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1 **Development of a model for the alcoholic fermentation of cocoa beans by a *Saccharomyces***  
2 ***cerevisiae* strain**

3 **Christelle Kouamé<sup>1,2</sup>, Gérard Loiseau<sup>2\*</sup>, Joël Grabulos<sup>1,2</sup> Renaud Boulanger<sup>1,2</sup>, Christian**  
4 **Mestres<sup>1,2</sup>**

5 1 CIRAD, UMR QualiSud, F-34398 Montpellier, France

6 2 QualiSud, Univ Montpellier, CIRAD, Montpellier SupAgro, Univ Avignon, Univ Réunion, Montpellier, France

7 \* Correspondance: [gerard.loiseau@supagro.fr](mailto:gerard.loiseau@supagro.fr); Tel.: +33-04-67-61-57-13

8 **Abstract:**

9 The aromatic quality of chocolate requires the use of cocoa with high aromatic potential, this being  
10 acquired during the fermentation of cocoa beans. Traditional fermentation is still often carried out  
11 on a small scale with wild strains of yeasts and acetic bacteria and under poorly controlled  
12 conditions leading to cocoa quality ranging from best to worst. This study is the first part of a  
13 project aiming to control quality of cocoa to produce high aromatic quality chocolate by using a  
14 mixed starter of selected strains of yeast and acetic bacteria and by controlling the conditions of  
15 fermentation. To achieve this objective, a mathematical model of the alcoholic fermentation of  
16 cocoa beans has been developed. The growth, glucose consumption and ethanol production of  
17 *Saccharomyces cerevisiae* LM strain in synthetic broth were modelled for the most important  
18 intrinsic (pH, glucose, ethanol, free nitrogen and oxygen levels) and extrinsic (temperature, oxygen  
19 level) fermentation parameters. The model was developed by combining the effects of individual  
20 conditions in a multiplicative way using the gamma concept. The model was validated in liquid  
21 synthetic medium at two different inoculation levels  $10^4$  and  $10^6$  CFU/mL with an increase in  
22 temperature that recorded during spontaneous fermentations. The model clearly shows that the level  
23 of inoculation and the speed of the increase in temperature clearly drive yeast growth, while other

24 factors including pH and ethanol, free nitrogen and oxygen levels have no significant impact on  
25 yeast growth.

26 **Keywords:** yeast; starter; chocolate; gamma concept; predictive microbiology

27

## 28 1. Introduction

29 Flavor is the essential attribute of the quality of chocolate. It is directly linked to that of the raw  
30 cocoa beans that depends essentially on the post-harvest processing of beans, namely fermentation  
31 and drying. Post-harvest processing is performed on-farm or in nearby small-scale processing units  
32 and is poorly controlled leading to variable and/or poor quality cocoa beans (Aprotosoie et al.,  
33 2016). Obtaining good quality marketable raw cocoa beans requires good agricultural practices  
34 (selection of plants, selection of cocoa pods/beans, maintenance of fermentation equipment,  
35 removal of the placenta) and good fermentation practices. Fermentation indeed plays a key role in  
36 developing chocolate flavor precursors (Afoakwa et al., 2008; Beckett, 2003; Camu et al., 2008;  
37 Owusu, 2010; Pereira et al., 2017; Ravelomanana et al., 1985; Schwan and Wheals, 2004). Before  
38 fermentation, cocoa beans are removed from the pod. They are surrounded by a white mucilaginous  
39 pulp; rich in pectin, citric acid and sugars (Pettipher, 1986). Generally, in Côte d'Ivoire, the beans  
40 are piled in heaps or placed in wooden boxes covered with banana leaves. The cocoa beans then  
41 begin to ferment. Fermentation occurs spontaneously and usually lasts from five to seven days. It  
42 has two main phases. The first is anaerobic and lasts an average of two days during which yeasts  
43 convert the sugars in the pulp into ethanol by alcoholic fermentation with an accompanying slight  
44 increase in the temperature that can reach 35 °C. At the same time, the pulp is hydrolyzed; this  
45 allows the entry of air into the mass, creating aerobic conditions maintained by periodic turning of  
46 the heaps of beans that favor the start of the second phase. During the second phase, oxygen is  
47 consumed by acetic bacteria to oxidize ethanol into acetic acid, which can be over-oxidized to  
48 carbon dioxide and water. As these reactions are exothermic, the mass temperature increases to

49 reach or even exceed 50 °C. At the same time, ethanol and acetic acid diffuse in the beans leading  
50 to the death of the embryo but also to biochemical changes in the cotyledons such as the reduction  
51 of the sapid compounds responsible for bitterness and astringency, changing the color of the beans  
52 and the production of the precursors of the chocolate flavor (Kadow et al., 2013; Trognitz et al.,  
53 2013). The presence of a large lactic population during the fermentation of cocoa beans has been  
54 reported for many years. In addition, some authors identify a lactic phase, and lactic acid bacteria  
55 would play a relevant positive or negative role. Overall, if the yeasts *S. cerevisiae* and *H. opuntiae*,  
56 the lactic acid bacteria *L. fermentum* and the acetic acid bacteria *A. pasteurianus* lead the  
57 fermentation, good marketable beans are generally obtained (Camu et al., 2008, 2007; Daniel et al.,  
58 2009; Nielsen et al., 2007; Papalexandratou et al., 2011; Papalexandratou and De Vuyst, 2011). For  
59 these authors, lactic acid bacteria by consuming more fructose than glucose and citric acid to  
60 produce lactic acid, acetic acid, ethanol and mannitol would be essential for the proper progress of  
61 acetic fermentation and the aromatic quality of fermented beans. However, for Gálvez et al. (2007)  
62 and Ho et al. (2015), lactic acid bacteria would compete for glucose with yeasts which would lead  
63 to poorly ethanolic fermentation of beans and to beans with rich lactic acid content; this excessive  
64 acidification can depreciate chocolate quality.

65 Inoculation with selected microorganisms has long been practiced to control the quality of  
66 fermented foods including wine, beer and bread (Leroy and De Vuyst, 2004). Recently several  
67 authors tested selected starters for the fermentation of cocoa beans. Inoculated fermentation at  
68 laboratory or pilot scale levels revealed the potential of this approach. The inoculation levels are  
69 generally  $10^5$  or  $10^6$  CFU/g for yeasts. These levels lead to regular fermentation and the  
70 development of desirable characteristics of the cocoa beans; higher inoculation levels would be  
71 prohibitive for large-scale commercial use. Inoculated fermentation could also reduce the  
72 fermentation period to between three and five days instead of five to seven days for natural  
73 fermentation (Lefeber et al., 2012; Meersman et al., 2015; Moreira et al., 2017; Sandhya et al.,

2016; Schwan, 1998). The quality of the inoculated beans was generally better than the quality obtained from spontaneous fermentation. Leal et al. (2008), during controlled fermentation of 45 kg cocoa beans inoculated with a strain of *K. marxianus* with high pectinolytic activity observed that this strain was present throughout the fermentations characterized by a faster and more intense pulp hydrolysis and a higher (+2°C) increase in temperature of beans mass. Inoculated cocoa beans showed a higher level of cotyledonary storage proteins degradation, a lower level of titratable acidity and a better cut-test score, indicating a higher proportion of well fermented beans. Sensorial analysis of the chocolate produced from cocoa beans fermented with *K. marxianus* gives better results in terms of the flavor attributes and global acceptability. Crafacck et al. (2013) used a mixed starter culture composed of *P. kluyveri* or *K. marxianus* associated with *Lb. fermentum* and *A. pasteurianus*. During fermentations of 20 kg of cocoa beans inoculated with a mixed starter a faster consumption of citric acid was observed compared to spontaneous fermentation, whereas no major differences in the pulp concentrations of acetic acid, lactic acid and mannitol were observed between the inoculated and spontaneous fermentations. After sensory analysis, chocolate produced from inoculated beans was found to be sweeter, fruity and with a full cocoa flavor. However, tests are still required at industrial scale to define the required conditions for inoculated fermentation as the raw material is subject to great variability (the origin of the plants, selection of the cocoa pods/beans, their maturity, the delay between pod opening and fermentation) and the environmental conditions. Proper inoculation with different strains (at different levels and at different times) and fermentation thus remain difficult to achieve without understanding the trophic competition and the succession of microbial strains, and the impact of the changes in environmental conditions, especially temperature, on the development of the strains. A modeling approach could help take all these parameters into account and help control the fermentation process.

The use of tools and methods of predictive microbiology (Augustin, 2005) has enabled the development of mathematical models of microbial growth under different environmental

99 conditions, including the fermentation of Gowé, by Mestres et al. (2017) and Munanga et al. (2016).  
100 Modeling makes it possible to run simulations to evaluate the ability of the strains to control  
101 fermentation, to define the relative rates of the different microbial species that are used in the  
102 starter, the conditions of inoculation, and finally to define the parameters to control the process  
103 including temperature, pH, and substrate concentration (Arellano-Plaza et al., 2007; Mestres et al.,  
104 2017; Munanga et al., 2016).

105 This study was carried out as part of a project to control the production of premium chocolate  
106 characterized by floral and fruity notes. To achieve this goal, it was planned using a mixed starter  
107 consisting of selected strains of yeast and acetic bacteria and to develop a global model of the cocoa  
108 beans. The expected global model should be able to describe the growth, the use of substrates and  
109 the production of metabolites under various environmental conditions (temperature, pH, O<sub>2</sub>, organic  
110 acids content and ethanol) of strains constituting the starter. The global model will result of  
111 assembling two sub-models, that of alcoholic fermentation, and that of acetic fermentation. This  
112 global model will be built progressively, step by step, by progressively assembling sub-models  
113 recognized as being robust without their integration in the global model modifying the architecture  
114 of this one. The model of alcoholic fermentation developed here accounts for the most important  
115 intrinsic (glucose, ethanol, and nitrogen contents, pH) and extrinsic (temperature, oxygen level)  
116 fermentation parameters and can predict the growth of the yeast strain under the increasing  
117 temperature observed during cocoa bean fermentation in real conditions. This model provides  
118 insights into the parameters that drive the relay of the yeast population to that of acetic bacteria  
119 observed during cocoa beans fermentation.

## 120 **2. Material and methods**

### 121 **2.1. Microbial strain**

122 The *Saccharomyces cerevisiae* LM strain was provided by Lallemand S.A.S. (Blagnac, France)  
123 as active dry yeast at a viable cell concentration of  $10^{10}$  CFU/g. The yeast was stored at 4 °C.  
124 Before experiments, yeast was reactivated in sterile water at 35 °C for 30 minutes.

## 125 **2.2. Batch culture**

126 One liter Lambda Minifor fermenters equipped with a cold water condenser on air exit pipes.  
127 (LAMBDA Instruments GmbH, Baar-Switzerland), were used. Agitation, temperature, pO<sub>2</sub>,  
128 airflow, and pH were monitored and controlled. 1 M hydroxide sodium and 0.5 M sulfuric acid was  
129 used to adjust the pH. Yeast growth was monitored using an infrared in-line turbidity sensor (Optek  
130 FC20-ASD19-, Elscolab, Arcueil, France).

## 131 **2.3. Microplate assays**

132 Culture tests were carried out with a Bioscreen C MBR (Labsystems, Helsinki, Finland), in  
133 sterile microplates under controlled stirring and temperature (30 °C). Turbidity was measured at  
134 wavelength 600 nm. The growth rate was determined according to Membré et al. (2002).

## 135 **2.4. Plate counting methods**

136 Tenfold serial dilution of samples (0.1 mL) was carried out in 9 g/L NaCl water and plated on  
137 Sabouraud agar (Biokar-diagnostics, Beauvais, France). Plates were incubated at 30 °C for 48 h.  
138 Counts are based on triplicate plating.

## 139 **2.5. Chemical analyses**

140 Glucose, ethanol and glycerol contents were measured by HPLC (Dionex Ultimate 3000,  
141 Thermo Electron SAS, Courtaboeuf, France) with separation on an Aminex HPX87H column  
142 (Biorad, Hemel Hempstead, UK) and detection with both refractive-index and ultraviolet detectors  
143 (210 nm). Elution was performed at 30 °C with 5 mM sulfuric acid at a flow rate of 0.6 mL/min.

## 144 **2.6. Modeling**

### 145 **2.6.1. Primary model**

146 The logistic model with delay and rupture (Rosso, 1995) was used to describe microbial  
 147 growth, according to Equation (1).

$$148 \quad \frac{dNt}{dt} = \begin{cases} 0 & , t \leq \lambda \\ \mu_{max} Nt \left(1 - \frac{Nt}{N_{max}}\right) & , t > \lambda \end{cases} \quad (1)$$

149 where  $\lambda$  is the lag phase (h),  $\mu_{max}$  the maximum **specific** growth rate ( $h^{-1}$ ),  $Nt$  and  $N_{max}$  (CFU/mL) the  
 150 microbial count at time t and at the stationary growth phase.

### 151 **2.6.2. Modeling the lag phase ( $\lambda$ )**

152 In our conditions, i.e. direct inoculation with the *Saccharomyces cerevisiae* LM strain in the  
 153 form of active yeast, the duration of the latency phase ( $\lambda$ ) in hours, increases linearly with  
 154 temperature (T, °C) according to empirical Equation (2).

$$155 \quad \lambda = a * T + b \quad (2)$$

### 156 **2.6.3. Secondary model**

157 The gamma concept secondary model (Zwietering et al., 1993) was used to describe the impact  
 158 of temperature, pH, and glucose, ethanol, nitrogen content on the growth rate ( $\mu_{max}$ ), according to  
 159 Equation (3).

$$160 \quad \mu_{max} = \gamma(T^{\circ}C) * \gamma(pH) * \gamma([Glucose]) * \gamma([Ethanol]) * \gamma([Nitrogen]) \quad (3)$$

161 with  $\gamma$  values ranging from 0 to 1.

### 162 ***Impact of temperature and pH on yeast growth***

163 The values of gamma factor for temperature and pH, namely the cardinal temperature and pH  
 164 model (CTPM), proposed by (Rosso et al., 1995), were calculated according to equation (4)

$$165 \quad CTPM_n(X) = \begin{cases} 0 & , X \leq X_{min} \\ \frac{(X-X_{max})(X-X_{min})^n}{(X_{opt}-X_{min})^{n-1} \{(X_{opt}-X_{min})(X-X_{opt})-(X_{opt}-X_{max})[(n-1)X_{opt}+X_{min}-nX]\}} & , X_{min} < X < X_{max} \\ 0 & , X \geq X_{max} \end{cases} \quad (4)$$



166 where  $CTPM_n$  values range between 0 and 1.  $X$  corresponds to temperature or pH and the indices  
167 min, opt, max, to the minimum, optimum and maximum values for temperature or pH.  $n$  is a shape  
168 parameter that equals 1 for pH and 2 for temperature.

169 Temperature test cultures were performed in a one-liter glass fermenter using yeast nitrogen  
170 base (YNB) without amino acids (Biokar-Diagnostics, Beauvais, France) supplemented with 20 g/L  
171 glucose at a controlled pH of 4. Temperatures from 10 °C to 45 °C were tested.

172 pH test cultures were carried out Bioscreen C microplates according to Membré et al. (2002), in  
173 YNB without amino acids supplemented with 20 g/L glucose. The pH was adjusted from 3 to 8 (in  
174 progressive steps of 0.5) by adding sterile 1 M sodium hydroxide and 0.5 M sulfuric acid. The  
175 experimental temperature was set at 30 °C and all the tests were conducted in duplicate.

#### 176 *Effect of glucose*

177 In agreement with the substrate inhibition model of Andrews (1968), the value of the gamma  
178 factor for glucose and was calculated according to equation (5).

$$179 \quad \gamma([S]) = [S] / (Ks + [S] + [S]^2/Ki) \quad (5)$$

180 where  $\gamma([S])$  is the gamma factor of glucose ranging between 0 and 1,  $[S]$  is the initial glucose  
181 concentration (g/L),  $Ks$  (g/L) is the saturation constant for glucose and  $Ki$ , the inhibition constant  
182 (g/L).

183 Glucose culture assays were performed in sterile Bioscreen C microplates at pH 4 and 30 °C, in  
184 YNB without amino acids, supplemented with concentrations of glucose ranging from 0 to 50 g/L.  
185 Two tests were performed per glucose concentration.

#### 186 *Nitrogen content on yeast growth rate*

187 In agreement with the Monod model (Liu, 2006), the value of the gamma factor for nitrogen  
188 was calculated according to equation (6).

189 
$$\gamma([S]) = [S] / (K_n + [S]) \quad (6)$$

190 where  $\gamma([S])$  is the gamma factor of nitrogen, ranging between 0 and 1,  $[S]$  is the initial nitrogen  
191 concentration (mg/L) and  $K_n$  (mg/L) is the saturation constant of the Monod model for nitrogen.

192 Nitrogen culture tests were carried out on sterile Bioscreen C microplates at pH 4 and 30 ° C, in  
193 YNB broth without amino acids and without ammonium sulfate added with 20 g / L of glucose and  
194 nitrogen at concentrations ranging from 0 to 20 mg / L by adding di ammonium sulfate salt.

### 195 *Effect of ethanol content on yeast growth*

196 The effect of ethanol on yeast growth was determined using equation (7) proposed by Coroller  
197 et al. (2005).

198 
$$\gamma([SI]) = 1 - \left( \frac{[SI]}{MIC} \right) \quad (7)$$

199 where  $[SI]$  is the ethanol concentration in g/L and  $MIC$  is the minimum inhibitory concentration of  
200 ethanol.

201 Ethanol culture assays were performed in sterile Bioscreen C microplates at pH 4 and 30 °C,  
202 using YNB broth without amino acids supplemented with 20 g/L of glucose and with ethanol  
203 concentrations ranging from 0 to 75 g/L, in duplicate.

### 204 *Kinetics of ethanol production*

205 The kinetics of ethanol production was directly proportional to yeast growth by equation (8),  
206 using the model of Luedeking and Piret in which maintenance energy is disregarded (Van Impe et  
207 al., 2005).

208 
$$\frac{d[EtOH]}{dt} = Y_{(EtOH/N)} * \mu N \quad (8)$$

209 where [EtOH] is the concentration of ethanol (g/L) and  $Y_{(EtOH/N)}$  is the yield of ethanol in relation to  
210 the yeast population (CFU/mL) and  $\mu$  is the specific growth rate ( $h^{-1}$ ). After integration, this  
211 equation gives:

$$212 \quad [EtOH] = Y_{(EtOH/N)} * (N_t - N_0) \quad (9)$$

213 Where  $N_0$  (CFU/mL) is the initial the population

### 214 **Glucose consumption**

215 Similarly, glucose consumption is directly proportional to the yeast population and glucose  
216 content can be modeled by equation (10).

$$217 \quad [Glucose] = [Glucose]_0 - Y_{(glucose/N)} * (N_t - N_0) \quad (10)$$

218 where  $[Glucose]_0$  is the initial glucose content and  $Y_{(glucose/N)}$ , is glucose consumption in relation  
219 to the yeast population.

### 220 **2.7. Effect of oxygen**

221 The effect of the oxygen concentration in the culture medium on the *Saccharomyces cerevisiae*  
222 LM strain growth was studied in one-liter Lambda glass fermenter containing YNB broth without  
223 amino acid supplemented with 20 g/L glucose. The pH was set at 4 and the temperature at 30 °C.  
224 Three levels of oxygenation (0, 4 and 8 mg/L) were tested.

### 225 **2.8. Validation of the yeast growth model**

226 The model was validated using the one-liter Lambda glass fermenter with YNB broth without  
227 amino acid supplemented with 60 g/L glucose, at pH 4. A temperature profile (from 32 °C to 45 °C)  
228 was programmed to follow as faithfully as possible the same increases in temperature as those  
229 recorded during spontaneous semi-industrial fermentation of cocoa beans in Côte d'Ivoire (Fig. 1).  
230 Two inoculation rates ( $10^4$  or  $10^6$  CFU/mL) were tested in duplicate. Infrared absorbance was

231 monitored throughout fermentation and samples were collected at hourly intervals to assess the  
 232 metabolites.

233

234

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236

237 **Table 1: Parameters of the growth model in YNB medium taken into account**  
 238 **and environmental conditions.**

Parameters	<ul style="list-style-type: none"> <li>• <math>\mu_{opt}</math></li> <li>• <math>t_{lag} (\lambda)</math></li> <li>• <math>T_{min}, T_{opt}, T_{max}</math></li> <li>• <math>pH_{min}, pH_{opt}, pH_{max}</math></li> <li>• <math>MIC_{EtOH}</math></li> <li>• <math>K_S \text{ glucose}</math></li> <li>• <math>Y_{gluc}/CFU/mL</math></li> <li>• <math>Y_{ethanol}/CFU/mL</math></li> </ul>
Environmental conditions	<ul style="list-style-type: none"> <li>• <math>[Glucose]_{initial} = 60 \text{ g/L}</math></li> <li>• <math>pH = 4</math></li> <li>• <math>PO_2 = 0 \text{ mg/L}</math></li> <li>• <math>Stirring = 3 \text{ Hz}</math></li> <li>• <i>Temperature steps:</i> <ul style="list-style-type: none"> <li>- from 0 h to 23 h : 32 °C to 36 °C</li> <li>- from 23 h to 42 h : 36 °C to 45° C</li> </ul> </li> </ul>

239

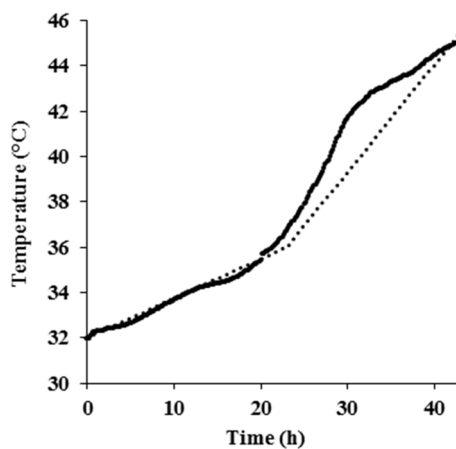
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246 **Figure 1: Temperature programmed for validation tests during model validation (dashed curve). Temperatures recorded during spontaneous fermentation in an ivory wooden box Coast, (solid curve).**

247

## 247 2.9. Data processing

248 The parameters of the models and their standard errors were adjusted with Origin® (OriginLab,  
249 Northampton, MA, United States) software using linear or non-linear curve fitting with the  
250 Levenberg and Marquardt iteration algorithm. Simulink® (MATLAB and Statistics Toolbox  
251 Release 2012b, The MathWorks, Inc., Natick, Massachusetts, United States.) software was used to  
252 build the global model of yeast growth.

## 253 3. Results and discussion

### 254 3.1. Modeling growth kinetics on a synthetic medium

255 Table 2 lists the values of growth kinetics parameters for the *Saccharomyces cerevisiae* LM  
256 strain. The cardinal temperature values and pH were estimated by adjusting the cardinal model (Eq.  
257 4) to the experimental data sets by minimizing the residual standard root mean square error  
258 (RMSE).

259 *Table 2: Secondary model parameter values ± standard errors for Saccharomyces*  
260 *cerevisiae LM strain*

261

Modeled Variable	Equation	Parameter	Value
$\mu_{max}$ (h <sup>-1</sup> )	(4)	pHmin	2.20 ± 0.14
		pHmax	7.92 ± 0.19
		pHopt	4.54 ± 0.14
		$\mu_{opt}$	0.46 ± 0.01
		Tmin (°C)	8.2 ± 2.5
		Tmax (°C)	41.8 ± 0.3
		T opt (°C)	33.3 ± 0.5
$\lambda(\mathbf{h})$	(2)	a (h.°C <sup>-1</sup> )	-0.61 ± 0.06
		b (h)	27.5 ± 1.8

$MIC$ (g/L)	(7)	[Ethanol]	$92.2 \pm 3.8$
$K_S$ (g/L)	(5)	[Glucose]	$0.27 \pm 0.02$
$K_i$ (g/L)			$209 \pm 43$
$K_N$ (mg/L)	(6)	[Nitrogen]	$0.04 \pm 0.01$
$Y_{(ethanol/N)}$ (mg/CFU)	(9)	Ethanol production ( $10^{-7}$ mg/CFU)	$1.59 \pm 0.08$
$Y_{(glucose/N)}$ (mg/CFU)	(10)	Glucose consumption ( $10^{-7}$ mg/CFU)	$3.34 \pm 0.10$

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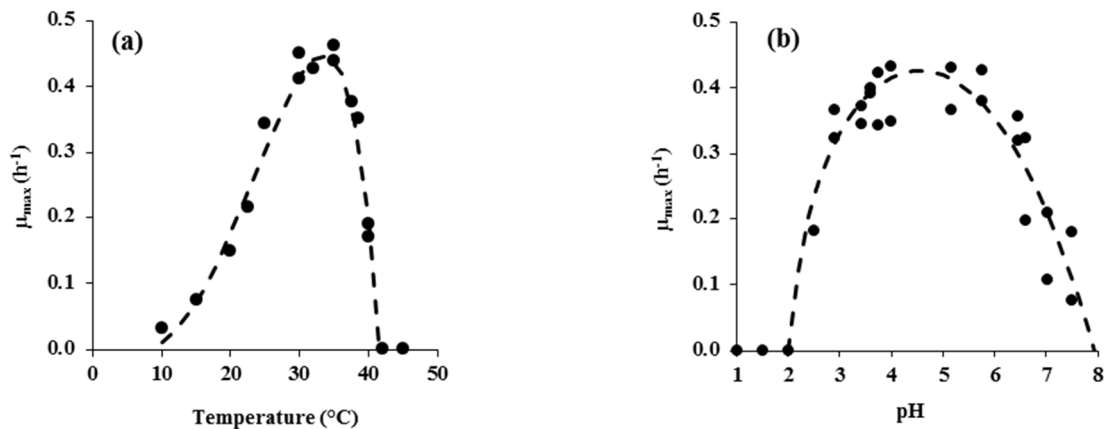
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**Figure 2: Effect of the temperature at pH =4 (a) and of the pH (b) at 30 °C on the maximum growth rate of *Saccharomyces cerevisiae* LM strain.**

273 As shown in Figure 2, the CTPM secondary model fit the experimental data well (RMSE 0.04).

274 The optimal growth temperature of the *Saccharomyces cerevisiae* LM strain was 33.3  $^{\circ}C$ , with  $\mu_{opt}$

275 of 0.46  $h^{-1}$  (Fig. 2a). These values are in agreement with those in the literature. Walsh and Martin

276 (1977) reported a  $T_{opt}$  range of 30  $^{\circ}C$  to 35  $^{\circ}C$  for *S. cerevisiae* and (Arroyo-López et al., 2009),

277 34.1  $^{\circ}C$ . According to Torija et al. (2003), the optimal growth temperatures for oenological yeast

278 strains are between 30  $^{\circ}C$  and 35  $^{\circ}C$ . The *Saccharomyces cerevisiae* LM strain appeared slightly

279 thermotolerant, with a  $T_{max}$  of 41.6  $^{\circ}C$ . In a comparative study of 27 strains of the genus

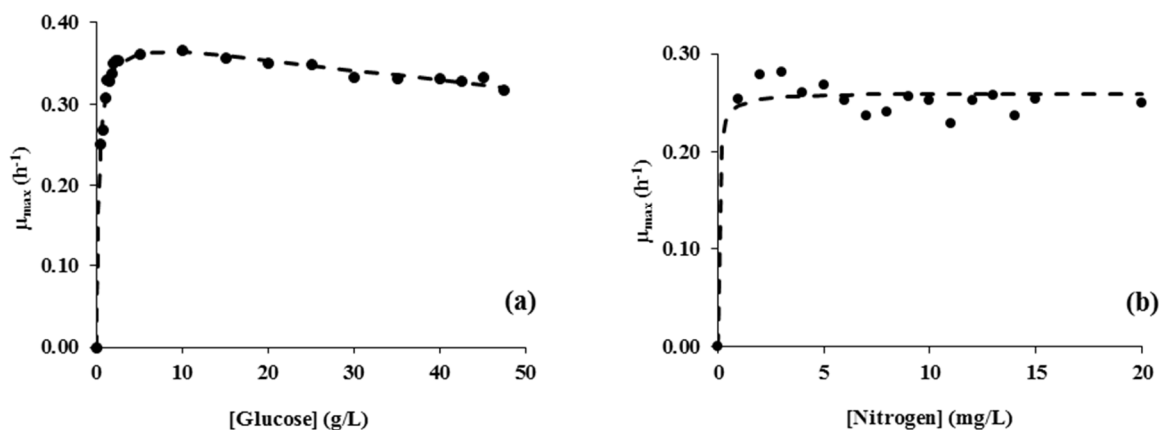
280 *Saccharomyces* and non-*Saccharomyces*, (Salvadó et al., 2011) reported that *S. cerevisiae* was the

281 most thermotolerant species with an optimum temperature close to 33 °C with a  $\mu_{max}$  of 0.45 h<sup>-1</sup> and  
282 a maximum temperature of 45 °C. Daniel et al. (2009) isolated from fermenting cocoa beans 91  
283 yeast strains able to grow between 25 °C and 35 °C, with the exception of a thermotolerant strain of  
284 *P. kudriavzevii* predominant in all fermentations able to grow at 45 °C. The relative  
285 thermotolerance of the *Saccharomyces cerevisiae* LM strain should allow it to dominate other  
286 strains of yeast and more particularly non-*Saccharomyces*, as suggested by Salvadó et al. (2011) as  
287 a temperature of 42 °C is usually reached after the beginning of the acetic fermentation. Indeed,  
288 Meersman et al. (2015) selected thermotolerant hybrids (that can grow at 42 °C) which partly  
289 contributed to their ability to dominate spontaneous pilot scale fermentations of cocoa beans.

290 The *Saccharomyces cerevisiae* LM strain appeared to be acid-tolerant with a  $pH_{opt}$  value of 4.5  
291 and an almost constant maximum growth rate of 0.32 to 0.38 h<sup>-1</sup> with an increase in pH from 2.9 to  
292 5.7. Absence of variation in the growth rate was also reported by for *S. cerevisiae*. Charoenchai et  
293 al. (1998) showed that a pH between 3 and 4 did not significantly affect the growth rate and cell  
294 biomass of wine yeasts. At pH 6.5 and above, there was a marked decrease in the growth rate of the  
295 *Saccharomyces cerevisiae* LM strain and at pH 8, growth stopped (Fig. 2b). During the alcoholic  
296 fermentation of cocoa beans, the pH value is fairly stable, i.e. between 3.5 and 4, at the end of the  
297 fermentation but sometimes reaches 5.5 (Gálvez et al., 2007). This suggests that pH has little effect  
298 on the growth of the *Saccharomyces cerevisiae* LM strain during fermentation.

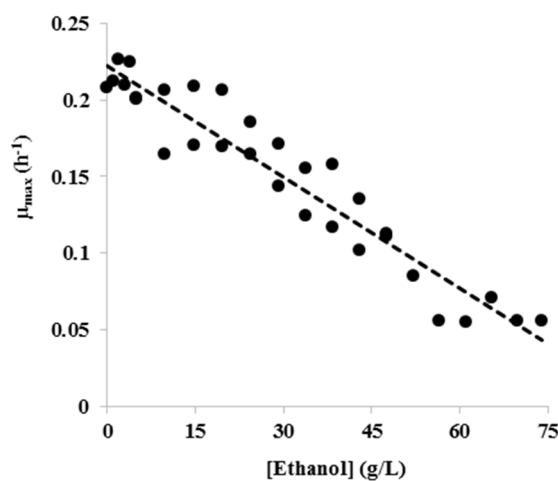
299 The  $K_s$  and  $K_i$  values for glucose are respectively of 0.27 g/L and 209 g/L were determined by  
300 adjusting the Andrew model (Eq. 5) to the experimental data (Fig. 3a). These results indicate that  
301 the growth of the *Saccharomyces cerevisiae* LM strain is very slightly impacted by the glucose  
302 concentration, which has been reported for glucose concentrations above 200 g/L by many authors  
303 including Charoenchai et al. (1998). Considering the low level of inhibition of the growth of the  
304 *Saccharomyces cerevisiae* LM strain for initial glucose concentrations close to 60 g / L, this effect  
305 will not be taken into account in the model.

306 Considering the impact of the concentration of mineral nitrogen on the growth rate of  
 307 *Saccharomyces cerevisiae* LM strain,  $K_n$  value for the Monod model (Eq. 6) was very low (fitted at  
 308 0.04 mg/L of free nitrogen, with a quite large uncertainty) (Fig. 3b). There are more free amino  
 309 acids in pulp and their content increase from 1 to 5 mg/g upon fermentation (Camu et al., 2007).  
 310 This shows that the amount of free nitrogen is not a limiting factor in the fermentation of cocoa  
 311 beans and this parameter will consequently not be taken into account in the model.



312  
 313 **Figure 3: Effect of the initial glucose concentration (a) and the nitrogen concentration (b)**  
 314 **on the growth rate of the *Saccharomyces cerevisiae* LM strain. Experimental data (black**  
 315 **dots) and fitted (dashed line).**

316  
 317 Equation (7) satisfactorily describes the decrease in  $\mu_{max}$  with an increase in the ethanol  
 318 concentration (Fig. 4).



326 **Figure 3: Inhibitory effect of ethanol on growth of the *Saccharomyces***  
***cerevisiae* LM strain. Experimental data (dots), fitted data (dashed line).**



327  
328 A  $MIC_{Ethanol}$  of 92.5 g/L was calculated for the *Saccharomyces cerevisiae* LM strain at 30 °C  
329 and pH 4. The *Saccharomyces cerevisiae* LM strain appears to be fairly resistant to ethanol. This  
330 result is consistent with the values reported in the literature by Rose (1980) ranging from 55 g/L to  
331 103 g/L, by Arroyo-López et al. (2010) values ranging from 95.6 to 141.4 g/L and by Antoce et al.  
332 (1997) values ranging from 88.8 to 107.7 g/L. Samagaci et al. (2014) isolated 18 out of 36  
333 pectinolytic strains isolated from cocoa able to grow at 30 °C at an ethanol concentration of 79 g/L  
334 and 7 strains able to grow up to 94.8 g/L. The maximum concentration of ethanol is less than 20 g/L  
335 (Camu et al., 2008, 2007; Lefeber et al., 2012; Schwan, 1998) during cocoa bean fermentation and  
336 is in any case much lower than the  $MIC_{Ethanol}$  of the *Saccharomyces cerevisiae* LM strain; the  
337 ethanol concentration consequently has very little impact on the growth of this strain. Ethanol can  
338 also have an antagonistic effect of temperature as high ethanol concentrations decrease the optimum  
339 growth temperature of yeast strains and increase the lag phase (Medawar et al., 2003). Sá-Correia  
340 and Van Uden (1983) reported that with an ethanol concentration close to 11% (v/v) the maximum  
341 growth temperature of a *S. cerevisiae* strain drops from 42 °C to 27 °C. Samagaci et al. (2014)  
342 reported that 36 yeast pectinolytic strains isolated from fermented cocoa beans showing both  
343 tolerance to alcohol (10-12% alcohol) and to temperature (up to 40 °C) failed to grow at only 35 °C  
344 with less than 8-10% alcohol. The maximum level of ethanol recorded during cacao fermentation  
345 being 5 to 10 times lower (less than 2%), the interaction between ethanol level and temperature can  
346 be neglected in the model.

347 The value of the maximum growth rate was between 0.41 and 0.43 h<sup>-1</sup> (results not shown) for  
348 dissolved oxygen concentrations 0; 4 and 8 mg/L. This factor was thus not taken into account in the  
349 model.

350 Figure 5 shows the consumption of glucose and the production of ethanol by the  
351 *Saccharomyces cerevisiae* LM strain. The mass yield of ethanol from glucose ( $Y_{p/s}$ ) is 0.46 g/g,

352 which is close to the theoretical value 0.51 g of ethanol per gram of glucose consumed. This  
353 theoretical yield is never reached as part of the glucose is used for the synthesis of biomass and  
354 secondary metabolites such as glycerol. In *S. cerevisiae* under oenological conditions, the observed  
355 yields are between 0.47 g/g and 0.48 g/g (Lonvaud et al., 2010). Similarly, Laopaiboon et al. (2007)  
356 found values ranging to 0.42 g/g to 0.48 g/g by fermenting sweet sorghum juice.

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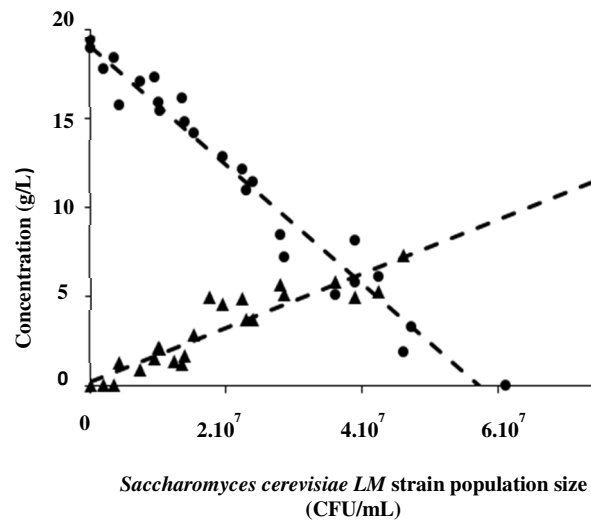
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365 **Figure 4: Relation between the concentration of glucose (●), ethanol (▲) and**  
366 **the yeast population size.**

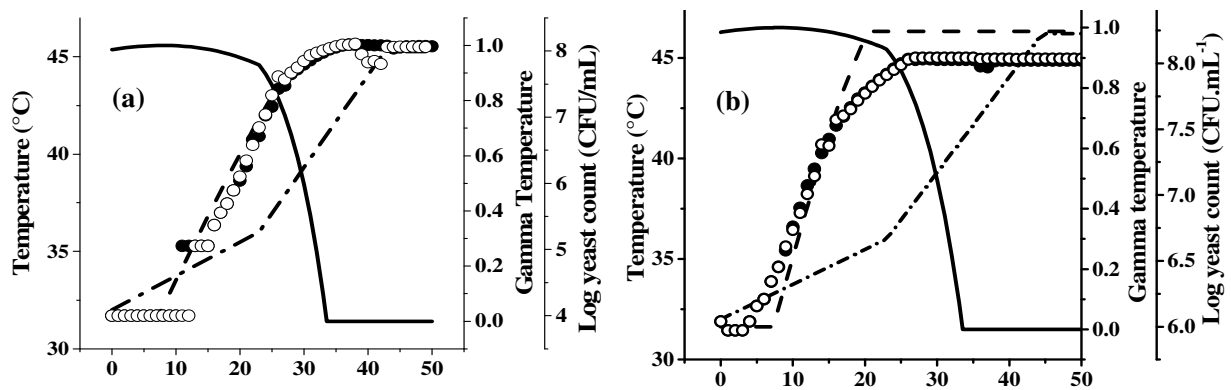
### 366 3.1. Validation of the model on synthetic medium

367 The global alcoholic fermentation model was developed using the values of the parameters and  
368 environmental conditions listed in Table 1. During the beans fermentation, some amount of  
369 glucose is also consumed by lactic acid bacteria, this is not included in this model, only built to  
370 predict the behavior of the yeast strain. The *Saccharomyces cerevisiae* LM strain was cultured  
371 in yeast nitrogen base without amino acids, supplemented with 60 g/L glucose at pH 4, in  
372 conditions approaching those observed at the beginning of cocoa bean fermentations  
373 characterized by absence of sucrose, but an equal amount of glucose and fructose (Ho et al.,  
374 2015; Papalexandratou et al., 2011). We only added glucose as the *Saccharomyces cerevisiae*  
375 LM strain can grow indifferently on glucose or fructose (results not shown). The temperature  
376 was regulated to mimic the measured profile observed in a wooden box during spontaneous  
377 fermentation in Côte d'Ivoire (Fig. 1) and close to the temperature profile observed in Ghana  
378 and the Dominican Republic (Camu et al., 2007; Gálvez et al., 2007; Pettipher, 1986). Two  
379 levels of inoculation, 10<sup>4</sup> and 10<sup>6</sup> CFU/mL, were tested to assess the influence of the initial  
380 yeast population on the course of the fermentation, given that this range is often used for the

381 inoculation of cocoa beans (Meersman et al., 2015; Ramos et al., 2014). In comparison, the  
 382 initial populations in natural cocoa fermentations are generally between  $10^3$  and  $10^7$  CFU/mL  
 383 (Ardhana and Fleet, 2003; Batista et al., 2015; Camu et al., 2007; Nielsen et al., 2007). It is  
 384 indeed likely that pod ripeness and postharvest pod age (due to transport and temporary  
 385 storage) determine the initial amounts of yeasts and LAB (Camu et al., 2007).

386 Figure 6 compares the experimental population measured during duplicated fermentation with  
 387 the population predicted by the model under the same conditions.

388



389

390 **Figure 5: *Saccharomyces cerevisiae* LM strain growth (○ rep1 and ● rep2); simulated**  
 391 **growth of yeasts (- - -); temperature profile (-.-.-) and  $\gamma$ -temperature (—) with**  
 392 **inoculation level at  $10^4$  CFU/mL (a) and  $10^6$  CFU/mL (b).**

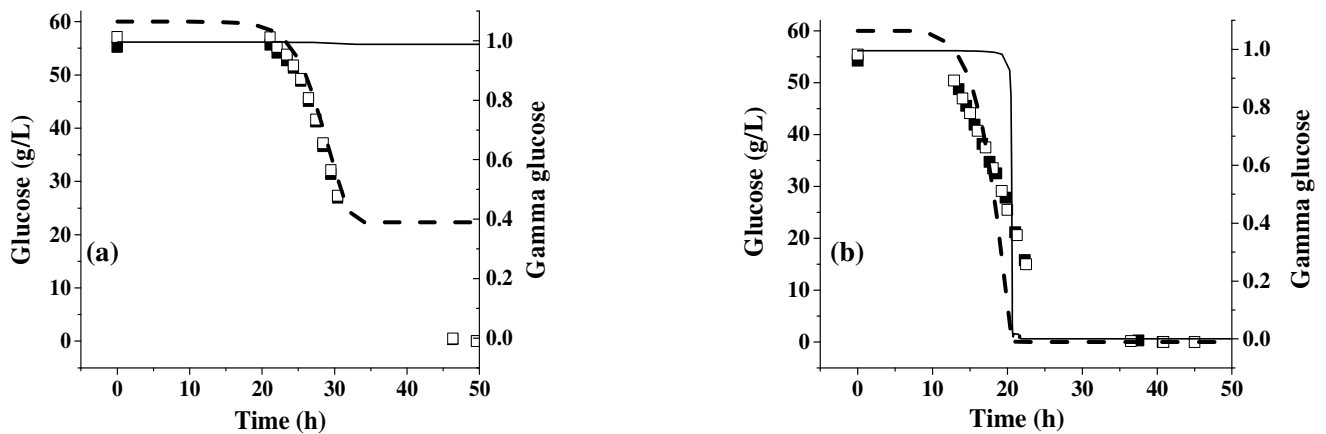
392 The model predicted a lag phase of nearly 7 h for both fermentations whereas it was longer,  
 393 close to 10 h with an inoculation level of  $10^4$  CFU/mL and shorter, close to 3 h with an inoculation  
 394 level of  $10^6$  CFU/mL. The lag phase is indeed always difficult to predict (Augustin and Carrier,  
 395 2000; Medawar et al., 2003; Munanga et al., 2016) and the model presented a RSE of 2.1 h for the  
 396 lag phase. This difficulty in measuring and predicting the lag phase has several possible  
 397 explanations, the heterogeneity of the population, the balance between the individual latency of  
 398 each cell and the latency of the global population, or the phenomena of intermediate latency  
 399 explained by Swinnen et al. (2004). Nevertheless, the differences between the predicted and  
 400 experimental populations are less than one log unit, and one can thus consider that the model

401 predicts the growth of the *Saccharomyces cerevisiae* LM strain fairly well. However, the model  
402 could be improved by a better determination of the lag phase.

403 With an inoculation level of  $10^4$  CFU / mL, the population increased for approximately 23 h  
404 and reached a stationary phase of 33 h, while the model predicts growth arrest at 33.6 h when the  
405 temperature reached 41 °C, i.e. the  $T_{max}$  of the *Saccharomyces cerevisiae* LM strain and when the  $\gamma$   
406 temperature became null (Fig. 6a). These results are consistent with those of Mensonides et al.  
407 (2002), who showed that increasing the culture temperature of a strain of *S. cerevisiae* from 28 °C  
408 to 43 °C resulted in a sharp decrease in the growth rate at 40 °C, a complete end to growth at 42 °C,  
409 and total loss of viability above 43 °C. On the other hand, with an inoculation level of  $10^6$  CFU/mL,  
410 the growth of the population stopped after 21 h according to the model and at 26 h in the  
411 experiment when the temperature was close to 37.5 °C, which corresponds to a  $\gamma$ temperature of 0.7  
412 (Fig. 6b). Consequently, another parameter was responsible for stopping growth.

413 Figure 7 shows the changes in glucose concentration predicted by the model and those  
414 observed during the growth of the yeast strain. With an inoculation level of  $10^4$  CFU/mL, the model  
415 predicted changes in the glucose concentration fairly well up to 33 h when the temperature reached  
416 41 °C. While the model predicted glucose consumption would stop at that time, in the experiment,  
417 the consumption of glucose continued after 33 h and was only complete after 48 h (Fig. 7a).

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With an inoculation level of  $10^6$  CFU/mL, the consumption of glucose by the *Saccharomyces cerevisiae* LM strain was well predicted by the model up to 18 h when the temperature approaches 36 °C. The model predicted total exhaustion of glucose at 20 h, while in the experiment glucose was exhausted at 25 h. During this slight discrepancy in the fermentation

**Figure 6: Changes in glucose concentration ( $\square$  rep1 and  $\blacksquare$  rep 2); simulated changes in glucose (- - -) and  $\gamma$ -glucose (—) during fermentation with inoculation levels of  $10^4$  CFU/mL (a) and  $10^6$  CFU/mL (b).**

LM strain, both in the model ( $\gamma$ -glucose of 0) and in real conditions.

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Concerning ethanol production, with an inoculation level of  $10^4$  CFU/mL the experimental and predicted data matched very well (Fig. 8a). With an inoculation level of  $10^6$  CFU/mL (Fig. 8b), the model predicted over-production of ethanol after 18 h (by 10 g/L), as it predicted over consumption of glucose. These discrepancies appear to be linked to slight overestimation of the growth and final population by the model ( $5 \cdot 10^7$  CFU/mL); the slight difference in the populations indeed represents an extra ethanol production of 9 g/L. Anyway, the final concentrations of ethanol in the validation tests were in the range of the peak ethanol production (between 10 and 24 g/L at 24-48 h) observed in natural or inoculated fermentations of cocoa (Crafack et al., 2013).

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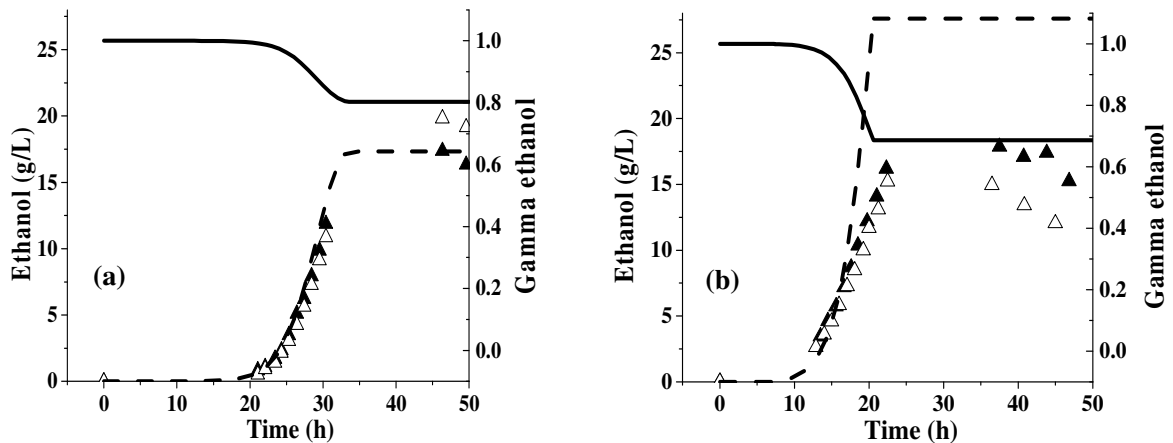
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**Figure 7: Changes in ethanol concentrations ( $\Delta$  rep1 and  $\blacktriangle$  rep 2); simulated changes in ethanol (- - -) and  $\gamma$ -ethanol (—) during fermentation with inoculation levels of  $10^4$  CFU/mL (a) and  $10^6$  CFU/mL (b).**

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The model predicts yeast growth, glucose consumption and ethanol production fairly well, except at the end of the exponential phase. This is particularly true with the lower inoculation level when the temperature reaches  $T_{max}$  of the strain. This can be attributed to the fact that we did not take decoupling between the production of ethanol and the consumption of glucose into account in the model, which reduces the yield of biomass and ethanol. This phenomenon is widely reported in the literature as being the result of the adaptation of yeast to heat stress; as a consequence, the energy required by the cell for maintenance increases due to the production of heat shock proteins and metabolites such as trehalose and glycerol (Aldiguier et al., 2004; Kukec et al., 2003; Mensonides et al., 2014; Omori et al., 1996). The concentrations of glycerol measured after total glucose consumption were 4.15 g/L and 4.76 g/L respectively for inoculation levels of  $10^4$  and  $10^6$  CFU/mL, i.e. nearly one quarter of the total amount ethanol produced. The ratio of glycerol produced to glucose consumed (60 g/L) was between 0.069 g/g and 0.079 g/g. This ratio is higher than that calculated for non-botrytized wines in which between 4 and 9 g/L of glycerol is produced from an initial total sugar contents in grape juice of 200 g/L giving a ratio of between 0.02 g/g and

462 0.045 g/g (Remize et al., 2000). To improve the accuracy of the model, it thus appears necessary to  
463 take into account the maintenance energy and the production of glycerol, particularly when the  
464 model temperature increases. This is all the more important as, according to Illeghems et al. (2016),  
465 glycerol could allow the growth of *A. pasteurianus* strains often found during the acetic  
466 fermentation of the beans.

467         The model and the validation sets, performed in conditions that mimic cocoa fermentation,  
468 clearly demonstrate that the inoculation level and the speed of increase of temperature drive the  
469 yeast growth while other factors such as pH and ethanol, free nitrogen and oxygen levels do not  
470 have a significant impact on yeast growth. If the inoculation level is high and/or the increase in  
471 temperature is low, glucose is completely exhausted after 1 day, while with a low yeast inoculation  
472 level and/or a rapid increase in temperature, yeast stops growing before the glucose is exhausted.  
473 Similar observations have been made for cocoa fermentation, i.e. complete exhaustion of glucose  
474 after high level of yeast inoculation and 1-2 day fermentation has been reported (Crafack et al.,  
475 2013; Lefeber et al., 2012; Sandhya et al., 2016) while residual glucose has been reported after  
476 natural fermentation or with a low level of yeast inoculation. The former leads to high transient  
477 ethanol content during cocoa fermentation, but to acidic, astringent chocolate with a quite low  
478 hedonic rating (Sandhya et al., 2016) while the latter can lead to chocolate with a high hedonic rate.  
479 In any case, the death of yeasts at the end of the anaerobic phase is largely documented (De Vuyst  
480 and Weckx, 2016) and attributed to depletion of fermentable sugar, an increase in temperature and  
481 in pH and/or ethanol inhibition (Crafack et al., 2013; De Vuyst and Weckx, 2016). The model  
482 developed here and the validation tests confirmed that the increase in the concentration of ethanol  
483 and the change in pH do not have a significant impact on yeast growth or death during cocoa  
484 fermentation, since most yeasts showed the same behavior as the *Saccharomyces cerevisiae* LM  
485 strain. The increase in temperature and glucose exhaustion thus clearly drive yeast growth and death  
486 during the anaerobic phase of cocoa fermentation. It should be noted that we did not observe yeast



487 death as the yeast population was calculated from measured absorbance and this increases with  
488 biomass whether the biomass is alive or dead. As already mentioned above, maintenance  
489 metabolism and energy consumption during the stationary phase were not included in the model,  
490 neither was yeast adaptation or the kinetics of death depending of the speed of the temperature  
491 increase. Taking these phenomena into account could improve the predictability of the model,  
492 particularly the consumption of glucose at the end of the growth phase when temperature is the  
493 limiting growth factor, for example, with low inoculation levels. This work is a first step for  
494 developing a global model of cocoa pulp fermentation that can help for controlling cocoa  
495 fermentation. The development of the model of alcoholic fermentation of the pulp surrounding  
496 cocoa beans by *Saccharomyces cerevisiae* LM strain was based on the use of predictive  
497 microbiology models and particularly by using the gamma concept. The next step is under  
498 development; a similar approach is being used to model acetic fermentation using a strain of acetic  
499 bacteria isolated from an industrial fermentation in Côte d'Ivoire. Once validated, it will be  
500 combined with the alcoholic fermentation model to obtain the overall model of cocoa bean  
501 fermentation. It will thus be validated at a laboratory scale, with a pilot scale fermenter, and on an  
502 industrial scale in a fermentation center in Côte d'Ivoire. In addition, to take into account the  
503 consumption of the hexoses by LAB strains, an existing model of the lactic fermentation (Mestres  
504 et al., 2017; Munanga et al., 2016) can be integrated into the global model of the fermentation of  
505 cacao.

## 506 ***Conclusion***

507 This study is the first step of development of a global model of the cocoa beans fermentation of  
508 a project whose goal is to control the aromatic quality of chocolate by using a mixed microbial  
509 starter under controlled fermentation conditions. A model of alcoholic fermentation of the pulp  
510 surrounding cocoa beans by *Saccharomyces cerevisiae* LM strain, based on the use of the gamma  
511 concept, has been developed. Simulations and validations tests on a laboratory scale carried out

512 with the model during fermentations under conditions mimicking those recorded during industrial  
513 fermentations in Côte d’Ivoire reveal the potential of modeling for the control of the anaerobic  
514 phase of cocoa fermentation. The model clearly show that the level of inoculation and the speed of  
515 the increase in temperature clearly drive yeast growth, while other factors including pH and ethanol,  
516 free nitrogen and oxygen levels have no significant impact on yeast growth.

517 The next step is developing, with a similar approach, a model acetic fermentation using a strain  
518 of acetic bacteria isolated from an industrial fermentation in Côte d’Ivoire and to combine both  
519 models to design a global model of the fermentation of the pulp of cocoa for predicting the  
520 evolution of substrates (glucose, fructose, ethanol) and metabolites (ethanol, acetic acid) that can  
521 have a direct impact of cocoa quality.

522

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529 and facilities; Christelle Kouame and Joël Grabulos carried out the experiments; Christelle Kouamé,  
530 Christian Mestres and Gérard Loiseau analyzed the data. They wrote the article before submission  
531 and revised it according to the comments of the editor and reviewers.

532 **Conflicts of interest:** Authors declare they have no conflicts of interest.

533 **References**

534 Afoakwa, E.O., Paterson, A., Fowler, M., Ryan, A., 2008. Flavor formation and character in cocoa and  
535 chocolate: a critical review. *Crit. Rev. Food Sci. Nutr.* 48, 840–857.  
536 <https://doi.org/10.1080/10408390701719272>

537 Aldiguier, A.S., Alfenore, S., Cameleyre, X., Goma, G., Uribelarrea, J.L., Guillouet, S.E., Molina-Jouve, C.,  
538 2004. Synergistic temperature and ethanol effect on *Saccharomyces cerevisiae* dynamic behaviour in  
539 ethanol bio-fuel production. *Bioprocess Biosyst. Eng.* 26. [https://doi.org/10.1007/s00449-004-0352-](https://doi.org/10.1007/s00449-004-0352-6)  
540 6

541 Andrews, J.F., 1968. A mathematical model for the continuous culture of microorganisms utilizing inhibitory  
542 substrates. *Biotechnol. Bioeng.* 10, 707–723. <https://doi.org/10.1002/bit.260100602>

543 Antoce, O.-A., Antoce, V., Takahashi, K., Yoshizako, F., 1997. Quantitative study of yeast growth in the  
544 presence of added ethanol and methanol using a calorimetric approach. *Biosci. Biotechnol. Biochem.*  
545 61, 664–669. <https://doi.org/10.1271/bbb.61.664>

546 Aprotosoai, A.C., Luca, S.V., Miron, A., 2016. Flavor chemistry of cocoa and cocoa products-an overview.  
547 *Compr. Rev. Food Sci. Food Saf.* 15, 73–91. <https://doi.org/10.1111/1541-4337.12180>

548 Ardhana, M.M., Fleet, G.H., 2003. The microbial ecology of cocoa bean fermentations in Indonesia. *Int. J.*  
549 *Food Microbiol.* 86, 87–99. [https://doi.org/10.1016/S0168-1605\(03\)00081-3](https://doi.org/10.1016/S0168-1605(03)00081-3)

550 Arellano-Plaza, M., Herrera-López, E.J., Díaz-Montaña, D.M., Moran, A., Ramírez-Córdova, J.J., 2007.  
551 Unstructured kinetic model for tequila batch fermentation. *Int. J. Math. Comput. Simul.* 1, 6.

552 Arroyo-López, F.N., Orlić, S., Querol, A., Barrio, E., 2009. Effects of temperature, pH and sugar  
553 concentration on the growth parameters of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their  
554 interspecific hybrid. *Int. J. Food Microbiol.* 131, 120–127.  
555 <https://doi.org/10.1016/j.ijfoodmicro.2009.01.035>

556 Arroyo-López, F.N., Salvadó, Z., Tronchoni, J., Guillamón, J.M., Barrio, E., Querol, A., 2010. Susceptibility  
557 and resistance to ethanol in *Saccharomyces* strains isolated from wild and fermentative  
558 environments. *Yeast* 27, 1005–1015. <https://doi.org/10.1002/yea.1809>

559 Augustin, J.-C., 2005. Modélisation de la croissance microbienne et gestion de la sécurité sanitaire des  
560 aliments. Université Paris XII Val de Marne, Paris, France.

561 Augustin, J.-C., Carlier, V., 2000. Mathematical modelling of the growth rate and lag time for *Listeria*  
562 *monocytogenes*. *Int. J. Food Microbiol.* 56, 29–51. [https://doi.org/10.1016/S0168-1605\(00\)00223-3](https://doi.org/10.1016/S0168-1605(00)00223-3)

563 Batista, N.N., Ramos, C.L., Ribeiro, D.D., Pinheiro, A.C.M., Schwan, R.F., 2015. Dynamic behavior of  
564 *Saccharomyces cerevisiae*, *Pichia kluyveri* and *Hanseniaspora uvarum* during spontaneous and  
565 inoculated cocoa fermentations and their effect on sensory characteristics of chocolate. *LWT - Food*  
566 *Sci. Technol.* 63, 221–227. <https://doi.org/10.1016/j.lwt.2015.03.051>

567 Beckett, S.T., 2003. Is the taste of British milk chocolate different? *Int. J. Dairy Technol.* 56, 139–142.

568 Camu, N., De Winter, T., Addo, S.K., Takrama, J.S., Bernaert, H., De Vuyst, L., 2008. Fermentation of  
569 cocoa beans: influence of microbial activities and polyphenol concentrations on the flavour of  
570 chocolate. *J. Sci. Food Agric.* 88, 2288–2297. <https://doi.org/10.1002/jsfa.3349>

571 Camu, N., De Winter, T., Verbrugge, K., Cleenwerck, I., Vandamme, P., Takrama, J.S., Vancanneyt, M.,  
572 De Vuyst, L., 2007. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid  
573 bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. *Appl. Environ.*  
574 *Microbiol.* 73, 1809–1824. <https://doi.org/10.1128/AEM.02189-06>

575 Charoenchai, C., Fleet, H., Henschke, P., 1998. Effects of temperature, pH, and sugar concentration on the  
576 growth rates and cell biomass of wine yeasts. *Am. J. Enol. Vitic.* 49, 283–288.

577 Coroller, L., Guerrot, V., Huchet, V., Lemarc, Y., Mafart, P., Sohier, D., Thuault, D., 2005. Modelling the  
578 influence of single acid and mixture on bacterial growth. *Int. J. Food Microbiol.* 100, 167–178.  
579 <https://doi.org/10.1016/j.ijfoodmicro.2004.10.014>

580 Crafacek, M., Mikkelsen, M.B., Saerens, S., Knudsen, M., Blennow, A., Lowor, S., Takrama, J., Swiegers,  
581 J.H., Petersen, G.B., Heimdal, H., Nielsen, D.S., 2013. Influencing cocoa flavour using *Pichia*  
582 *kluyveri* and *Kluyveromyces marxianus* in a defined mixed starter culture for cocoa fermentation. *Int.*  
583 *J. Food Microbiol.* 167, 103–116. <https://doi.org/10.1016/j.ijfoodmicro.2013.06.024>

584 D'Amato, D., Corbo, M.R., Nobile, M.A.D., Sinigaglia, M., 2006. Effects of temperature, ammonium and  
585 glucose concentrations on yeast growth in a model wine system. *Int. J. Food Sci. Technol.* 41, 1152–  
586 1157. <https://doi.org/10.1111/j.1365-2621.2005.01128.x>

587 Daniel, H.-M., Vrancken, G., Takrama, J.F., Camu, N., De Vos, P., De Vuyst, L., 2009. Yeast diversity of  
588 Ghanaian cocoa bean heap fermentations. *FEMS Yeast Res.* 9, 774–783.  
589 <https://doi.org/10.1111/j.1567-1364.2009.00520.x>

590 da Veiga Moreira, I. M., Miguel, M. G. D. C. P., Duarte, W. F., Dias, D. R., & Schwan, R. F. (2013).  
591 Microbial succession and the dynamics of metabolites and sugars during the fermentation of three  
592 different cocoa (*Theobroma cacao* L.) hybrids. *Food Research International*, 54(1), 9-17.

593 De Vuyst, L., Weckx, S., 2016. The cocoa bean fermentation process: from ecosystem analysis to starter  
594 culture development. *J. Appl. Microbiol.* 121, 5–17. <https://doi.org/10.1111/jam.13045>

595 Gálvez, S.L., Loiseau, G., Paredes, J.L., Barel, M., Guiraud, J.-P., 2007. Study on the microflora and  
596 biochemistry of cocoa fermentation in the Dominican Republic. *Int. J. Food Microbiol.* 114, 124–  
597 130. <https://doi.org/10.1016/j.ijfoodmicro.2006.10.041>

598 Ho, V.T.T., Zhao, J., Fleet, G., 2015. The effect of lactic acid bacteria on cocoa bean fermentation. *Int. J.*  
599 *Food Microbiol.* 205, 54–67. <https://doi.org/10.1016/j.ijfoodmicro.2015.03.031>

600 Illegheems, K., Pelicaen, R., De Vuyst, L., Weckx, S., 2016. Assessment of the contribution of cocoa-derived  
601 strains of *Acetobacter ghanensis* and *Acetobacter senegalensis* to the cocoa bean fermentation  
602 process through a genomic approach. *Food Microbiol.* 58, 68–78.  
603 <https://doi.org/10.1016/j.fm.2016.03.013>

604 Kadow, D., Bohlmann, J., Phillips, W., Lieberei, R., 2013. Identification of main fine flavour components in  
605 two genotypes of the cocoa tree (*Theobroma cacao* L.). *J. Appl. Bot. Food Qual.* 86.

606 Kukec, A., Berovic, M., Wondra, M., Kos, T., 2003. Influence of temperature shock on the glycerol  
607 production in cv. Sauvignon Blanc fermentation 205–206.

608 Laopaiboon, L., Thanonkeo, P., Jaisil, P., Laopaiboon, P., 2007. Ethanol production from sweet sorghum  
609 juice in batch and fed-batch fermentations by *Saccharomyces cerevisiae*. *World J. Microbiol.*  
610 *Biotechnol.* 23, 1497–1501. <https://doi.org/10.1007/s11274-007-9383-x>

611 Leal, G.A., Gomes, L.H., Efraim, P., de Almeida Tavares, F.C., Figueira, A., 2008. Fermentation of cacao  
612 (*Theobroma cacao* L.) seeds with a hybrid *Kluyveromyces marxianus* strain improved product  
613 quality attributes. *FEMS Yeast Res.* 8, 788–798. <https://doi.org/10.1111/j.1567-1364.2008.00405.x>

614 Lefeber, T., Papalexandratou, Z., Gobert, W., Camu, N., De Vuyst, L., 2012. On-farm implementation of a  
615 starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates  
616 produced thereof. *Food Microbiol.* 30, 379–392. <https://doi.org/10.1016/j.fm.2011.12.021>

617 Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation  
618 industry. *Trends Food Sci. Technol.* 15, 67–78. <https://doi.org/10.1016/j.tifs.2003.09.004>

619 Liu, Y., 2006. A simple thermodynamic approach for derivation of a general Monod equation for microbial  
620 growth. *Biochem. Eng. J.* 31, 102–105. <https://doi.org/10.1016/j.bej.2006.05.022>

621 Lonvaud, A., Renouf, V., Strehaiano, P., 2010. *Microbiologie du vin : bases fondamentales et applications*,  
622 Ed. Tec & Doc Lavoisier, Cachan, France.

623 Medawar, W., Strehaiano, P., Délia, M.-L., 2003. Yeast growth: lag phase modelling in alcoholic media.  
624 *Food Microbiol.* 20, 527–532. [https://doi.org/10.1016/S0740-0020\(02\)00170-3](https://doi.org/10.1016/S0740-0020(02)00170-3)

625 Meersman, E., Steensels, J., Paulus, T., Struyf, N., Saels, V., Mathawan, M., Koffi, J., Vrancken, G.,  
626 Verstrepen, K.J., 2015. Breeding strategy to generate robust yeast starter cultures for cocoa pulp  
627 fermentations. *Appl. Environ. Microbiol.* 81, 6166–6176. <https://doi.org/10.1128/AEM.00133-15>

628 Membré, J.M., Leporq, B., Vialette, M., Mettler, E., Perrier, L., Zwietering, M.H., 2002. Experimental  
629 protocols and strain variability of cardinal values (pH and aw) of bacteria using Bioscreen C:  
630 microbial and statistical aspects. In *Food Micro 2002: Microbial adaptation to changing*  
631 *environments: 18th Symposium of the International Committee on Food Microbiology and Hygiene*,  
632 Lillehammer, Norway, 2002 (pp. 143-146). Matforsk.

633 Menonides, F.I.C., Schuurmans, J.M., Teixeira de Mattos, M.J., Hellingwerf, K.J., Brul, S., 2002. The  
634 metabolic response of *Saccharomyces cerevisiae* to continuous heat stress. *Mol. Biol. Rep.* 29, 103–  
635 106.

636 Menonides, F.I.C., Brul, S., Hellingwerf, K.J., Bakker, B.M., Teixeira de Mattos, M.J., 2014. A kinetic  
637 model of catabolic adaptation and protein reprofiling in *Saccharomyces cerevisiae* during  
638 temperature shifts. *FEBS J.* 281, 825–841. <https://doi.org/10.1111/febs.12649>

639 Mestres, C., Bettencourt, M. de J.C., Loiseau, G., Matignon, B., Grabulos, J., Achir, N., 2017. Modeling  
640 cereal starch hydrolysis during simultaneous saccharification and lactic acid fermentation; case of a

641 sorghum-based fermented beverage, gowé. *Food Res. Int.* 100, 102–111.  
642 <https://doi.org/10.1016/j.foodres.2017.06.059>

643 Moreira, I.M. da V., Vilela, L. de F., Miguel, M.G. da C.P., Santos, C., Lima, N., Schwan, R.F., 2017.  
644 Impact of a microbial cocktail used as a starter culture on cocoa fermentation and chocolate flavor.  
645 *Molecules* 22, 766. <https://doi.org/10.3390/molecules22050766>

646 Munanga, B. de J.C., Loiseau, G., Grabulos, J., Mestres, C., 2016. Modeling lactic fermentation of Gowé  
647 using *Lactobacillus* starter culture. *Microorganisms* 4, 44.  
648 <https://doi.org/10.3390/microorganisms4040044>

649 Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Andersson, T.S., Holzapfel, W.H., 2007. The  
650 microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-  
651 independent methods. *Int. J. Food Microbiol.* 114, 168–186.  
652 <https://doi.org/10.1016/j.ijfoodmicro.2006.09.010>

653 Omori, T., Ogawa, K., Umemoto, Y., Yuki, K., Kajihara, Y., Shimoda, M., Wada, H., 1996. Enhancement of  
654 glycerol production by brewing yeast (*Saccharomyces cerevisiae*) with heat shock treatment. *J.*  
655 *Ferment. Bioeng.* 82, 187–190. [https://doi.org/10.1016/0922-338X\(96\)85048-3](https://doi.org/10.1016/0922-338X(96)85048-3)

656 Owusu, M., 2010. Influence of raw material and processing on aroma in chocolate. University of  
657 Copenhagen, Faculty of Life Science.

658 Papalexandratou, Z., Camu, N., Falony, G., De Vuyst, L., 2011. Comparison of the bacterial species  
659 diversity of spontaneous cocoa bean fermentations carried out at selected farms in Ivory Coast and  
660 Brazil. *Food Microbiol.* 28, 964–973. <https://doi.org/10.1016/j.fm.2011.01.010>

661 Papalexandratou, Z., De Vuyst, L., 2011. Assessment of the yeast species composition of cocoa bean  
662 fermentations in different cocoa-producing regions using denaturing gradient gel electrophoresis.  
663 *FEMS Yeast Res.* 11, 564–574. <https://doi.org/10.1111/j.1567-1364.2011.00747.x>

664 Papalexandratou, Z., Vrancken, G., De Bruyne, K., Vandamme, P., & De Vuyst, L. (2011). Spontaneous  
665 organic cocoa bean box fermentations in Brazil are characterized by a restricted species diversity of  
666 lactic acid bacteria and acetic acid bacteria. *Food Microbiology*, 28(7), 1326-1338.  
667 <https://doi.org/10.1016/j.fm.2011.06.003>

668 Pereira, G.V., Alvarez, J.P., Neto, D.P. de C., Socco, V.T., Tanobe, V.O.A., Rogez, H., Góes-Neto, A.,  
669 Socol, C.R., 2017. Great intraspecies diversity of *Pichia kudriavzevii* in cocoa fermentation  
670 highlights the importance of yeast strain selection for flavor modulation of cocoa beans. Food Sci.  
671 Technol. <https://doi.org/10.1016/j.lwt.2017.05.073>

672 Pettipher, G.L., 1986. Analysis of cocoa pulp and the formulation of a standardised artificial cocoa pulp  
673 medium. J. Sci. Food Agric. 37, 297–309.

674 Ramos, C.L., Dias, D.R., Miguel, M.G. da C.P., Schwan, R.F., 2014. Impact of different cocoa hybrids  
675 (*Theobroma cacao L.*) and *S. cerevisiae* UFLA CA11 inoculation on microbial communities and  
676 volatile compounds of cocoa fermentation. Food Res. Int. 64, 908–918.  
677 <https://doi.org/10.1016/j.foodres.2014.08.033>

678 Ravelomanana, R., Guiraud, J.P., Vincent, J.C., Galzy, P., 1985. The yeast flora of cocoa bean fermentation  
679 in the Ivory Coast. World J. Microbiol. Biotechnol. 1, 319–326.

680 Remize, F., Sablayrolles, J., Dequin, S., 2000. Re-assessment of the influence of yeast strain and  
681 environmental factors on glycerol production in wine. J. Appl. Microbiol. 88, 371–378.

682 Rose, A.H., 1980. Recent research on industrially important strains of *Saccharomyces cerevisiae*, in: Biology  
683 and Activities of Yeast. Presented at the The Society for Applied Bacteriology Symposium Series.  
684 Academic Press, Skinner, FA, Rassmore, SM, and Daven, RR (eds.), London, p. 103.

685 Rosso, L., 1995. Modélisation et microbiologie prévisionnelle: Élaboration d'un nouvel outil pour l'agro-  
686 alimentaire. Université Claude Bernard-Lyon I, Lyon, France.

687 Rosso, L., Lobry, J.R., Bajard, S., Flandrois, J.P., 1995. Convenient model to describe the combined effects  
688 of temperature and pH on microbial growth. Appl. Environ. Microbiol. 61, 610–616.

689 Sá-Correia, I., Van Uden, N., 1983. Temperature profiles of ethanol tolerance: Effects of ethanol on the  
690 minimum and the maximum temperatures for growth of the yeasts *Saccharomyces cerevisiae* and  
691 *Kluyveromyces fragilis*. Biotechnol. Bioeng. 25, 1665–1667. <https://doi.org/10.1002/bit.260250620>

692 Salvadó, Z., Arroyo-López, F.N., Barrio, E., Querol, A., Guillamón, J.M., 2011. Quantifying the individual  
693 effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*. Food  
694 Microbiol. 28, 1155–1161. <https://doi.org/10.1016/j.fm.2011.03.008>



695 Samagaci, L., Ouattara, H., Goualie, B., Niamke, S., 2014. Growth capacity of yeasts potential starter strains  
696 under cocoa fermentation stress conditions in Ivory Coast. *Emir. J. Food Agric.* 26, 861.  
697 <https://doi.org/10.9755/ejfa.v26i10.18114>

698 Sandhya, M.V.S., Yallappa, B.S., Varadaraj, M.C., Puranaik, J., Rao, L.J., Janardhan, P., Murthy, P.S., 2016.  
699 Inoculum of the starter consortia and interactive metabolic process in enhancing quality of cocoa  
700 bean (*Theobroma cacao*) fermentation. *LWT - Food Sci. Technol.* 65, 731–738.  
701 <https://doi.org/10.1016/j.lwt.2015.09.002>

702 Schwan, R.F., 1998. Cocoa fermentations conducted with a defined microbial cocktail inoculum. *Appl.*  
703 *Environ. Microbiol.* 64, 1477–1483.

704 Schwan, R.F., Wheals, A.E., 2004. The microbiology of cocoa fermentation and its role in chocolate quality.  
705 *Crit. Rev. Food Sci. Nutr.* 44, 205–221. <https://doi.org/10.1080/10408690490464104>

706 Serra, A., Strehaiano, P., Taillandier, P., 2005. Influence of temperature and pH on *Saccharomyces bayanus*  
707 *var. uvarum* growth; impact of a wine yeast interspecific hybridization on these parameters. *Int. J.*  
708 *Food Microbiol.* 104, 257–265.

709 Swinnen, I.A.M., Bernaerts, K., Dens, E.J.J., Geeraerd, A.H., Van Impe, J.F., 2004. Predictive modelling of  
710 the microbial lag phase: a review. *Int. J. Food Microbiol.* 94, 137–159.  
711 <https://doi.org/10.1016/j.ijfoodmicro.2004.01.006>

712 Torija, M.J., Rozes, N., Poblet, M., Guillamón, J.M., Mas, A., 2003. Effects of fermentation temperature on  
713 the strain population of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 80, 47–53.

714 Trognitz, B., Cros, E., Assemat, S., Davrieux, F., Forestier-Chiron, N., Ayestas, E., Kuant, A., Scheldeman,  
715 X., Hermann, M., 2013. Diversity of cacao trees in Waslala, Nicaragua: Associations between  
716 genotype spectra, product quality and yield potential. *PLoS ONE* 8, e54079.  
717 <https://doi.org/10.1371/journal.pone.0054079>

718 Van Impe, J., Poschet, F., Geeraerd, A., Vereecken, K., 2005. Towards a novel class of predictive microbial  
719 growth models. *Int. J. Food Microbiol.* 100, 97–105.  
720 <https://doi.org/10.1016/j.ijfoodmicro.2004.10.007>

721 Walsh, R.M., Martin, P.A., 1977. Growth of *Saccharomyces cerevisiae* and *Saccharomyces uvarum* in a  
722 temperature gradient incubator. *J. Inst. Brew.* 83, 169–172.

723 Zwietering, M.H., Wijtzes, T., Rombouts, F.M., Riet, van't K., 1993. A decision support system for  
724 prediction of microbial spoilage in foods. *J. Ind. Microbiol. Biotechnol.* 12, 324–329.