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Hybrid in situ product recovery technique applied to (A)IBE fermentation

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11 **Abstract**

12 Fermentative production of alcohols is a promising alternative to petroleum-based fuels
13 industry. However, the development of a competitive biological process implies the
14 achievement of higher production titer and enhanced productivity. In the case of butanol
15 production by solventogenic strains, In Situ Product Recovery (ISPR) techniques help
16 to overcome the inhibition threshold of the microorganism and to reduce the overall cost
17 of downstream process. In this work, gas stripping (GS) was preliminary studied in an
18 abiotic system and high flow rates (vvm - vessel volume minute⁻¹ > 2 min⁻¹) were needed
19 to achieve higher butanol stripping rate than its production rate. Combined liquid
20 extraction (LE) with pulse Gas Stripping (GS) were applied to batch fermentations
21 resulting in a synergic effect giving a higher glucose consumption rate and a lower
22 aqueous butanol concentration. Moreover, in this hybrid system, biphasic bioreactor
23 acted as a “liquid-liquid equilibrium step” and both phases were stripped concomitantly.

24

25 **Keywords:** IBE; extractive fermentation; gas stripping; *Clostridium beijerinckii*.

26

27 **Introduction**

28 Production of fuels and chemicals from sugars has attracted much attention during the
29 last decades, becoming a global priority as atmospheric CO₂ levels and oil prices
30 continue to increase. Alcohols obtained by fermentation processes are included among
31 the most promising petroleum substitutes due to their wide range of applications:
32 advanced biofuels, industrial solvents, chemical feedstock, bioplastics production, etc.

33 The development of an economically competitive biological process implies the
34 achievement of higher production titers of alcohol in the bioreactor. However,
35 microorganisms usually have limited tolerance for certain products and may be
36 inhibited by excessively high concentrations occurring when the threshold concentration
37 is exceeded. Particularly, ABE (acetone, butanol, ethanol) and IBE (isopropanol,
38 butanol, ethanol) fermentations are subject to strong inhibition by products [1], which
39 leads to low final product concentration, high recovery costs and large volumes of
40 wastewater generation. Several options have been reported to produce metabolites from
41 fermentation beyond their inhibition threshold. One possible solution would deal with
42 the improvement of the biocatalyst, in terms of higher selectivity and tolerance towards
43 the main fermentation product. Another alternative focuses on the biocatalyst
44 environment and concerns the decrease of product inhibition by continuous product
45 removal and recovery from the fermentation medium. This can be achieved by an In
46 Situ Product Removal system. The later approach allows the recovery of butanol as fast
47 as it is produced during the fermentation, and thus it would keep the butanol
48 concentration under the inhibition threshold. This allows the generation of more
49 concentrated butanol stream which is more easily recovered [2, 3, 4, 5, 6] and results in
50 several benefits impacting the overall process performance: smaller reactor volume,

51 lower downstream cost, less wastewater generation. The integrated product recovery of
52 butanol (and other alcohols) from aqueous broth can be based on the differences
53 between physical and chemical properties of the compounds to be separated, or on their
54 interaction with an auxiliary agent or material. In any case, the total recovery of butanol
55 is not necessarily achieved in the pre-concentration step and thus further purification
56 may be required. Separation techniques for *in situ* product recovery that have been
57 explored over the last few decades are adsorption, gas stripping, liquid-liquid extraction,
58 pervaporation, etc.

59 Among different possibilities, gas stripping (GS) arises as a relatively simple technique
60 that can effectively remove AIBE compounds from the fermentation broth [7]. Gases
61 generated during AIBE fermentation -or external agents like nitrogen - are used in this
62 technique to separate the solvents. These gases are sparged into the bioreactor and
63 volatile compounds are recovered and subsequently condensed. Gas stripping can be
64 used either *in situ* or in stream configurations, with or without previous removal of
65 solids. Several AIBE configurations (batch, fed batch and continuous mode) have been
66 tested in laboratory scale, and in all cases both the product yield and productivity have
67 improved. The effects of several operating parameter such as the gas recycle rate, the
68 bubble size or the amount of antifoam added to the broth have been studied [8, 9] for a
69 gas stripping system coupled or not to fermentation. Ezeji *et al.*, 2005 [10] observed that
70 *C. beijerinckii* was not affected adversely by GS and productivities and yields were
71 increased to 200 and 118% respectively, as compared to control in batch fermentation.
72 Xue *et al.*, 2012 [11] applied gas stripping in fed-batch mode to a system combining
73 free and immobilized cells doubling its reference productivity, obtaining highly
74 concentrated condensates and reducing about 90 % of the estimated energy cost for the

75 overall process. However, even if GS technique improves in all cases fermentation
76 performances, before its industrial application, several aspects will need to be faced up,
77 such as: excessive foaming during fermentation that leads to operational problems, high
78 cost of stripped alcohols recovery, huge compressors needed.

79 *In situ* butanol recovery technique by liquid extraction (LE) coupled to a fermentation
80 system, an insoluble extracting compound is added to the fermentation broth. Both
81 phases are then easily separated. The optimal solvent is a compromise between high
82 alcohol distribution coefficient and no toxicity towards microorganisms [6, 12]. In
83 practice, biocompatible solvents present low butanol extraction capacity, therefore high
84 volumetric ratio of solvents to aqueous phase is needed in order to keep butanol below
85 the inhibition threshold concentration, which increases the operational volume
86 necessary in the bioreactor.

87 Combination of both *in situ* recovery techniques (GS and LE) and their applications for
88 batch and fed-batch ABE fermentations were first proposed by Lu and Li, 2016 [13]. In
89 their work, the authors applied nitrogen stripping directly through the organic phase
90 (oleyl alcohol) in a biphasic reactor. They obtained an ABE productivity of 40 % higher
91 at $vvm = 1.6 \text{ min}^{-1}$ in batch mode and increased up to 95-105 % in fed batch mode [14]
92 applying 1.6 and 3.3 min^{-1} of gas flow rate, respectively. Moreover, glucose
93 consumption was boosted consequently.

94 In this work, a hybrid system combining gas stripping and liquid extraction was applied
95 to batch IBE fermentations. Vegetable oil based mixture solvent was used in a biphasic
96 reactor and the integrated technique (GS-LLE) performance was compared to control
97 assays (individual ISPR technique). Previously, gas stripping was studied in an abiotic
98 set up (representative synthetic fermentation medium, no cells) in order to evaluate a

99 first order stripping rate model and to establish the optimal operating window for this
100 system.

101 **1. Material and Methods**

102 ***2.1 Experimental set-up for abiotic IBE gas stripping***

103 A schematic of the experimental apparatus for gas stripping used in this study is shown
104 in **Fig. 1**. End fermentation representative solutions of 1-butanol-isopropanol-ethanol
105 (11/5/0.5 g/L) (PubChem CID: 263, 3776, 702, respectively) were prepared in
106 demineralized water and placed in 1 L bioreactor (500 mL of working volume) with a
107 Rushton impeller and a sparger placed on the bottom of the vessel below the impeller.
108 Temperature inside the bioreactor, agitation rate and gas flow rate were controlled
109 variables in the system. For collecting condensates, two cold traps were arranged and
110 cooled at 4 and -15 °C, respectively. Sampling was done in the reactor and also in both
111 cold traps, so the performance of total alcohol stripping process could be estimated.
112 Nitrogen (PubChem CID: 947) was sparged through the aqueous solution at a fixed
113 flow rate before it was led to the condenser flasks and then through a flask with water
114 containing ice before it was released to the atmosphere. Temperature set point in the
115 bioreactor was fixed at 37 °C while agitation rate was set at 300 rpm. Gas stripping rate
116 was evaluated at several vvm (1 vvm = 1 L of N₂ per 1 L of liquid volume per minute):
117 0.5, 2 and 3 min⁻¹. Each experiment was carried out twice.

118 ***2.2 Mathematical development***

119 The system was described by means of a simple macroscopic model in order to quantify
120 the stripping process. At gas-liquid interface, the steady state flux balance can be
121 expressed as follows according to Whitman double film theory:

122 $\phi_{fluxGL} = k_L (C_{i,l} - C_{i,l}^*) = k (C_{i,g}^* - C_{i,g}) = K_{LG} (HC_{i,l} - C_{i,g}^*)$ (mol/m² s) Eq. A.1

123 Where k_L , k_G are respectively the individual mass transfer coefficient based on liquid
 124 and gas phases (m/s), K_{LG} is the overall mass transfer coefficient based in gas phase
 125 (m/s), $C_{i,l}$, $C_{i,g}$ the bulk liquid and gas composition for compound i (mol/m³),
 126 respectively. $C_{i,l}^*$, $C_{i,g}^*$ are the corresponding equilibrium composition with bulk
 127 composition in each phase (mol/m³). Moreover, H denotes the Henry coefficient
 128 (dimensionless). Therefore, the gas phase balance is expressed as follows:

129 $V_g \frac{d(C_{i,g})}{dt} = -Q_g (C_{i,g}) + V_T K_{LG} a (HC_{i,l} - C_{i,g}^*)$ (mol/s) Eq. A.2

130 Where V_g is the gas phase volume in the bioreactor (m³), V_T the total volume (m³), Q_g
 131 denotes the flow gas rate through the bioreactor (m³/h). Gas composition is assumed to
 132 be solute free at the bioreactor inlet. Eq.A.2 may be simplified with a quasi-steady state
 133 approximation in the gas phase as following:

134 $C_{i,g} = \frac{V_T K_{LG} a H}{Q_g + V_T K_{LG} a} C_{i,l}$ (mol/m³) ...Eq. A.3

135 In liquid phase, mass balance for solute i being stripped can be expressed as:

136 $V_l \frac{d(C_{i,l})}{dt} = (-r_i) V_l + Q (C_{i,l,0} - C_{i,l}) - V_T K_{LG} a (HC_{i,l} - C_{i,g}^*)$ (mol/m³) Eq.A.4

137 Where V_l is the liquid phase volume in the bioreactor (m³), V_T the total volume
 138 (therefore $V_T = V_l + V_g$) (m³), Q denotes the liquid flow rate through the bioreactor (m³/h)
 139 and r_i the reaction rate for compound i inside the bioreactor (mol/Lh). The first two
 140 terms are assumed to be equal to zero (closed system for liquid phase, and no reaction
 141 inside since abiotic system was employed). Considering these assumptions and

142 combining previous equations, the simplified expression for the variation of aqueous
143 phase composition during gas stripping for compound I was obtained:

$$144 \quad \frac{d(C_{i,l})}{dt} = -\frac{K_{LG} a Q_g H}{\frac{V_L}{V_T} Q_g + V_L K_{LG} a} C_{i,l} = -\beta C_{i,l} \quad (\text{mol/m}^3 \text{ s}) \quad \text{Eq. A.5}$$

145 β denotes the stripping factor which finally encloses thermodynamics (H) and transfer
146 ($K_{LG}a$) effects. If we assume that the system does not have any mass transfer limitations,
147 previous equation may be simplified to:

$$148 \quad \frac{d(C_{i,l})}{dt} = -\frac{Q_g H}{V_L} C_{i,l} = -\beta C_{i,l} \quad (\text{mol/m}^3 \text{ s}) \quad \text{Eq. A.6}$$

149 According to Eq. A.6, in a thermodynamic controlled system stripping factor can only
150 be improved by applying higher vessels volume per minute to the system.

151 In parallel, dynamic simulation of a successive liquid-vapor flash operations occurring
152 inside the bioreactor has been developed with SIMULIS Thermodynamics software.
153 The aim of this model is the prediction of the system thermodynamics, in other words,
154 the maximal attainable stripping rate at operating conditions, in case of no gas-liquid
155 transfer limitations exist. This model also includes liquid-liquid-vapor flash calculus
156 applied to outlet gas flow, in order to simulate cold trap units and to predict condensates
157 composition.

158

159 **2.3 Microorganism and culture media**

160 *C. beijerinckii* DSMZ 6423 spores were stored in 150 µL saline suspension cryotubes at
161 -60 °C. All experiments started with a heat-shock of the spores for 1 min at 100 °C to
162 induce germination and subsequently they were used to inoculate 10 ml of
163 potato/glucose preculture medium previously sterilized (121 °C, 20 min). The
164 preculture media was incubated anaerobically at 36 °C, 24 h. The abiotic culture
165 medium was regenerated at 100 °C for 10 min previously and placed inside an
166 Anaerocult jar (Oxoid) for 72 h to guarantee anoxic conditions at the beginning of the
167 fermentation. The potato/glucose preculture media contained the following
168 composition: 250 g/L boiled potatoes; 2 g/L (NH₄)₂SO₄ (PubChem CID:6097028); 2
169 g/L CaCO₃ (PubChem CID: 10112), 10 g/L glucose (PubChem CID: 53782692) . The
170 pre-culture medium was transferred to 100 ml of culture medium disposed in 250 mL
171 sealed biphasic bioreactor using Schott bottle, previously purged with nitrogen
172 (PubChem CID: 947) during 20 min. The culture medium composition for IBE
173 fermentations was: 6 mg/L FeSO₄ 7H₂O (PubChem CID: 62662); 1 g/L MgSO₄ 7H₂O
174 (PubChem CID: 24083); 1 g/L KH₂PO₄ (PubChem CID: 516951); 0.6 g/L K₂HPO₄
175 (PubChem CID: 24450); 2.4 g/L CH₃COONH₄ (PubChem CID: 176); 0.1 g/L p-
176 aminobenzoic (PubChem CID: 978); 2.5 g/L yeast extract (PubChem CID: 24973165)
177 and 60 g/L glucose (PubChem CID: 53782692). Biphasic bioreactors were incubated at
178 36 °C and low orbital agitation (50 rpm).

179

180 **2.4 Pulse gas stripping, extractive and integrated gas stripping-extractive** 181 **fermentations**

182 Batch IBE fermentations were performed to study and compare the effect of pulsed gas
183 stripping, liquid liquid extraction and integrated gas stripping-liquid liquid extraction
184 system. All fermentations were carried out in 500 mL schott bottles, filled with 200 mL
185 of culture media and initial glucose concentration of 90 g/L. The culture medium was
186 inoculated with 20 mL of cells in their maximal growth rate (same inoculum for all the
187 bottles). The system was previously purged with N₂. The temperature was fixed at 36 °C
188 and gentle agitation was kept at 50 rpm. The whole system was autoclaved at 121 °C
189 during 20 minutes before inoculation, pH value was set to 6 at the beginning of the
190 fermentation and then it varied freely through the fermentation according to the acids
191 generation and subsequent consumption.

192 Gas stripping fermentations were carried out by the application of four nitrogen
193 stripping pulses of 1.5 vvm for 30 min at 25, 46, 51 and 118 h of fermentation,
194 respectively. Schott bottles were adapted with a gas sparger in order to optimize the
195 droplet distribution inside the bioreactor. A vegetable oil base mixture composed of
196 sunflower oil (90 % v/v) and a C₁₂ based Guerbet alcohol (2-Butyl -1-Octanol, 2B1O)
197 (PubChem CID: 19800) was tested as extractive agent in biphasic fermentations. The
198 ratio organic: aqueous phase was fixed at 1:1 (v/v). Low agitation allowed maintaining
199 a clear separation between phases in all fermentations. The bioreactor was adapted with
200 a sampling device for both aqueous and organic phases. The integrated system
201 (GS+LLE) was carried out at the operating conditions described above. Two duplicates
202 of each system were carried out at the same time. Only one of the duplicates was
203 sampled periodically and the other one was kept closed and not sampled until the end of
204 fermentation.

205 ***2.5 Analytical methods***

206 Samples were centrifuged at 4000 rpm for 20 min, therefore in the supernatant were
207 measured: pH (Toledo mettle; Columbus OH-USA), glucose consumption (YSI 2700
208 Select; Yellow Springs OH -USA). IBE products in the aqueous phase were quantified
209 by Gas Chromatography (Agilent Technologies 7890B GC System; Santa Clara CA-
210 USA), equipped with an Agilent VF-624ms column using He (PubChem CID: 23987)
211 as the carrier gas and a flame ionization detector (FID), temperature of the oven was 35
212 °C and it was increased at a gradient of 2 °C/min until 60 °C and subsequently increased
213 up 15 °C/min to 200 °C for 10 min. Alcohols were quantified in the organic phase with
214 a back flush (reversal flow) system consisting of an 10 m HP-PONA precolumn
215 (Agilent Technologies) with a pressure ramp of 38.1 psi for 30, 40, 45 and 50 min
216 followed by 5 psi/min until 10 psi, and 0 psi during 0-10 min. The HP-PONA
217 precolumn was connected to a 45 m HP-PONA column (Agilent Technologies) with a
218 pressure of 34.5 psi and a temperature ramp of 35 °C for 10 min, 1.1 °C/min until 130
219 °C, then 15 °C/min until 280 °C and finally to 280 °C for 0-15 min. The flame
220 ionization detector temperature was at 300-310 °C. Both columns used He as the carrier
221 gas. In aqueous samples, free growing cell evolution was estimated measuring optical
222 density at 600 nm (Spectrophotometer Shimadzu UV-1240; Kyoto, Japan).

223

224 **2. Results and Discussion**

225 ***3.1 Butanol stripping rate***

226 The butanol stripping rate was investigated at three different gas flow rates: 0.25, 1 and
227 1.5 L/min (corresponding to 0.5, 2 and 3 vvm, respectively). The bioreactor was filled
228 with synthetic aqueous solution representing the final IBE fermentation composition
229 (11, 5 and 2 g/L for butanol, isopropanol and ethanol, respectively) at the beginning of

230 each experiment. Temperature was first raised up to 37 °C inside the reactor and stirring
 231 rate was fixed at 300 rpm. Aqueous solution was sampled each 2 h for calculating
 232 alcohol stripping rate. At the same time intervals, condensates were collected in the cold
 233 traps for further analyses and quantification. Experimental obtained data were first
 234 compared with SIMULIS thermodynamic modeling results for alcohols stripping rate
 235 inside the bioreactor (butanol aqueous concentration evolution in **Fig. 2**). It can be seen
 236 that thermodynamic prediction for aqueous butanol depletion rate is in good agreement
 237 with experimental data for 0.5 and 2 vvm. These results confirmed the absence of mass
 238 transfer limitation in the bioreactor under experimental conditions. In other words,
 239 thermodynamic controlled the process and therefore acquired data will give the
 240 maximal attainable butanol stripping rate or the minimal vvm that should be applied for
 241 a given extraction rate. Data obtained with 3 vvm showed that butanol was stripped
 242 slightly faster than thermodynamic prediction. This can be attributed to non-negligible
 243 physical entrainment of water droplets in outlet lines when high flowrates were applied.
 244 Based on these experimental results and previous discussion, Eq. A.7 was regressed for
 245 estimation of stripping rate constant (β , h⁻¹) at different conditions (**Table 1**) as follows:

$$\beta = \frac{\ln\left(\frac{C_{i,0}}{C_i}\right)}{t} \quad \dots\text{Eq. A.7}$$

247 Butanol and isopropanol have similar stripping rate constants (slightly higher for
 248 butanol), and they are systematically two times higher than ethanol stripping rate
 249 constant. These results are in agreement with Vrije *et al.*, 2013 [9], which studied the
 250 gas stripping with IBE model solutions at fixed vvm = 1 min⁻¹. Stripping rate from
 251 aqueous medium will depend not only on stripping coefficient (which encloses

252 thermodynamics – Henry coefficient- and mass transfer –KLG-) but also on
253 concentration ratio of alcohols in aqueous system [9, 11]. Stripping rate order has been
254 kept constant in our experiments: butanol was stripped higher than isopropanol, while
255 stripping rate of ethanol was lower. Relative stripping rate of alcohols is difficult to
256 predict as Henry coefficients from literature present high variability for these
257 compounds in such diluted solutions.

258 Alcohols stripping rates generally diminished with decreasing IBE concentrations in
259 aqueous solution (**Table 2**). In batch process fermentations, butanol sets the inhibition
260 threshold because it is the main inhibitory metabolite and its removal rate should be at
261 least equal or higher than the specific productivity of butanol in the bioreactor, in order
262 to avoid its accumulation. A macroscopic mode inspired in ABE literature was
263 developed (data not shown) in order to estimate instantaneous butanol productivity in a
264 batch fermentation. Maximal butanol productivity was close to 0.5 g/Lh after 30 h of
265 fermentation. These data were compared in **Table 2** and it was observed for each gas
266 flow rate applied (or vvm) a minimal butanol concentration in the aqueous phase inside
267 the bioreactor that it was needed in order to equalize butanol productivity and butanol
268 stripping rate. According to these results, a vvm=0.5 min⁻¹ would never be enough to
269 follow biological production in studied conditions (37 °C). Minimal concentration of 5
270 g/L of butanol inside the bioreactor must be reached before applying a gas stripping of
271 vvm=2 min⁻¹ in order to strip butanol as fast as its maximal productivity rate. Butanol
272 inhibition threshold imposes the maximal concentration that would be suggested in
273 order to maximize productivity during operation.

274

275 3.2 Selectivity

276 Selectivity is defined here as mass unity of stripped alcohol per mass unity of stripped
277 water in the gas outlet of the bioreactor. In a batch operation system, selectivity varies
278 as a function of water and inlet gas stripping composition, since the ratio water/alcohol
279 in the gas stream is governed by system thermodynamics. In this experimental work,
280 only end point selectivity is obtained, as a result of an overall mass balance of water and
281 alcohol in the system; it would be therefore a mean value corresponding to the whole
282 batch assay. In **Fig. 3** selectivity was calculated by thermodynamic simulation
283 (SIMULIS software, using specific in-house thermodynamic model) and was plotted as
284 a function of butanol concentration in the aqueous phase inside the bioreactor at fixed
285 operating conditions (37 °C and 2 vvm). Experimental data corresponding to the end of
286 the batch assay at 2 vvm (at minimal butanol concentration) were higher than the
287 estimated ones, since these data corresponded to the mean total selectivity considering
288 higher concentration of butanol in the aqueous phase from the beginning of the
289 experimental test, as it was stated previously.

290 Butanol selectivity by nitrogen stripping technique was low: which means that outlet
291 gas left the bioreactor with non-negligible water quantity (even if physical entrainment
292 was not considered here). This behavior will directly impact the operational cost of the
293 process and will determine the recovery system of alcohols in gas loop.

294 Stripping rate of water scarcely varied in function of butanol aqueous concentration
295 while stripping rate of butanol increased proportionally to its stripping coefficient and
296 the local aqueous butanol composition [8]. Then, the asymptotic diminution of the
297 process selectivity when the medium was increasingly diluted could be explained. These

298 results showed that gas stripping process became interesting only with a fixed butanol
299 concentration in the aqueous phase [11].

300 *3.3 Condensation rate and condensates composition*

301 In a gas stripping-fermentation coupled industrial process, not only stripping rate of the
302 inhibitory metabolite should be kept at least equal to its production rate inside the
303 bioreactor but also stripped alcohols should be fully recovered from the gas loop before
304 being recycled, in order to renew and maintain their stripping capacity through the
305 operation.

306 The experimental unit used in these experiments (**Fig. 1**) had two condensers in cascade
307 working at 4 and -15 °C, respectively. Almost the totality of condensates were
308 recovered from the first cold step at 4 °C. Only at the end of each batch assay (when
309 alcohol concentration was lower in the aqueous phase) a mass of condensates could be
310 quantified from cold step at -15 °C. Strong linearity existed between butanol
311 concentration in the condensates and butanol concentration in the aqueous phase (**Fig.**
312 **4**). Experimental data corresponding to GC analysis of condensates were obtained from
313 the batch assays carried out at $vvm= 2 \text{ min}^{-1}$ (represented in **Fig. 4**) experimental data
314 showed good agreement with the simulated ones obtained by SIMULIS model. Besides,
315 if the aqueous butanol concentration was higher than its limit solubility (7.7 % wt. at 20
316 °C) a phase demixing zone could force an additional separation of the condensate liquid
317 phase (simulated data **Fig. 4**). Moreover, this behavior has been already proved in ABE
318 fermentation-GS coupled technique [11]. Additionally, it would be possible to recover
319 one or two liquid phase alcohols in the condensates collector (cold trap at 4 °C) which
320 were highly concentrated (> 10-100 times the initial concentration in the bioreactor) but
321 the collected volume could represent only the 5% of bioreactor initial volume.

323 ***3.4 Suggested operation***

324 The first objective of the gas stripping recovery technique is the end product inhibition
325 alleviation by partial stripping of main inhibitory metabolites in a process. By means of
326 combined abiotic experimentation with synthetic fermentation broth and
327 thermodynamic simulation work, it has been proved that this technique becomes
328 interesting at higher alcohol concentration in aqueous phase (remained under inhibition
329 threshold). Indeed, not only butanol stripping rate was maximized when its
330 concentration was the highest in aqueous phase, but also selectivity of the process (g
331 alcohol stripped/ g water stripped) decreased asymptotically when the medium was
332 diluted. In this work, alcohol concentration in condensates from the outlet gas was
333 linearly dependent on the stripped aqueous phase concentration and above solubility
334 limit of butanol in water (~7.7 % wt. at 20 °C) where additional separation by demixion
335 zone appeared. From previous statements, it was suggested the application of gas
336 stripping technique for IBE fermentation in a pulse-mode (or intermittent mode): gas
337 stripping would be activated only when butanol attains a predefined concentration in
338 order to boost extraction performances.

339

340 ***3.5 Pulse gas stripping, extractive and integrated gas stripping-extractive coupling*** 341 ***fermentations***

342 IBE fermentations coupled to different separation techniques were carried out in batch
343 conditions. GS, LLE and hybrid GS-LLE system are compared to control fermentation
344 (no separation technique). For GS and GS-LLE system, pulse-mode operation for gas

345 injection (nitrogen) was applied at three predefined time intervals since aqueous butanol
346 was not known on real time.

347 For extractive fermentations, a vegetable oil based mixture composed of sunflower oil
348 (90% v/v) and a C12 based Guerbet alcohol (2 butyl-1octanol, 2B1O) was used as the
349 extractive agent. **Fig. 5** represents two discriminatory parameters in the performance of
350 IBE fermentation with GS and LLE coupling techniques: the first one represents the
351 glucose consumption and the second one the butanol concentration in aqueous phase.
352 Synergic effect was reached with GS-LLE coupling technique based on higher glucose
353 consumption rate and lower aqueous butanol concentration during the operation because
354 butanol extraction rate from aqueous phase was more increased than individual ISPR
355 techniques and control assays. Overconsumption of 23% glucose was observed when
356 GS or LLE performed individually; while hybrid integrated technique showed 45% of
357 overconsumption of glucose related to control fermentation. Total solvent
358 concentrations were not quantified during these experiences because gas phase was not
359 analyzed in these experiments. Nevertheless, total solvent IBE production could be
360 estimated and ranked from sugar consumption and constant IBE yield of fermentation
361 (0.35 g/g). These data are synthetized in **Table 3**. On the other hand, in **Fig. 5** it is
362 observed that bioactivity was stopped approximately at 60 h for fermentations
363 containing the extracting phase inside the bioreactor, even if non-inhibitory butanol
364 concentration (<4 g/L) was measured in the aqueous phase and remaining glucose
365 concentration could be quantified. This could be attributed to midterm toxicity of the
366 solvent used in LLE-fermentations towards the specific microorganism employed. This
367 needs to be further investigated. This solvent showed biocompatibility at the beginning
368 of the fermentation in previous screening work (data not shown). Glucose consumption

369 rate and biomass formation were enhanced during the first 50 h (**Fig. 5**), which means
370 that biocompatibility of this solvent is not an issue during the first part of the
371 fermentation. Kollerup and Daugulis (1985) [15] classified the modes of cell inhibition
372 in extractive fermentations into different mechanisms depending on the initial effect of
373 the solvent into the metabolic and enzymes activity during the fermentation. In our case,
374 middle term toxicity was observed with vegetable oil mixture and 2B1O, respectively.
375 These results should be confirmed in future experiments.

376 Respective theoretical equilibrium concentrations of butanol and isopropanol in organic
377 and aqueous phase are estimated from experimental partition coefficient of the
378 extracting solvent previously determined and considered organic and aqueous volumes
379 at each sampling time. These data were plotted in **Fig. 6** with experimental data
380 corresponding to the evolution and distribution of butanol and isopropanol
381 concentrations in aqueous and organic phases inside the biphasic bioreactor. Both series
382 data (experimental -lines- and theoretical equilibrium distribution -dots-) were in
383 agreement so it could be concluded that GS-LLE system behaved as an equilibrium
384 stage in the experimental conditions studied for this particular set up configuration. In
385 other words, the mass liquid-liquid transfer rate was higher than the main metabolite
386 production rate. This behavior has already been observed for ABE fermentation [12].
387 On the other hand, butanol in organic phase in hybrid GS-LLE system seemed to be
388 partially stripped when compared to LLE single technique, since its concentration
389 slightly decreased from 50 h of fermentation. This can be explained by a phase transfer
390 phenomena from organic phase to aqueous phase while aqueous phase has been
391 stripped. Butanol in aqueous phase forms butanol/water azeotrope which is more
392 volatile than butanol and water alone. When gas stripping technique was applied, the

393 azeotrope was stripped because of its lower boiling point than water. Interestingly,
394 organic phase acted in this case as a butanol storage to both limit and control butanol in
395 aqueous phase, while gas stripping technique removed the extra water concentration.
396 The concomitant gas stripping with this configuration has already been mentioned [14].
397 The authors applied in their system oleyl alcohol as an extracting agent, which has four
398 times higher partition coefficient for butanol than the vegetable oil based mixture used
399 in this work. As a result, it had a positive effect for liquid extraction but made it more
400 difficult for solvent regeneration (more alcohol affinity in organic phase). Continuous
401 gas stripping technique was applied directly in the organic phase system from 48 h of
402 fermentation in order to boost final glucose consumption, while pulse-fedbatch gas
403 stripping was applied in this work from the early step of fermentation in order to
404 increase glucose consumption and production rates.

405

406 **3. Conclusions**

407 Gas stripping was studied in abiotic representative system for (A)IBE batch
408 fermentation. High flow rates ($v_{vm} > 2 \text{ min}^{-1}$) were needed in order to achieve
409 stripping rate of butanol higher than biological production rate. A pulse-GS mode was
410 suggested, allowing to maximize selectivity (g butanol/g water stripped) and alcohol
411 concentration in condensates. Combined separation techniques (pulse –GS-LLE) were
412 then applied to batch fermentations. A synergic effect appeared when using the
413 integrated technique, resulting in the highest butanol extraction rate and productivity.
414 Moreover, biphasic bioreactor acted as an equilibrium step and both phases were
415 stripped concomitantly.

416

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480 **Legends**

481

482 **Table 1.** Stripping rate constant (β) at 0.5, 2 and 3 vvm for synthetic IBE aqueous
483 solutions.

484

485 **Table 2.** Butanol removal rate from model.

486

487 **Table 3.** Estimated IBE concentration (g/L) in aqueous phase under ISPR (*In situ*
488 *product recovery* technique): gas stripping (GS), liquid-liquid extraction (LLE) and
489 coupling GS-LLE fermentations.

490

491 **Fig.1.** Experimental system for abiotic IBE gas stripping used in this study.

492

493 **Fig.2.** Butanol aqueous concentration evolution (experimental data: dots,
494 thermodynamic simulation: lines) vvm=0.5 min⁻¹ (■); vvm=2 min⁻¹ (▲); vvm=3 min⁻¹
495 (●).

496

497 **Fig.3.** Selectivity variation of the batch abiotic gas stripping process, experimental data
498 (■), thermodynamic simulation (line).

499

500 **Fig.4.** Condensates composition at 4 °C versus aqueous butanol concentration,
501 experimental data (▲), total condensate simulation (line), demixed condensates
502 simulation (o).

503

504 **Fig.5.** Glucose, butanol concentration and absorbance in aqueous phase with ISPR
505 techniques. (▲GS, ◆LLE, ●GS+LLE, x control).

506

507 **Fig.6.** Total butanol and isopropanol (g/L) concentration in aqueous and organic phases
508 with LLE and GS-LLE coupling fermentations. Calculated butanol (○); calculated
509 isopropanol (Δ); experimental butanol (●); experimental isopropanol (▲).

510

511

512

513 **Tables**

514

515 **Table 1.** Stripping rate constant (β) at 0.5, 2 and 3 vvm for synthetic IBE aqueous
516 solutions.

Alcohols	Stripping rate constant, β (h^{-1})		
	vvm 0.5 min^{-1}	vvm 2 min^{-1}	vvm 3 min^{-1}
Butanol	0.042±0.009	0.107±0.009	0.184±0.010
Isopropanol	0.040±0.01	0.098±0.009	0.170±0.002
Ethanol	0.021±0.005	0.053±0.006	0.108±0.010

517

518

519 **Table 2.** Butanol removal rate from model.

Butanol concentration in aqueuse phase (g/L)	Butanol stripping rate (g/Lh)		
	vvm=0,5 min^{-1}	vvm= 2 min^{-1}	vvm=3 min^{-1}
0	0	0	0
2	0.08	0.21	0.37
4	0.17	0.43	0.74
6	0.25	0.64	1.10
8	0.34	0.86	1.47
10	0.42	1.07	1.84

520

521 **Table 3.** Estimated IBE concentration (g/L) in aqueous phase under ISPR (*In Situ*
522 *Product Recovery* technique): gas stripping (GS), liquid-liquid extraction (LLE) and
523 coupling GS-LLE fermentations.

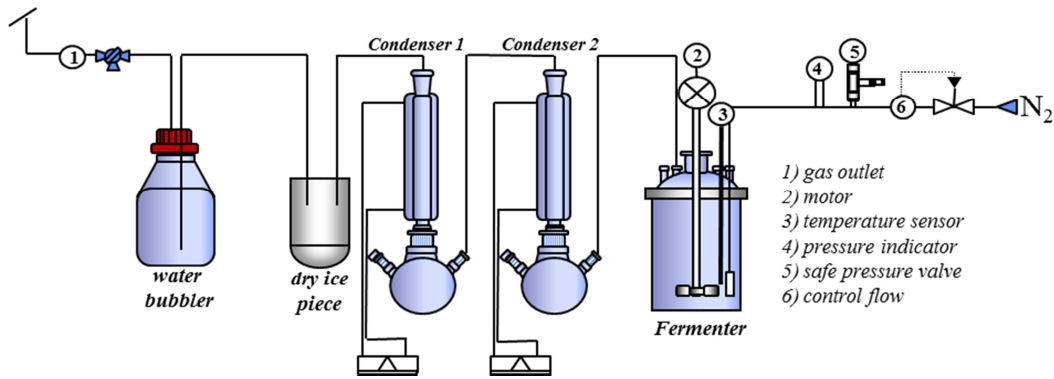
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	Estimated IBE concentration (g/L)
Control	12.41 ± 0.38
GS	13.01 ± 1.61
LLE	13.82 ± 0.65
GS+LLE	16.46 ± 0.24

525

526 **Figures**

527

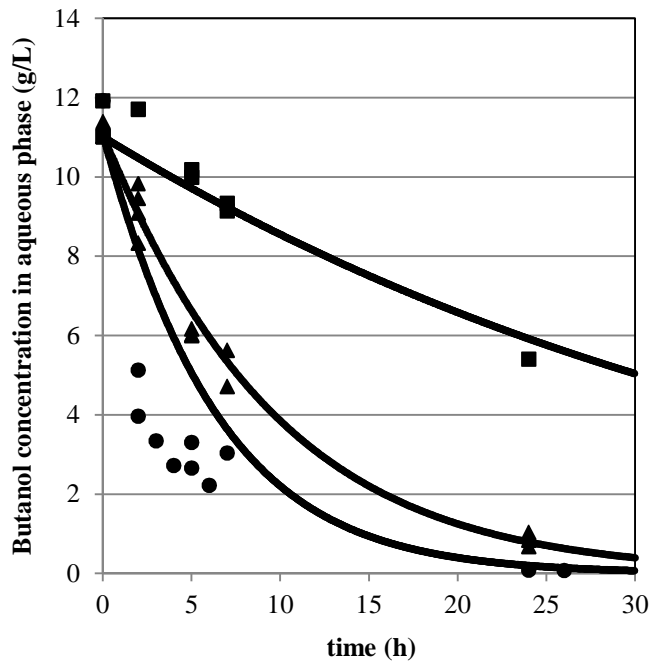


528

529 **Fig. 1.** Experimental system for abiotic IBE gas stripping used in this study.

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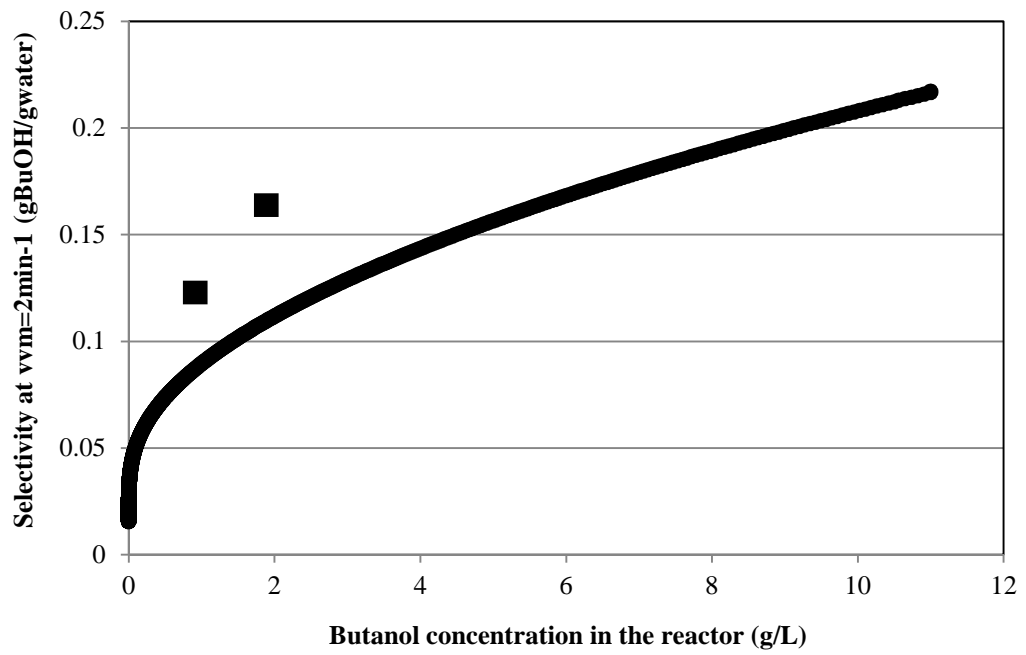
532

533 **Fig. 2.** Butanol aqueous concentration evolution (experimental data: dots,

534 thermodynamic simulation: lines) vvm=0.5 min⁻¹ (■); vvm=2 min⁻¹ (▲); vvm=3 min⁻¹

535 (●).

536



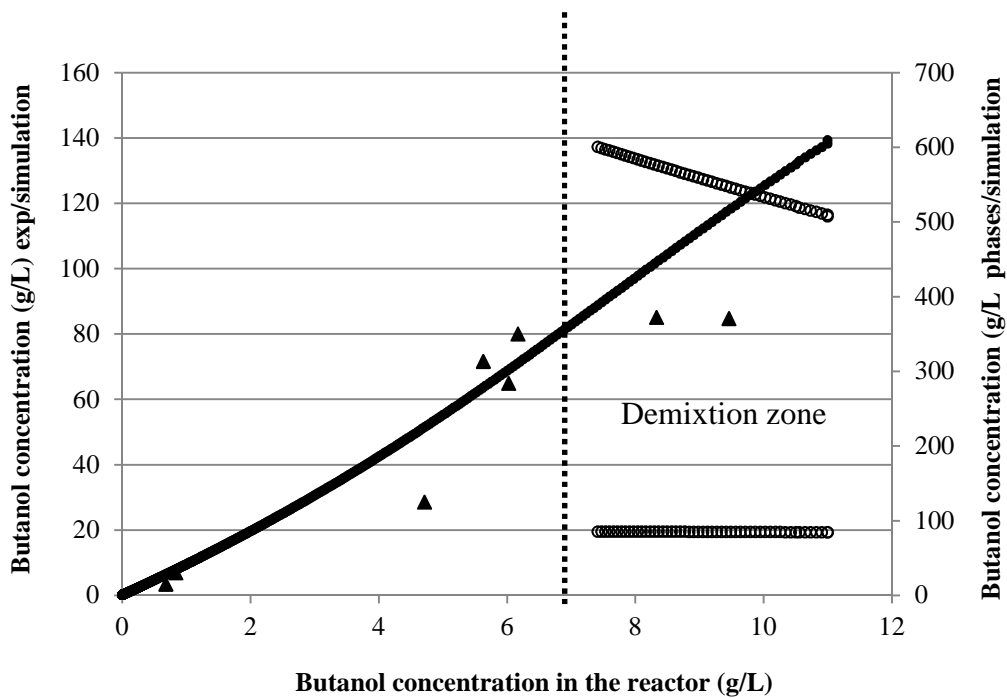
537

538 **Fig. 3.** Selectivity variation of the batch abiotic gas stripping process, experimental data

539 (■), thermodynamic simulation (line).

540

541



542

543 **Fig. 4.** Condensates composition at 4 °C versus aqueous butanol concentration,
 544 experimental data (▲), total condensate simulation (line), demixed condensates
 545 simulation (o).

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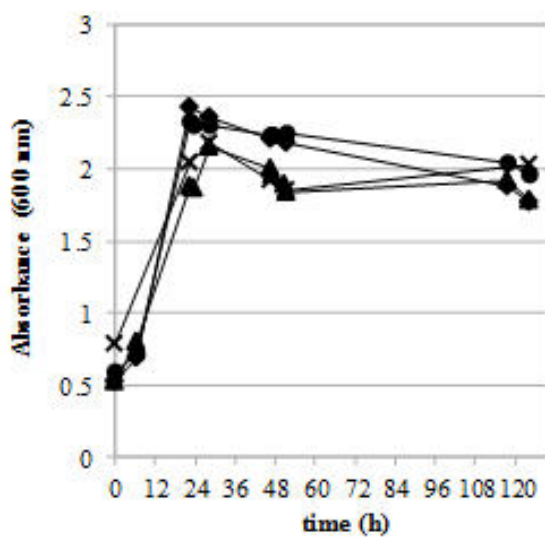
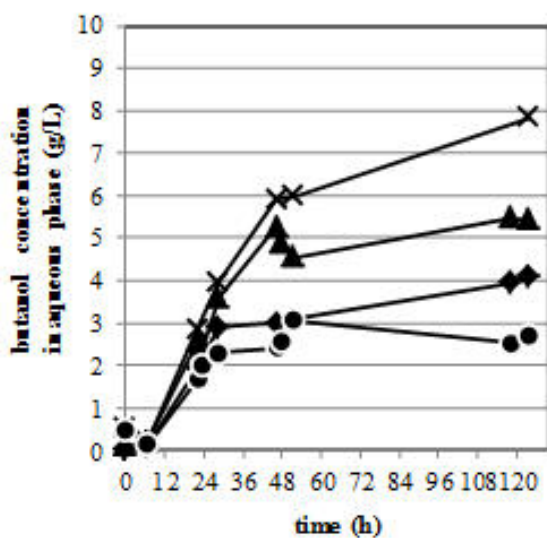
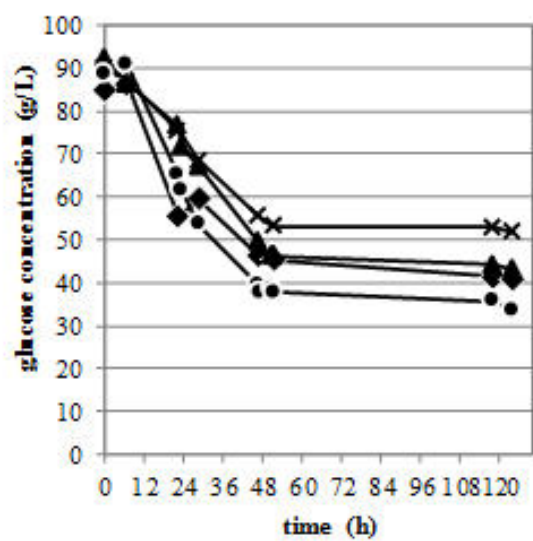
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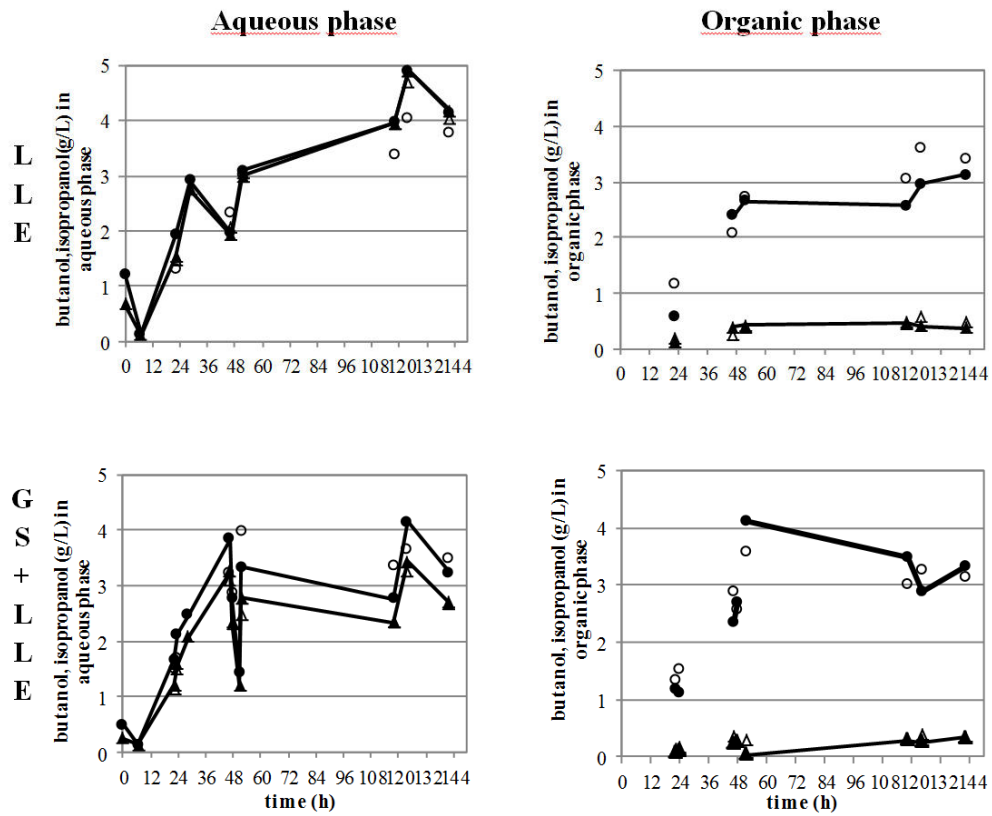
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555
 556 **Fig. 5.** Glucose, butanol concentration and absorbance in aqueous phase with ISPR
 557 techniques. (▲ GS, ◆ LLE, ● GS-LLE, x control).



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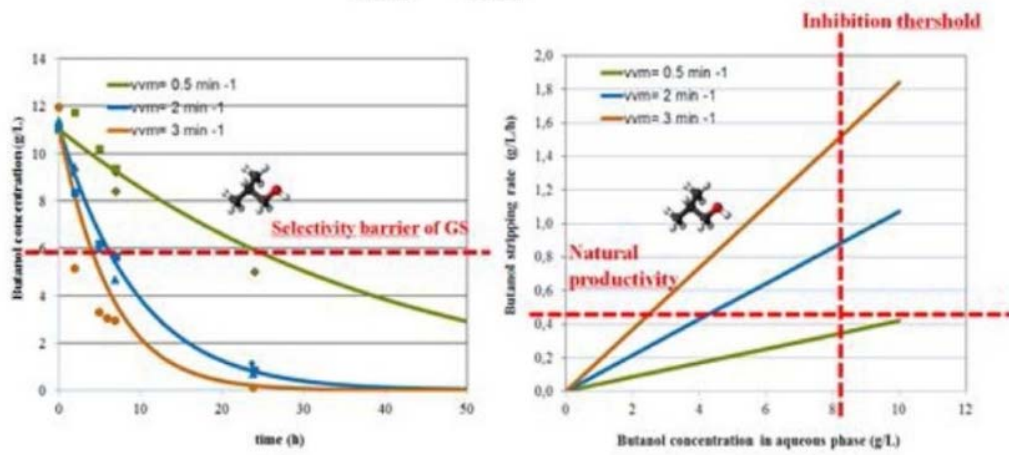
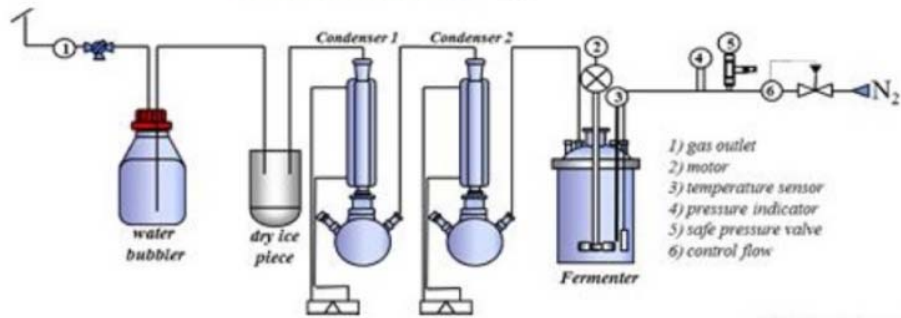
560 **Fig. 6.** Total butanol and isopropanol (g/L) concentration in aqueous and organic phases

561 with LLE and GS-LLE coupling fermentations. Calculated butanol (○); calculated

562 isopropanol (△); experimental butanol (●); experimental isopropanol (▲).

563

Abiotic system



Integrated GS+LLE



LLE w/v: 1_{aq}:1_{org}

GS: pulses
(15 min, 1.5 vvm)
t= 46h, 51h, 118h

