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**Modeling of isoamyl acetate production by fermentation with *Pichia fermentans*
in an aerated system coupled to *in situ* extraction**

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Abstract

This study deals with the production of isoamyl acetate (IAA) by fermentation of sugar cane molasses with the strain *Pichia fermentans* ITD00165, using L-leucine as precursor. A mathematical model that describes the experimental data from fermentation was developed for its use as a tool for further process optimization. The fermentation system was constantly aerated and coupled to liquid-liquid *in situ* extraction with decane as the recovery solvent. Thus, the model integrates the biological production of IAA, its partition coefficient in the two liquid phase system and the stripping effect of aeration. A productivity of 26-mg-L⁻¹h⁻¹ was obtained with addition of 4-g-L⁻¹ of L-leucine at 12-h of fermentation. The use of the model for process optimization was explored. According to it, the maximum theoretical productivity that can be obtained is 63-mg-L⁻¹h⁻¹. The model was used to determine that 1.6-g-L⁻¹ is the minimum concentration of L-leucine that can be added without significantly reducing IAA production. Also, it makes possible to propose an adequate decane/culture medium ratio, to have a desired final concentration and amount of recovered IAA. This value can be adjusted based on the needs of further purification steps and is useful to define a global economic optimum of the process.

Keywords: Aroma production, fermentation kinetics, partition coefficient, simulation, natural flavoring substance.

Chemical compounds studied in this article

Isoamyl acetate (PubChem CID: 31276); Decane (PubChem CID: 15600); L-leucine (PubChem CID: 6106).

1 **Introduction**

2 Esters of short-chain fatty acids are important flavor and fragrance compounds
3 widely used in the food and beverage industries. Isoamyl acetate (IAA) is
4 characterized by its strong smell of banana which gives it a very important place in
5 food, pharmaceutical and perfumery industries with a demand of 75 tons per year in
6 USA alone in 2010 [1–3], increasing over the years. This substance is obtained by
7 chemical synthesis, extraction from natural sources, or fermentation [4].

8 In recent years, the interest towards the production of flavor compounds through
9 white biotechnology processes over traditional methods has increased. Principal
10 reasons are that chemical synthesis often consists in an environmentally unfriendly
11 production process, with important drawbacks such as poor reactions selectivity
12 resulting in racemic mixtures, low yields, and high downstream costs [5,6]. Also,
13 consumers have developed an apprehensive attitude towards these synthetic
14 compounds, especially if the products are related to food or domestic usage.
15 Moreover, the extraction of flavoring compounds from natural sources gives very low
16 yields and has potential difficulties with obtaining the raw material [3,4,7]. Contrary
17 to chemical synthesis, flavors obtained by fermentation can have the “natural
18 flavoring substance” classification by the European (EC No 1334/2008) and U.S.
19 (21CFR101.22) regulations, which allows using them safely as an additive in food
20 and beverages. This has been the main reason for developing biochemical processes
21 over the years, but presently, after years of research, the inherent advantages of white
22 biotechnology processes are the driving force for its application: operation under mild

23 and more environmentally friendly conditions, as well as chemical and stereo
24 specificity of the obtained compounds, are the most important ones [8].

25 There are several published studies focused on IAA biological production. They
26 include enzymatic synthesis using lipases and esterases [1,3,9–15] and fermentation
27 process by microorganisms, especially yeasts strains [6,16–20]. Whole cell
28 fermentation can be a more economical method than enzymatic systems and easier to
29 scale up to industrial level. There are some encouraging studies with interesting IAA
30 productivities in the literature; for example, Yilmaztekin *et al.* [6] used a *Williopsis*
31 *saturnus* strain in a medium composed of beet molasses and fusel alcohols as a source
32 of isoamyl alcohol (IAOH), a precursor of the ester. A productivity of $2.46 \text{ mg L}^{-1} \text{ h}^{-1}$
33 was obtained by adding 1% of fusel alcohol at 72 h. Quilter *et al.* [19], obtained 1.16
34 $\text{mg L}^{-1} \text{ h}^{-1}$ using a mutant strain of *Saccharomyces cerevisiae* and testing several
35 fermentation conditions. However, these values are not high enough to make the
36 process attractive at industrial level and further optimization is needed.

37 In fermentative processes, the production of IAA is strongly affected by the medium
38 composition, fermentation conditions and the microorganism used [6,18,19].
39 However, a very important factor to consider is physical behavior of IAA in
40 fermentation medium once produced. IAA is a very volatile compound and its
41 production presents some difficulties including phase separation and challenging
42 product recovery. Moreover, IAA production could be favored by chemical
43 equilibrium shift through *in situ* extraction. A way to improve the performance of
44 fermentative processes is the direct recovery of the desired product, also called *In Situ*
45 Product Recovery (ISPR). This technique can overcome the inhibition by product

46 accumulation and also decreases the loss of cellular viability caused by product
47 toxicity [21,22]. Liquid-liquid extraction with organic solvents is a promising method
48 for IAA recovery, since IAA is more soluble in them than in aqueous solutions. For
49 the same reasons, lipase-catalyzed esterification is carried out in solvent systems
50 [12,14,15,23].

51 Modeling and simulation has become the most used tool for processes optimization,
52 since it makes possible to explore a wider range of parameter values at a minimum
53 price, compared to experimental approach. Thus, the aim of this work is to develop a
54 model able to describe the production and extraction of IAA from sugar cane
55 molasses, with L-leucine added as precursor, by fermentation with the strain *Pichia*
56 *fermentans* ITD00165. The model includes the behavior of IAA in an aerated
57 fermentation system coupled to an *in situ* extraction with a solvent (decane). As the
58 aeration factor affects the retention of IAA in the system due to a stripping effect, the
59 model is useful as an optimization tool to find the conditions in which the highest
60 amount of IAA could be produced and recovered.

61 **Materials and methods**

62 *1.1. Fermentations*

63 *1.1.1. Yeast strain*

64 The strain *Pichia fermentans* ITD00165 was used. It was one of the strains isolated
65 from a spontaneous fermentation of *Agave duranguensis* obtained from the Microbial
66 Biotechnology Lab's Culture Collection at the Durango Institute of Technology. It
67 was chosen because of its high IAA production capacity [24].

68 1.1.2. *Isoamyl acetate production*

69 Fermentations were carried out in 1 L glass containers with a diameter/height ratio of
70 1:2, where 600 mL of culture medium were inoculated with an initial concentration of
71 1×10^7 cells mL⁻¹ of a 24 h pre-grown culture. The pre-culture was inoculated in 100
72 mL of the sugar cane molasses medium in 250 mL Erlenmeyer flasks, with agitation
73 of 120 rpm at 28°C. In order to retain the IAA produced, 150 mL of decane (Sigma-
74 Aldrich, USA) was put at the top of the culture medium, creating an *in situ*
75 entrapping system. Compressed air was fed into the medium with a flow rate of 600
76 mL min⁻¹ (1 VVM) at the bottom of the vessel, in order to provide oxygen to the cells
77 and to agitate the culture medium. No other agitation system was used, thus liquid
78 phases were not mixed, only a slight local dispersion at liquid/liquid interface was
79 observed. The temperature was maintained at 28 °C and the fermentation was
80 monitored during 24 h. In fermentations, the effect of addition of the amino acid L-
81 leucine (Sigma-Aldrich, USA) as a precursor of IAA was tested. Therefore, three
82 experiments were carried out: 1) Fermentation with no addition of L-leucine, 2) The
83 addition of 4 g L⁻¹ of L-leucine at the beginning of fermentation (t_0) and 3) adding 4 g
84 L⁻¹ after 12 h of fermentation (t_{12}). All experiments were performed in duplicate.

85 1.1.3. *Process monitoring*

86 Samples of 1 mL of medium and decane phases were taken for 24 hours, every 4
87 hours for experiments 1 and 3, and every 2 hours for experiment 2. Monitoring
88 consisted in measurements of biomass by staining viable count with methylene blue
89 in a Neubauer chamber and dry weight; reducing sugars by DNS technique [25]; L-
90 leucine and the nitrogen (N) source in molasses by a colorimetric method with

91 ninhydrin [26]; and IAA production measured in the decane phase by gas
92 chromatography on a Gas Chromatograph 6890N (Agilent Technologies, USA)
93 equipped with a flame ionization detector (FID) and a HP-Innowax column (Hewlett-
94 Packard, USA) (length, 30 m; inside diameter, 0.25 mm; film thickness, 0.25 μm).
95 Temperatures were as used by Rojas *et al.*[20] with some modifications: injector
96 block and detector 220 and 300 $^{\circ}\text{C}$, respectively. The oven temperature was
97 programmed as follows: equilibrated at 60 $^{\circ}\text{C}$ for 10 min, followed by a ramp of 20 $^{\circ}\text{C}$
98 min^{-1} , up to 250 $^{\circ}\text{C}$. Ethanol and IAOH were analyzed in culture medium by gas
99 chromatography with a method designed for alcohols [27]: injector block and
100 detector 250 and 300 $^{\circ}\text{C}$, respectively; 1:10 split ratio; oven temperature equilibrated
101 at 40 $^{\circ}\text{C}$ for 5 min, followed by a ramp of 10 $^{\circ}\text{C min}^{-1}$, up to 260 $^{\circ}\text{C}$.

102 1.2. *Aeration effect on retention of isoamyl acetate in decane*

103 Experiments were carried out in the same conditions as fermentations but without cell
104 inoculation. Instead, IAA was added to the system at a concentration of 1 g L^{-1} in two
105 different conditions: 1) Adding IAA into the culture medium and 2) the same amount
106 of IAA was added into the decane phase. Samples of decane were taken periodically
107 and were analyzed by gas chromatography as mentioned before. All experiments
108 were performed in duplicate.

109 1.3. *Molasses medium analysis by HPLC*

110 A sample of fermentation medium, consisting in molasses diluted at 100 g L^{-1} with
111 distilled water was analyzed by HPLC in order to determine the sugars and
112 aminoacids content. Sugars were analyzed using a column Biorad Aminex with a

stationary phase of sulfonated divinyl benzene-styrene (HPX-87H, 300 mm x 7.8 mm x 9 μ m), at a temperature of 35°C, UV detection was made at 210 nm. For aminoacids, anAccQ-Fluor Reagent Kit was injected in a Waters column (ACCQ-TAG, 150mm x 3.9 x 4 μ m), at a temperature of 34°C, and fluorescent UV detection was made at 250 nm for excitation and 395 nm for emission.

1.4. Determination of partition coefficients

1.4.1. Gas-liquid partition coefficient (K_{gq})

The Phase Ratio Variation (PRV) method was used as described by Morakul *et al.*, [28]. In order to evaluate the effect of the changes in medium composition during fermentation, 8 different solutions of IAA at 1 g L⁻¹ were prepared in different aqueous matrices, simulating different stages of fermentation during IAA production: 1) water, 2) sucrose 100 g L⁻¹, 3) ethanol 10 g L⁻¹, 4) sucrose 150 g L⁻¹, 5) ethanol 15 g L⁻¹, 6) sucrose 50 g L⁻¹ + ethanol 5 g L⁻¹, 7) sucrose 25 g L⁻¹ + ethanol 7.5 g L⁻¹, and 8) sugar cane molasses 100 g L⁻¹. These conditions were proposed considering a maximum production of ethanol of 15 g L⁻¹ and a maximum sugar concentration of 100 g L⁻¹ in the fermentation medium during the process. Aliquots of 50 μ L to 2 mL from each solution were introduced into four different headspace vials (22 mL, Chromacol, France) closed with Teflon/silicone septa in metallic caps, giving volume ratios between gas and liquid phases (β) of 10 to 439. The vials were then equilibrated at 28°C for at least 1 h (the time required to reach equilibrium in static conditions). Once equilibrium was reached, a 500 μ L sample of headspace gas was taken with a gastight syringe, preheated to 40 °C, in an automatic headspace sampler CTC Pal and injected with a 100 μ L s⁻¹ rate to a gas chromatograph (Agilent

G1530A, Germany) using FID detector. A HP-INNOWax column (30 m × 0.53 mm × 1.00µm) from Agilent (stationary phase: polyethylene glycol) was used, using helium as a carrier gas with a flow rate of 10 mL min⁻¹. The oven temperature started at 60 °C followed by a ramp of 10 °C min⁻¹ up to 220 °C. Injector and FID detector temperature was 250°C. Peaks areas were acquired with Agilent GC Chemstation software. A mass balance equation was used for partition coefficients calculation, as described by Ettre *et al.* [29]. The gas-liquid partition coefficient for IAA was expressed as the concentration ratio (K_{gq}):

$$K_{gq} = \frac{C_g^*}{C_q^*} \quad (1)$$

with C_g^* and C_q^* being IAA concentration at equilibrium in the gas and aqueous phases respectively.

1.4.2. Liquid-liquid partition coefficient (K_{dq})

In this determination, different IAA solutions of 0.1 g L⁻¹ in aqueous phase were also prepared changing the matrix composition in order to simulate the changes in the medium during fermentation. The solutions were 1) water, 2) sucrose 100 g L⁻¹, 3) ethanol 10 g L⁻¹, 4) sucrose 25 g L⁻¹ + ethanol 7.5 g L⁻¹, 5) sucrose 50 g L⁻¹ + ethanol 5 g L⁻¹, 6) sucrose 70 g L⁻¹ + ethanol 10 g L⁻¹, and 7) sugar cane molasses 100 g L⁻¹. Some solutions had different sucrose and ethanol concentrations from those used in the gas-liquid partition coefficient measurements (K_{gq}), in order to have intermediate concentration values. Then, 25 mL of each solution were put in contact with 25 mL of decane in 60 mL separation funnels. They were left to reach the equilibrium in static conditions for at least 48 h at a temperature of 28°C.

157 After equilibrium was reached, 1.5 mL samples of aqueous and decane phase were
158 taken in 2 mL vials closed with Teflon/silicone septa in metallic caps and stored in
159 the tray at 4°C. A sample of 1 µL was taken from each vial with a water-tight syringe,
160 in an automatic headspace sampler CTC Pal and injected with a 5 µL s⁻¹ rate to the
161 gas chromatograph (G 1530A, Germany), equipped with the same column. For the
162 analysis method, oven temperature started at 35 °C followed by a ramp of 5°C min⁻¹
163 to 60 °C and another one of 15°C min⁻¹ up to 220 °C. Injector and FID detector
164 temperature was set to 250 °C.

165 IAA concentrations in both phases were determined using adequate calibration
166 curves. Partition coefficient K_{dq} was calculated as the ratio at equilibrium of IAA in
167 decane C_d^* and in aqueous solution C_q^* :

$$K_{dq} = \frac{C_d^*}{C_q^*} \quad (2)$$

168 1.4.3. Statistical analysis

169 Partition coefficient values were compared with a One Way Analysis of Variance
170 (ANOVA) and the Fisher Least Significant Difference (LSD) post-hoc test with the
171 program Statistica version 7.0 (StatSoft, USA).

172 1.5. Dynamic model

173 The equations of the mathematical model were implemented in a program written
174 under Matlab 2013b (The Mathworks Inc, Natick, MA). The parameters were
175 identified by linear and nonlinear regression, using the Statistic Toolbox.

176 1.5.1. Modeling partition coefficients

177 The values of gas-liquid and liquid-liquid partition coefficients were fitted to a
 178 statistical model that accounts for dependence on the medium composition (at
 179 constant temperature), as described by Mouret *et al.* [30]. Sugar and ethanol
 180 concentrations, their interaction and the effect of molasses were considered:

$$\log K_{dq} = p_a + p_b S_t + p_c E + p_d S_t E + p_e M \quad (3)$$

$$\log K_{gq} = p_1 + p_2 S_t + p_3 E + p_4 S_t E + p_5 M \quad (4)$$

181 where K_{gq} and K_{dq} are the gas-liquid and liquid-liquid partition coefficients
 182 respectively, E is the ethanol concentration (g L^{-1}), S_t is the sugar concentration (g L^{-1}),
 183 and M is the presence of molasses (0 or 1) in aqueous phase. Model parameters
 184 $p_1 \dots p_5$ and $p_a \dots p_e$ are constants depending on the considered compound and
 185 temperature, in this case IAA at 28°C. Parameter values were determined by stepwise
 186 descending linear regression, starting with the complete model and removing
 187 statistically not significant terms one by one, starting with the ones with the largest
 188 coefficient of variation.

189 1.5.2. Modeling the isoamyl acetate loss in gas phase by stripping

190 IAA transfer in the system was considered as follows: it is transferred from the
 191 aqueous medium (q), where it is synthesized by the cells, simultaneously to 1) the
 192 decane phase (d); and 2) to the gas phase (g). Also, a fraction of the IAA in decane
 193 phase is transferred to the gas phase. Medium and decane phases are very little
 194 dispersed into each other, so the transfer to the gas phase is different in each liquid
 195 phase. The IAA transferred to the air fed in the fermentation system was considered
 196 as lost due to a stripping effect. Therefore, this could be described by the model

197 developed by Marin *et al.*[31] with some modifications (Equation 5), considering
 198 IAA present in both decane and fermentation medium, and the removal by the air
 199 flow passing through the system. The global mass balance between the liquids,
 200 namely decane (d) + aqueous medium (q) and the stripping gas (g) was written as:

$$\frac{d(C_d V_d + C_q V_q)}{dt} = -C_g \cdot Q_g \quad (5)$$

201 where C_d , C_q and C_g are IAA concentration in decane, aqueous and gas phase
 202 respectively, V_d and V_q are the volumes (mL) of decane and aqueous phase
 203 respectively, and Q_g is the air flow rate through the system (mL h⁻¹). It was assumed
 204 that the liquid and gas phases were not in equilibrium, and the expression of mass
 205 transfer from the two liquid phases (d , q) to the air flow (gas phase, g) is:

$$C_g \cdot Q_g = k_q A_q \left(C_q - \frac{C_g}{K_{gq}} \right) + k_d A_d \left(C_d - \frac{C_g}{K_{gd}} \right) \quad (6)$$

206 Here k_q and k_d are the overall mass transfer coefficients of IAA between the gas and
 207 the aqueous and decane phases respectively, and A_q and A_d are the interfacial areas
 208 of each phase with air bubbles [31]. K_{gd} is the partition coefficient between the gas
 209 and decane phases determined from Equations 1 and 2 according to Equation 7.

$$K_{gd} \stackrel{\text{def}}{=} \frac{C_g^*}{C_d^*} = \frac{K_{gq}}{K_{dq}} \quad (7)$$

210 At time scale of IAA production process by fermentation (12 h), equilibrium was
 211 considered between the aqueous phase and decane:

$$C_q = \frac{C_d}{K_{dq}} \quad (8)$$

212 After substituting C_q from Equation 8, equation 6 was solved for C_g , giving:

$$C_g = \frac{k_l a_l}{\left(D_g + \frac{k_l a_l}{K_{gd}}\right)} \cdot C_d \quad (9)$$

213 where

$$k_l a_l = \frac{\left(\frac{k_q A_q}{K_{dq}} + k_d A_d\right)}{V_q} \quad \text{and} \quad D_g = \frac{Q_g}{V_q} \quad (10)$$

214 The product $k_l a_l$ is a volumetric mass transfer coefficient which characterizes
 215 transfer between the liquid phase (decane + aqueous medium) as a whole and the gas
 216 phase and D_g is the specific aeration rate of the medium.

217 Substituting Equation 9 in 5, the loss rate of IAA, considering decane as the reference
 218 medium, is obtained:

$$\frac{dC_d}{dt} = - \frac{k_l a_l \cdot D_g}{\left(D_g + \frac{k_l a_l}{K_{gd}}\right) \left(\frac{V_d}{V_q} + \frac{1}{K_{dq}}\right)} \cdot C_d \quad (11)$$

219 Equation (11) can be solved easily assuming K_i , k_i , A_i and V_i to be constant in
 220 isothermal conditions, leading to Equation 12 that gives the residual concentration of
 221 IAA in decane considering the stripping effect of the air fed into the system:

$$C_d = C_{d0} \exp \left(- \frac{k_l a_l \cdot D_g}{\left(D_g + \frac{k_l a_l}{K_{gd}} \right) \left(\frac{V_d}{V_q} + \frac{1}{K_{dq}} \right)} t \right) \quad (12)$$

222

223 The only unknown parameter in this equation is the volumetric mass transfer
 224 coefficient ($k_l a_l$). In order to verify the assumption that liquid phases are not in
 225 equilibrium with the gas phase and IAA stripping is limited by mass transfer, a
 226 variant of the model considering equilibrium between the gas and the liquid phases
 227 was also considered for comparison, corresponding to a very large $k_l a_l$ value. In that
 228 situation, Equation 12 reduces to Equation 13:

$$C_d = C_{d0} \exp \left[-K_{gd} \cdot \frac{D_g}{\left(\frac{V_d}{V_q} + \frac{1}{K_{dq}} \right)} t \right] \quad (13)$$

229 1.6. Modeling the isoamyl acetate production by fermentation

230 To develop the fermentation model, the metabolic pathway involved in IAA synthesis
 231 by yeast was considered (Figure 1) [32]. IAA is formed from esterification of IAOH
 232 and the acetyl group from acetyl coenzyme A. Depending on medium composition,
 233 IAOH could be produced *de novo* from sugars consumption or by a catabolic
 234 pathway from degradation of the aminoacid L-leucine, called Ehrlich pathway [33].
 235 The acetyl-CoA formed during metabolism comes mainly from sugar consumption
 236 and is used in several other reactions like biosynthesis of lipids, amino acids, fatty
 237 acids, and is also involved in the tricarboxylic acids cycle [32].

Table 1 shows the equations used to describe the production and consumption of the compounds considered in the fermentation model, without considering extraction by decane and losses by stripping. Biomass, ethanol, IAOH and IAA are the produced compounds, while fermentable reducing sugar, L-leucine and IAOH as well, are the consumed ones. To describe biomass production, the Monod equation was used (Equations 14 and 20). In that case biomass production is proportional to fermentable sugars consumption. Therefore, fermentable sugars consumption was described by Equation 18, $Y_{X/S}$ being the yield of biomass production related to sugar consumption. Ethanol production was also considered to be proportional to sugar consumption (Equation 15), using the corresponding yield ($Y_{EtOH/S}$).

According to the metabolic pathway (Figure 1), IAOH is formed from both sugars and L-leucine consumption, with the corresponding specific production rates ξ_S and ξ_L and is consumed for IAA synthesis with yield $Y_{IAA/IAOH}$ (Equation 16). For IAA production from IAOH, an analogous of the Monod equation was used, considering both fermentable sugars and IAOH as limiting substrates (Equations 17 and 23). L-leucine consumption is described by Equation 19, the specific consumption rate also involving fermentable sugar as limiting substrate (Equation 22).

1.6.1. Coupled model

In order to represent the IAA retained in the fermentation system with decane and stripping, the mass balance equation for IAA production from the fermentation model (Equation 17) was modified to include IAA partition between the fermentation

259 medium and decane as well as IAA losses by stripping (Equation 11) leading to
 260 equation 24:

$$\frac{dC_d}{dt} = vX \frac{V_q}{V_d + \frac{V_q}{K_{dq}}} - \frac{k_l a_l \cdot D_g}{\left(D_g + \frac{k_l a_l}{K_{gd}}\right) \left(\frac{V_d}{V_q} + \frac{1}{K_{dq}}\right)} C_d \quad (24)$$

261 The result was expressed in terms of IAA concentration in decane (assuming
 262 equilibrium between fermentation medium and decane, Equation 8) because all
 263 concentration measurements were performed in this phase.

Results and discussion

1.7. Model development

1.7.1. Gas-liquid (K_{gq}) and liquid-liquid (K_{dq}) partition coefficients

Figure 2 shows the K_{gq} values at different concentrations of ethanol and sucrose, compounds that were chosen to simulate the medium composition during fermentation. It can be observed that an increment on ethanol concentration increased the solubility of the esters in the aqueous phase, decreasing their volatility. On the other side, sugar concentration has a ‘salting out’ effect, increasing the volatility of aroma compound. Thus, K_{gq} value tends to decrease during fermentation. The same tendencies were observed in the work of Morakul *et al.* [28].

Figure 3 shows liquid-liquid partition coefficient (K_{dq}) values. As it can be observed, changes in medium composition have a much stronger impact than on K_{gq} . Sugar concentration has a higher “salting out” effect than on K_{gq} . In the case of molasses solution, the K_{dq} value is significantly smaller than for model solutions. This could be because in molasses other components such as lipids, amino acids or minerals are present, which could affect more importantly the K_{dq} value.

Table 2 shows the parameter values obtained from the stepwise linear regression of K_{gq} and K_{dq} with the different simulated medium compositions. Parameters that are not significantly different from zero were iteratively removed from the model, beginning with the factor with the highest coefficient of variation [30]. For K_{dq} regression, ethanol and molasses had a significant effect. Sugar concentration coefficient resulted not significant. Note that solutions prepared to represent

fermentation conditions were a mixture of ethanol and sugar, inversely correlated as in a real fermentation medium, which makes it difficult to differentiate each effect. Thus, the absence of sugar coefficient in Equation 3 does not necessarily mean that there is no effect. In the K_{dq} equation, sugar effect could have been absorbed by the ethanol coefficient. For example, in the work of Morakul *et al.* [34] the partition coefficient equation is expressed only in terms of ethanol concentration but it represents also sugar concentration in the medium, since they are inversely related in fermentation conditions.

As it was expected, the concentration of molasses had an important effect in K_{dq} model, but is negligible in K_{gq} model. These results show that complex medium (molasses) composition has a stronger effect on K_{dq} than on K_{gq} . Identifying which compounds of the molasses solution have significant effects on K_{dq} requires further investigations.

1.7.2. Isoamyl acetate loss by air stripping

Figure 4 displays the experimental data obtained in stripping experiments after adding IAA in aqueous and decane phase, together with model predictions. Determination coefficient (R^2) values for both experiments were 0.913 and 0.996 respectively. In fermentation conditions, 2.5% of IAA per hour is lost due to aeration, and the value of $k_l a_l$ coefficient that represents mass transfer from liquid to gas phase was $0.0117 \pm 0.0003 \text{ h}^{-1}$ (fitted using Equation 12). The relatively low value of the volumetric mass transfer coefficient $k_l a_l$ for isoamyl acetate corresponds to reduced IAA losses by stripping from the considered device, a desired feature in this case.

Although aeration has an important effect on IAA loss from fermentation system, Rojas *et al.* [20] observed that its production by non-*Saccharomyces* strains is improved in highly aerobic culture conditions (shaking at 120 rpm), compared with minimally aerobic conditions (without shaking). From all the strain tested, *Pichia anomala* had the best IAA production and it had a 21 fold increase in aerobic conditions. Also, Inoue *et al.* [35] reported a large amount of IAA produced by a *Hansenula mrakii* strain in aerobic conditions.

For modeling purposes, it was assumed that decane and aqueous phase were in equilibrium at the fermentation time scale. This assumption appears to be verified by the experimental data shown in Figure 4. The experiment of IAA added into the culture medium shows that after 1 h of aeration, IAA transferred into decane phase, and in the following measurements a decrease of concentration due to aeration can be observed. Thus, it appeared that within 1 h, equilibrium between the two phases was reached.

Figure 4 also shows in dashed lines the calculated loss of IAA with the assumption that liquid and gaseous phases are in equilibrium (Equation 13). IAA losses are clearly overestimated, meaning that the equilibrium between gas and liquid phases is far from being reached, probably because the residence time of the air bubbles fed in the system is very short. This corroborates the assumption that the stripping phenomenon due to air feed is limited by mass transfer between liquid and gas phase, and validates the model built on that basis.

1.7.3. Parameter estimation for the fermentation model

Data obtained from fermentation experiments 1 (no addition of L-leucine) and 2 (addition of L-leucine at t_0) were used to calculate the fermentation model parameters (Table 3), while experiment 3 was used for validation of the model. Initial conditions were taken from experimental values. In case of L-leucine in experiments 1 and 3, the initial value was obtained from HPLC analysis of molasses, showing that there was a small amount of this aminoacid (0.01 g L^{-1}). However, this amount is too small to explain the IAA production with no addition of L-leucine into the medium, meaning that there must be a pathway from sugar consumption to IAA synthesis as described by Piendl and Geiger [32] and considered in the model.

The maximum specific growth rate μ_{max} was calculated by linear regression of the logarithm of biomass produced ($\log X$) versus time (t) values in the exponential growth phase of fermentation. The value obtained ($\mu_{max} = 0.230 \pm 0.006 \text{ h}^{-1}$) is somewhat lower than the ones found in other works dealing with yeast growth in sugar cane molasses, where μ_{max} can vary from 0.24 - 0.45 h^{-1} due to the difference in nutrient or inhibitor composition in molasses, ambient conditions and the yeast strain used [36–38]. Yields values $Y_{X/S}$ and $Y_{EtOH/S}$ were calculated by linear regression of biomass and ethanol concentrations respectively vs. reducing sugars concentration. For $Y_{IAA/IAOH}$ value, the stoichiometric value $130/88$ was considered (ratio of IAA and IAOH molecular weights). Values of ξ_{Smax} , ξ_{Lmax} , v_{max} , K_S and $Y_{IAOH/L}$ were determined by fitting the model simultaneously to biomass, sugar, L-Leucine, IAOH concentration measurements in the fermentation medium and IAA concentration measurements in decane.

In the case of K_{SIA} , K_L and K_{IAOH} values, it was found that in a range from 0 to 0.05 g L⁻¹ their value had a minor effect on the model predictions. It was not possible to determine exact values for these parameters with the experimental data available, because experiments were performed in batch mode and corresponding substrates (sugar, L-leucine, IAOH) were rate limiting during too short time periods. These parameters were thus fixed to 0.02 g L⁻¹. For K_S , a value of 6.41 ± 0.57 g L⁻¹ was found. This value depends on numerous factors such as environmental conditions, composition of the medium and the strain used, so the range of values found in the literature is quite extensive. For example Das *et al.* [38] determined a K_S of 1.53g L⁻¹ in fermentation with *Pichia fermentans* in sugar cane bagasse extract, Ponce *et al.* [37] determined a value of 4.1 g L⁻¹ for *Saccharomyces cerevisiae* in fermentation of sugar cane molasses. However, the value obtained in the present study is higher than the works mentioned before, which confirms that substrate limitation coefficients cannot be reliably determined in batch fermentations. Other micronutrients might become limiting before complete sugar exhaustion.

1.8. Model validation

Once all model parameters were determined, data obtained from Experiment 3 (addition of L-leucine at t_{12}) was used for model validation. Figure 5 shows the model predictions compared to experimental data. In general, it was obtained a good agreement for all considered compounds. Sugar consumption was quite well described by the model ($R^2=0.986$). The amount of the fermentable sugars was taken as a fixed fraction of the total sugar concentration present in molasses, determined as 35% in Experiments 1 and 2. Biomass and ethanol are produced proportionally to the

sugar consumption, in agreement with the modeling assumptions; ethanol production was well described by the model ($R^2=0.977$). However, biomass is slightly underestimated by about 13% ($R^2=0.917$), which means that the growth rate μ_{max} and yield $Y_{X/S}$ values were slightly higher in Experiment 3 than in Experiments 1 and 2.

Figure 5 shows that before 12 h of fermentation, IAOH was produced from sugars at a low specific rate ξ_S , and almost entirely converted to IAA. After L-leucine addition, the production rate of IAOH increased significantly since the Ehrlich pathway participated to the synthesis with a higher specific rate ξ_L . This is consistent with parameter values reported in Table 3, where $\xi_{Lmax} > \xi_{Smax}$. Most of IAOH produced was converted to IAA at a specific rate v , which is consistent with $v_{max} > \xi_{Lmax}$ in Table 3. However, it can be observed that there is a fraction of IAOH accumulated in the fermentation medium, also described by the model ($R^2=0.819$).

IAOH accumulation is an important point to pay attention, since it has been proved that this compound has an inhibitory effect on yeast cells growth. *Saccharomyces cerevisiae* growth is slower at a concentration of about 4 g L⁻¹ [39,40]; and *Williopsis saturnus* growth is inhibited at concentrations of fusel oil greater than 2% (about 8 g L⁻¹ of IAOH). In this study, IAOH concentration determined in all the experiments was less than 0.74 g L⁻¹, a small value compared to those found in the literature. This is an advantage of maintaining a low IAA concentration in fermentation medium by ISPR, which accelerates IAOH esterification, reducing its accumulation.

The accumulation of IAA was quite well described by the model ($R^2=0.952$) which also calculated its actual production without losses by stripping (Figure 5). Model calculations indicate an amount of about 17% of IAA lost by stripping in these fermentation conditions. The highest IAA productivity obtained in experimental conditions was $26 \text{ mg L}^{-1} \text{ h}^{-1}$ with addition of 4 g L^{-1} of L-leucine at 12 h of fermentation, which is higher than other values reported in the literature obtained by yeast fermentation without *in situ* extraction [6,41].

1.9. Improvement strategies for isoamyl acetate production and retention

The developed model can be used as a tool to simulate the process of IAA production in different conditions in order to find those that results in a better IAA production and retention, with a more efficient use of L-leucine and decane. Key conditions that can be modified in order to improve production and retention in the system are: i) the time and amount of L-leucine addition, and ii) the decane to culture medium volume ratio in fermentation system.

L-leucine was added to the medium at a concentration of 4 g L^{-1} , however, experimental data shows that it was not completely consumed by the cells, either added from the beginning of fermentation or after 12 h. The model was used to test different initial concentrations of L-leucine and simulate resulting IAA concentration in decane. It was found that at concentrations of 1.6 g L^{-1} of L-leucine or higher, the IAA final concentration is not significantly different from IAA production with 4 g L^{-1} of L-leucine, as shown in Figure 6.

Figure 7a shows IAA distribution in the fermentation-extraction system at different decane/medium ratios. It can be observed that the amount of IAA recovered in decane phase increases by increasing the decane volume, as one may expect. A better extraction percentage from the fermentation medium and lower losses by air stripping are obtained. Although the amount of product retained increases with the amount of decane used, final concentration of IAA tends to decrease as shown in Figure 7b, which means obtaining an increasingly diluted solution and higher costs of subsequent product purification. The model is thus a useful decision making tool to determine the appropriate amount of decane, taking into account losses, the retained IAA fraction and the type of downstream process to be used for purification. In order to have an idea of the maximum potential production of the process, a simulation was performed with a very high decane to culture medium ratio (100:1), assuming that there will be no significant IAA loss in this condition. According to the model, with 1.6 g L⁻¹ of L-leucine one can obtain a maximum theoretical productivity of 63 mg L⁻¹ h⁻¹, based on IAA recovered in decane data. The actual value of solvent/medium ratio has to be selected considering the need for further purification steps. The model is thus a useful tool to perform an economic evaluation of the process, in order to optimize costs.

Conclusion

A model able to describe isoamyl acetate (IAA) production from sugar cane molasses and L-leucine as precursor, by the strain *Pichia fermentans* ITD00165 was developed and successfully used to predict the ester production indifferent conditions. It reproduces quite well the coupled phenomena of the *in situ* production/extraction process, and allows identifying the key points that can be improved to increase production. The *in situ* extraction with decane resulted in several advantages: it increased IAA production by decreasing product inhibition and IAOH accumulation in the medium, which has been proven to inhibit yeast cell growth. Decane had a good biocompatibility with the strain used. Moreover, it has a good affinity with IAA which is very volatile and poorly soluble in water, so it decreased the loss of IAA by stripping. In addition, thanks to the model it is easier to monitor IAA production from a single measurement in the decane phase and to calculate the total amount produced.

The model describes not only the biological production of IAA, but also includes the physical distribution of the ester according to key factors, like aeration, medium composition changes through fermentation and volume ratio of both liquid phases. It provides a more complete panorama of the process, allowing to test different configurations and find the best compromise between fermentation conditions and IAA retention to get the highest possible amount of product at acceptable purification costs.

Further investigation is needed in order to better understand the whole process. For example, aeration is a very important factor for IAA production, but it also increases

457 the loss by stripping, so it is a key process factor that has to be controlled. The
458 developed model is able to determine the rate of isoamyl acetate loss due to aeration,
459 and it could be further extended to include the effect of different air flow rates on
460 IAA production and thus indicate the best compromise. After testing model
461 predictions in a wide range of situations of practical interest, its implementation in
462 industrial processes could make it a valuable tool for process optimization and
463 provide a solid basis for choosing and developing a suitable product recovery
464 strategy.

465

Nomenclature		Units
A	Gas-liquid contact area	cm^2
C	Isoamyl acetate concentration	g L^{-1}
D_g	Specific aeration rate of the medium	h^{-1}
$EtOH$	Ethanol concentration	g L^{-1}
$IAOH$	Isoamyl alcohol concentration	g L^{-1}
K_{dq}	Liquid-liquid partition coefficient	g g^{-1}
K_{gq}	Gas-liquid partition coefficient	g g^{-1}
K_{IAOH}	Isoamyl alcohol saturation coefficient for isoamyl acetate production	g L^{-1}
K_L	L-Leucine saturation coefficient for isoamyl alcohol production	g L^{-1}
K_S	Sugar saturation coefficient for biomass production	g L^{-1}
K_{SIA}	Sugar saturation coefficient for isoamyl alcohol production	g L^{-1}
k_d	Overall mass transfer coefficient of isoamyl acetate between the decane and gas phase	cm h^{-1}
k_q	Overall mass transfer coefficient of isoamyl acetate between the aqueous and gas phase	cm h^{-1}
$k_l a_l$	Volumetric mass transfer coefficient between both liquid	h^{-1}

	phases as a whole and the gas phase	
L	L-leucine concentration	g L^{-1}
$p_1 \dots p_5$	Parameters of isoamyl acetate K_{dq} model	-
$p_a \dots p_e$	Parameters of isoamyl acetate K_{gq} model	-
Q_g	Aeration flowrate	mL h^{-1}
S	Sugar concentration	g L^{-1}
X	Biomass concentration	g L^{-1}
$Y_{EtOH/S}$	Ethanol yield per sugar consumption	g g^{-1}
$Y_{IAA/IAOH}$	Isoamyl acetate yield per isoamyl alcohol consumption	g g^{-1}
$Y_{IAOH/L}$	Isoamyl alcohol yield per L-leucine consumption	g g^{-1}
$Y_{X/S}$	Biomass yield per sugar consumption	g g^{-1}
μ_{max}	Maximum specific cellular growth rate	h^{-1}
ν_{max}	Maximum specific isoamyl acetate production rate	h^{-1}
ξ_{Lmax}	Maximum specific isoamyl alcohol production rate (from L-leucine)	h^{-1}
ξ_{Smax}	Maximum specific isoamyl alcohol production rate (from sugars)	h^{-1}

<i>Subscripts</i>	
d	Decane phase
g	Gas phase
q	Aqueous phase
f	Fermentable
t	Total
0	Initial value
*	Value at equilibrium
<i>Abbreviations</i>	
IAA	Isoamyl acetate

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598

599 **Figure legends**

600 **Figure 1.** Schematic representation of the metabolic pathways involved in isoamyl
601 acetate synthesis [32].

602 **Figure 2.** Gas-aqueous phase partition coefficient (K_{gq}) values of isoamyl acetate for
603 different fermentation medium compositions. Error bars represent standard deviation
604 ($n = 2$), different letters correspond to significantly different values.

605 **Figure 3.** Decane–aqueous phase partition coefficient (K_{dq}) values of isoamyl acetate
606 for different fermentation medium compositions. Error bars represent standard
607 deviation ($n=2$), different letters correspond to significantly different values.

608 **Figure 4.** Isoamyl acetate stripping due to air flow. Symbols correspond to
609 experimental measurements and error bars represent standard error ($n = 2$). Solid
610 lines represent model predictions considering gas-liquid mass transfer resistance;
611 dashed lines represent model predictions assuming gas and liquid phases in
612 equilibrium.

613 **Figure 5.** Comparison between model predictions and measured data in the validation
614 experiment, with L-leucine addition at 12 hours of fermentation. Symbols:
615 experimental measurements, solid lines: model predictions, dotted line: produced
616 isoamyl acetate without considering losses by air stripping.

617 **Figure 6.** Final isoamyl acetate concentration in decane at different concentrations of
618 L-leucine.

619 **Figure 7.**a) Isoamyl acetate distribution in the fermentation-extraction system and
620 losses by stripping for different decane/medium ratios, b) Isoamyl acetate recovery
621 and final concentration in decane at different decane/medium ratios. Results obtained
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 647 isoamyl acetate without considering losses by air stripping.

648

Figure 6. Final isoamyl acetate concentration in decane predicted by the model for different initial concentrations of L-leucine.

Figure 7. a) Isoamyl acetate distribution in the fermentation-extraction system and losses by stripping for different decane/medium ratios, b) Isoamyl acetate recovery and final concentration in decane at different decane/medium ratios. Results obtained from model simulations.

Table 1. Mass balance and kinetic equations for the fermentation model, without considering isoamyl acetate extraction by decane and losses by stripping

Compounds	Equations	
Biomass	$\frac{dX}{dt} = \mu X$	(14)
Ethanol	$\frac{dEtOH}{dt} = \frac{Y_{EtOH/S}}{Y_{X/S}} \mu X$	(15)
Isoamyl alcohol	$\frac{dIAOH}{dt} = \xi_S X + \xi_L X - \frac{1}{Y_{IAA/IAOH}} \nu X$	(16)
Isoamyl acetate	$\frac{dC}{dt} = \nu X$	(17)
Fermentable reducing sugars	$\frac{dS_f}{dt} = -\frac{1}{Y_{X/S}} \mu X$	(18)
Leucine	$\frac{dL}{dt} = -\frac{1}{Y_{IAOH/L}} \xi_L X$	(19)
Kinetic parameters	Equations	
Specific growth rate	$\mu = \mu_{max} \frac{S_f}{K_S + S_f}$	(20)
Specific isoamyl alcohol production rate (with sugar consumption)	$\xi_S = \xi_{Smax} \frac{S_f}{K_{SIA} + S_f}$	(21)
Specific isoamyl alcohol production rate (with L-leucine consumption)	$\xi_L = \xi_{Lmax} \frac{L}{K_L + L} \frac{S_f}{K_{SIA} + S_f}$	(22)
Specific isoamyl acetate production rate	$\nu = \nu_{max} \frac{IAOH}{K_{IAOH} + IAOH} \frac{S_f}{K_{SIA} + S_f}$	(23)

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Table 2. Parameter values for isoamyl acetate partition coefficient in the fermentation system.

Partition coefficient	Parameter	Factor	Value $\pm$ standard error
Gas-liquid	p_1	-	-3.716 $\pm$ 0.026
	p_2	S_t	0.0014 $\pm$ 0.0004
	p_3	E	0
	p_4	$S_t E$	0
	p_5	M	0
Liquid-liquid	p_a	-	4.211 $\pm$ 0.083
	p_b	S_t	0
	p_c	E	-0.031 $\pm$ 0.012
	p_d	$S_t E$	0
	p_e	M	-2.326 $\pm$ 0.145

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Table 3. Model parameters and experimental initial conditions

Parameters	Value± standard error			Unit
μ_{max}	0.230 ± 0.006			h ⁻¹
ξ_{Smax}	0.0013 ± 0.0003			h ⁻¹
ξ_{Lmax}	0.0046 ± 0.0005			h ⁻¹
ν_{max}	0.0062 ± 0.0004			h ⁻¹
$Y_{X/S}$	0.854 ± 0.050			g g ⁻¹
$Y_{EtOH/S}$	0.373 ± 0.020			g g ⁻¹
$Y_{IAOH/L}$	0.452 ± 0.055			g g ⁻¹
$Y_{IAA/IAOH}$	130/88			g g ⁻¹
K_S	6.41 ± 0.57			g L ⁻¹
K_{SIA}	0.02			g L ⁻¹
K_L	0.02			g L ⁻¹
K_{IAOH}	0.02			g L ⁻¹
$k_l a_l$	0.0117 ± 0.0003			h ⁻¹
Initial conditions	1) No addition	2) Addition at t_0	3) Addition at t_{12}	
X_0	0.86 ± 0.12	0.89 ± 0.04	1.01 ± 0.01	g L ⁻¹
S_0	35.18 ± 0.53	32.24 ± 1.88	36.40 ± 1.03	g L ⁻¹
L_0	0.0093	4.03 ± 0.02	0.0093	g L ⁻¹
$EtOH_0$	0.21 ± 0.03	0.17 ± 0.02	0.23 ± 0.05	g L ⁻¹
$IAOH_0$	0	0	0	g L ⁻¹
C_{d0}	0	0	0	g L ⁻¹