Effectiveness of various bioreactors for thraustochytrid culture and production (*Aurantiochytruim limacinum* BUCHAXM 122)

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This study aimed to develop bioreactors for cultivation of thraustochytrid, *Aurantiochytrium limacinum* BUCHAXM 122, that are low in cost and simple to operate. Obtaining maximum biomass and fatty acid production was a prerequisite. Three bioreactor designs were used: stirred tank bioreactor (STB), bubble bioreactor (BB) and internal loop airlift bioreactor (ILAB). The bioreactors were evaluated for their influence on oxygen mass transfer coefficient (k_La), using various spargers, mixing speed, and aeration rates. Biomass and DHA production from STB, BB, ILAB were then compared with an incubator shaker, using batch culture experiments. Results showed that a bundle of eight super-fine pore air stones was the best type of aeration sparger for all three bioreactors. Optimal culture conditions in STB were 600 rpm agitation speed and 2 vvm aeration rate, while 2 vvm and 1.5 vvm aeration provided highest biomass productivity in BB and ILAB, respectively. Antifoam agent was needed for all reactor types in order to reduce excessive foaming. Results indicated that with optimized conditions, these bioreactors are capable of thraustochytrid cultivation with a similar efficiency as cultivation using a rotary shaker. STB had the highest k_La and provided the highest biomass of 43.05 ± 0.35 g/L at 48 h. BB was simple in design, had low operating costs and was easy to build, but yielded the lowest biomass (27.50 ± 1.56 g/L). ILAB, on the other hand, had lower k_La than STB, but provided highest fatty acid productivity, of 35.36 ± 2.51% TFA.

1 **Effectiveness of various bioreactors for thraustochytrid culture and**

2 **production (*Aurantiochytruim limacinum* BUCHAXM 122)**

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20 ABSTRACT

21 This study aimed to develop bioreactors for cultivation of thraustochytrid, *Aurantiochytrium*

22 *limacinum* BUCHAXM 122, that are low in cost and simple to operate. Obtaining maximum

23 biomass and fatty acid production was a prerequisite. Three bioreactor designs were used: stirred

24 tank bioreactor (STB), bubble bioreactor (BB) and internal loop airlift bioreactor (ILAB). The

25 bioreactors were evaluated for their influence on oxygen mass transfer coefficient (k_La), using

26 various spargers, mixing speed, and aeration rates. Biomass and DHA production from STB, BB,

27 ILAB were then compared with an incubator shaker, using batch culture experiments. Results

28 showed that a bundle of eight super-fine pore air stones was the best type of aeration sparger for

29 all three bioreactors. Optimal culture conditions in STB were 600 rpm agitation speed and 2 vvm

30 aeration rate, while 2 vvm and 1.5 vvm aeration provided highest biomass productivity in BB

31 and ILAB, respectively. Antifoam agent was needed for all reactor types in order to reduce

32 excessive foaming. Results indicated that with optimized conditions, these bioreactors are

33 capable of thraustochytrid cultivation with a similar efficiency as cultivation using a rotary

34 shaker. STB had the highest k_La and provided the highest biomass of 43.05 ± 0.35 g/L at 48 h.

35 BB was simple in design, had low operating costs and was easy to build, but yielded the lowest

36   biomass (27.50 ± 1.56 g/L). ILAB, on the other hand, had lower $k_L a$ than STB, but provided

37   highest fatty acid productivity, of 35.36 ± 2.51% TFA.

39   **INTRODUCTION**

40   Thraustochytrids are unicellular, eukaryote, heterotrophic, and obligate marine microorganisms.

41   They are widely distributed and play an important role as ecological decomposers in marine

42   environments (*Marchan et al., 2018*). They have the ability to produce high amounts of long-

43   chain polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and

44   docosapentaenoic acid (DPA), essential fatty acids for marine animals (*Ryosuke & Takeshi,*

45   *2015*; *Unagul et al., 2017*; *Jaritkhuan & Suanjit, 2018*). These fatty acids are also crucial for

46   human health and well-being (*Horrocks & Yeo, 1999*; *Osendarp, 2011*; *Asztalos et al., 2016*).

47   Thraustochytrids can provide an alternative energy source, as through palmitic acid (PA), used in

48   biodiesel production (*Lee Chang et al., 2013*; *Kim et al., 2013*). Production of PUFAs using

49   thraustochytrids has been developed; thus, these organisms represent a promising and significant

50   source of PUFAs for biotechnological applications.

51   For mass culturing, thraustochytrids require a high oxygen mass transfer (*Michelin et al.,*

52   *2013*) to produce primary metabolites during the growth phase (*Qu et al., 2010*). Oxygen is a key

53   substrate for aerobic bioprocesses and has low solubility in aqueous solutions. Therefore, a

54   continuous oxygen supply is needed and the oxygen transfer rate must be known to achieve an

55   optimum design operation in batch bioreactors (*Garcia-Ochoa & Gomez, 2009*; *Chang et al.,*

56   *2013*). Bioreactors have already been developed for culturing microbial cells. Therefore, aerobic

57   cell cultivation using bioreactors should be an effective approach to enhance mass production of

58   thraustochytrids. However, optimizing an appropriate bioreactor will be key in achieving

59   successful cultivation of this microorganism.

60   Currently, many different types of bioreactors are in use for cell culture, including stirred

61   tank bioreactor (STB), bubble bioreactor (BB) and internal loop airlift bioreactor (ILAB). STB,

62   the most widely-used bioreactor, is equipped with an impeller for homogenizing culture media

63   and a sparger for delivering oxygen to the cells. STBs are primarily used to scale-up the culture

64   process from a research and development scale to a manufacturing scale. BBs are basically

65   cylindrical bioreactors, with a gas distributor at the bottom; whereby the gas is sparged, in the

66   form of bubbles into either a liquid phase or a liquid-solid suspension. The BB method is

67   normally used for cell growth and oxygen supply; because it doesn't use a complex mixing

68   system, these reactors can be compact and have low operating and maintenance costs (*Kantarci,*

69   *Borak & Ulgen, 2005*). ILAB is one type of bubble reactor, having an internal draft-tube that

70   promotes gas-liquid mass transfer and mixing (*Saravanan, Pakshirajan & Saha, 2008*). Today's

71   bioreactors for mass culture of microorganisms are still expensive, even without automated

72   production control systems. In addition, knowledge is limited on thraustochytrid culture in these

73   bioreactor types.

74   Growth of *A. limacinum* is limited mostly by oxygen availability in the reactor.

75   Increasing oxygen mass transfer in bioreactors can enhance growth and fatty acids production of

76   *A. limacinum* to levels equivalent to a shaking flask (commonly used a reference). This research

77   was conducted in order to develop and modify a simple, low-cost bioreactor that is reliable and

78   easy to operate for mass production of the thraustochytrid *A. limacinum* BUCHAXM 122. Three

79   kinds of bioreactors, stirred tank bioreactor (STB), bubble bioreactor (BB) and internal loop

80   airlift bioreactor (ILAB) were modified for mass production of *A. limacinum* BUCHAXM 122,

81   and maximizing biomass and DHA production. The objectives of this research were to evaluate

82   the influence of various spargers, mixing rates, and aeration rates on oxygen mass transfer

83   coefficient (k_La), and then to compare biomass and DHA production in STB, BB, ILAB and

84   incubator shaker in batch culture experiments. It should be of great interest for the

85   biopharmaceutical industry to further continue and develop more efficient cell cultivation

86   processes with higher product quality at lower manufacturing costs and simple operating

87   conditions.

88

MATERIALS AND METHODS

Experimental design

91   Three bioreactor types, stirred tank bioreactor (STB), bubble bioreactor (BB) and internal loop

92   airlift bioreactor (ILAB) were built and modified with the concept of low-cost and simple

93   operation. These improved bioreactors were then evaluated for their influence on oxygen mass

94   transfer coefficient (k_La), using various spargers, mixing rates and aeration rates, then compared

95   with an incubator shaker (section 1, evaluated using distilled water without microorganisms).

96   Best conditions for cultivation of *A. limacinum* BUCHAXM 122 found in section 1 (maximum

97   k_La) for each developed bioreactor were determined, and compared with standard incubator

98   shaker (section 2). A schematic overview of this study is summarized as Fig. 1.

99

100   **Bioreactor configuration and operating conditions**

101   In this study, three types of bioreactors were designed and operated using low cost materials:

102   stirred tank, bubble and internal loop airlift (Fig. 2). The stirred tank bioreactor (STB) was

103   modified from *Wegrich & Shurter (1953)*, *Scragg (1991)*, and *Stanbury, Whitaker & Hall*

104   *(1995)*. Our STB consisted of a culture vessel made from a 2-L laboratory bottle (working

105   volume of 1 L), four stainless steel baffles (each baffle 1.25 cm wide x 12.5 cm high, located 0.5

106   cm away from the tank wall) and one set of disk turbines (5 cm in diameter, with eight 0.9 x 1

107   cm blades). The impeller shaft was made of a 30-cm carbon rod. Impeller rotation was controlled

108   by a microcontroller system (Arduino). Motor speed was regulated through a Proportional-

109   Integral-Derivative Controller (PID Controller). The aeration system consisted of a sparger

110   installed under the impeller. Air inlet-outlet and sampling tubes were installed at the top of the

111   bioreactor (Fig. 2A).

112   The bubble bioreactor (BB) was constructed using a 2-L laboratory bottle with a working

113   volume of 1 L. The culture vessel was 13 cm in diameter and 21 cm in height. Its aeration system

114   consisted of a sparger bundle located at the bottom of the reactor. The sparger provided uniform

115   aeration throughout the vessel. Air inlet, outlet and sampling tubes were installed, similar to the

116   STB (Fig. 2B).

117   The internal loop airlift bioreactor (ILAB) was redesigned with modification from *Cerri*

118   *& Badino (2010)*. The culture vessel was 9 cm in diameter and 45 cm in height, with a working

119   volume of 2 L. The internal draft tube was made of borosilicate glass with 26 cm height and 5

120   cm diameter. Aeration was provided through a sparger bundle at the bottom. Air was bubbled

121   from the bottom through the draft tube (riser), creating water flow downward through the

122   downcomer channel, between the draft tube and outer wall (Fig. 2C).

123   Air purification was performed using a series of two filters (0.3 μm diameter polyethylene

124   filter and a 0.2 μm diameter filter) and 12-watt UV lamp. The filters and UV lamp were

125   connected by a silicone tube. Prior to operation, the tube outlet end from each bioreactor was

126   immersed in 1,000 ppm copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) to prevent outside

127   contamination (*Reyes-Jara et al., 2016*) (Fig. 3). Aeration rate was controlled by Rotameter

128   (Dwyer, Michigan City, Indiana, USA)**.**

129

130   **Investigation of oxygen mass transfer**

131   ***Effects of sparger configuration on oxygen mass transfer efficiency***

132   Six sparger configurations for aeration were investigated for oxygen mass transfer capability in

133   the bioreactors: 1) silicone tube with 4 mm internal diameter, without sparger (control), 2)

134   spherical coarse-bubble air stone, 3) spherical fine-bubble air stone, 4) four spherical fine-bubble

135   air stones, 5) four cylindrical super-fine bubble air stones and 6) eight cylindrical super-fine

136   bubble air stones (Fig. 4). The coarse and fine air stones were 25 mm in diameter with pore size

137   of 200-500 µm and 150-300 µm, respectively, while the cylindrical air stones were 10 mm in

138   diameter and 20 mm in length, with a pore size of 10-20 µm. These air stones are widely used in

139   aquariums. Oxygen mass transfer capability by spargers was indicated by volumetric mass

140   transfer coefficient (k_La). k_La value was measured in all bioreactors using distilled water, and

141   then compared to the value obtained for an incubator shaker (GALLENKAMP, UK), using 250-

142   ml Erlenmeyer flasks agitated at 200 rpm. Testing was performed using five trials. k_La was

143   measured using a DO meter (YSI 550A, steady-state Clark type polarographic, USA) at room

144   temperature, with zero oxygen water generated by the sulfite method (modified from *Puskeiler &*

145   *Weuster-Botz, 2005*; *Bouaifi, et al., 2001*). Atmospheric air containing approximately 21 percent

146   oxygen was then introduced into all bioreactors and shakers. All dissolved oxygen measurements

147   were continuously measured from zero oxygen concentration until they reached saturation

148   (average 7.04± 0.16 ppm). Data collection was performed by video recording of the DO meter

149   display. During measurement of dissolved oxygen in the shaker, the flask was locked to the

150   shaking platform and the oxygen probe was securely fixed with cotton wrapped in foil and

151   masking tape at the mouth of the flask, with the tip not touching the flask bottom. In STB and

152   BB reactors, the oxygen probe was located at the bottom of the tank, with the tip not touching the

153   bottom. For the ILAB, the oxygen probe was affixed to the middle of the riser.

154

155

156   The k_La value was measured by employing the dynamic method (non-fermentative

157   method) (*Garcia-Ochoa & Gomez, 2009*). The relationships of k_La to the oxygen transfer rate

158 (OTR) and the oxygen uptake rate (OUR) of a growing cell were described by the following

159 equations, (Eq. 1-3). These relationships represent one of the foundations of bioreactor design.

160

161
$$\frac{dC}{dt} = \text{OTR - OUR} \qquad (1)$$

162
$$\text{OTR} = k_L a(C_s - C) \qquad (2)$$

163 Substituting Eq. (2) into Eq. (1) results in

164
$$\frac{dC}{dt} = k_L a(C_s - C) - OUR \qquad (3)$$

165 where C_s is the saturated oxygen concentration in the liquid phase, and C is the DO

166 concentration, OUR=0. Integration of Eq. (3) from C_0 to C gives:

167
$$\ln (Cs - C)/(Cs - C0) = - kLa \cdot t \qquad (4)$$

168 where C_0 is the initial DO concentration of dissolved oxygen at t=0,

169

170   The $k_L a$ value was calculated from slope by plotting $\ln (C_s-C)/(C_s-C_0)$ against time

171 according to the equation. The $k_L a$ obtained was calculated from the linear slope of oxygen

172 increase during aeration. These measurements were performed for around 30-90 seconds for all

173 treatments. Calculation of $k_L a$ was based on the principle that R-squared is closest to 1.

174

175   In STB, all sparger types were tested, using an agitation speed of 450 rpm and aeration

176 rate of 3 vvm (*Chang et al., 2014*). In BB and ILAB, all sparger configurations were tested with

177 3 vvm aeration (*Özcan, Sargın & Göksungur 2014*). The bundle of four fine-bubble air stones

178 was not investigated in ILAB, because the space under the draft tube was too small.

179

180 ***Effects of agitation speed and aeration rate on oxygen mass transfer efficiency***

181 The experiment was performed using sparger types that provided highest $k_L a$ from the previous

182 experiment. Bioreactor aeration was further evaluated with different agitation speeds and

183 aeration rates. Agitation speeds tested in STB were 450 and 600 rpm (*Chang et al., 2013*; *Chang*

184 *et al., 2014*), in combination with aeration rates of 0.5, 1, 2 and 3 vvm, BB and ILAB were

185 operated at identical aeration rates.

186

187 **Cultivation of *A. limacinum* BUCHAXM 122 in bioreactors**

188 ***Stock culture and inoculation of A. limacinum BUCHAXM 122***

189 *A. limacinum* BUCHAXM 122 was isolated from mangrove forests located in the Mangrove

190 Resources Development Station 2, Tha Son, Chanthaburi Province, Thailand. *A. limacinum*

191 BUCHAXM 122 inoculum was prepared in 250 mL Erlenmeyer flasks containing 50 mL of

192 GYP liquid medium, composed of 60 g/L glucose, 10 g/L yeast extract and 10 g/L peptone in 15

193 PSU seawater. Cultures were incubated at 25 °C with shaking (200 rpm) for 48 h, before being

194 used as the inoculum.

195

196 ***Comparison of bioreactor types for A. limacinum BUCHAXM 122 cultivation***

197 Five percent (5%, v/v) *A. limacinum* BUCHAXM 122 inoculum was transferred and cultured in

198 each bioreactor, using GYP medium with the optimum sparger type, aeration rate and agitation

199 speed obtained from previously described experiments. Shaking flask cultures were carried out

200 in 250 mL Erlenmeyer flasks containing 50 mL of GYP medium with 200 rpm agitation, then

201 assigned as the control. All experiments were performed in duplicate. Cell samples were

202 collected every 24 h throughout the 168-h cultivation period, to determine biomass and DHA

203 production. Dissolved oxygen was measured by DO meter (YSI 550A). Reduced sugar was

204 determined by the dinitrosalicylic acid (DNS) method (*Miller, 1959*).

205 Dry biomass was determined by sampling 2 mL culture media from each bioreactor or

206 culture flask, using sterile procedures. Samples were filtered through Whatman GF/C (1.2 µm)

207 and rinsed with PBS solution. Filtered cells were then freeze dried at -80 °C (using freeze-dryer

208 Heto LyoLab 3000, Heto-Holten A/S, Allerød, Denmark), and weighed. Average dry cell weight

209 was plotted on a growth curve. Direct transmethylation (modified from *Shimizu et al., 1988*) was

210 performed in order to analyze fatty acid composition of *A. limacinum* BUCHAXM 122.

211 Extracted fatty acid methyl esters (FAME) were analyzed using gas chromatograph (HP 6890

212 Series GC System, Wilmington, DE, USA) equipped with a flame ionization detector (FID) and

213 fitted with a column using capillary column HP-INNO Wax polyethylene glycol (60 m x 250 µm

214 x 0.25 µm). Initial temperature was 50 °C for 1 minute, increasing at a rate of 50 °C/minute to

215 200 °C, holding for 1 minute, then increasing by 2 °C/minute to 210 °C, and holding for 14

216 minutes. Helium was used as the carrier gas. Both detector and injector temperatures were set at

217 250 °C. Fatty acid content was calculated according to peak area of the chromatogram, in

218 accordance with the internal standard (nonadecanoic acid, 19:0, Fluka, Buchs, Switzerland).

219

220 **Statistical analysis**

221 Data were evaluated using one-way analysis of variance (ANOVA) and LSD for post hoc

222 comparisons, at a confidence level of 5% ($p < 0.05$). Multiple linear regression (MLR) was

223 performed with the stepwise method in order to show relationships between $k_L a$ and specific

224 growth rate, productivity, and sugar consumption.

225

226 **RESULTS**

227 **Effect of sparger configuration on oxygen mass transfer capability**

228 As shown in Fig. 5, aeration through a bundle of eight super-fine air stones resulted in the

229 highest $k_L a$ value for STB (123.66 ± 15.47 h^{-1}), BB (122.99 ± 21.04 h^{-1}) and ILAB ($123.53 \pm$

230 4.57 h^{-1}). $k_L a$ values for eight super-fine air stones in STB and BB were significantly higher than

231 for other spargers. $k_L a$ values were similar for all air stone configurations in ILAB. Using

232 optimized conditions, the $k_L a$ values obtained from eight super-fine air stones were not

233 significantly different ($p > 0.05$) from Erlenmeyer flasks in the rotary shaker at 200 rpm (132.98

234 ± 18.75 h^{-1}) (Table S1).

235

236 **Effects of agitation speed and aeration rate on oxygen mass transfer**

237 $k_L a$ values in all bioreactors increased with increasing aeration rate (Table 1). An aeration rate of

238 3 vvm in BB and ILAB resulted in significantly higher $k_L a$ values than with other rates ($p <$

239 0.05). In STB, the highest $k_L a$ value of 155.92 ± 18.52 h^{-1} ($p < 0.05$) was found at 600 rpm with

240 3 vvm; however, there were no significant differences in $k_L a$ values using aeration rates of 2 and

241 3 vvm or agitation speeds of 450 and 600 rpm ($p > 0.05$).

242

243 **Growth and biomass production of *A. limacinum* BUCHAXM 122 in bioreactors**

244 When all bioreactors culturing *A. limacinum* BUCHAXM 122 were aerated at a high rate, i.e., 3

245 vvm, there was a problem with foam formation on top of the culture medium. Therefore, the

246 experiment was repeated with reduced aeration rates of 2 vvm for STB and BB and 1.5 vvm for

247 ILAB in combination with 0.49% polypropylene glycol (SIGMA-ALDRICH) antifoaming agent.

248   Biomass in STB peaked within 48 h (43.05 ± 0.35 g/L); thereafter, the biomass decreased and

249   remained stable from 96-120 h (30.70 ± 1.84 - 30.65 ± 1.20 g/L), with 2.07 ± 0.12 g/L reducing

250   sugar residue. For ILAB, biomass rapidly increased within 48 h (35.50 ± 1.84 g/L), after which it

251   slowly and continuously increased until 120 h (37.60 ± 3.82 g/L), with 3.61 ± 2.67 g/L reducing

252   sugar residue. For the shaker, biomass rapidly increased within 48 h (29.55 ± 1.77 g/L); after that

253   the biomass slowly increased between 72 h-120 h (31.30 ± 0.28 - 32.90 ± 0.28 g/L), with 2.04 ±

254   0.46 g/L sugar residue. BB yielded the lowest biomass, rapidly increasing at 24-48 h, then slowly

255   and continuously increasing until 120 h (38.55 ± 4.60 g/L), with 41.87 ± 0.10 g/L reducing sugar

256   residue. Highest biomass and residual glucose for each bioreactor are shown in Fig. 6.

257   Comparing all bioreactors at 48 h, the highest biomass was for STB, which had significantly

258   higher yields ($p < 0.05$) than ILAB, shaker, and BB. Sugar was nearly depleted at the end of the

259   experiment in STB, ILAB and the shaker, but not for BB (Table S2).

260   Highest DHA production was obtained from ILAB at 120 h (199.02 ± 0.41 mg/g DW,

261   35.36 ± 2.51% TFA). However, there was no significant difference in DHA production when

262   comparing this treatment to ILAB at 72 h (189.95 ± 9.98 mg/g DW, 36.93 ± 0.64% TFA), ILAB

263   at 96 h (189.77 ± 7.65 mg/g DW, 38.15 ± 0.70% TFA), and the shaker at 96 h (190.50 ± 10.64

264   mg/g DW, 35.15 ± 2.31% TFA) ($p > 0.05$) (Fig. 7, Table S3). Initial DO in the culture medium

265   was 0.03 ± 0.01 mg/L. DO values at the end of fermentation in STB (8.20 ± 0.09 mg/L) and

266   ILAB (8.23 ± 0.36 mg/L) were significantly higher than in the shaker (6.59 ± 0.08 mg/L) and

267   BCB (6.95 ± 0.16 mg/L).

268   A further experiment was conducted, reducing aeration rates to 0.1 vvm for all

269   bioreactors and the shaker, and without addition of anti-foaming agent. Highest biomass was

270   observed in STB (25.00 ± 0.99 g/L), showing no significant difference from the shaker (24.40 ±

271   0.85 g/L) or ILAB (23.41 ± 0.29 g/L) ($p > 0.05$). Biomass in STB was significantly higher than

272   BB (21.15 ± 1.48 g/L) ($p < 0.05$). Moreover, there were no significant differences in DHA

273   production among the shaker, STB and ILAB ($p > 0.05$) (Table 2). STB, shaker and ILAB

274   consumed more glucose than BB. However, at the end of the experiment, glucose was not

275   completely consumed in any of the treatments (Fig. 6, Table S4).

276   DO values before and at the end of fermentation in the shaker (6.55 ± 0.03 mg/L, 6.54 ±

277   0.01 mg/L), STB (6.48 ± 0.02 mg/L, 6.45 ± 0.06 mg/L) and ILAB (6.45 ± 0.05 mg/L, 5.95 ±

278 0.21 mg/L) were quite similar, while DO for BB (4.99 ± 0.05 mg/L, 1.03 ± 0.11 mg/L), was

279 significantly lower.

280   Comparison between k_La and aeration rate revealed that aeration rates in STB and BB at

281 2 vvm and in ILAB at 3 vvm resulted in k_La values equivalent to that in the shaker (Fig. 8).

282 Multiple regression analysis results showed that specific growth rates of *A. limacinum*

283 BUCHAXM 122 was positively correlated with the k_La value.

284

DISCUSSION

285

Bioreactor configuration and operating conditions

286

287 Advantages of using STB, BB and ILAB bioreactors for microbial production have been

288 reported in previous studies (*Stanbury, Whitaker & Hall 1995*; *Cerri & Badino, 2010*). The

289 noteworthy feature of STB is that the sparger is placed under the impeller, producing the highest

290 k_La value and resulting in a high biomass. The STB, although widely used in biological

291 production, is well known to have shearing problems, which can cause cell damage (*Kim, Kang*

292 *& Lee, 1997*). In this study, the BB design was modified by using 2-L laboratory glass bottles. It

293 has low maintenance costs and reduces overall cost of oxygen delivery by 10-20 percent, relative

294 to the STB (*Humbrid, Davis & McMillan, 2017*). However, in this study, its simplified design

295 caused sedimentation in the culture vessel, resulting in the lowest biomass production. This was

296 due to the combined effects of the culture media flow properties and the gas content, which

297 relates back to the diameter of the reactor (*Kantarci, Borak & Ulgen, 2005*).

298   Results of this study showed that ILAB had a lower k_La value than STB; on the other

299 hand, liquid circulation and mixing was better than in BB. ILAB is generally used in applications

300 involving low viscosity fluids, not requiring mechanical agitation, and providing low shear

301 forces (*Fontana, Polidoro & da Silveira, 2009*). This study suggests that ILAB might suitably

302 support high fat microbial culture. For instance, the ILAB bioreactor has been used to cultivate

303 oleaginous yeast *Rhodotorula glutinis* (*Yen & Liu, 2014*). However, ILAB has limited capability

304 when used in high aeration applications (*Qu et al., 2010*) due to foam formation. Reduced

305 aeration rates and lower oxygen mass transfer might affect the oxygen availability for cell

306 growth.

307

Effect of sparger configuration on capability of oxygen mass transfer

308

309   This study shows that the bundle of eight super-fine air stones was the best sparger type for all

310   three bioreactors, since it produced a k_La value equivalent to the standard 200 rpm rotary shaker

311   (132.98 ± 18.75 h^{-1}). The k_La value measured from rotary shaker was similarly to the k_La value

312   (108-180 h^{-1}) obtained by shaking in a 50-ml sodium sulfite system at 120-200 rpm (*Kato et al.,*

313   *2005*). The super-fine pores provide numerous small air bubbles, with uniform distribution by

314   using eight stones, leading to a homogeneous flow. This homogeneous flow facilitates the

315   biochemical reactions involved in oxygen utilization and mass transfer of oxygen to cells

316   (*Garcia-Ochoa et al., 2010*), while larger bubbles lead to coalescing and unsteady heterogeneous

317   flow creation (*Hoseinkhani et al., 2019*). Results of this study are consistent with a previous

318   report, which found that increasing surface area of the sparger and number of pores resulted in

319   higher k_La (*De Wilde et al., 2014*) by improving bubble dispersion, preventing bubble overflow

320   and reducing gas flow rate. Similarly, cultivation of *Schizochytrium* sp. using a microbubble-type

321   sparger was found to have 8.6-fold higher k_La values than with a general type sparger (*Ju et al.,*

322   *2019*). However, it was also based on the pore size of sparger; if the pore size is too large, the

323   k_La difference may not be seen. For instance, the k_La value was not significantly different when

324   using spargers with different numbers of holes (1, 4, and 9) and hole diameters (6, 3 and 2 mm)

325   (*Karimi et al., 2013*). Conversely, the sparger affects bubble size, and bubble size is specific to

326   certain oleaginous microalgae culturing. For instance, culturing *Crypthecodinium cohnii* in BB

327   using bubbles of 0.18 cm diameter resulted in higher biomass than bubbles of 0.09 and 0.36 cm

328   diameter. However, the 0.36 cm bubbles gave highest DHA production, followed by 0.18 and

329   0.09 cm, respectively (*Hoseinkhani et al., 2019*). This indicates that there is an optimal bubble

330   size leading to growth and production of healthy fatty acids. For this reason, further studies to

331   find the appropriate sizing of air bubbles for *A. limacinum* BUCHAXM 122 production should

332   be initiated.

333

334   **Effect of agitation speed and aeration rate on oxygen mass transfer**

335   This study shows that increasing agitation speed and aeration rate resulted in higher k_La values.

336   Similar results have been reported for a 30-L STB system; when agitation speed was increased

337   from 300 rpm and 0.6 m^3/h to 600 rpm and 2 m^3/h, k_La value increased from 143 ± 19 h^{-1} to

338    $1,802 \pm 105$ h^{-1} (*Chang et al., 2013*). In addition, higher agitation speeds (400-800 rpm) with 1-3

339   L/m^3 have been found to be the most efficient for oxygen mass transfer in STB. Similar results

340 have been reported for agitation speeds of 450 and 600 rpm, with aeration rate of 1-3 vvm

341 (*Karimi et al., 2013*). Agitation rates below 300 rpm resulted in insufficient agitation to capture

342 air bubbles in the bioreactor, and consequently may limit mass transfer of oxygen. Our data show

343 that aeration rate has an effect on the k_La value in ILAB and BB, as well. Likewise, in the

344 cultivation of *Rhodotorula glutinis* in ILAB, it was found that k_La value increased with aeration

345 rate (1.0, 1.5, 2.0 and 2.5 vvm), and the aeration rate at 1.5-2.5 vvm was able to provide high

346 biomass and fatty acid yields (*Yen & Liu., 2014*). Higher k_La values have also been obtained in

347 bubble column bioreactor (BCB) by increasing the aeration rate (*Kumar & Vinod, 2014*).

348   Effects of agitation speed and aeration rate on k_La can be explained by the relation of

349 stirrer speed and specific interfacial area values in STB, and by bubble size in all bioreactor

350 types. Therefore, speed of the stirrer and mixing play an important role in breaking up the air

351 bubbles. Also, turbulence and shear are caused by baffles (*Kawase & Moo-Young, 1990; Garcia-*

352 *Ochoa & Gomez, 2009*). Air bubble dispersion is also related to gas dispersion, gas hold-up and

353 residence time (*Joshi, Pandit & Sharma, 1982; Barigou & Greaves, 1996; Garcia-Ochoa &*

354 *Gomez, 2009*). Dispersion is also related to other factors, such as liquid properties, gas velocity,

355 gas injection rate, operating pressure, injection tube diameter and column diameter (*Kawase,*

356 *Halard & Moo-Young, 1992; Garcia-Ochoa & Gomez, 2009; Garcia-Ochoa et al., 2010;*

357 *Mohagheghian & Elbing, 2018*).

358

359 **Biomass and DHA production of *A. limacinum* BUCHAXM 122 in bioreactors**

360 Production of *A. limacinum* BUCHAXM 122 in STB showed faster growth and higher biomass

361 yield than in the rotary shaker, ILAB and BB. These findings are consistent with batch

362 fermentation of *Aspergillus oryzae* in STB, with agitation rates of 200-700 rpm and aeration

363 rates of 0.5 -2.5 vvm (14.1 ± 0.13 g/L) which is higher than ILAB at 0.5-2.5 vvm (10.1 ± 0.03

364 g/L) (*Fontana, Polidoro & da Silveira, 2009*). In general, increases in k_La lead to more biomass

365 production (*Hoseinkhani et al., 2019*). This is due to oxygen demand, particularly in the ATP

366 production process. Cell density increases rapidly during the exponential phase, producing a

367 large number of primary substances (primary metabolites), such as enzymes, nucleic acids and

368 other proteins from ATP by aerobic respiration (*Qu et al., 2010*). Conversely, when DO

369 concentrations decrease, metabolic rate of microorganisms displays a significant decrease (*Sinha,*

370 *Dautz & Hader, 2001*). From experiments with an STB system, fed-batch cultures of

371   *Schizochytrium* sp. S31 with agitation rates of 300, 450 and 600 rpm revealed that 600 rpm

372   provided highest oxygen uptake rates and yielded the highest biomass (*Chang et al., 2014*).

373   These findings are consistent with our study. Comparing two other bioreactors, *Özcan, Sargin &*

374   *Göksungur (2014)* demonstrated that *Aureobasidium pullulans* produced higher biomass from

375   ILAB, with aeration rates at 1 vvm than from BCB at 0.2, 1.1 and 2 vvm.

376   According to a previous study, cell growth (μ; 0.090/h) and glucose consumption rate (qs;

377   0.115/h) in airlift-type bioreactors were higher than from fermentation in stirrer-type vessels

378   (0.057/h and 0.064/h, respectively) (*Hong et al., 2013*). Our results clearly indicate that biomass

379   production from bioreactors is related to glucose consumption. The highest biomass was in STB,

380   which also had the highest glucose consumption. BB, on the other hand, yielded the lowest

381   biomass, with its glucose consumption being lower than the other bioreactors and the rotary

382   shaker. This shows that efficiency of the bioreactor affects the remaining glucose in the reactor;

383   with glucose remaining in a reactor tank being the starting point for further development of

384   suitable culture media recipes. In addition, cell culture techniques in the log phase and reduction

385   of oxygen during the stationary phase of fatty acid production should be further developed (*Qu et*

386   *al., 2010; Rosa et al., 2010*).

387   One of the limitations of using a high aeration rate is that it produces large amounts of

388   foam. In our cultures of *A. limacinum* BUCHAXM 122, higher biomass was obtained at 2 vvm

389   in STB and BB and at 1.5 vvm in ILAB. However, if an antifoaming agent is required to avoid

390   overflowing, it can cause a decrease in $k_L a$ values (*Sundaramurthy, Srivastava & Mishra, 2011*).

391   Consequently, cell cultivation in STB could be carried out at aeration rates of 0.1 vvm, since

392   biomass concentrations for 72 h (25.00 ± 0.99 g/L) were similar to the shaker (22.35 ± 3.89 g/L),

393   and were higher than the range of values reported for *A. limacinum* (range of 9.39-20.71 g/L)

394   (*Jaritkhuan & Suanjit, 2018*).

395   DHA production obtained from ILAB and the rotary shaker were relatively similar in

396   yield. Highest DHA content in ILAB with 1.5 vvm aeration rate was significantly higher than

397   both STB and BB. It was found that high agitation speeds negatively impacted DHA production

398   in STB. As reported by Chang *et al.* (2014) for *Schizochytrium* sp. S31, an agitation speed of 600

399   rpm produced lower DHA (29.37 ± 0.89% TFA) than 300 rpm (36.04 ± 1.78% TFA). Our

400   experiments also showed that using an agitation speed of 600 rpm, STB produced lower DHA

401   yield than other bioreactor types.

402   It is known that microbial cells are sensitive to mechanical shear stress, particularly from
403   high agitation intensity, as it causes cell damage (*Wase et al., 1985*; *Hong et al., 2013*). The cell
404   wall of thraustochytrid organisms is non-cellulosic and thin, ranging from 2 to 3 nm thick
405   (*Marchan et al., 2018*), and can be affected by relatively high shear stress, consistent with the
406   report by *Wang & Lan (2018)*. In addition, cells with flagella are more shear sensitive than
407   unicellular algae cells (*Wang & Lan, 2018*). Thraustochytrids can move during the zoospore
408   stage using two flagella. They may be affected by shear, resulting in less fatty acid production,
409   since zoospores also store fatty acids (*Morita et al., 2006*).

410   A low aeration rate is more favorable for efficient DHA synthesis (*Qu et al., 2010*;
411   *Hoseinkhani et al., 2019*). This study demonstrates that low aeration rates, i.e., 0.1 vvm, provide
412   highest DHA yield during late stationary phase (168 h) in all bioreactors. ILAB, operated under
413   low aeration rate (with less shear stress), may be an appropriate choice for DHA production.
414   High oxygen content during the cell stage accumulates fatty acids, allowing cells to use carbon
415   sources (glucose) in the breathing process and metabolism, rather than being used in lipid
416   accumulation processes. The appropriate amount of primary metabolite production by oxygen
417   limitation produces a sufficient quantity of Acetyl-CoA (a precursor to the synthesis of fatty
418   acids). This also results in higher cell accumulation of fatty acids. Therefore, the DHA content in
419   thraustochytrids depends on both amount of biomass (log and early stationary phase) and
420   production of fatty acids (stationary phase). According to this study, both ILAB and shaker have
421   lower shear forces than STB, which has a positive effect on maintaining cellular integrity. The
422   cells are not damaged, resulting in a high DHA content. Moreover, the results show that k_La
423   value in ILAB was relatively lower than in the shaker. It is also associated with synthesis of fatty
424   acids for each strain of thraustochytrid, as a pathway of with or without oxygen, to comply with
425   the in-cell enzyme system (*Jakobsen et al., 2008*).

426   Higher DHA production may be related to the activity of anaerobic fatty acid pathways
427   (PUFA synthase or PKS), highly active under low dissolved oxygen (*Matsuda et al., 2012*; *Metz*
428   *et al., 2001*). Studies on transcriptome and gene expression analysis of *Schizochytrium* sp. under
429   different oxygen supply conditions have shown that high oxygen supply is used in NADPH and
430   acetyl-CoA production for cell growth, rather than for DHA production (*Bi et al., 2018*).
431   Consequently, high k_La is required for growth, while DHA production does not require a very
432   high oxygen supply (*Chang et al., 2014*).

433   Therefore, ILAB can be used as an alternative to the STB bioreactor, with production

434   performance similar to the standard rotary shaker. However, STB is still the most suitable

435   bioreactor for thraustochytrids if biomass yield is the major priority, followed by production of

436   intracellular products (*Hoseinkhani et al., 2019*). The simplified and low-cost ILAB design used

437   in this study shows favorable production from *Aurantiochytrium* with 23.41-36.05 g/L biomass

438   and 36.93-40.29% DHA in total fatty acids. This production yield is comparable to previous

439   studies, which reported ranges of 13-59.2 g/L biomass (72 and 125 h) and 31.09-52.3% TFA (72

440   and 36 h) (Table 3) (*Yaguchi et al., 1997*; *Rosa et al., 2010; Ethier et al., 2011*; *Hong et al.,*

441   *2013*; *Lee Chang et al., 2013*).

442

CONCLUSIONS

444   Three designs of low-cost bioreactors, stirred tank (STB), bubble (BB) and air-lift (ILAB) were

445   evaluated for cultivation of *Aurantiochytrium limacinum* and DHA production, in combination

446   with optimized sparger (eight super-fine bubble air stones) and aeration rate (between 1.5-2

447   vvm). All reactors are capable of thraustochytrid cultivation, with growth and yield comparable

448   to a 200-rpm rotary shaking flask. Each bioreactor type has unique advantages. STB had the

449   highest k_La values (155.92 ± 18.52 h^{-1}), producing the highest biomass (43.05 ± 0.35 g/L).

450   However, agitation by the impeller causes shear stress damage to the cells, and may affect fatty

451   acid production. BB is simple in design, easiest to build, and lowest cost. However,

452   thraustochytrid cultivation in BB requires a high airflow rate in order to avoid cell sedimentation

453   during cultivation. ILAB provided moderate cell density (37.60 ± 3.82 g/L) but the highest

454   amount of fatty acids (199.02 ± 0.41 mg/g DW, 35.36 ± 2.51% TFA). With ILAB, the uniform

455   flow pattern through the riser and downcomer in the bioreactor allows homogeneous mixing, and

456   hence is appropriate for oleaginous cell growth. For further study, other equipment designs

457   should be investigated that could reduce cell sedimentation, as found in BB and ILAB

458   bioreactors. Moreover, the shape of ILAB might be redesigned, in order to accommodate the fine

459   bubbles formed and higher air flow rate, which induces high amounts of foaming.

460

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641

Figure 1

Experimental design.

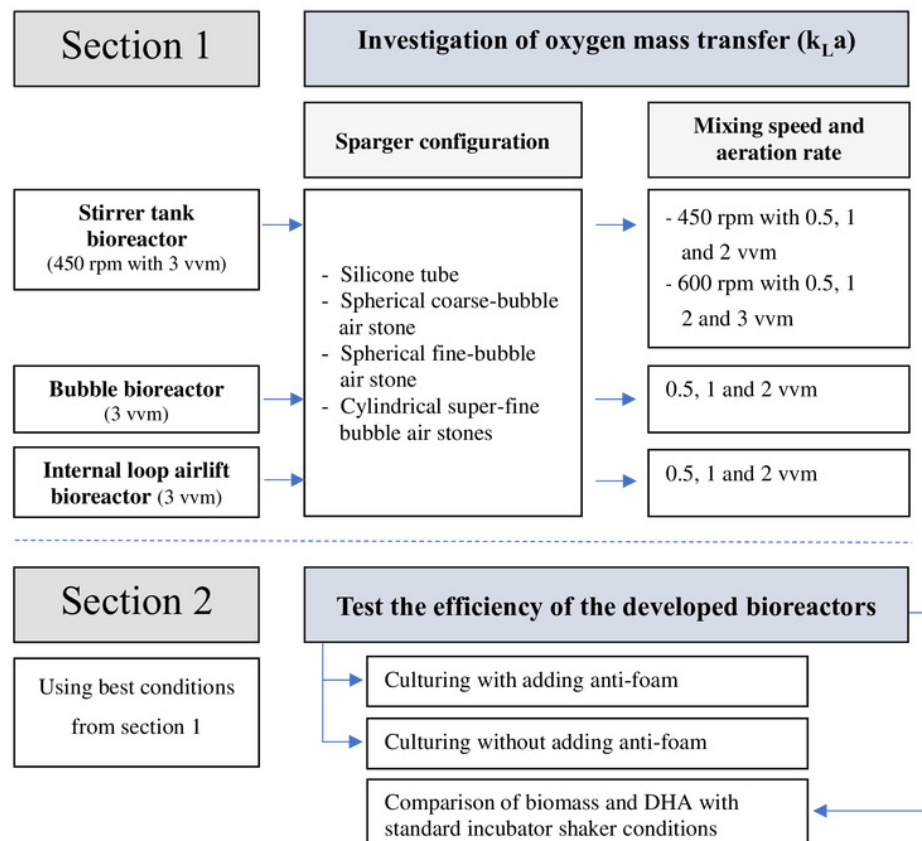



Figure 2

Photographs and configurations of three bioreactors with spargers.

(A) Stirred tank bioreactor (STB), (B) Bubble bioreactor (BB), (C) Internal loop airlift bioreactor (ILAB).

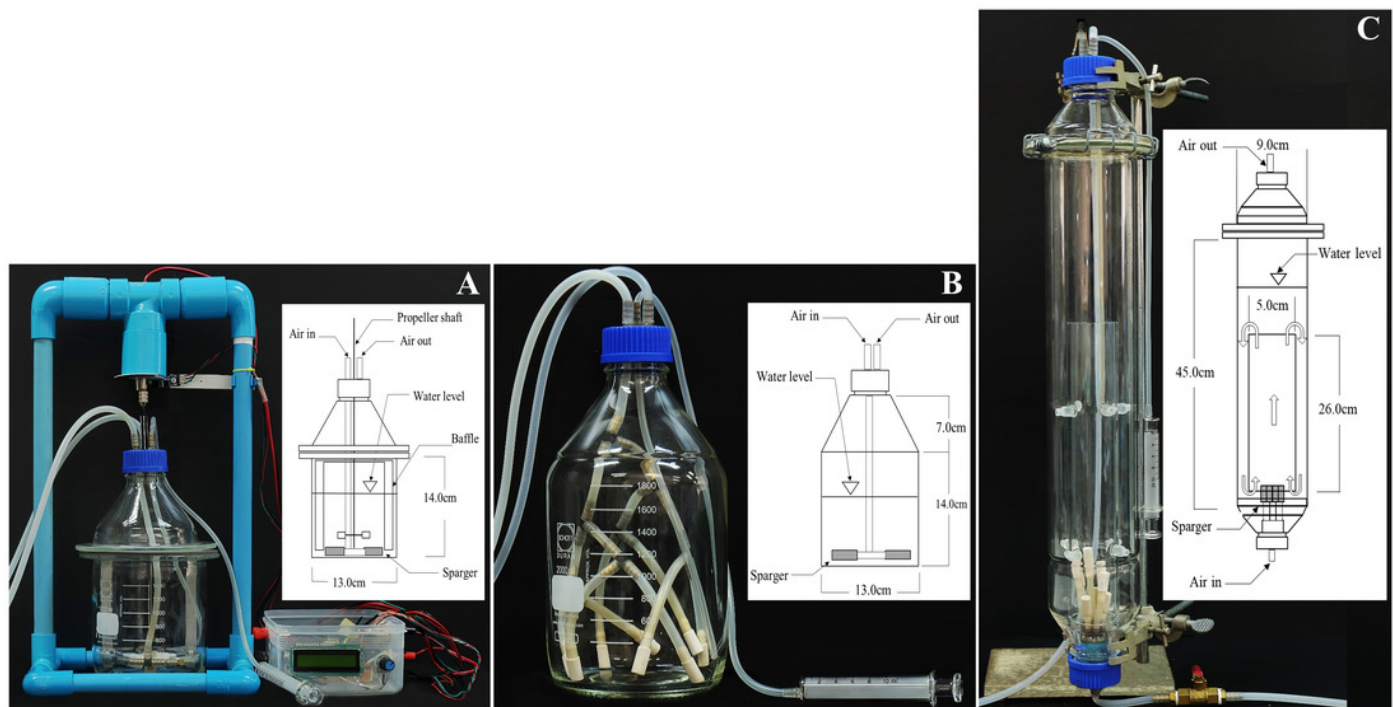



Figure 3

Bioreactor aeration system.

Air out from the air compressor passes through two filters and UV light faces all bioreactors; aeration rate is controlled by the rotameter.

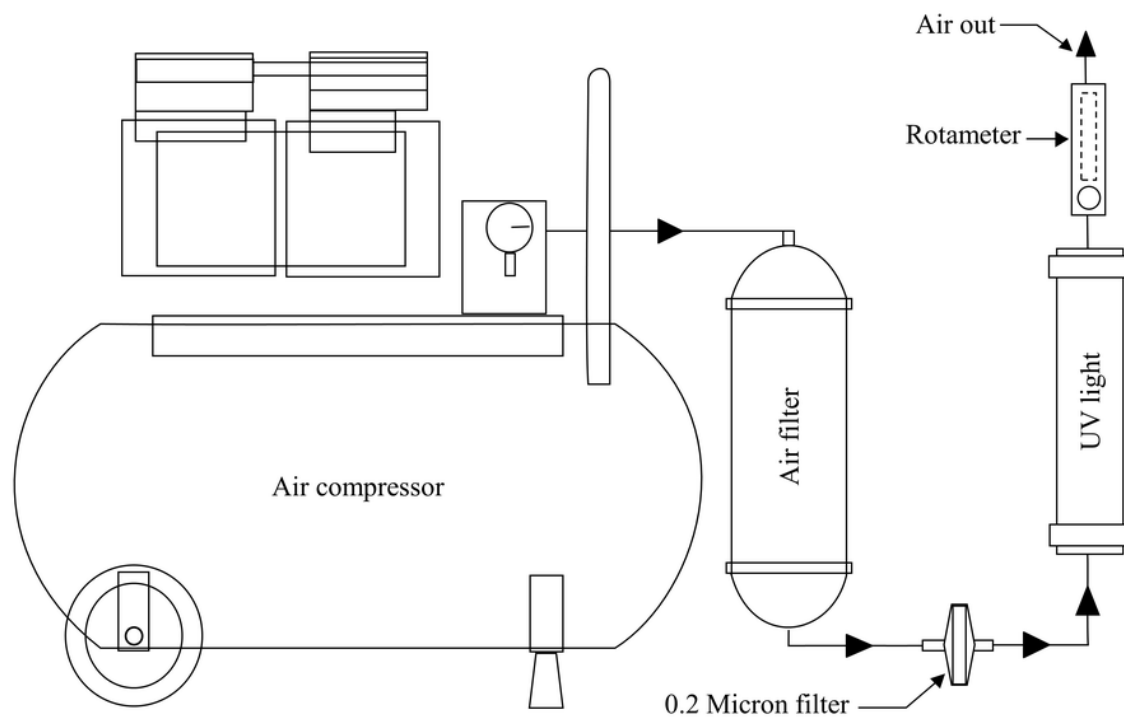



Figure 4

Sparger types.

(A) silicone tube, (B) spherical coarse-bubble air stone, (C) spherical fine-bubble air stone, (D) cylindrical super-fine bubble air stone.

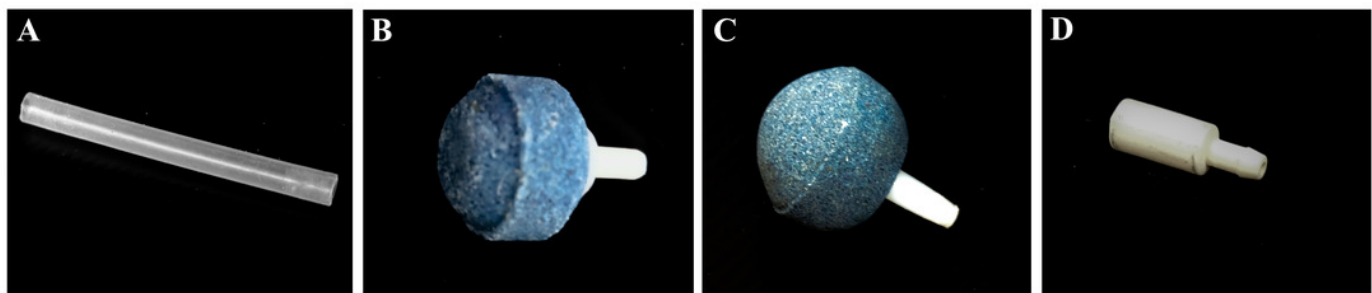



Figure 5

$k_L a$ values in three bioreactors using different sparger configurations.

All sparger types were tested, using aeration rate of 3 vvm in bubble, internal loop airlift and stirred tank bioreactor, with an agitation speed of 450 rpm.

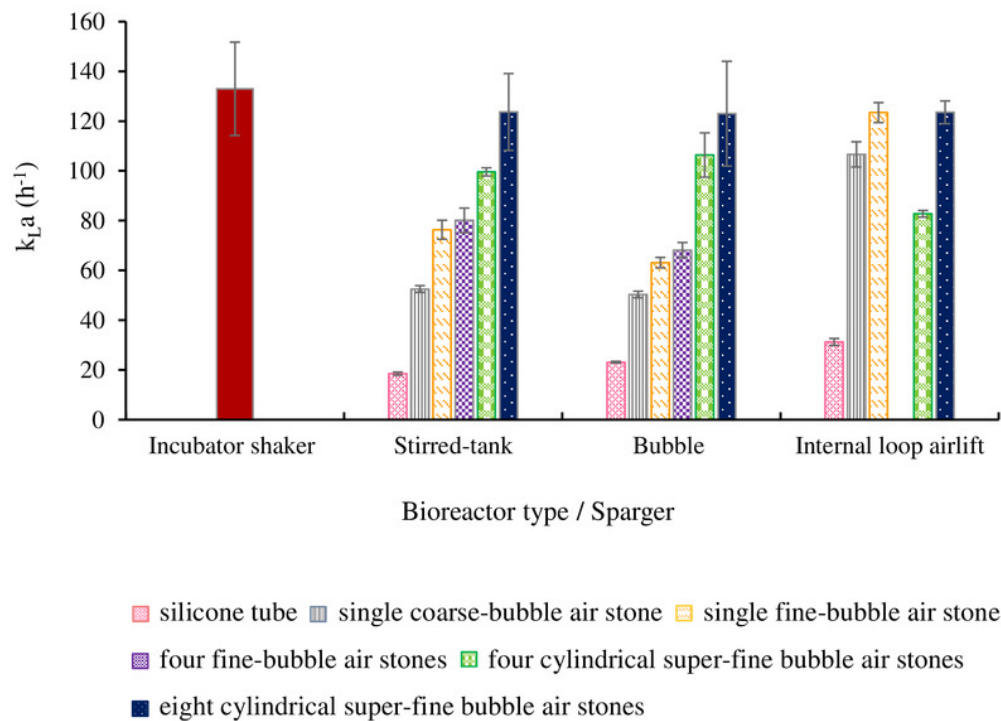



Figure 6

Biomass and total sugar during 120 h and168 h fermentation of *A. limacinum* BUCHAXM 122 in different bioreactors with constant initial sugar concentration (60 g/L).

(A, B) Shaker at 200 rpm; (C, D) Stirred tank bioreactor at 2 vvm and 0.1 vvm; (E, F) Bubble bioreactor at 2 vvm and 0.1 vvm; (G, H) Internal loop airlift bioreactor at 1.5 vvm and 0.1 vvm.

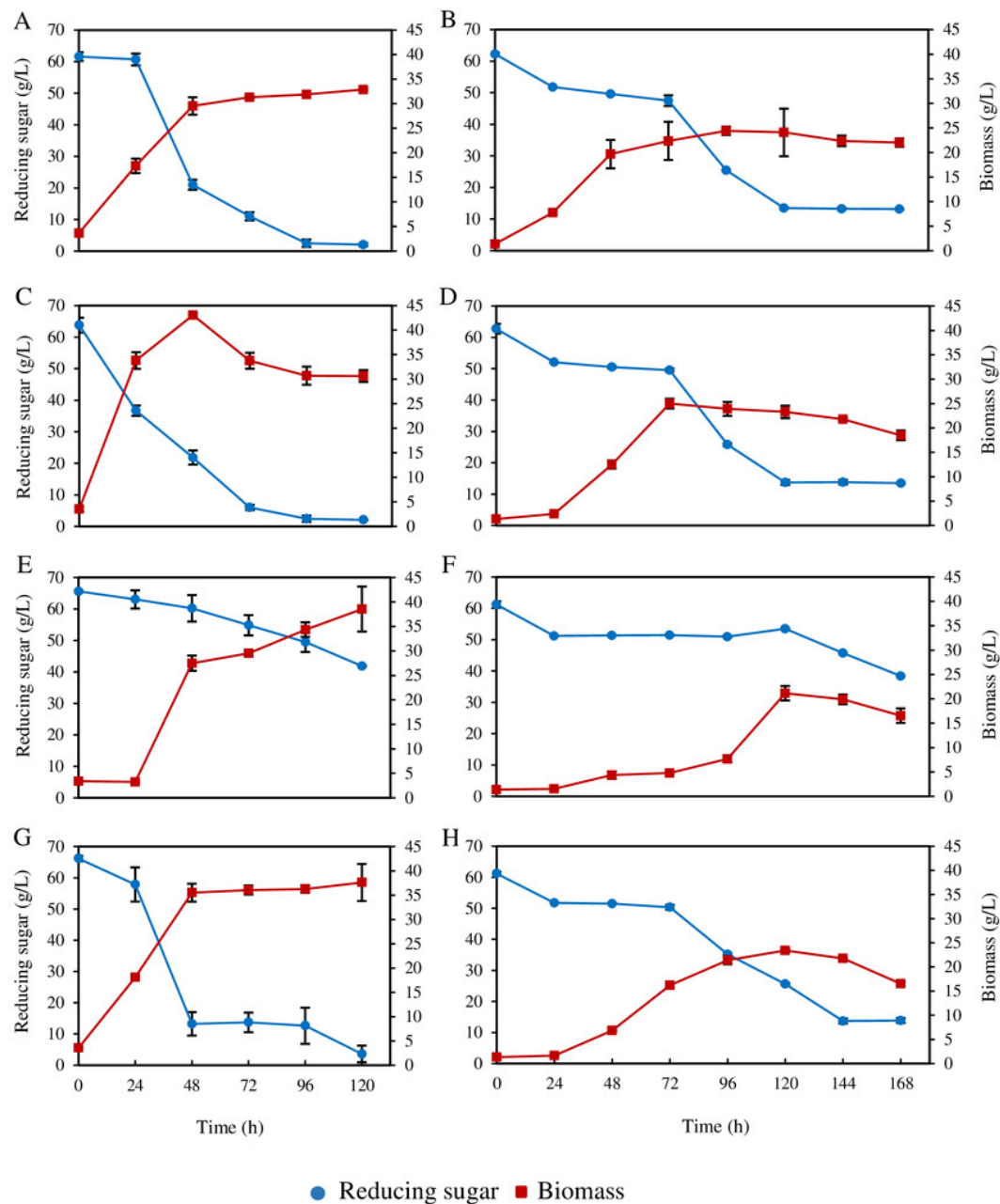



Figure 7

DHA (mg/g DW) (A) and fatty acid (% TFA) (B) production by *A. limacinum* BUCHAXM 122 during 120 h in different bioreactor types.

Aeration provided at 2 vvm for bubble and stirred tank bioreactors with agitation speed at 600 rpm and 1.5 vvm for internal loop airlift bioreactor.

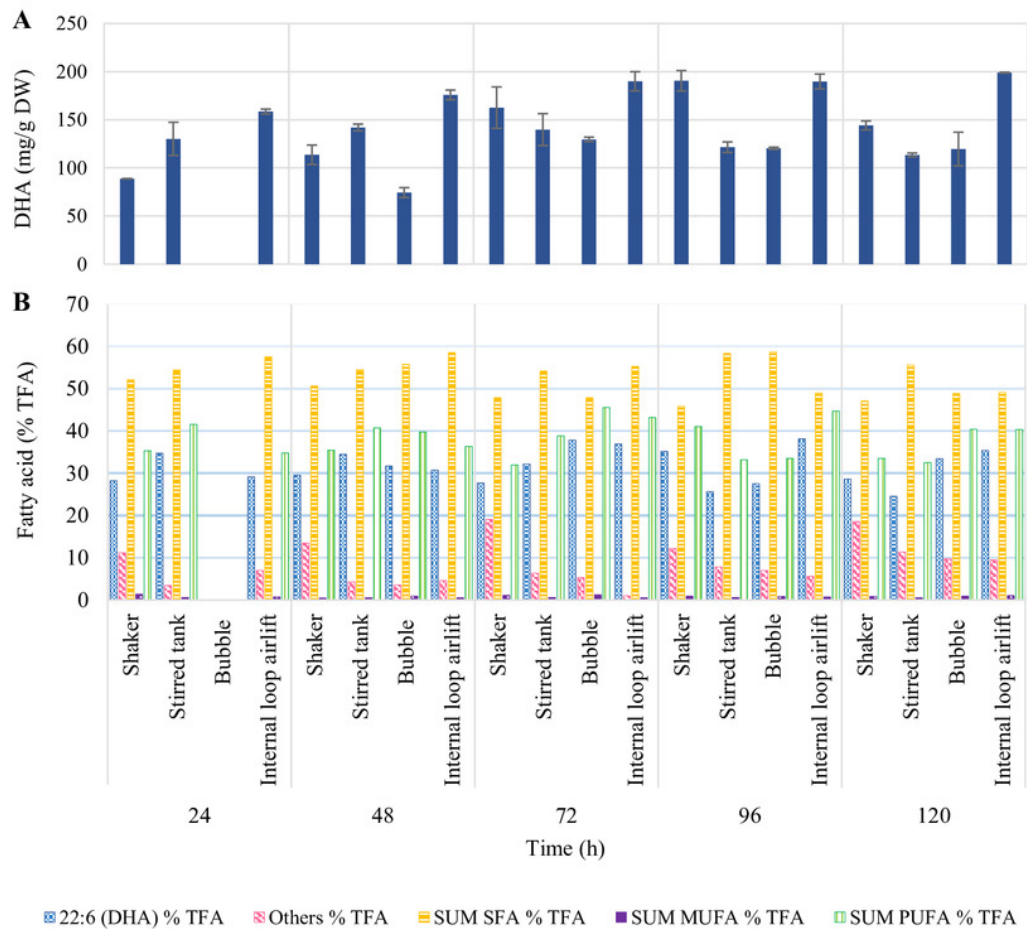
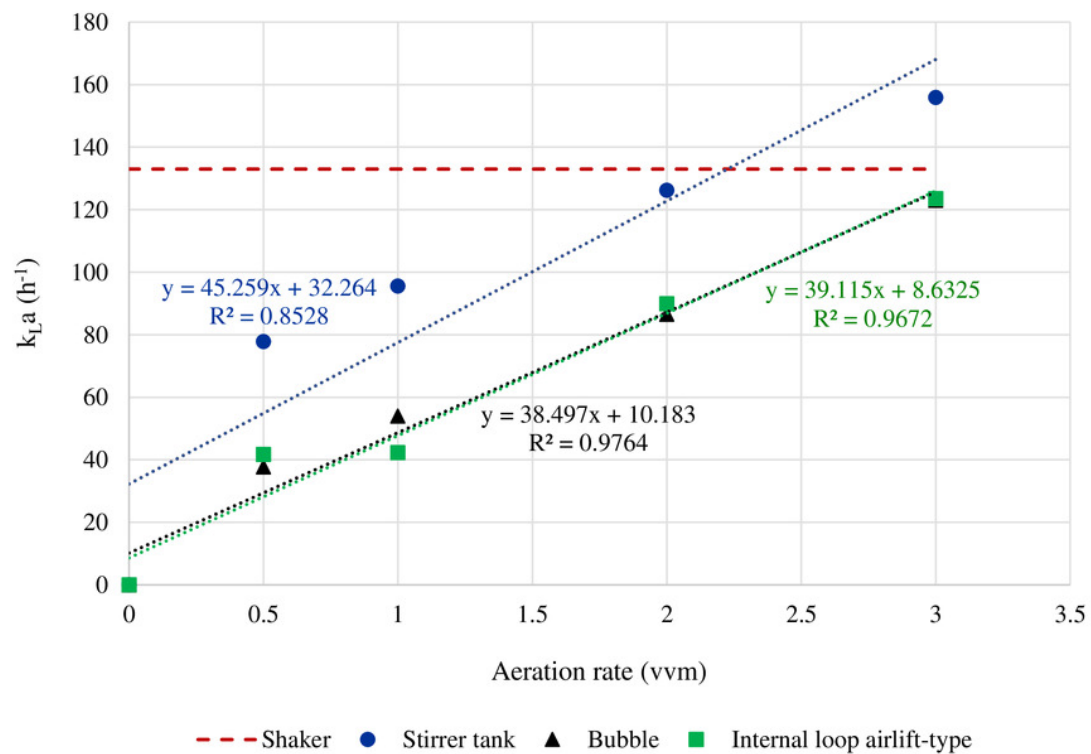



Figure 8

Exponential relationship between aeration rate and $k_L a$ in different bioreactors and shaker.

The $k_L a$ values at 0.5, 1, 2 and 3 vvm in bubble, internal loop airlift and stirred tank bioreactors with agitation speeds of 600 rpm and 200 rpm in shaker.

$k_L a$ values for bioreactors at different agitation speeds and aeration rates.

Aeration Rate (vvm)	k_La (h^{-1})			
	Stirred		Bubble	Internal loop airlift
	450 rpm	600 rpm		
0.5	51.03±2.72[a]	77.18±5.10[b]	37.70±1.45[a]	41.68±1.32[a]
1.0	82.38±3.38[b]	95.57±4.86[c]	53.96±1.13[b]	42.33±2.16[a]
2.0	129.71±5.03[d]	126.21±7.65[d]	86.50±2.93[c]	89.87±3.58[b]
3.0	123.66±15.47[d]	155.92±18.52[e]	122.99±21.04[d]	123.53±4.57[c]

2   Mean for each bioreactor type with different superscript letters are significantly different at

3   $p < 0.05$.

DHA production at 168 h in different bioreactor types, provided with aeration at 0.1 vvm.

Fatty acid		Shaker	Stirred tank	Bubble	Internal loop airlift
22:6 (DHA)	mg/g DW	237.78 ± 35.04[a]	217.08 ± 6.85[a]	75.53 ± 15.79[b]	250.48 ± 18.51[a]
	% TFA	39.05 ± 3.44[a]	34.04 ± 0.79[a]	19.84 ± 2.86[b]	40.20 ± 6.09[a]
Others	% TFA	2.34 ± 0.10[a]	4.38 ± 4.12[a]	2.89 ± 0.22[a]	11.08 ± 13.87[a]
SUM SFA	% TFA	49.85 ± 3.61[a]	52.64 ± 4.58[ab]	68.69 ± 0.83[b]	42. 82 ± 10.47[a]
SUM MUFA	% TFA	1.13 ± 0.05[a]	1.25 ± 0.01[a]	3.57 ± 4.38[a]	1.57 ± 0.37[a]
SUM PUFA	% TFA	46.68 ± 3.66[a]	41.72 ± 0.47[a]	24.86 ± 3.33[b]	44.52 ± 3.03[a]

2 Means with different superscript letters (in rows) are significantly different ($p < 0.05$);

3 FA= saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty

4 acids.

Biomass and DHA yields for *Aurantiochytrium* sp. grown with different bioreactor types and culture media.

Bioreactor type	Strain	Carbon and nitrogen source	Fermentation volume (L) and Conditions	Biomass (g/L)	DHA (% TFA)	Reference
Stirrer tank	*A. limacinum* SR21[a]	Glucose (NH$_4$)2SO$_4$ Corn steep liquor	3 L (Fed batch) 300 rpm	21.9-59.2 (62-125 h)	33.3-38.6 (92 h)	Yaguchi et al. (1997)
	A. limacinum SR21[a]	Glucose Corn steep liquor Ammonium acetate	3.5 L 300-500 rpm, 1.71 vvm, 28 °C	51.95 (72 h)	-	Rosa et al. 2010
	A. limacinum SR21[a] (ATCC MYA-1381)	Crude glycerol YP	4.5 L (Continuous) 0.1 vvm	13 (after 72 h)	31.09 (after 72 h)	Ethier et al. (2011)
	Aurantiochytrium sp. KRS101	GY	3,000 L	30.5 (after 48 h)	39.5 (after 48 h)	Hong et al. (2013)
	Aurantiochytrium sp. TC20	GYP	1.6 L (Fed batch) 300-1,150 rpm, 0.3-1.5 vvm, 20°C	56.6 (69 h)	43.2 (69 h)	Lee Chang et al. (2013)
	A. limacinum BUCHAXM 122	GYP	1 L 600 rpm, 25 °C 0.1 vvm	25.00 (72 h)	34.04 (168 h)	This study
			2 vvm	43.05 (48 h)	34.47 (48 h)	
Bubble column	-	-	-	-	-	-
	A. limacinum BUCHAXM 122	GYP	1 L 25 °C, 0.1 vvm	21.15 (120 h)	19.84 (168 h)	This study
			2 vvm	29.50 (72 h)	37.82 (72 h)	
Internal loop airlift-type	*Aurantiochytrium* sp. KRS101	GY	3,000 L	25 (after 24 h)	52.3 (36)	Hong et al. (2013)
	A. limacinum BUCHAXM 122	GYP	2 L, 25 °C, 0.1 vvm	23.41 (120 h)	40.29 (168 h)	This study
			1.5 vvm	36.05 (72 h)	36.93 (72 h)	

1   a = *Schizochytrium* (now called *Aurantiochytrium*), Numbers in parentheses following biomass and DHA yields represent

2   fermentation time (hours).