1 **Effect of Tris-(hydroxymethyl)-amino methane on microalgae**

2 **biomass growth in a photobioreactor**

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17 **Abstract**

18 One of the buffers namely Tris (Tris-(hydroxymethyl)-amino methane) was used to

19 increase the growth of microalgae by stabilizing the pH value in microalgae cultures.

20 The objective of this research is to determine the growth rate and biomass productivity

21 of *Chlorella* sp. with and without Tris addition. Both conditions function at various N:P

22 ratios cultured in photobioreactors (carbon dioxide of 5% (v/v), light intensity of 3.3

23 Klux). Daily variations in nutrient removal (nitrogen and phosphorus), cell

24 concentration, DO, temperature and pH were measured for data analysis. The results

25 show that the largest yield of biomass was achieved at the N:P ratio of 15:1 with and

26 without Tris. After cultivation lasting 92 hours, the algae concentration at this ratio was

27 1250 mg L^{-1} and 3568 mg L^{-1} with and without Tris, respectively. This indicates that

28 adding Tris to the photobioreactor greatly reduces algae biomass due to bacterial

29 competition.

30 *Keywords:chlorella* sp.*,* tris (Tris-(hydroxymethyl)-amino methane, photobioreactor,

31 N:P ratio, microalgae

32

33 1. Introduction

34 The rising global demand for energy is a serious issue with respect to fossil fuel

35 depletion and carbon dioxide emissionslinked to global greenhouse scenarios. Carbon

36 dioxide is the most important anthropogenic greenhouse gas. Biofixation is the only

37 economically feasible and environmentally sustainable technology in the long-term

38 (Kumar et al., 2010). This biomass which is produced by converting carbon dioxide can

39 be used to create products of high value, such as fatty acids, biodiesel, biogas, ethanol

40 and organic fertilizers (Lopes et al., 2008; Giordano et al., 2014). It is worth noting that

41 microalgae with 70% oil by weight can produce 23 times more oil compared to oil

42 palm, the current major biofuel producer (Wang et al., 2009). The principle of

43 biological fixation is that carbon dioxide is used to provide the carbon source for

44 microalgae in the photobioreactor. Microalgae are known to contain large amounts of

45 lipids within their cell structure, and so they are increasingly attracting interest as a

46 biofuel feedstock. Microalgae photosynthesis will not produce any additional carbon

47 dioxide while energy production and nutrient utilization for their growth can be

48 achieved sustainably (Kumar et al., 2010; Pittman et al., 2011).

49

50   *Chlorella* sp., a genus of green unicellular microalgae which is highly efficiency at

51   removing the nutrient from wastewater (Shi et al., 2007; Imaizumi et al., 2014). It is

52   spherical in shape, about 2-9 µm in diameter and without flagella. Although no useful

53   microalgae have been found to be useful for carbon dioxide sequestration, *Chlorella* sp.

54   has good commercial values and can grow under a high carbon dioxide concentration of

55   40% (Sakai et al., 1995). It is regarded as one of the most important energy microalgae

56   due to its high protein accumulation, high lipid content and product variation (Das et al.,

57   2011). The lipid content of *Chlorella* sp is about $32 \pm 34\%$ of the dry weight (Chiu et

58   al., 2008). In addition, the carbon dioxide fixation rate is $0.68 \text{ mg L}^{-1} \text{ day}^{-1}$ (David and

59   Prabakaran, 2012). They produce approximately half of the atmospheric oxygen and

60   when used simultaneously the greenhouse gas carbon dioxide can grow

61   photoautotrophically (Melanie, 2013). Indeed, *Chlorella* sp. is suitable microalgae for

62   CO_2 mitigation and biodiesel production.

63   The process of microalgae cultivation is influenced by many factors. Nitrogen and

64   phosphorous are two of the key factors in algae growth. The relative amounts of such

65   essential nutrients required for growth and reproduce differ among algae species.

66   Furthermore, the type of cultivation conditions for microalgae also need to be

67   considered significantly. Phototrophic cultivation is the most commonly used

68   cultivation condition for microalgae growth (Yoo et al., 2010). Namely photobioreactor

69   is a closed system which is used to cultivate a single-species culture of microalgae for

70   prolonged duration. It produces a large amount of microalgae biomass, which is about

71   13 times as concentrated as the biomass found in a raceway pond (Chisti, 2007). Bubble

72   column reactors owe their many uses to their excellent mass and heat transfer

73   characteristics. It is easy to construct and operate and has high surface area to volume

74   ratio (Ugwu et al., 2008), and requires only low maintenance costs. However, there may

75   be significant phased back-mixing occurring. It is difficult to scale-up due to the

76   complex interaction between the faces. The sole source of agitation is provided by the

77   isothermal expansion of sparged gas (Chisti et al., 2006).

78   In previous studies, the cultivation of microalgae has incorporated the use of organic

79   buffers to increase microalgae growth. Tris (Tris-(hydroxymethyl)-amino methane) is a

80   buffer used to stabilize pH in microalgae cultures (Suzana et al., 2008). However, Tris is

81   very controversial because the efficiency of pH stabilization has not been clearly

82   demonstrated (Fabregas et al., 1993). In addition, the harmful effects of Tris have been

83   observed in some phytoplankton species and freshwater algae (Harrison et al., 1980).

84   Previous studies have indicated that Tris impacts on photosynthesis by inhibiting

85   mechanisms such as the transportation of HCO_3^- across the plasma membrane

86   (Axelsson et al., 2000). This buffer also stimulates the growth of bacteria, leading to

87   cultivation being severely curtailed (Fabregas et al., 1993). Nonetheless, it is

88   questionable whether the beneficial effects of Tris are more or less than its effects on

89   photosynthesis. Thus, this research aims to determine the growth rate and biomass

90   productivity of *Chlorella* sp. with and without Tris. Both conditions are operated at

91   various N:P ratios cultured in a photobioreactor.

92

93   **2. Material and methods**

94   *2.1. Microalgae strain and culture medium*

95   The microalgae strain used in this study was *Chlorella* sp. which was supplied by The

96   Research Center of Aquaculture II. Ruan et al. (2011) cultivated *Chlorella* sp. in the

97   culture medium with the following solid ingredients: 100 mg L^{-1} $MgSO_4.7H_2O$; 50 mg

98   L^{-1} $CaCl_2.2H_2O$. The liquid chemicals include: 1 mL L^{-1} glacial acetic acid; 1 mL L^{-1}

99   trace elements solution consisted of 50 g L^{-1} Na_2EDTA; 22 g $L^{-1}ZnSO_4.7H_2O$; 0.05 g L^-

100   ^{1} $CaCl_2.2H_2O$; 11.4 g L^{-1} H_3BO_3; 5.06 g $L^{-1}MnCl_2.4H_2O$; 4.99 g L^{-1} $FeSO_4.7H_2O$; 1.61

101   g L^{-1} $CoCl_2.6H_2O$; 1.57 g L^{-1} $CuSO_4.5H_2O$; 1.10 g L^{-1} $(NH_4)6Mo_7O_{24}.4H_2O$ and 16 g L^-

102   ^{1} KOH.

103   *2.2. Mass ratio adjustment*

104   In this study involving an experiment to produce a *Chlorella* sp. biomass, the

105   concentration of Tris and NH_4Cl was adjusted to suit the different N:P ratios (10:1,

106   15:1, 20:1, 25:1). The remaining chemical component remains unchanged. The

107   concentration of Tris and NH_4Cl was altered but not the concentration of K_2HPO_4 and

108   KH_2PO_4 in the culture medium. The final concentrations of Tris, NH_4Cl, K_2HPO_4, and

109   KH_2PO_4 in the medium are presented in Table 1.

110   Table 1. Components of synthetic medium

111

112   *2.3. Bubble column photobioreactor*

113   A diagram of the experimental pilot used in this study is illustrated in Fig. 1. The

114   photobioreactor was covered with a thick wood cover (5 mm) to retain a constant

115   temperature and prevent outside light from affecting it, and to concentrate the light

116   illuminated by three 18W lamps which were set up in the box. The microalgae were

117   cultivated in two identical columns - scale photobioreactors with a diameter of 100 mm

118   and a height of 600 mm. The working volume in the photobioreactor column was 4000

119   mL. The aeration system for the reactor consisted of a 20 mm diameter air diffuser

120   which was located at the bottom of the column. The system was operated under the

121   following conditions: temperature of $29 \pm 2^{o}C$, 3 Klux of light intensity and 24:0 light-

122   dark cycles (continuous illumination provided by three cool white lamps). Air mixture

123   flow into the photobioreactor was provided via an air pump and a pure carbon dioxide

124   tank through a 6 mm gas tube. With three rotameters which measured the air's flow

125   (from the air pump), the carbon dioxide gas and gas mixture, respectively, the carbon

126   dioxide /air mixture at 2.0 L min^{-1} flow rate was adjusted to achieve an air stream with

127   5% (v/v) of carbon dioxide. All experiments were carried out in batch mode.

128

129   Fig. 1 Bubble column photobioreactor system diagram

130

131   *2.4. Relationship between cells density and dry mass*

132   By using both methods together, cells density of *Chlorella* sp. was measured with a

133   counting method and dry biomass was measured by filtering a known volume of culture

134   medium through a 0.45 micrometer filter. It was then dried at $60^{o}C$ for 24 h, and the

135   standard curve of cell density and dry biomass were done. The formula of the standard

136   curve equation: y = 29989 x - 749565 (x: dry biomass, mg L^{-1}; y: cell density, cell mL^{-}

137   ^{1}).

138   *2.5. Biomass concentration analyses*

139   Cell density was determined each day using a hemocytometer (Germany) under a

140   microscope (Eclipse E50i; Nikon, Tokyo, Japan). Free-living algal growth was

141   determined daily (4 times per day). Cell density was measured by putting an algae

142   sample onto the mirrored surface of the Neubauer counting chamber. Then it was placed

143   under the microscope for cell counting, according to the Fuchs-Rosenthal and Burker

144   method. The formula to calculate the cell density after counting is α x 0.25 x 10^6, with α

145   being the average number of cells in 4 squares. When the cell density from the above

146   method was obtained, calculating the dry mass was done using the formula for the

147   standard curve equation.

148

149   **3. Results and discussion**

150   *3.1. Growth of Chlorella* sp. *with and without Tris in photobioreactor*

151   The growth curves of *Chlorella* sp. with and without Tris are shown in Fig. 2. It was

152   observed that the lag phase of culture conditions was lasted 20 h for the first cultivation,

153   reaching the logarithmic phase when the 20th hour of cultivation began. This was

154   followed by a stationary phase and then a death phase. Fig. 2 shows that a larger

155   maximum dry biomass without Tris was achieved than with Tris at the various N:P

156   ratios.

157   In the cultivation of N:P ratio of 10:1, the maximum dry biomass without Tris was 2.4

158   times larger than with Tris, achieving a concentration of 1404 mg L^{-1} and 584 mg L^{-1}

159   after cultivation lasting 92 h and 80 h, respectively. Similar to the N:P ratio of 20:1 and

160   25:1, the maximum dry biomass without Tris was 1134 mg L^{-1} and 1033 mg L^{-1}

161   whereas with Tris it was 800 mg L^{-1} and 742 mg L^{-1}, respectively. Results of the

162   previous study conducted by Cabanelas et al. (2013) reported that the maximum dry

163   biomass of *Chlorella* sp. was approximately 1160 mg L^{-1} and 1500 mg L^{-1} at the N:P

164   ratio of 12:1; and 14:1, respectively. This indicates that optimal the N:P ratio for

165   microalgae biomass production tends to the N:P ratio of 15:1.

166   In this study, with the cultivation at N:P ratio of 15:1, the dry biomass without Tris was

167   also 2.9 times higher than with Tris, leading to the biomass concentrations of 3568 mg

168   L^{-1} and 1250 mg L^{-1} under cultivating duration of 92 h and 96 h respectively. Similar

169   results were obtained by Agwa et al. (2012) when cultivated *Chlorella* sp. in the same

170   medium, dry biomass achieving at 3070 mg L^{-1}. Cho et al. (2013) also revealed that

171   *Chlorella* sp. cultivation could be achieved at the maximum dry biomass of

172   approximately 3010 mg L^{-1} at the N:P ratio of 15:1, using mixed wastewater between

173   from 10 % anaerobic digestion effluent and from 90% sludge dewatered supernatant.

174   Reversely, the maximum dry biomass of *Chlorella* sp. obtained by the study of Chiu et

175   al. (2008) was only approximately 899 mg L^{-1} with 5% of carbon dioxide and high

176   density cell. However, the optimal N:P ratio of 15:1 for microalgae biomass production

177   found from this study is contrast to typical N:P ratio of 8:1 conducted by USDA (1992).

178

179   Fig. 2 Dry biomass of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P

180   ratios

181

182   After a maximum of 5 days, the growth curves indicated characteristics of the stationary

183   phase where the amount of newly formed cells are equal to that for dying cells. Similar

184   results were obtained by Ruan et al. (2011) in the cultivation of *Chlorella* sp. Guerrero

185   et al. (1999) reported that a lack of lag phase is due to high carbon and inorganic

186   nutrient availability. However, the lag phase of this study occupies 20 h of the first

187   cultivation. This indicated that the concentration of carbon and inorganic nutrient was

188   still low.

189   In the logarithmic phase, the growth was carried out continuously between 20 h and 72

190   h. The maximum dry biomass of 3568 mg L^{-1} (equivalent to cell density of 1.05 x 10^8

191   cells mL^{-1}) obtained from the N:P ratio of 15:1 was found without Tris. Results of this

192   study indicated that *Chlorella* sp. could be cultivated in the following conditions that

193   enhanced biomass production: temperature of $29 \pm 2^{o}C$, light intensity of 3 Klux, N:P

194   ratio of 15:1 and without Tris.

195   Indeed, when cultivating the N:P ratio of 15:1, the maximum dry biomass was achieved

196   both with and without Tris. This is closely similar to results reported by Cho et al.

197   (2013) and Cabanelas et at. (2013). The above results indicated that a significant

198   decrease in the dry biomass occurs with increasing applied nitrogen ratio (i.e., ratio of

199   N:P greater than 15:1). For example, the study of Chiu et al. (2014) reported that the

200   N:P ratios higher than 17:1 contributed to lower biomass production.

201

202   *3.2. pH and DO curves of Chlorella sp. with and without Tris in photobioreactor*

203   Fig. 3 illustrates the representative variation of pH with and without Tris as a function

204   of residence time. Without Tris, during the 8 h lag phase, the pH value was stable in the

205   range of 6.5 - 6.7 at the various N:P ratios. However, in the logarithmic phase, the pH

206   value decreased slightly from 6.4 to 6.0. Especially at N:P ratio of 15:1, there was a

207   significantly reduction in pH from the 56th hour to the 96th hour. Lazzaro et al. (2008)

208   reported that the culture medium consisting of the predominant form of CO_3^{2-} will lead

209   to an inefficient production of biomass. Thus, the initial pH adjusted in the range of 6.5

210   - 7.0 increased to form HCO_3^{2-} which easily uptaked by *Chlorella* sp. (Beardall et al.,

211   1998). For this reason, in this study the consumption of HCO_3^- started increasing during

212   the logarithmic phase when pH have a downward trend by generating H^+. In addition,

213   the pH value of the culture medium decreased according to the uptake of ammonia

214   (Park et al., 1997). Tan et al. (2016) noted that the growth of *Chlorella* sp. was slower if

215   e pH fell to 5.0 and this is the pH limitation value of *Chlorella* sp. With Tris, the pH

216   value remained stable from 7.0 to 7.3 during the cultivation phase because Tris is one of

217   the buffers often used to stabilize pH in microalgae cultures (Fabregas et al., 1993).

218

219   Fig. 3 pH curves of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P ratios

220

221   Fig. 4 shows the relationship of the concentration of dissolved oxygen with and without

222   Tris over time. Dissolved oxygen concentration increases dramatically during the

223   cultivation time for with and without Tris. It is generally agreed that photosynthetic

224   oxygen is a product of photosynthesis. The daily DO peak increased gradually with the

225   increase of cell mass when the algae were in the exponential growth phase. With the

226   best N:P ratio cultured, the highest achievement of DO was 6.3 mg L^{-1} and 8.6 mg L^{-1}

227   with and without Tris, respectively. The concentration of dissolved oxygen can be used

228   to indicate good algal growth. However, residual dissolved oxygen may cause oxygen

229   accumulation which can damage and reduce cell growth. Dissolved oxygen

230   concentration should not reach a saturation level of 35 mg L^{-1} (Carvalho et al., 2006).

231   Therefore, it is important to have good mass transfer in the photobioreactor, which

232   highlights the importance of photobioreactor design. Bubble column photobioreactor in

233   this study is not concerned with the accumulation of oxygen inside it (Das et al., 2011).

234   DO peak will continue to increase from the start of the logarithmic phase until the

235   stationary phase begins. All cultures confirmed such behavior and the dissolved oxygen

236   concentration declines after reaching the stationary phase. This finding is similar to the

237   results obtained by Chai et al. (2012). Dissolved oxygen level drops in the stationary

238   phase when the amount of dying cells is equal to that for newly formed cells.

239

240   Fig. 4 DO curves of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P ratios

241

242   *3.3. Presence of protozoa (Paramecium) in during the cultivation of Chlorella sp.*

243   Without Tris, there were no organic nutrients evident during the period of and no

244   *Paramecium* was present. However, with Tris, the presence of *Paramecium* was evident

245   at the various ratios of N:P. More specifically, they were observed by 100X

246   magnification and defined after cultivation lasting 44 h. Results obtained in this study

247   indicated that Tris is one of the most important factors determining the presence of

248   *Paramecium* in the cultivation of *Chlorella* sp. This conclusion is similar to that

249   reported by Fabregas et al. (1993). Furthermore, *Paramecium* disrupts the growth of

250   freshwater algae, and typically *Chlorella* sp. is the main food source for them

251   (Tillimann, 2004).

252

253   **4. Conclusions**

254   Some concluding remarks can be made regarding the impact of Tris on microalgae

255   biomass growth in the photobioreactor as follows. Firstly, under operation the

256   conditions of N:P =15:1 ratio, *Chlorella* sp. performed the best either with or without

257   Tris, achieving a high biomass concentration after cultivation of 92 h and 96 h.

258   Secondly, the dry biomass without Tris is 3 times larger than that with Tris. Thirdly,

259   Tris is one of the factors that can determine the presence of *Paramecium* in the

260   cultivation of *Chlorella* sp.

261

262   **Acknowledgements**

266

267   **References**

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365   **Figure captions**

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367   **Fig.1.** Bubble column photobioreactor system diagram

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369   **Fig.2.** Dry biomass of *Chlorella* sp. with and without Tris at various N:P ratios

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371   **Fig.3.** pH curves of *Chlorella* sp. with and without Tris at various N:P ratios

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373   **Fig.4.** DO curves of *Chlorella* sp. with and without Tris at various N:P ratios

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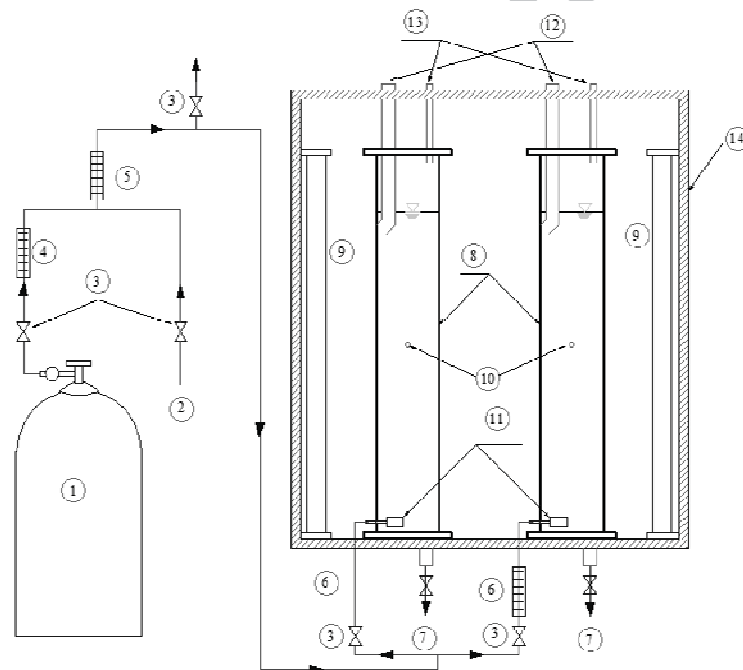
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Fig.1. Bubble column photobioreactor system diagram

1: CO_2 tank; 2: air pump; 3: air valves; 4,5,6: rotameter; 7,10: liquid valves; 9: cool white lamps; 11: gas diffuser; 12: p H, temperature and DO analyzer; 13: gas outlet; 14: opaque cover
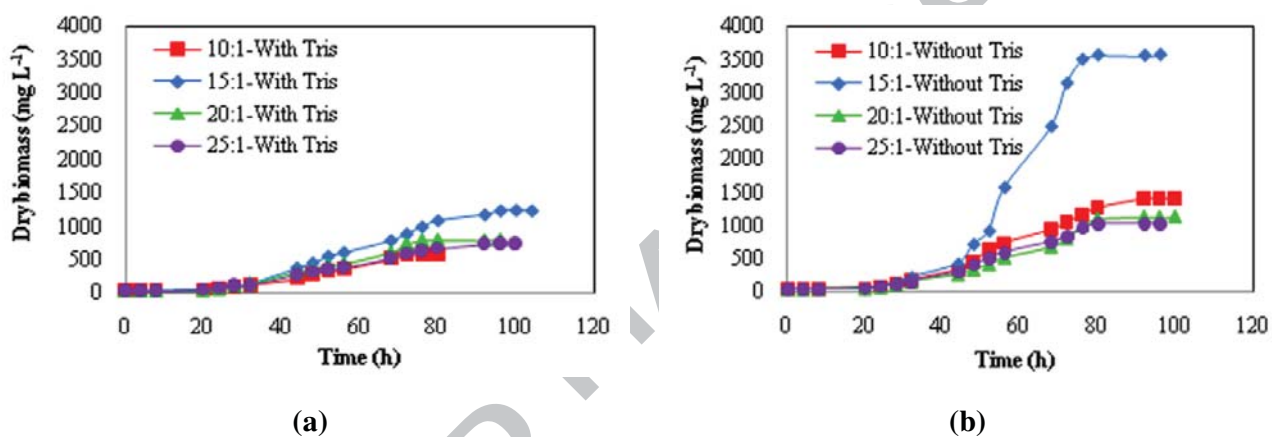
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(a) (b)

407 **Fig.2.** Dry biomass of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P
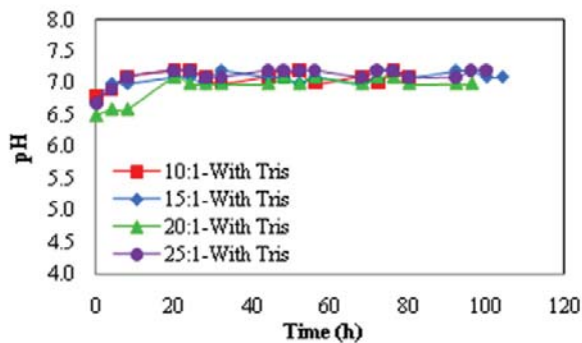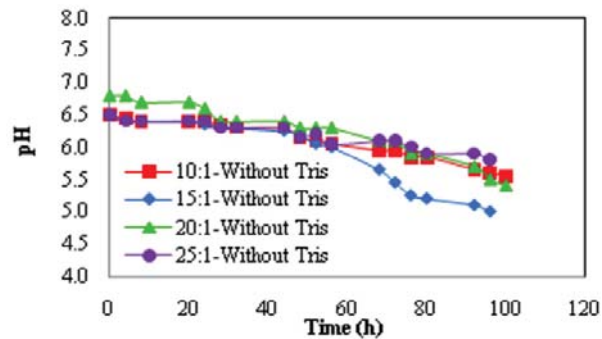408 ratios

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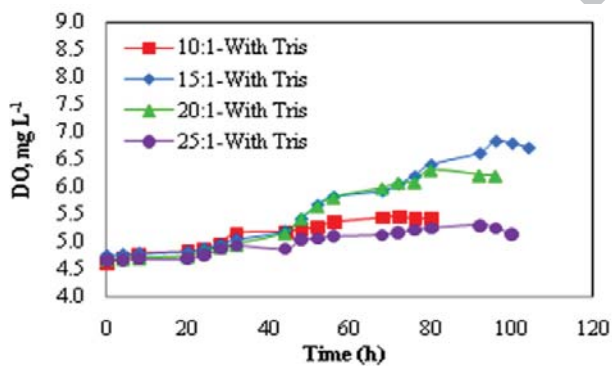
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(a)   **(b)**

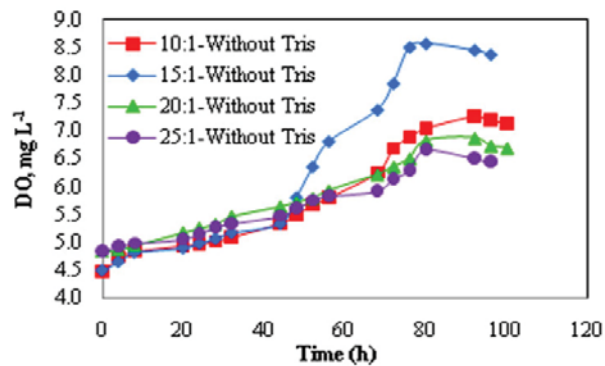420   **Fig.3.** pH curves of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P ratios

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(a)   **(b)**

423   **Fig.4.** DO curves of *Chlorella* sp. with Tris (a) and without Tris (b) at various N:P
424   ratios

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429   **Table 1.** Components of synthetic medium

Components	N:P ratio			
	10:1	15:1	20:1	25:1
	Without Tris			
NH_4Cl (mg L^{-1})	1339.9	2009.4	2679.3	3349.1
K_2HPO_4 (mg L^{-1})		120		
KH_2PO_4 (mg L^{-1})		60		
	With Tris			
Tris-$H_2NC(CH_2OH)_3$	1346.3	2019.4	2692.5	3365.6
NH_4Cl (mg L^{-1})	744.3	1116.5	1488.7	1860.9
K_2HPO_4 (mg L^{-1})		120		
KH_2PO_4 (mg L^{-1})		60		

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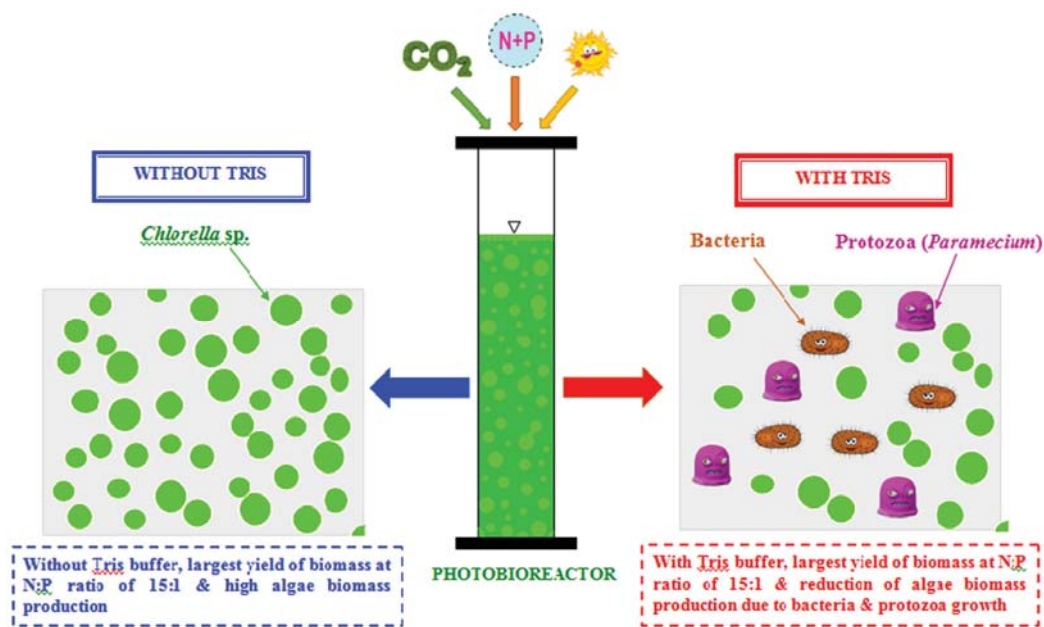
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443   Graphical abstract

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Highlights

448   • Effect of Tris on microalgae growth was investigated at different N:P ratios.
449   • *Chlorella* sp. performed well in both conditions with and without Tris at N/P of
450   15.
451   • Dry microalgae biomass without Tris was 3-fold higher than that with Tris.
452   • Tris can determine the presence of *Paramecium* in the cultivation of *Chlorella*
453   sp.

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