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Homeoviscous response of *Clostridium pasteurianum* to butanol toxicity during glycerol fermentation

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

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17 **Abstract:**

18 *Clostridium pasteurianum* has been shown to ferment glycerol into butanol at higher
19 yields than when sugars are used as the carbon source. *C. pasteurianum*'s potential to use
20 biodiesel-derived crude glycerol as the carbon source has been gaining importance in the recent
21 past. This study investigated the homeoviscous response of *C. pasteurianum* during butanol
22 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 ^1H -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 ^1H -NMR spectra were recorded on a VarianTM 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].

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

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 90°C 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 °C 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 °C 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 containing 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 $\% \text{ FAME} = \frac{\text{Area}_{\text{FAME}}}{\sum \text{FAME}_{\text{area}}} \times 100$ (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 $\pm 1\mu\text{N/m}$ 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.

194 **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.

203 **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 $^1\text{H-NMR}$, fluorescent anisotropy and GC-MS . The $^1\text{H-NMR}$ 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 $^1\text{H-}$
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 $^1\text{H-NMR}$. The fluorescence anisotropy data at 37°C shows a decrease in
223 anisotropy with increase in the butanol dose. The anisotropy, $\langle r \rangle$, of the unexposed control was
224 found to be 0.120 and $\langle r \rangle$ 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 $^1\text{H-NMR}$ 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 (C₁₉ to C₂₂), 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 C₁₁ to C₁₆ were found to decrease proportionately with increase in butanol dose. Similarly,
242 the longer fatty acids, C₁₉ to C₂₂, 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 C₁₂ to C₁₉ (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). Initially, 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 $^1\text{H-NMR}$ 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 $^1\text{H-NMR}$ 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 $^1\text{H-}$
287 NMR (Figure 3). The anisotropy, $\langle r \rangle$, 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 grown 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

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, $\Pi > \Pi_{\text{Collapse}}^{\text{protein}}$ 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 *II-A* isotherms
407 and the presence of hydrophobic membrane proteins in the lipid membrane of *C. pasteurianum*
408 during EB2.

409 At 25°C *II-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 *II-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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