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Differential Homeoviscous Response of *Clostridium Pasteurianum* by Membrane Composition and Structural Adaptations to Butanol Toxicity

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Abstract:

Clostridium pasteurianum has been shown to ferment glycerol into butanol at higher

yields than when sugars are used as the carbon source. *C. pasteurianum'*s potential to use

biodiesel-derived crude glycerol as the carbon source has been gaining importance in the recent

past. This study investigated the homeoviscous response of *C. pasteurianum* during butanol

stress. *C. pasteurianum*'s lipid composition of the plasma membrane during butanol challenge

23   was analyzed. *C. pasteurianum* was found to exert two different homeoviscous responses by

24   altering the composition of the lipid membranes in an attempt to counteract the butanol toxicity.

25   Addition of exogenous butanol to a fermentation, when *C. pasteurianum* produced endogenous

26   butanol, led to an increase in the ratio of saturated to unsaturated fatty acids. On the other hand,

27   addition of exogenous butanol to fermentation, when *C. pasteurianum* did not produce any

28   endogenous butanol, led to a decrease in the ratio of saturated to unsaturated fatty acids. This

29   differential response for exogenous butanol during the presence and absence of an active butanol

30   biosynthesis indicates that *C. pasteurianum* is a versatile micro-organism that has the potential to

31   be engineered as an industrial butanol producer using crude glycerol, a promising low cost

32   feedstock for butanol production.

33   **Keywords**

34   *Clostridium pasteurianum*; Glycerol; Fermentation; Butanol; Lipid composition; Homeoviscous

35   response

36

37   **Introduction**

38   *Clostridium pasteurianum,* an anaerobic spore-forming firmicute, ferments glycerol as

39   the sole substrate, resulting in a mixture of butanol, ethanol, and 1,3-propanediol (PDO) along

40   with acetate and butyrate (*1-4*). *C. pasteurianum* is of particular interest for butanol production,

41   as it has also been shown to ferment biodiesel-derived crude glycerol as the sole substrate (*3, 4*).

42   Butanol is toxic to cells, as it partitions into the cell membrane and affects both the

43   structural and functional integrity of the cell. The extent of solvent toxicity correlates to the log

44 P value. Solvents with a log P value less than 4 partitions into the lipid membrane bilayer and

45 are considered extremely toxic. Butanol has a log P value of 0.8 and is considered to be one of

46 the most toxic solvents (*5*). When *Clostridia* are exposed to solvents, the solvents exhibit a

47 fluidizing effect on the phospholipid bilayer, which causes the organism to alter the lipid

48 composition of the bilayer. This response of bacteria to tolerate toxic solvents by altering the

49 composition of the lipid bilayer is known as homeoviscous response (membrane viscosity is

50 proportional fluidity). To compensate for the fluidizing effects of butanol, *Clostridia* increase

51 the ratio of saturated to unsaturated fatty acids (SFA/UFA) in the lipid membrane, thereby

52 reducing the fluidity of the membrane and increasing butanol tolerance (*6-16*). The fluidity of

53 the lipid membrane is directly proportional to the amount of saturated fatty acids in the tail of the

54 lipid bilayer (*11*). Hence, the bacteria that tolerate more butanol have a much higher SFA/UFA

55 ratio in the lipid bilayer (*11*). This has been observed to be an essential biophysical process of

56 tolerating butanol in various butanol producing organisms from the genus *Clostridium (6-8, 10,*

57 *12, 13)*. It has been established that lipid composition and distribution on cell membrane play an

58 essential role not only in maintaining membrane stability, curvature and membrane fluidity but

59 also in modulating protein function and insertion on the membranes.(*17-19*) Membrane fluidity

60 is essential for maintaining the proper distribution and diffusion of embedded proteins in

61 membrane.(*20*)

62   Membrane lipid composition changes in response to alcohol toxicity by increasing the

63 distribution of unsaturated fatty acids observed in *Lactobacillus heterohiochii, Lactobacillus*

64 *homohiochii, Escherichia coli*, and *Saccharomyces cereeisiae*.(*21-23*) However, an opposite

65 effect (decreasing in the amount of saturated fatty acids) in response to alcohol toxicity is also

66 observed in several other microorganisms; *Clostridium acetobutylicum* (*10*) and *Bacillus*

67 *subtilis*. Timmons et al has studied a comparison of wild-type and ethanol-adapted (EA)

68 *Clostridium thermocellum*.(*24*) EA cells preserve the optimum level of fluidity in response to

69 ethanol toxicity with increasing the fatty acids chain lengths of lipid tails and decreasing the

70 unsaturation index in the cell membrane; resulting in higher membrane rigidity (*10*).

71   Butanol has been shown to affect the membrane by increasing fluidity and hence

72 reducing lipid ordering(*10, 25-27*). Also, butanol's toxic effects lead to the formation of

73 interdigitated phases and phase separation(*25, 28*). Overall, butanol can compromise cellular

74 function of the membrane by altering cell fission, fusion, budding, vesicle formation and cell

75 signaling (*28, 29*). The passive and active transport of substrates and products is also affected,

76 along with the structure and function of integral membrane proteins. This can hinder the ability

77 of the cell to maintain an internal pH and inhibits membrane-bound ATPases and the uptake of

78 glucose (if present), which subsequently inhibits energy generation (*9*). Membrane bound

79 ATPases are one such examples, which maintain a transmembrane pH for ATP generation.

80 Butanol inhibits the ATPases and reduces the transmembrane pH resulting in lower ATP

81 formation (*30*).

82   To our knowledge, the surface mechanical properties of butanol-tolerant cell

83 membrane producing solvents have not yet been examined. In this study, the membrane extracts

84 of *Clostridia Pasteurianum* exposed to exogenous addition of different butanol concentrations

85 were used to investigate membrane composition, membrane phase behavior, and membrane

86 fluidization.

87

88 2 Materials and Methods

89 2.1 Materials

90 All chemicals were purchased from Fisher Scientific and Sigma-Aldrich. Deuterated

91 chloroform (CDCl$_3$) was obtained from Cambridge Isotope Laboratory. Synthetic lipids, 1,2-

92 Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, >99% purity) and 1,2-dioleoyl-*sn*-glycero-3-

93 phosphocholine (DOPC, >99% purity) were obtained from Avanti Polar Lipids, Inc.

94 2.2 Bacterial Strain

95 The bacterial strain *C. pasteurianum* ATCC 6013 was purchased from American Type

96 Culture Collection and the glycerol stock was maintained as described earlier [8].

97 2.3 Effect of n-Butanol

98 The effect of n-butanol (n-butanol was used throughout all experiments) on the bacterial growth

99 and the stability and change in composition of the membrane were studied by adding butanol to

100 the media containing either glucose or glycerol as the sole carbon source. For investigating the

101 effect of butanol, exogenous butanol was added to the media varied from 0 to 1% (w/v) (0 gL^{-1}

102 to 10 gL^{-1}) containing either glycerol (25 gL^{-1}) or glucose (50 gL^{-1}) in Biebl media [6] were used

103 to study the effect of exogenous butanol during an active butanol biosynthesis on glycerol and its

104 absence when grown in glucose, as *C. pasteurianum* does not produce butanol when growing on

105 glucose. The cells were allowed to grow in the presence of butanol for 24 hours after which the

106 membrane was extracted. All experiments were conducted with 10% (v/v) inoculum, pre-grown

107 in RCM.

108

109 2.4 Extraction of the Cell Membrane

110   Cell membranes were extracted using the modified protocol of Bligh and Dyer using

111   dichloromethane/methanol mixtures [29]. The cells were harvested (0.5 mL cell suspension) by

112   centrifugation at 13000 rpm for 15 minutes and the pellets were resuspended in 0.5 mL of sterile

113   1.0% (w/v) NaCl in a 10 mL glass sample tube with PTFE lined caps. To the resuspended

114   pellets, 2 mL of dichloromethane/methanol mixture (1:2 v/v) was added and shaken vigorously

115   for 15 minutes. It was followed by a 2 hour incubation at RT, followed by centrifugation at 2500

116   rpm and the supernatant (S1) was collected in a fresh tube. The pellet was again resuspended in

117   0.5 mL 1.0% NaCl and 2 mL of a dichloromethane/methanol mixture (2:1 v/v) was added to the

118   resuspended pellet and shaken vigorously for 15 minutes. Following a 2 hour incubation, the

119   samples were centrifuged at 2500 rpm and the supernatant (S2) was collected. Supernatants S1

120   and S2 were combined and 1 mL of dichloromethane and 1 mL of sterile 1.0% NaCl were added.

121   The top phase (aqueous) was removed and the bottom (organic) phase was retained. The solvent

122   was evaporated under a gentle nitrogen stream. Once a dry film was obtained, the headspace

123   was flushed with nitrogen, capped tightly, and stored for further analysis.

124

125   **2.5   NMR Analysis**

126   The dry film of the membrane was dissolved in $CDCl_3$ for ^{1}H-NMR analysis. Synthetic

127   lipids, DPPC and DOPC were used as standards for NMR analysis. Various ratios of DPPC and

128   DOPC (1:0, 3:1, 1:1, 1:3 and 0:1) in a total lipid concentration of 10 mM were used for

129   calibration (Supplementary Material and Figure S1). The synthetic lipids were dried and

130   dissolved in $CDCl_3$. All ^{1}H-NMR spectra were recorded on a Varian^{TM} Unity Inova 500 (500

131   MHz) spectrometer equipped with a 5 mm triple resonance inverse detectable probe. The

132   percentage of unsaturation in the membrane lipid samples was calculated by multiplying the ratio

133   of the intensity of the peak corresponding to the olefinic hydrogen (at 5.31 ppm) to total intensity

134   of all the peaks from the lipid sample by 100 (Eq 1) [30].

135    $$\%unsaturation = \frac{Intensity_{olefenic}}{Intensity_{total}} \times 100 \quad \textbf{(Eq 1)}$$

136

137   **2.6   Liposome Preparation and Fluorescence Anisotropy**

138   The dry film of the reconstituted cell membrane and 0.1 mM 1,6-diphenyl 1,3,5-

139   hexatriene (DPH) dissolved in chloroform were mixed and co-evaporated under a gentle stream

140   of nitrogen until a dry reconstituted cell membrane/DPH film remained. Vacuum was used (~ 60

141   min) to remove residual solvent from the film. The film was then hydrated with distilled water

142   and maintained at 50°C in a water bath for 1 hour before shaking. The reconstituted

143   membrane/DPH film was suspended as multilamellar liposomes by vigorously shaking for

144   approximately 1 hour. The liposomes were sonicated before use for 60 min at 50°C, which has

145   been previously shown to yield unilamellar liposomes. DPPC liposomes at 10 mM lipid

146   generated by the same procedure were used as control. Fluorescence anisotropy (Perkin Elmer

147   LS 55) and melting temperature were measured using the L-format configuration with DPH as

148   the hydrophobic bilayer probe from 25 to 50°C at a rate of 1°C/min under continuous mixing as

149   described previously (25).

150

151   **2.5   GC/MS Analysis**

152   The lipid samples were methylated for GC/MS analysis. The dried lipid samples were

153   saponified using 1 mL of 3N sodium hydroxide at 90ºC for an hour and then cooled to RT. The

154   excess sodium hydroxide was neutralized with 1.8 mL of 3.6 N hydrochloric acid at 90 ºC for 10

155   minutes and cooled to RT. The free fatty acids were extracted using 1 mL of hexane and diethyl

156   ether (1:1 v/v). The organic phase was then separated into a clean and dry round bottom flask.

157   The fatty acid hydrolyzates were dried using a rotoevaporator and stored under Argon in

158   desiccators.

159   The dried fatty acid hydrolyzates were derivatized using a 5 mL borontrifluoride-

160   methanol complex at 60 ºC for 5 minutes and then cooled to RT. To the cooled solution, 1 mL

161   water and 1 mL hexane were added and the container shaken multiple times to ensure the

162   transfer of esters into the non-polar solvent. The upper organic layer was removed and

163   transferred into an Erlenmeyer flask conataining 5 g of anhydrous sodium sulfate. The flask was

164   incubated at RT to dry overnight. The sodium sulfate was filtered and the hexane solution was

165   transferred into a clean round bottom flask and dried in a rotoevaporator and the samples were

166   stored under Argon. For GC-MS analysis, samples were dissolved in 250 µL of

167   dichloromethane.

168   Fatty acid methyl esters (FAME) derivatives were analyzed by a Shimadzu GC-MS QP

169   2010 system using a SHR5xLB silica capillary column (30m× 0.25mm ID, composed of 100%

170   dimethyl polysiloxane). Manufacturers protocol were followed for FAME analysis.The

171   compounds were analyzed with GC-MS solution and identified by comparison with the data in

172   the NIST libraries. The distribution of FAMEs of *C. Pasteurianum* was determined by dividing

173   area counts for each FAME species by the total FAME area, as defined below:

174   % FAME $= \frac{Area_{FAME}}{\sum FAME_{area}} x100$ **(Eq 2)**

175   2.6   Surface pressure-area (Π-A) isotherms

176   *Π-A* isotherms were conducted in a Langmuir trough (model 102M, Nima technology

177   Ltd, UK) with a deposition area of 70 cm² at 25°C, 37°C and at 50°C. A Wilhelmy plate was used

178   to measure surface pressure with an accuracy of ± 1μN/m connected to an electronic micro-

179   balance. Isotherms were monitored by NIMA TR 7.4 .vi software. The external water bath

180   system was used to control the sub phase temperature. Monolayers were obtained by spreading

181   diluted solutions of dry lipid sample in chloroform at the air/water interface using a 5 μl

182   microsyringe.

183   The chloroform was allowed to evaporate and the lipid films to spread at air/water for 15 min.

184   The system was the equilibrated for 15 min before being compressed at a speed of 10 cm²/min.

185   2.7   Elastic modulus / area compressibility modulus

186   Mechanical properties of the monolayer films were determined by the compressibility

187   modulus. The elastic modulus (C_s^{-1}) / area compressibility modulus is the reciprocal of the

188   compressibility (C_s).(*31*) The elastic modulus corresponds to the elasticity of the Langmuir films

189   under the compression force. C_s^{-1} values were defined as:

190       $C_s^{-1} = -A \left(\frac{\partial \pi}{\partial A} \right)_T$ (Eq 3)

191 where, A is the area per molecule at the particular surface pressure and Π is the corresponding

192 surface pressure. The maximum compressibility or elasticity ($C_{s\,Max}^{-1}$) provides information on

193 the onset of the plastic region, and the maximum packing condition of monolayer.

Results and Discussion

195 Clostridial species produce various metabolites in the form of acids and solvents, and

196 have to modify the composition of their lipid membranes to tolerate the toxic effects of the

197 produced metabolites. The fundamental studies to identify the effect of the toxic metabolites on

198 the lipid composition of during the homeoviscous response can be investigated using the tools of

199 chemical biology. This study is focused on investigating the effect of butanol in C. pasteurianum

200 leading to the tolerance response involving changes in the lipid membrane composition using

201 tools to analyze the physical and structural compositions of the membranes along with the

202 membrane phase behavior.

Effect of Exogenous Butanol

204 The homeoviscous response of *C. pasteurianum* to the addition of exogenous butanol was

205 studied under two conditions, where the first condition involved the addition of exogenous

206 butanol during the production of butanol by *C. pasteurianum* during glycerol fermentation

207 (EB1), while the second condition involved the addition of exogenous butanol while no butanol

208 was produced, during the fermentation of glucose (EB2). *C. pasteurianum* cultures do not

209 undergo solventogenesis during the fermentation of glucose, as the fermentation is

210 predominantly only in the acidogenic phase, resulting in butyric acid as the major fermentation

211   product with no butanol formation.

212   Butanol was added at concentrations of 0 g/L (control), 2.5 g/L (0.0335 M), 5 g/L (0.067

213   M), 7.5 g/L (0.105 M) and 10 g/L (0.134 M), respectively, once the cells reached mid

214   exponential phase. The membranes were extracted after 24 hours of butanol exposure and

215   analyzed using ^{1}H-NMR, fluorescent anisotropy and GC-MS . The ^{1}H-NMR and fluorescent

216   anisotropy results are summarized in Figure 1, while Figure 2 summarizes the data from the GC-

217   MS analysis on the fatty acid composition of the membranes.

218   The addition of butanol during glycerol fermentation resulted in a homeoviscous

219   response due to a decrease in the degree of unsaturation in the membrane as determined by ^{1}H-

220   NMR (Figure 1) of the extracted lipid of cells exposed to different butanol concentrations. The

221   fluorescent anisotropy of the reconstituted *C. pasteurianum* cell membranes supports the results

222   obtained from ^{1}H-NMR. The fluorescence anisotropy data at 37°C shows a decrease in

223   anisotropy with increase in the butanol dose. The anisotropy, <r>, of the unexposed control was

224   found to be 0.120 and <r> was found to reduce as the butanol dose increased in the media. The

225   anisotropy of the membrane had a relatively constant value with a minor increase at higher

226   concentrations of butanol at 7.5 g/L and 10 g/L. The fluorescence anisotropy of DPH in the

227   membranes is inversely related to the fluidity of the reconstituted lipid membranes. The decrease

228   in the anisotropy indicates the fluidizing effect of butanol on the membrane lipids. The

229   stabilization of the anisotropy at higher butanol concentrations can be explained as the response

230   of the bacteria to tolerate the fluidizing effects of butanol. The overlap of the anisotropy data

231   with the ^{1}H-NMR data substantiates the existence of a dominant homeoviscous response leading

232   to a compositional change in the lipid membranes. This can be further confirmed by analyzing

233   the fatty acid composition of the membrane.

234   The data from GC-MS on the fatty acid composition (Figure 2) of the membranes

235   corroborates the presence of the homeoviscous response to butanol. Furthermore, there was an

236   increase in the percentage of higher carbon saturated fatty acids (C_{19} to C_{22}), which are almost

237   completely absent in the control samples with no external butanol present. A similar trend was

238   also observed in the disappearance or reduction in the shorter chain fatty acids with an increase

239   in the dose of butanol stress (Figure 2). The shorter length fatty acids C10, were completely

240   absent in the cells exposed to higher butanol concentrations, while the other shorter fatty acids

241   from C_{11} to C_{16} were found to decrease proportionately with increase in butanol dose. Similarly,

242   the longer fatty acids, C_{19} to C_{22}, were not observed in the control which was not exposed to

243   butanol and had a proportionate increase in the percentage with corresponding increase in the

244   butanol dosage. The compositional analysis through GC-MS not only supports the results from

245   NMR and fluorescence anisotropy, but also substantiates that *C. pasteurianum* exerts a

246   homeoviscous response to butanol in EB1 condition by two major changes in the fatty acid

247   composition to counteract the fluidity of the toxic butanol. First, it increases the percentage of

248   longer chain fatty acids at the expense of shorter chain fatty acids and secondly by increasing the

249   ratio of saturated to unsaturated fatty acids.

250   The effect of butanol on the lipid composition and the fluidity of the lipid membrane has

251   also been reported for *C. acetobutylicum* (*6, 7, 10, 12*). *C. acetobutylicum* produces butanol by

252   fermenting glucose and the effect of butanol challenge was studied during the growth of *C.*

253   *acetobutylicum* in glucose (butanol producing media) (*6, 7, 10, 12*). *C. acetobutylicum* tolerates

254   butanol through a homeoviscous response that predominantly involves an increase of saturated

255 fatty acids at the expense of unsaturated fatty acids in the lipid membrane (*6, 7, 10, 12*). Lepage

256 et al reported the composition of the *C. acetobutylicum*'s fatty acid composition in the lipid

257 membrane, which consisted of fatty acids from C_{12} to C_{19} (*10*). The ratio of the unsaturated to

258 saturated fatty acids was found to be close to 1 without any butanol exposure but the ratio was

259 reduced to 0.87 and 0.77 respectively with an exposure to 4 g/L and 8 g/L butanol respectively,

260 due to the reduction in unsaturation and an increase in saturated fatty acids in the membrane

261 lipids, driven by a homeoviscous response.

262   As a control, the change in the degree of unsaturation and anisotropy of the lipid

263 membrane during the production of endogenous butanol was studied and compared

264 (Supplementary material and Figure S2 and S3). The addition of butanol during glycerol

265 fermentation results in a similar result as observed during the endogenous butanol production.

266 The only change was observed in the anisotropy data which accounts for the fluidizing effect of

267 the exogenous butanol on the membrane. The difference observed in the anisotropy data is

268 consistent with the previously reported fluidizing effect of butanol observed through

269 fluorescence anisotropy of synthetic lipids and reconstituted membrane lipids (*8, 25*).

270 **The Differential Response to Butanol Toxicity**

271   The effect of butanol during EB1 showed a conventional homeoviscous response by

272 increasing the fatty acid length and the ratio of saturated to unsaturated fatty acids. To further

273 investigate the sole effect of butanol toxicity the cells grown in glucose were exposed to butanol

274 (EB2). Intially, the EB2 experiment was conducted to match the same butanol stress dosage from

275 0 g/L to 10 g/L but was also performed at higher concentrations of butanol of up to 20 g/L.

276   As explained earlier, the extracted membranes were studied using ^{1}H-NMR and

277   fluorescent anisotropy. Figure 3 summarizes the change in the fluidity of the membrane in terms

278   of anisotropy and the degree of unsaturation in the membranes of *C. pasteurianum* grown on

279   glucose. The ^{1}H-NMR results indicated an increase in the percentage of unsaturation in the fatty

280   acid tails of the lipid membranes extracted from the cells stressed with exogenous butanol. This

281   result is completely contrasting to the results obtained earlier during butanol stress on glycerol

282   fermentation. The degree of unsaturation in the lipid membranes increased proportionately with

283   increasing concentration of butanol in the media. For an exogenous butanol concentration of 5

284   g/L, a small drop in the percentage of unsaturation in the fatty acid tail was observed when

285   compared to the control with no external butanol. The data obtained from fluorescent anisotropy

286   of the reconstituted membrane, using DPH as the probe, supported the data obtained from ^{1}H-

287   NMR (Figure 3). The anisotropy, <r>, measured at 37°C decreased gradually with an increase in

288   the concentration of butanol in the media.

289   The lipid membranes extracted from the cells were also analyzed by GC-MS to determine

290   the constituent fatty acids. Figure 3 summarizes the results from GC- MS analysis of the cells

291   stressed with exogenous butanol. Thirteen different fatty acids were identified using GC-MS, of

292   which 11 were saturated. The data on the composition of the fatty acids from Figure 4 shows not

293   only an increase in the degree of unsaturation in the fatty acids, but also an increase in the

294   percentage of fatty acid length ($\geq C_{16}$) with an increase in the concentration of exogenous

295   butanol.

296   The degree of unsaturation was found to increase (Figure 3 and 4) in the presence of

297   exogenous butanol, but without endogenous butanol production (cells gown on glucose). An

298   increase in unsaturation coincided with an increase in membrane fluidity (Figure 4). This is in

299   contrast with the response observed for EB1(Figure 2).

300   It has been shown that an increase in the percentage of saturated lipids in the model

301   liposomes (model membranes from DPPC and DOPC) results in an increase in the anisotropy of

302   the liposomes (*25*). Furthermore, the addition of butanol to model membranes comprised of

303   DPPC, DOPC or mixture of the two have shown a decrease in anisotropy due to the fluidizing

304   effects of butanol on the lipid membrane (*25, 29*). Butanol fluidizes membranes by reducing

305   inter lipid interactions and the surface tension within the membrane. Increase in the degree of

306   unsaturation in lipid membranes has been shown to augment the fluidizing effects of butanol

307   (*25*). Hence, the results obtained from the addition of exogenous butanol to the cells of *C.*

308   *pasteurianum* in EB2 contradict the results obtained during EB1. This led to the question of

309   whether other non-lipid entities could be involved in the homeoviscous adaptation of the

310   membrane in EB2.

311   The fatty acid composition of the cells also varied considerably when the cells are grown

312   on glycerol and glucose . The cells grown on glucose and exposed to exogenous butanol show

313   neither a decrease in shorter (C_6 to C_{10}) fatty acids nor an increase in longer (C_{19} and greater)

314   fatty acids (Figure 4). The fatty acid composition of the cells are also different under the two

315   condition, glucose and glycerol fermentation. The fatty acid chain length is as low as 6 carbons

316   during glucose fermentation, while the the lowest fatty acid in glycerol fermentation is 10.

317   Similar distinction is also observed in the maximum chain length as well for the two carbon

318   sources. Moreover, the percentage of unsaturated fatty acids increased with increasing

319   concentrations of exogenous butanol during EB2 (Figure 4).

320   The reduction in shorter chain fatty acids and an increase in longer chain fatty acids in *C.*

321   *acetobutylicum* resulted in a decrease of membrane fluidity (*6, 10*).   It was also observed in *C.*

322   *acetobutylicum* that challenging the cells with butanol resulted in the formation of longer chain

323   fatty acids at the expense of shorter chain fatty acids (*6, 10*).

324   The increase in unsaturation (exogenous butanol on glucose) observed with all three

325   different analytical methods cannot be explained by a homeoviscous adaptation, as it led to an

326   increase in the fluidity of the membrane consistent with the toxic effect of butanol (Figure 3 and

327   4). However, an increase in unsaturation in the lipid membrane of cells exposed to solvent stress

328   has been reported for *E. coli* during ethanol stress and for *C. butyricum* during 1,3-PDO stress

329   (*14, 32*). Dombek and Ingram reported that the plasma membrane became more rigid during

330   ethanol challenge experiments, but the extracted lipid membrane exhibited higher fluidity in

331   comparison to the control cells that were unexposed to ethanol. This shows that the rigidity of

332   the membrane is not only dependent on the ratio of saturated to unsaturated fatty acids, or the

333   presence of shorter or longer fatty acids, but is also dependent on the lipid to protein ratio in the

334   membrane *(34)*. Membrane proteins can rigidify the membrane despite irrespective of the lipid

335   composition. Hence, an increase in protein:lipid ratio can account for the net increase in the

336   rigidity of the membrane and compensates for the increase in fluidity as a result of the increased

337   unsaturation of the lipid tails (Figure 3).

338   During the formation of butanol (EB1), the bacteria must be synthesizing butanol efflux

339   pumps in the membrane that serve as butanol transporters to the extracellular environment (*33*).

340   Dunlop *et al.* have shown that cloning and expressing of efflux pumps from different

341   microorganisms for various solvents resulted in an increase of solvent tolerance. But,

342 overexpression of butanol and iso-butanol efflux pumps did not improve butanol tolerance

343 leading to a conclusion that the toleration of butanol is a much more complex phenomenon (*33*).

344 There should also be a correlation between the transcriptional regulation of the genes involved in

345 butanol production and the genes responsible for butanol tolerance through homeoviscous

346 adaptation. If there was no correlation, the homeoviscous adaptation of *C. pasteurianum* must be

347 similar during both, endogenous production (from glycerol) and exogenous addition (growth on

348 glucose).

349 *Π–A* **isotherms of reconstituted cell membrane (RCM) monolayers**

350 To gain more understanding of the interaction within lipid-lipid and lipid-protein, it is

351 essential to first characterize the phase behavior of the RCM monolayers spread at the air/water

352 interface. The *Π–A* isotherms of RCM is plotted in Figure 5. Three different temperatures (25,

353 37, and 50 °C) and sample extract at different conditions (without butanol and with 10 g/l

354 butanol) have been examined. Without exogenous butanol in media at 25°C, the experimental

355 isotherms exhibited a liquid expanded (LE) phase and a collapse phase. The trends in *Π–A*

356 isotherms were slightly different with those of unsaturated phospholipids(*34*) and to *E. coli* lipid

357 extract(*17*). *Π* of RCM monolayers was much lower than the unsaturated lipids and the *E. coli*

358 lipid extract and lift off area was not observable on RCM monolayers. The collapse of the RCM

359 monolayers took place at ~25 mN/m indicating that the monolayers had low viscosity due to

360 strong lipid interaction and a plausible displacement of relaxation membrane protein. At 37°C

361 RCM monolayers isotherms existed in single *LE* phase and collapse phase disappeared that could

362 be attributed to the increase of disordered lipid tails and disordered organic membrane protein

363 lowering lipid-protein interactions in membrane monolayers. With increasing temperature up to

17

364   50 °C, Π–A isotherms occurred at lower pressure. The appearance of the lift-off area was able to

365   observe. A plateau region on Π–A isotherms indicates that the *G-LE* phase transition occurred.

366   Increases temperature lowered hydrogen bonding between lipid head groups and hydrophobic

367   interactions between lipid tails and extended the relaxation phenomena in membrane proteins.

368   In addition, the length of plateau region could be associated with the ratio of

369   unsaturated/saturated lipid (U/S). A short plateau region displayed as a high ratio of saturated

370   lipid. The addition of 10 g/l butanol in media had markedly different effects on the Π–A

371   isotherms (Figure 5) and the maximum elastic modulus, C_{Max}^{-1} (Figure 6). With temperature

372   changes (25, 37, and 50 °C) Π–A isotherms of RCM monolayer existed in the *LE* phase and

373   occurred at higher surface pressure than without butanol present in media. This reveals that the

374   content of *hydrophobic proteins* (Supplementary material and Figure S4) adsorbed *at air/water*

375   *interface had increased creating a higher Π-A* and an increased C_{Max}^{-1} (more rigid). The C_{Max}^{-1}

376   decreased proportionally with increasing temperature due to disordering structure of lipid and

377   expanded relaxation phenomena.

378   Based on our interpretation data, the effect of protein can be interpreted as shown in

379   Figure 7. At low surface pressure ($\Pi < \Pi_{Collapse}^{protein}$) 25°C, hydrophobic membrane protein from

380   cell grew in media without butanol exhibited relaxation phenomena within lipid membrane

381   monolayers and water molecules were between the hydrophilic head groups of lipids. As a result,

382   repulsive contribution at polar headgroup was high, and attractive van der Waals interactions

383   between tails of lipids and interfacial interaction at head/tail of tails were low promoting a weak

384   molecular packing. At high surface pressure, $\Pi > \Pi_{Collapse}^{protein}$ external pressure compressed the

385   protein network until it failed and displaced from the interface, forming collapse protein

386   multilayers in the aqueous bulk phase near to the interface; thus, the collapse phase appeared. In

387   the case of the reconstituted cell membranes extracted from cells grew in media with butanol, the

388   content of hydrophobic membrane protein was increased resulted in longer relaxation

389   phenomena. Therefore, the collapse pressure appeared higher. It is clear that homeoviscous *C.*

390   *pasteurianum* producing solvents altered the lipid membrane composition to modulate protein

391   function and insertion on the membranes in determining membrane integrity and activity.

392

393   **4. Conclusion**

394   This study is the first report of the analysis of the homeoviscous adaptation to butanol by

395   *C. pasteurianum* and this study is also the first study that quantifies lipid composition in *C.*

396   *pasteurianum*. The analysis of the extracted membranes from cells exposed to various

397   concentration of butanol in two different media, butanol producing medium containing glycerol

398   and non-butanol producing medium containing glucose, resulted in two completely different

399   responses with respect to the changes in the composition of the lipids. During butanol

400   production, when stressed with exogenous butanol, *C. pasteurianum* responds by increasing the

401   ratio of the saturated to unsaturated fatty acids in the membrane. This is also the response found

402   in other butanol producing species of *Clostridia*, along with the response exhibited by other

403   microbes towards tolerating toxic organic compounds (*14-16, 35*). *C. pasteurianum* exhibits a

404   different response when grown on glucose (no butanol produced), but challenged with exogenous

405 butanol, which can be explained on the hypothesis of altering the ratio of the protein to lipids in

406 the membrane (Figure 7 and 8). This hypothesis was further confirmed using by Π-A isotherms

407 and the presence of hydrophobic membrane proteins in the lipid membrane of C> apsteurinum

408 during EB2.

409   At 25°C Π-A isotherms of the membrane monolayer were observed in the *LE*

410 phase and the collapse phase. The collapse phase on membrane monolayers was observed

411 because of a greater attractive interaction of saturated lipids and a displacement of lipid

412 monolayers on collapse protein network at the interface. At 50°C Π-A isotherms membrane

413 monolayer exhibited a *G-LE* phase transition and *LE* phase. The *G-LE* phase transition appeared

414 due to a low interaction of lipid-lipid and lipid-protein associating with disordering protein and

415 lipid structure. This study implies the effect of unsaturated lipids and proteins on membrane

416 monolayers and helps our understanding of the mechanism of butanol tolerant membrane by *C.*

417 *Pasteurianum* could be utilized to develop cultures in higher concentrations of butanol leading to

418 more cost-effective and more efficient on butanol production.

419   The result from this study substantiates the assumption that a correlation exists between

420 the modes of homeoviscous response, which are in turn, dependent on the activation of butanol

421 production pathway. Furthermore, the existence of two different homeoviscous adaptations to

422 butanol challenge in *C. pasteurianum*, demonstrates the potential of this organism to be studied

423 further in terms of proteomics, functional genomics and metabolic engineering for the

424 development of an industrial strain. The unavailability of the genome sequence and the

425 proteome data must be addressed to explore butanol production and tolerance in *C.*

426 *pasteurianum*. In the meantime, methods to use genomic data from closely related species can

427   be explored to establish a platform that can be used to perform transcriptional analysis of *C.*

428   *pasteurianum* during butanol stress.

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