase   [47].

380   Furthermore, composition of the lipid profile is in general correlated to culturing

381   conditions, and this may be another reason for low fatty acid content in the algal

382   biomass produced in the present work. In contrast to polar lipids (e.g. membrane

383   components), neutral lipids are responsible for energy storage in cells and are precursors

384   for FAME production. It has been shown that different nutritional conditions can affect

385   the percentage of neutral lipids within the total lipid content varying from 2.9% to 60%

386   [36]. In addition, low irradiation, as in the present study, induces the formation of polar

387   lipids, whereas the formation of triacylglycerols is favoured at high light intensity

388   conditions [48]. Also, although results show that available organic carbon source was

389   consumed, nitrogen and phosphorus were still abundant in the effluent of culture

390   (Figure 5). Therefore, microalgae in this condition were not stressed by nutrient

391   limitation and thus tended to invest carbon and energy for cell growth. The high protein

392   content 38.82% (w/w) in the algal biomass is an indicator for the active proliferation. In

393   conclusion, in the present work the high growth rate (supported by sufficient nutrient

394   supplement) was probably the reason for the relatively low fatty acid yield. Clearly,

395   there is a tradeoff between biomass productivity and lipid content that cannot be

396   achieved simultaneously. This is why two-phase cultivation strategies are a possible

397   solution for the economics of algae cultivation [49, 50].

398

399   *Estimation of biomass value and economic potential*

400   The revenue generated from cultivationg *C. sorokiniana* in this specific wastewater is

401   estimated to be 3.27 € kg^{-1} dry biomass, which includes 2.63 € kg^{-1} (80.4%) from the

402   production of valuable bioproducts and 0.64 € kg^{-1} (19.6%) from removal of nutrients

403   from wastewater as an environmental service (Table 9).

404   More specifically, chlorophyllin accounts for 59.7% of the total value, whereas

405   the share of biodiesel is negligible (1.4%) as a consequence of the low FAME yield. As

406   per kilo of microalgae produced, roughly 1580 L wastewater can be treated at a dilution

407   rate of 2.41 d^{-1}, which makes significant contribution (19.6%) to the overall revenue.

408   However, the nutrient removal efficiencies in this condition are unsatisfactory for

409   treating wastewater. Removal efficiencies of only 52.1% for COD, 57.5% for nitrogen

410   and 68.8% for phosphorus were achieved. The cost for producing a kilo of microalgae

411   was estimated to be 12.46 € kg^{-1} comprising 94.5% for power for illumination, whereas

412   the remaining 5.5% was for CO_2 supply (2.7%), cost of cationic flocculant (0.4%),

413   power for harvest (2.1%) and aeration (0.3%).

414   As already mentioned, biodiesel is the least remunerative product. Despite the

415   fast growth of *C. sorokiniana*, the parallel low FAME production largely affects the

416   economics of the strategy presented in this study. Furthermore, coupling biomass

417   production and wastewater treatment contributes to the total revenue. However, the

418   COD and nutrients removal efficiencies at the dilution rate, 2.41 d^{-1} were poor.

419   Consequently, the resulted wastewater may not fulfill the quality for reuse and may

420   require additional steps for further treatment.

421   Finally, the economic potential in the case of utilizing artificial light is -9.19 €

422   kg^{-1}-biomass, showing economically unsustainable production.

423

424   ***Scenarios for potential cost reduction***

17

425 Economics of algal biomass production was assessed in four scenarios considering an

426 annual production of 330 days. The results indicate the economic potential can be

427 positive only when the cost for artificial light is eliminated (Figure 6). Results show that

428 the substitution of artificial light with sunlight can reduce production cost by 96.0%,

429 whereas the reduction resulted from using free CO_2 is 2.7%. The elimination of CO_2

430 cost has relatively little effect (+3.6%) on the overall cost reductions. By contrast,

431 economical potential can be increased by 116.1% and become positive as a result of

432 considerable drop in cost for artificial light.

433 On the other hand, the substitution of artificial light by sunlight hypothetically

434 causes 14% and 31% reduction in specific growth rate and cell density, respectively

435 [25], resulting in 40.7% reduction in biomass productivity. As a consequence, annual

436 revenue is reduced by 39.6%. In addition, because nitrogen removal is 56% less in a

437 light-dark cycle condition in comparison with continuous illumination [51], the shorter

438 illumination period leads to further decrease in nitrogen removal efficiency to 26.8%.

439 This analysis highlights that excluding use of artificial light is an imperative to

440 enable sustainable production of algal biomass for any purpose. In the base case, at least

441 76.5% of the cost for artificial light needs to be reduced to ensure breakeven for the

442 necessary utilities for biomass production (e.g. electricity, flocculant and CO_2). In the

443 case that excludes the costs for CO_2 and light, biomass cost is reduced to 424 € t^{-1},

444 which is substantially lower than 5,960 € t^{-1} as reported in [52] and 2,340 $ t^{-1} reported

445 in [53]. Exclusion of capital cost and operational cost such as labour and general plant

446 overhead is one major reason for the underestimation in our estimation. Furthermore,

447 some basic assumptions for the calculation are different. For example, aeration power

448 accounted for the biggest fraction of cost in Norsker et al.'s calculation, which is

449 relatively low in the present work.

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452 **Conclusion**

453 This work demonstrated that microalga *C. sorokiniana* can well adapt to the wastewater

454 chosen for this assessment and thus exhibits high biomass productivity. The cultivation

455 led to a significant but not optimal removal of COD, N and P. Nitrogen and phosphorus

456 removals were observed to be inversely proportional to dilution rates, while COD

457 removal was found to be constant. Microalgae cultivation can therefore be considered a

458 promising tool for partial nutrient recovery from wastewaters, but not yet an ideal tool

459 to meet wastewater treatment plants requirements. In this context, the nutrient recovery

460 translates in the production of valuable biomass that could make the entire process

461 profitable. The composition of the resulting biomass was determined in respect to lipids,

462 proteins and pigments content. The economic assessment performed on the entire

463 process showed that pigments in particular could play a pivotal role in economics of

464 algae production and should be the primary goal to pursue. It is noteworthy that the

465 cultivation conditions in the present study were generally chosen to ensure optimal

466 microalgae growth and optimal biomass productivity. However, the same conditions

467 translate in poor content of high value products in the same biomass. For this reason it

468 is advisable to develop two-phase cultivation strategies, in which microalgae are first

469 kept in optimal growth conditions to generate high biomass yield, and then stressed to

470 increase the high added value products content in the same biomass.

471 Finally the economic assessment performed on this specific species/wastewater

472 combination proved this cultivation strategy to be uneconomical, mostly due to the

473 energy consumption for artificial light, which accounts for 94.5% of the production

474 costs.

476   **Word count: 5,113**

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660   Table 1 Composition of KJ wastewater.

Indicator	Before sedimentation	After sedimentation
COD	442 mg O_2 l^{-1}	386.9 mg O_2 l^{-1}
N_{tot}	117 mg N l^{-1}	48.6 mg N l^{-1}
P_{tot}	10.5 mg P l^{-1}	7.2 mg P l^{-1}
NH_4-N	34.7 mg N l^{-1}	46.7 mg N l^{-1}

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685   Table 2. Parameter settings for PBR cultivation

Parameter	Setting
Temperature	37°C
pH	7.0
Light intensity	400 μmol m^{-2} s^{-1}
Air flow rate	160 ml min^{-1}
CO_2 flow rate	40 ml min^{-1}

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709   Table 3. Specifications and market prices of desirable products.

Product	Specification	Price	Reference
FAME	B100 biodiesel	734 € t^{-1}	Keysun Bio-Tech Co.Ltd
Amino acids	AA content: 54.4%	426 € t^{-1}	Seek Bio-Technology Co.Ltd
Lutein	80%	284 € kg^{-1}	Xi'an Lyphar Biotech Co.Ltd
Chlorophyllin	95%	165 € kg^{-1}	Xi'an Lyphar Biotech Co.Ltd
β-carotene	95%	411 € kg^{-1}	Xi'an Lyphar Biotech Co.Ltd

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736 Table 4. Comparison of experimental conditions and growth performance of *C.*
737 *sorokiniana* in flat panel PBR. PFD = photon flux density, D= dilution rate, C_X = biomass
738 concentration and P_b = biomass productivity.

Medium	PFD	D	C_X	P_b	Reference
	(μmol m^{-2} s^{-1})	(d^{-1})	(g l^{-1})	(g l^{-1} d^{-1})	
M8a	2100	5.76	2.2	12.2	[16]
IC effluent	2100	3.6	1.56	5.87	[17]
IC effluent	200	1.44	1.09	1,67	
KJ influent	400	2.41	0.60	1.52	This study

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761 Table 5. Comparison of media used for continuous cultivation of *C. sorokiniana* in flat
762 panel PBR.

Indicator	Unit	M8a	IC effluent	KJ influent
COD	mg O_2 l^{-1}	-	590	386.9
N_{tot}	mg N l^{-1}	1680	190	48.6
P_{tot}	mg P l^{-1}	641	11-12	7.2
NH_4-N	mg N l^{-1}	-	-	60.1

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788 Table 6. Productivities of desired bioproducts.

Product	Yield (%, w/w)	Productivity (mg l^{-1} d^{-1})
Biomass		1524
FAME	6.24	95
Protein	38.82	592
Lutein	0.103	1.57
Chlorophylls	1.182	18.01
β-carotene	0.044	0.671

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812   Table 7. Fatty acids profile of *C. sorokiniana*

Type of fatty acid		Percentage
Total FAs (% dw.)		6.24
Fatty acid (% total FAs)	Palmitic acid (C16:0)	20.22
	Palmitoleic acid (C16:1)	9.51
	Oleic acid (C18:1)	19.82
	Linolenic acid (C18:3)	8.39

813   Table 8. Characterization of *C. sorokiniana* biomass in literatures.

Research focus	Growth performance (d^{-1}/ g L^{-1} d^{-1})	Lipid content (%, w/w)	FAME yield (%, w/w)	Protein content (%, w/w)	Reference
Effect of temperature	-	~ 10%	1.3 – 6.1%	-	[35]
Effect of C/N ratio	-	13 – 46%	2.1 – 7.3%	-	[33]
Pigment composition	5.76 d^{-1}	10.0%	-	68.5%	[37]
Effect of biochemical stimulants	42 mg l^{-1}d^{-1}	5 – 7%	-	45 – 60%	[38]
Mixotrophic growth	0.44 d^{-1}	20 – 50%	-	10 – 32%	[39]
Effect of inoculum size	0.89 d^{-1}				[40]
Photoautotrophic/ heterotrophic growth	-	21 – 26% (P) 20 – 56% (H)	0.6 – 0.8% (P) 12 – 33.6% (H)	12 – 13% (P) 6.2 – 13% (H)	[36]
Cultivation with deep sea water	176.6 mg l^{-1}d^{-1}	51.7%	47.51%	-	[41]
Cultivation in cattle manure	12.77 mg l^{-1}d^{-1}	25 – 35%	12%	34%	[42]
Fed-batch cultivation	3.29 d^{-1}	14.5 – 38.7%	12.8 – 34.1%	-	[43]
Photoautotrophic/ heterotrophic/ mixotrophic growth	0.68 d^{-1} (P) 2.07 d^{-1} (H) 3.40 d^{-1} (M)	-	9.0% (P) 6.2 – 17.6% (H) 13.4 – 34.7% (M)	-	[34]
Cultivation in domestic wastewater	220 mg l^{-1}d^{-1}	48.31%	-	-	[44]
Mixotrophic growth	1.602 d^{-1}	20 – 27%	-	-	[45]
Effect of nitrogen limitation	3.21 d^{-1}	20 – 51%	-	-	[46]
Continuous cultivation	2.41 d^{-1}, 1.52 g l^{-1}d^{-1}		6.24%	38.8%	This study

814   (P: photoautotrophic; H: heterotrophic; M: mixotrophic)

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818 Table 9. Estimation of biomass value.

Product	Yield	Productivity	Revenue
Biomass		1.524 g $l^{-1}d^{-1}$	
FAME (B100)	0.0624 g g^{-1}	0.095 g $l^{-1}d^{-1}$	0.46 € kg^{-1}
Amino acid fertilizer (54.4%)	0.3882 g g^{-1}	0.592 g $l^{-1}d^{-1}$	0.162 € kg^{-1}
Lutein (80%)	1.03 mg g^{-1}	1.565 mg $l^{-1}d^{-1}$	0.292 € kg^{-1}
Chlorophyllin (95%)	11.81mg g^{-1}	18.014 mg $l^{-1}d^{-1}$	1.950 € kg^{-1}
β-carotene (95%)	0.44 mg g^{-1}	0.671 mg $l^{1}d^{-1}$	0.181 € kg^{-1}
Sum			2.630 € kg^{-1}
Wastewater treatment	Removal	Quantity	Revenue
Wastewater		1581.4 $L^{-3}kg^{-1}$	
COD	52.1%	0.319 kg kg^{-1}	0.042 € kg^{-1}
Nitrogen	57.5%	0.044 kg kg^{-1}	0.356 € kg^{-1}
Phosphorus	68.8%	0.008 kg kg^{-1}	0.242 € kg^{-1}
Sum			0.640 € kg^{-1}
Total revenue			3.271 € kg^{-1}

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