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Enhancing the bioproduction of value-added aroma compounds via solid-state fermentation of sugarcane bagasse and sugar beet molasses: Operational strategies and scaling-up of the process

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Abstract

Bioproduction of generally recognized as safe (GRAS) products starting with low-cost raw materials has become significant in the biorefinery concept. Thus, the solid-state fermentation (SSF) of agro-industrial residues using GRAS strains appears as alternative to obtain aroma compounds. Here, the SSF of the mixture sugarcane bagasse/sugar beet molasses was used for producing a mixture of value-added fruit-like compounds. The study aimed to enhance the production and ester selectivity evaluating three operational strategies at three scales (0.5, 4.5 and 22 L) using non-sterilized residues. While the average total volatile production was 120 mg_{Vol} per gram of dry substrate (g^{-1}_{ITS}), fed-batch operation promoted the highest increases in the ester content up to 57 $mg_{Est}\ g^{-1}_{ITS}$, an 88 and 59% more than in the static-batch and intermittent mixing modes respectively. Alternative operational strategies have compensated the scale-up adverse effects in the bioproduction, moving towards a sustainable large-scale application in a circular economy scheme.

Keywords

Kluyveromyces marxianus, waste valorization, Solid-state fermentation, sustainable process, scale-up.

1. Introduction

Aroma compounds are constituted by several volatile and nonvolatile components with specific physicochemical properties such that they produce odor perceptions in our brain (Berger, 2015). Natural aromas can be found in food, spices or essential oils, but also in flowers or plants (Sarma et al., 2014). Nowadays, their use as additives in food, cosmetic and fragrance industries is extensive due to their effect on the products, enhancing their organoleptic properties, and therefore, positively influencing the final consumer's perception and acceptance (Ziegler, 2007). Natural aromas are typically extracted from the matrices containing these compounds, but the low concentration of these species makes their recovery intricate and costly (Longo and Sanroman, 2006). Despite its cost, consumers prefer natural aromas than synthetic ones, since the latter tend to generate undesirable by-products imparting off-odors that change the organoleptic profiles of these (Etschmann et al., 2002). Consequently, the search for alternative routes for obtaining these compounds has become of major interest during the recent years (Dastager, 2009; de Oliveira Felipe et al., 2017). In this context, biotechnological routes appear as promising substitutes given the ability of some microorganisms to transform some raw materials into aroma compounds (Longo and Sanroman, 2006; Medeiros et al., 2010). In fact, these processes are encouraged by the current European and American legislation due to the qualification of Generally Recognized As Safe (GRAS), determining that products obtained by biotechnological routes are considered natural when the substrate used for this purpose also comes from a natural source (Dubal et al., 2008).

One of the biochemical process to obtain aroma compounds is the solid-state fermentation (SSF), which has been explored to produce value-added aromas like

vanillin (dos Santos et al., 2008), 2-phenethyl alcohol (Martínez et al., 2018), coconut-like aroma (Fadel et al., 2015) or fruit-like aromas (Martínez et al., 2017). Also, since the intrinsic characteristics of SSF made of it a suitable process to valorize solid organic waste (Castilho et al., 2009; Yazid et al., 2017), coupling SSF with agro-industrial residues becomes a good way for obtaining natural aroma compounds from a low-cost raw material (Sarma et al., 2014). Furthermore, since SSF is easy to operate and it requires relatively low capital investment (Idris et al., 2017), integration of wastes with SSF results in more sustainable, environmentally friendly and economic processes (de Oliveira Felipe et al., 2017). Obtaining aroma compounds via SSF of organic residues has been tested using different microorganisms: *Trichoderma* strains for producing coconut-like aroma (de Souza et al., 2008), *Saccharomyces cerevisiae* for aroma volatiles (Aggelopoulos et al., 2014), *Ceratocystis fimbriata* and *Kluyveromyces marxianus* for fruit-like compounds (Martínez et al., 2017; Rossi et al., 2009). *K. marxianus* characteristics (versatility, adaptability to grow under extreme conditions and in different solid media) make of this strain a promising volatiles producer (Lane and Morrissey, 2010; Morrisey et al., 2015).

Nevertheless, the efforts made to verify the feasibility of these bioprocesses are typically limited by constraints like the process scale, the sterilization of the substrates or the use of pure reagents as precursors, which in turn, restrict their use in large-scale applications (Aggelopoulos et al., 2014; Sarma et al., 2014). Besides, previous studies (Martínez et al., 2017) have shown the importance of the operating conditions in the selectivity of volatile compounds produced in SSF but, to our knowledge, there is no information about the effect of the operational strategies (operating modes) on the volatile compounds generation via SSF. In general, reported systems are limited to those

97 in which substrates are completely loaded at the beginning of the process and
98 considering that the solid media remains static during the fermentation time (a static-
99 batch). Since the nutrients availability is also a crucial parameter to the products
100 selectivity in SSF processes (Raimbault, 1998), the way the fermentation is performed,
101 could serve as a tool to modify this availability along the process. In this sense, the
102 intermittent mixing of the solid bed could improve process yield (Jiménez-Peñalver et
103 al., 2016) and fed-batch operation could enhance the microbial growth since the
104 nutrients are not entirely available at the beginning of the fermentation (Astolfi et al.,
105 2011). In the same way, the scale-up effect is a typical disadvantage of SSF process due
106 to the heat and mass transfer phenomena presented in the solid-liquid-gas interphases
107 (Cerda et al., 2017b; Soccol et al., 2017), hampering the development of the technology
108 at industrial scale. Since the behavior of the fermentation changes because of these
109 effects, productivity and selectivity are prone to be affected as well, so the
110 implementation of operating approaches able to minimize these negative effects (Astolfi
111 et al., 2011) becomes of major importance. Particularly, the bioproduction of aroma
112 compounds via SSF has been limited to lab and bench scales (Martínez et al., 2018,
113 2017), so the understanding of the process at higher scales is still very limited. As
114 described by some authors (de Oliveira Felipe et al., 2017; Sánchez et al., 2015), by
115 integrating more efficient strategies in SSF, it is also expected to contribute in the
116 improvement of the process sustainability, as well as in the industrial development of
117 the technology in the framework of the circular economy.
118 The aim of the study was to assess some operational strategies such as static-batch,
119 intermittent mixing and fed-batch to enhance the production of the aroma compounds
120 (fruit-like characteristics) in the SSF of the mixture sugarcane bagasse (SCB)/sugar beet

molasses (SBM) by means of *K. marxianus*. Also, it was investigated how these strategies affect the selectivity of the produced ester species (those most valuable for their high fruit profile). With this purpose, the selected strategies were tested using the non-sterilized substrate at three different scales (0.5, 4.5 and 22 L). Each scale was characterized by the potential effect in the fermentation temperature profile, produced due to the heat removal strategy of the process. Thus, at 0.5 L temperature was kept constant as a reference condition, at 4.5 L reactors worked at a near-isolated condition and at 22 L reactor was neither temperature-controlled nor isolated.

2. Materials and methods

2.1. Inoculum

Kluyveromyces marxianus (ATCC 10022) was purchased from Colección Española de Cultivos Tipo (CECT, Valencia, Spain). The strain was grown at sterile conditions (materials and media have been previously sterilized by autoclaving at 121°C for 30 min) at 30°C during 20 h on agar slants containing: glucose (40 g L^{-1}), yeast extract (5 g L^{-1}), soy peptone (5 g L^{-1}) and agar (20 g L^{-1}). *K. marxianus* was maintained in cryovials containing impregnated pearls with the strain at -80°C. Preparation of the inoculum consisted of adding one pearl into a 150 mL Erlenmeyer flask filled with 80 mL of a liquid medium consisting of glucose (40 g L^{-1}), yeast extract (5 g L^{-1}) and soy peptone (5 g L^{-1}). Then, using a rotary shaker, the culture was incubated for 20 h at 30°C and 180 rpm. Once grown, it was used to inoculate the non-sterilized substrate.

2.2. Substrate preparation

144 Sugarcane bagasse was supplied by Ingenio Ntra. Sra. del Carmen (Málaga, Spain) and
145 it has been dried at 60°C in an air oven during 24 h. Then, the substrate was ground to
146 achieve a particle size distribution in the range 0.5-32 mm by means of a granulator
147 mill. The dried and grounded substrate was stored at -20°C until it was used. Sugar beet
148 molasses were provided by the sugar company AB Azucarera Iberia S.A. (Madrid,
149 Spain) keeping them at 4°C until their addition to the substrate mixture. The substrate
150 mixture was prepared with sugarcane bagasse, adjusting the pH, moisture content, and
151 molasses content. This process was performed using a 1:1 (v:v) mixture of a phosphate
152 buffer pH 7 (0.1 M) and a nutrient solution containing 3.0 g L^{-1} $MgSO_4.7H_2O$, 0.4 g L^{-1}
153 $MnSO_4.4H_20$, 0.8 g L^{-1} $ZnSO_4.7H_2O$, 1.9 g L^{-1} $(NH_4)_2SO_4$ and 1.5 g L^{-1}
154 $Fe(NO_3)_3.9H_2O$. Following, molasses were added to the mixture above. Once they were
155 dissolved, they were mixed with the dried sugarcane bagasse. After preparation of the
156 substrate, it was inoculated using approximately 10^8 colony forming units (CFU) of *K.*
157 *marxianus* per gram of initial total solids content (ITS) of substrate (g_{ITS}).
158
159 2.3. SSF operational modes
160 Table 1 contains the main description of the performed experiments. As detailed,
161 evaluated strategies comprise: static-batch, intermittent mixing and fed-batch
162 operational modes. All them, tested at the three selected scales.
163 **Table 1**
164 2.3.1. Lab-scale 0.5 L and constant temperature
165 Lab scale tests were carried out in 0.5 L glass Erlenmeyers. The system consisted of a
166 triplicate (containing up to 96 g of prepared substrate) positioned in a temperature-
167 controlled water bath, such that independent mass flow controllers (Bronkhorst Hitec)

168 continuously supplied humidified air to the flask as detailed elsewhere (Ponsá et al.,
169 2010). The respirometric analysis consisted of computing the oxygen uptake rate
170 (OUR), and the cumulative oxygen consumption (COC) as stated by Ponsá et al. (2010).
171 When intermittent mixing or fed-batch approaches were tested, the content was
172 manually mixed into a 1 L glass beaker using a spatula and then quantitatively loaded
173 back into the reactor. Experiments were executed in triplicate and followed during 69 h.
174
175 2.3.2. Bench-scale near-isolated 4.5 L reactor
176 Experiments were performed in 4.5 L air-tight packed-bed reactors (adapted Dewar®
177 vessels) as described by Abraham et al. (2013) using up to 860g of the prepared
178 substrate. These reactors are thermally isolated, so they provide near-adiabatic condition
179 with negligible heat exchange with the surroundings. Thus, the process can proceed
180 with insignificant heat losses. The temperature of the solid media (measured at the
181 midpoint of the bed) (Pt-100 sensors, Sensotrans) and the exhausted gases oxygen
182 concentration (αLphase Ltd.) were monitored online using a self-made data acquisition
183 system (Arduino®-based), following the same respirometric analysis of section 2.3.1.
184 When intermittent mixing or fed-batch approaches were tested, the content was
185 manually mixed in 5 L plastic trays and then, quantitatively loaded back into the reactor.
186 Experiments were performed in duplicate and followed during 100 h.
187
188 2.3.3. Bench-scale non-isolated 22 L reactor
189 Experiments were performed in duplicate using a cylindrical 22 L reactor with an
190 automatic helical ribbon mixer and a removable inner basket where the substrate is
191 placed (Figure 1). The system was filled with a maximum of 2.5 kg of the prepared

192 substrate (90% capacity), and it was monitored similarly to the reactors described in
193 sections 2.3.1 and 2.3.2. Here, intermittent mixing was automatically set (14 rpm for 5
194 min) according to the established frequency as detailed in Table 1. Fed-batch additions
195 were accompanied by 5 min of mixing at the same mixing rate. Experiments were
196 monitored for 69 h.

197 **Figure 1**

198 2.4. Analytical methods

199 2.4.1. Determination of volatile compounds in the gas phase

200 The composition of the headspace in the SSF systems was determined by thermal
201 desorption gas chromatography mass spectrometry (TD-GC-MS) as described in
202 Martínez et al. (2017) (Supporting information). Total volatile productivity (P_{vol}^{t}) and
203 ester species productivity (P_{Est}^{t}) at time t, were computed as:

204

205 $$P_{vol}^{t}\left[\frac{mg_{Vol}}{h\ g_{TS}}\right] = \sum_{i=1}^{n} S_{AFR}\ C_i \quad (1)$$

206 $$P_{Est}^{t}\left[\frac{mg_{Est}}{h\ g_{TS}}\right] = \sum_{j=1}^{m} S_{AFR}\ C_j^{Est} \quad (2)$$

207 Where S_{AFR} is the specific air flow rate supplied to the reactor at time t (L h^{-1} g^{-1}_{ITS})
208 referred to the initial total solid content of the substrate, C_i and C_j^{Est} are the headspace
209 concentrations of the compound *i* and ester species *j* ($mg_{i,j}$ L^{-1}) at time t, *n* is the total
210 amount of quantified volatile compounds and m is total ester species quantified
211 (Supporting information).

212

213 2.4.2. *K. marxianus* cells counting

214 *K. marxianus* population was measured as described by Ballardo et al. (2016) adding 10
215 g of sample into a bottle containing 100 mL of a NaCl 9 g L^{-1} solution. The mixture was

shaken for 15 min at 200 rpm in an orbital shaker at 20°C. The supernatant was used to prepare dilutions in NaCl 9 g L^{-1} which were plated on Petri dishes (in triplicate) containing the same media used in the growing of the pure strain (that used for preparing the inoculum). Cultures were incubated at 30°C for 20 h, and the *K. marxianus* population was determined by counting the units. Results were expressed as the mean value in CFU of *K. marxianus* per gram of total solids at time t (g_{TS}).

2.4.3. Sugar content

Reducing sugars content of the fermented substrate were quantified using the DNS method (Miller, 1959). The analysis was performed using the supernatant produced after a solid-liquid extraction of the substrate with distilled water in a 1:7 (w/v) ratio at 50°C for 30 min. The liquid fraction was filtered through a 0.45 µm membrane filter, and it was properly diluted before its processing.

2.4.4. pH and moisture content

pH, total solids (TS), volatile solids (VS) and moisture content (MC) were measured following the standard procedures (The US Department of Agriculture and The US Composting Council, 2001).

2.5. Statistical analysis

For the evaluation of strategies, statistical differences were analyzed by one-way ANOVA ($p<0.05$ confidence) using the Tukey test. The results represent the mean value of at least two replicates. Data were analyzed with Minitab 16 (Minitab Inc.).

3. Results and discussion

Analysis of the process was performed by means of some performance indices: total cumulative volatile production (P_{vol}^{Acc}) [$mg_{vol}\ g^{-1}_{ITS}$] (calculated as the numerical integration of P_{vol}^{t}), total cumulative ester production (P_{Est}^{Acc}) [$mg_{Est}\ g^{-1}_{ITS}$] (computed as the numerical integration of P_{Est}^{t}), and space-time yield (Y_{s-t}) [$mg_{vol}\ L^{-1}\ h^{-1}$] (computed as the total productivity per reactor volume). Table 2 summarizes the main performance indices obtained for the different strategies at the selected scales.

Table 2.

3.1. Process behavior at lab scale

These experiments consisted of evaluating the effect of the operational strategies shown in Table 1 on the P_{vol}^{Acc} as well as on the selectivity to the ester species (measured as P_{Est}^{Acc}) at 0.5 L and constant temperature (T). Initial pH, MC, T, S_{AFR}, molasses load, and inoculum load were set at 5.3, 68%, 30°C, 0.11 L h^{-1} g^{-1}_{ITS}, 20% (dry basis) and 10^8 CFU g^{-1}_{TS} respectively according to previous findings (Martínez et al., 2017). In Figure 2(a) a comparison of the performance indices for the evaluated operational strategies at 0.5 L scale is presented. The first remarkable result that can be extracted is that there are no significant differences between the strategies when P_{vol}^{Acc} is analyzed according to the Tukey test, reaching a mean value of 107.6 $mg_{vol}\ g^{-1}_{ITS}$. On the contrary, the maximum space-time yield changes among the evaluated strategies, particularly when a fed-batch operation is used. These results suggest that operational strategies do not influence the total volatile production, and therefore, it could be expected that keeping the initial operating conditions unchanged, P_{vol}^{Acc} becomes a function of other variables like the nutrients availability. Instead, operational strategies have an evident effect on the selectivity of the species being produced in the fermentation. As observed in Figure

264 2(a), when the production is split into the three major groups of obtained species
265 (aldehydes, alcohols and esters) there is a change in the contribution of these species to
266 P_{vol}^{Acc} depending on the selected strategy. Thus, in the reference scenario (static-batch),
267 the mean contribution comes from the alcohol species (39.02±0.01%), followed by the
268 esters (36.02±0.02%) and finally the aldehydes (24.96±0.01%). When the intermittent
269 mixing is used, the contribution has the same pattern, but there is a subtle increase in the
270 amount of aldehydes and esters. Then, in the fed-batch strategy, the trend changes, and
271 this time the primary contribution is due to the ester species, with similar amounts of
272 alcohols and aldehydes (around 27% each). Based on these results it can be stated that
273 the intermittent mixing has produced negligible changes regarding P_{Vol}^{Acc} and P_{Est}^{Acc}
274 (Table 2) compared to the batch mode. Although it is expected an improvement in the
275 inoculum-nutrients interaction and lower compaction of the bed when using mixing
276 (Rodriguez-Fernandez et al., 2012), it seems the enhancement is limited due to the
277 scale. Also, by splitting the substrate load resulted in an efficient way to limit the
278 available nutrients in the media, forcing *K. marxianus* to use them to metabolize the
279 ester species preferentially.

280 **Figure 2**

281 In Figure 3, the time course of the SSF at 0.5 L and constant temperature in static-batch
282 (a) and fed-batch 33% (b) modes can be observed. The batch SSF shows a rapid
283 increase of biological activity (measured as OUR) until 24 h of processing when the
284 maximum activity is achieved, coinciding with the depletion of available sugars. At that
285 point, volatile production almost ceases, so the maximum $Y_{s\text{-}t}$ is also achieved. In the
286 fed-batch strategy, the process starts similarly than in batch mode with a rapid growth
287 until the maximum activity is achieved around 24 h (Figure 3(b)). At that point, the

second part of the substrate is fed, allowing the fermentation to proceed another 6 h at similar conditions than in the first hours of processing. At 30 h a second peak of activity is achieved, and the last addition of substrate is made; this time, the process takes another 6 h to reach the maximum activity. At that point, the productivity is almost null, and the accumulated volatile production hardly changes. As it is observed, fed-batch operation promotes P_{Est}^{Acc} by extending the *K. marxianus* activity (while in the static mode the activity grows during 24 h, in fed-batch it lasts 36 h), but as a consequence, a lower volatile productivity rate (*i.e.* P_{Vol}^{Acc} curve slope) than the batch mode is achieved. Therefore, a significant decrease in $Y_{s\text{-}t}$ is observed (Figure 2(a) and 3).

Figure 3

Results also show how the strain has adapted better to the solid media in the fed-batch strategy compared to the batch one. As seen in Figure 3(b), the maximum activity was achieved after the two successive additions of fresh material (21.3 g_{02} per kg of volatile solids (VS) per hour), even when the media contained a lower sugar availability than in preceding steps of the fermentation. Thus, *K. marxianus* was able to use this lower content of nutrients to grow even more than in the first steps of the process (1.48 10^9 CFU g^{-1}_{TS} at 24 h, 2.02 10^9 CFU g^{-1}_{TS} at 30 h, 3.53 10^9 CFU g^{-1}_{TS} at 36 h), and also more than in the batch scenario (up to 1.98 10^9 CFU g^{-1}_{TS} at 24 h).

Ester selectivity enhancement in fed-batch might be attributed to the synergistic effect of some factors. From a physical standpoint, operating a static-batch SSF implies a compression of the solid substrate inside the reactor, which is the result of several changes in the solid bed such as the increase in the moisture content or the loss of weight and porosity (Barrios-González, 2012). Typically, the loss of porosity would hinder in some degree the strain growth due to the lower accessibility to nutrients and

312 oxygen (Martínez et al., 2017), and therefore, it would limit the biotransformation.
313 When a fed-batch strategy is used, compression of the media is reduced since the partial
314 feeding implies a mixing in which the bed is readjusted to its initial condition, and
315 porosity of the bed is partially recovered. Another aspect of the fermentation influenced
316 by the strategy is the Crabtree effect. Because of this effect, in a static-batch SSF, the
317 yeast can use the available sugars to produce ethanol even under aerobic conditions,
318 spending a considerable amount of nutrients on it and limiting the ester production. In a
319 fed-batch strategy, this aspect is compensated when the fresh substrate is added since
320 the global effect is a dilution of the available nutrients (Carvalho et al., 2006). By doing
321 this, similarly than in a submerged fermentation (SmF), the amount of sugars in the
322 media is kept lower than in a static-batch SSF regarding the number of cells growing in
323 the media (Supporting information). Therefore, *K. marxianus* becomes less prone to
324 produce ethanol due to the Crabtree effect. Although some authors suggest *K.*
325 *marxianus* is Crabtree negative (Fonseca et al., 2008), many references indicate the
326 opposite (Etschmann et al., 2003; Lane and Morrissey, 2010) and given the results of
327 this study; it is clear that the studied strain is part of the group of Crabtree positive
328 yeasts.
329
330 3.2. Process in a 4.5 L near-isolated reactor
331 This set of experiments consisted of evaluating the effect of the operational strategies on
332 the P_{vol}^{Acc} and P_{Est}^{Acc} of the SSF considering the effect of working with variable
333 temperature in near-isolated reactors. By doing this, the process was assessed
334 resembling a typical large-scale SSF or composting operation, in which the mass of
335 substrate is large enough to create adiabatic zones due to the heat transfer limitations

inherent to the solid organic media (Barrena et al., 2006). In this way, a non-constant evolution of temperature is achieved, and the given increase in temperature (due to the metabolic activity) helps to the development of the fermentation (Santis-Navarro et al., 2011). Initial pH, MC, S_{AFR}, molasses load, and inoculum load were set at 5.3, 68%, 0.11 L h^{-1} g^{-1}_{ITS}, 20% (dry basis) and 10^8 CFU g^{-1}_{TS} respectively as done in section 3.1. Figure 2(b) contains a summary of the performance indices for the evaluated operational strategies at 4.5 L. In this case, both P_{vol}^{Acc} and Y_{s-t} present negligible differences among the evaluated strategies. While the mean P_{vol}^{Acc} has achieved 127.1 mg_{vol} g^{-1}_{ITS} for the set of strategies (18% above the value found at 0.5 L and constant temperature), the mean maximum Y_{s-t} was 121.5 mg_{Vol} L^{-1} h^{-1}. In this case, the distribution of the principal species has followed a similar trend to that in section 3.1. As observed, the static-batch operation promotes mainly the production of alcohols (44.26±0.03%) and just a 19.06±0.03% of esters. Once the intermittent mixing is integrated, the distribution changes, inducing an increase in ester accumulation (29.62±0.04%), with the corresponding reduction in alcohols species production (40.07±0.04%). Nonetheless, the most significant changes occur when the process is operated in fed-batch mode. In that case, the percentage of ester species reaches 33.67±0.03% while alcohols are reduced until 37.37±0.01%.

In this scenario, the operational strategies have affected the same factors aforementioned in section 3.1, but in addition, they have also influenced the temperature profile of the solid media, causing significant changes in the development of the fermentation. As seen in Figure 4(a), the time profile of the SSF in the near-isolated static mode presents a rapid OUR increase, temperature and volatile accumulation during the first 12 h of processing. At that point, the peak of maximum OUR (15 g_{02} kg^{-}

360 $^{1}{}_{VS}\ h^{-1}$) and temperature (48°C) are achieved. Then, a second peak is presented at 64 h
361 after a period of lower activity reaching up to 6 $g_{02}\ kg^{-1}{}_{VS}\ h^{-1}$ and 42°C, moment in
362 which the process starts decaying until its end. As observed, the differences among
363 static-batch processes at constant temperature and near-isolated are evident. In the latter,
364 the selectivity of volatiles suffers a vast change as a consequence of the temperature
365 (Martínez et al., 2017). In fact, since in the latter scenario the fermentation proceeds
366 most of the time at temperatures above 38°C (during 40.7 h), *K. marxianus* is prone to
367 produce alcohol species preferentially. On the contrary, when the fed-batch strategy is
368 used in the near-isolated conditions, the feeding points (at 12 and 19.5 h) help to reduce
369 temperature in around 20°C, allowing *K. marxianus* to grow at temperatures below
370 35°C for more extended periods than in a static mode (22 h against 14 h) (Figure 4(b)).
371 Consequently, the accumulation of esters becomes not just a function of the substrate
372 split as occurred at constant temperature, but mainly a function of the fermentation
373 temperature. Thus, P_{Est}^{Acc} can be improved keeping temperature controlled along the
374 fermentation. In this case, since two additions of fresh material are not enough to
375 control this variable, successive mixing where carried out each time temperature has
376 achieved a maximum (mixing without any substrate addition) to reduce the temperature
377 of the media (Figure 4(b)). By doing this, a higher activity for the *K. marxianus* and the
378 corresponding higher production of ester species was obtained. Since the operational
379 strategy by itself is not good enough to control the main variable affecting the adiabatic
380 process (temperature), a combination of operational strategy and reactor design could be
381 another approach to consider for large-scale applications.
382 **Figure 4**
383 3.3. Process in a 22 L non-isolated reactor

384 Experiments in this section involved the evaluation of the operational strategies on
385 P_{vol}^{Acc} and P_{Est}^{Acc} considering the effect of working with variable temperature in a non-
386 isolated steel 22 L reactor with automated mixing. Initial pH, MC, S_{AFR}, molasses load,
387 and inoculum load were set at 5.3, 66%, 0.11 L h^{-1} g^{-1}_{ITS}, 20% (dry basis) and 10^8 CFU
388 g^{-1}_{TS} respectively. Figure 2(c) shows the performance indices and the distribution of the
389 produced species for the different operational strategies at 22 L scale. As seen, there are
390 no significant differences among the P_{vol}^{Acc} for the assessed strategies, reaching a mean
391 of 119.9 mg_{vol} g^{-1}_{ITS}. Also, the distribution of aldehydes, alcohols and ester species
392 follows a similar trend when it is compared among the strategies. In the static-batch
393 mode, the major components of the produced volatiles are alcohols (40.93±0.02%), then
394 aldehydes (33.31±0.01%) and finally esters (25.76±0.04%). By means of intermittent
395 mixing the final distribution changes and in this time aldehydes are the main
396 components (38.50±0.03%), followed by alcohols (31.90±0.01%) and esters
397 (29.60±0.04%). In the case of fed-batch operation, the trend suddenly changes, and
398 47.45±0.02% of P_{vol}^{Acc} become ester species, while alcohols and aldehydes are almost
399 equally distributed with 25.51±0.01% and 27.04±0.05% respectively.
400 These results corroborate the trend found in the preceding sections in which the use of
401 more advanced operational strategies mainly improve the selectivity of the obtained
402 volatiles, enhancing the production of the ester species, those with a higher value-added
403 regarding the contribution to the fruity aroma profile (Longo and Sanroman, 2006). At
404 this scale, a static-batch SSF presents a time course (Figure 5(a)) starting with 11 h lag
405 phase, the time required to *K. marxianus* to increase the temperature of the media from
406 20°C to 24°C. At that point, a rapid growth allows reaching the maximum activity and
407 temperature after 19 h of processing (12 g_{02} kg^{-1}_{VS} h^{-1} and 40°C respectively). After an

activity fall (as befell in the near-adiabatic static-batch), a second peak is achieved around 35 h reaching 7.5 g_{02} kg^{-1}_{VS} h^{-1} and 36.5°C. During this phase, accumulation of volatiles is lower than in the rapid growth phase, and it almost stops after the second peak of biological activity, coinciding with the depletion of the available sugars and the maximum $Y_{s\text{-}t}$. In the fed-batch operation (Figure 5(b)), the process takes almost 15 h to start increasing both the activity and the temperature because the reactor is filled with only a third of the substrate mass (regarding the load used in the static-batch). It is only after 27 h when the first peak of maximum activity is achieved (14.5 g_{02} kg^{-1}_{VS} h^{-1}) reaching 30°C. At that point, the second addition of fresh material was performed, allowing the process to resume for another 5 h when the second peak of activity was achieved (13 g_{02} kg^{-1}_{VS} h^{-1} and 33°C). Then, the third addition of fresh substrate was performed, and as expected, falling of temperature together with the addition of new sugars in the medium. This allowed the strain to remain active for another 7 h (until 39 h), point when the process arrived at the maximum activity (18 g_{02} kg^{-1}_{VS} h^{-1}) and therefore, the maximum temperature (41°C). In this phase, the maximum $Y_{s\text{-}t}$ is also achieved (100.6 mg_{Vol} L^{-1} h^{-1}), but the availability of sugars has allowed the volatile productivity to remain unchanged until 49 h, maintaining the $Y_{s\text{-}t}$ almost constant during this 10 h of processing. Finally, once the available sugars are consumed, the process falls downs until its end after 69 h of processing, but in this phase, P_{vol}^{Acc} has remained almost unchanged and not significant volatile accumulation was obtained. Similarly than at 0.5 L, extending the *K marxianus* activity allowed producing more esters, but, it also induced a lower productivity in the growth phase which has carried to a significant decrease in $Y_{s\text{-}t}$ (Figure 2(c) and 5).

Figure 5

The same aspects affecting the esters selectivity at constant temperature could be perceived in the non-isolated reactor. Mainly, it was observed the better adaptation of the strain to the solid media when a fed-batch approach was used. Again, the partial feeding has allowed *K. marxianus* to grow better after the last addition of fresh material (1.15 10^9 CFU g^{-1}_{TS} at 27 h, 1.02 10^9 CFU g^{-1}_{TS} at 32 h, 1.93 10^9 CFU g^{-1}_{TS} at 39 h). Also, using intermittent mixing and fed-batch approaches seems to reduce the Crabtree effect (compared to the static-batch mode), since the available sugars regarding the number of cells in the media, resulted in just a fraction of the presented in the static-batch (Supporting information). As seen, the fed-batch strategy has positively affected the ester selectivity as occurred at lower scales, confirming its benefits on the process.

3.4. Scaling and operating strategy effects

As detailed in Figure 6, the scale-up effect, considering the inherent changes in the temperature evolution, have limited effects in P_{Vol}^{Acc}. In fact, the significant differences found among the evaluated scales suggest that the total volatile accumulation is an apparent function of the operating temperature as previously described by Martínez et al. (2017). Thus, the trend followed by P_{Vol}^{Acc} is similar than the average fermentation temperature at the evaluated scales. A different situation occurs for P_{Est}^{Acc}. In this case, changing the scale has different effects depending on the operating mode. For a static-batch, the increase in scale has promoted a reduction in the ester production without any improvement respect to the 0.5 L scenario. For the intermittent mixing, it could be stated that change in scale has served to improve the static-batch condition, but the improvement is not enough to reach the values found at 0.5 L. Finally, the analysis of

455 the fed-batch mode shows a higher improvement than in the mixing strategy and, at 22
456 L it is able to reach a level above the one achieved at 0.5 L.
457 Moreover, the results suggest that working with not-sterilized substrates produces, as
458 expected, a reduction in the volatile production. While in an optimized batch process
459 Martínez et al. (2017) have reached up to 161 $mg_{Vol}\ g^{-1}_{ITS}$ (40°C, 35% molasses and
460 0.14 L $h^{-1}g^{-1}_{ITS}$) and 47.6 $mg_{Ester}\ g^{-1}_{ITS}$ (30°C, 25% molasses and 0.11 L $h^{-1}g^{-1}_{ITS}$), here,
461 the ester content only reached 38.3 $mg_{Ester}\ g^{-1}_{ITS}$ in batch mode at the same conditions.
462 Similarly, the maximum volatile production reached here was 129.6 $mg_{Vol}\ g^{-1}_{ITS}$, far
463 from the results obtained at constant temperature and using sterilized substrates.
464 Nonetheless, the use of alternative operational strategies, particularly the fed-batch SSF,
465 points out that operating modes could act as practical tools for reducing this negative
466 effect. As seen, this strategy allowed to produce a maximum of 57 $mg_{Ester}\ g^{-1}_{ITS}$, a 20%
467 more than the best result found by Martínez et al. (2017).
468 **Figure 6**
469 On the other hand, regarding the operating strategies effects, it could be stated that, in
470 general, SSF processes are undertaken in batch mode, and the study of alternative SSF
471 operational strategies has been typically limited. However, the positive results in this
472 study, as well as some other SSF bench-scale applications (Astolfi et al., 2011; Cerda et
473 al., 2017a) using alternative operating strategies, possess in evidence their potential to
474 improve the SSF performance in a reliable and simple way. Particularly, considering the
475 fed-batch strategy, the positive impacts are not limited to the production and selectivity
476 aspects. As detailed in Table 2, although fed-batch strategy has the higher COC, it has
477 the lowest total air consumption per gram of processed substrate, with reductions
478 ranging between 10-29 % compared to the static-batch. Furthermore, this strategy

requires only a fraction of the inoculum needed in batch systems (*e.g.*, only 33% for FB-33), representing a considerable save of resources. As stated by Cheirsilp and Kitcha, (2015) the inoculum savings could play a key role in the implementation at industrial scale, positively influencing on economic and sustainability aspects of the SSF process.

4. Conclusions

Fruit-like compounds production via SSF was studied under different operational strategies at three scales using as substrate the non-sterilized mixture sugarcane bagasse/sugar beet molasses. The fed-batch strategy resulted in a more effective way to obtain these value-added compounds by enhancing the ester species content. The synergy among sugars availability and temperature control are the main responsible for fed-batch success for improving the esters selectivity. The importance of alternative operational strategies as tools for the enhancement of the SSF's efficiency and sustainability was also shown. However, for the industrial development of these strategies, a suitable downstream development needs to be assessed.

Acknowledgments

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Table 1. Characteristics of the evaluated operational strategies performed at 0.5, 4.5 and 22 L scale.

Experiment	Corresponding Strategy	Characteristic	Substrate load*
Static-batch	Static substrate (Reference condition)	Substrate remains static along the fermentation	100% at t_0
Mixing	Intermittent mixing	Improve interaction nutrients-strain 12 h fixed mixing interval	100% at t_0
FB-50[a]	Fed-batch operation + Intermittent mixing (at selected points[b])	Limiting nutrient availability + temperature control by means of a partial feeding	50% at t_0 50% at t_1
FB-33			33% at t_0 33% at t_1 33% at t_2

*t_0: At the beginning of the process; t_1: Point of maximum activity achieved after t_0; t_2: Point of maximum activity achieved after t_1. FB-50: fed-batch with a split of two; FB-33: fed-batch with a split of three. The reference for 100% load is the maximum amount of substrate used in the static-batch (*i.e.*, 96g for 0.5 L, 860g for 4.5 L and 2.5kg for 22L). [a]Fed-batch 50% was performed only at 0.5 L scale. [b]Mixing points in these strategies were defined based on the temperature profile; once the maximum temperature was achieved a mixing was performed.

641 **Table 2.** Performance parameters of the solid-state fermentation of sugarcane

642 bagasse/sugar beet molasses for producing aroma compounds.

Operational Strategy	Performance index*	Evaluated scale		
		0.5 L	4.5 L	22 L
Static-batch	P_{vol}^{Acc} (mg_{Vol} g^{-1}_{ITS})/(g_{Vol} L^{-1}_{r})	106.3/6.8	129.6/7.3	118.0/8.0
	P_{Est}^{Acc} (mg_{Est} g^{-1}_{ITS})/(g_{Est} L^{-1}_{r})	38.3/2.5	24.7/1.9	30.4/1.5
	C_{Air} (NL_{air} g^{-1}_{PTS})	7.4	7.5	8.0
	COC (g_{O2} kg^{-1}_{ITS})	254.8	238.0	217.8
Intermittent mixing	P_{vol}^{Acc} (mg_{Vol} g^{-1}_{ITS})/(g_{Vol} L^{-1}_{r})	105.4/6.8	122.4/7.5	121.3/7.6
	P_{Est}^{Acc} (mg_{Est} g^{-1}_{ITS})/(g_{Est} L^{-1}_{r})	38.6/2.5	36.3/2.2	35.9/2.2
	C_{Air} (NL_{air} g^{-1}_{PTS})	7.4	7.5	8.0
	COC (g_{O2} kg^{-1}_{ITS})	232.1	230.4	234.3
Fed-batch 33%	P_{vol}^{Acc} (mg_{Vol} g^{-1}_{ITS})/(g_{Vol} L^{-1}_{r})	109.3/7.0	129.3/7.5	120.3/8.0
	P_{Est}^{Acc} (mg_{Est} g^{-1}_{ITS})/(g_{Est} L^{-1}_{r})	49.3/3.2	43.5/3.5	57.1/2.7
	C_{Air} (NL_{air} g^{-1}_{PTS})	5.5	6.7	5.6
	COC (g_{O2} kg^{-1}_{ITS})	310.0	388.1	442.6

643 *Production indices have been referred to the initial total solid content, and to the reactor volume. P_{vol}^{Acc}:
644 Total cumulative volatile production, P_{Est}^{Acc}: Total cumulative ester production, C_{Air}: total air
645 consumption per gram of processed substrate (g_{PTS}); COC: cumulative oxygen consumption at the end of
646 the fermentation. L_r: reactor volume in L. Volumetric production is based on the apparent density of the
647 solid media (*i.e.*, at 0.5 L corresponds to 64 g_{ITS} L^{-1}_{r} and 62 g_{ITS} L^{-1}_{r} at 4.5 and 22 L scales)
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654 **Figure Captions**

655 **Figure 1.** Experimental set-up of the Solid-state fermentation not-isolated 22 L reactor.

656 **Figure 2.** Performance indices of the evaluated operational strategies. (a) Constant
657 temperature at 0.5 L; (b) Near-isolated 4.5 L reactor; (c) Non-isolated 22 L reactor.
658 P_{Vol}^{Acc}: Total cumulative volatile production, Max Y_{s-t}: Maximum space-time yield, FB-
659 50%: Fed-batch operation with two splits, FB-33%: Fed-batch operation with three
660 splits. Different capital letters indicate significant differences between the evaluated
661 groups ($p<0.05$).

662 **Figure 3.** Time course of the solid-state fermentation of sugarcane bagasse/sugar beet
663 molasses for producing fruit-like compounds at 0.5 L scale. (a) Static-batch, (b) Fed-
664 batch 33%. P_{Vol}^{Acc}: Total cumulative volatile production, P_{Est}^{Acc}: Total cumulative ester
665 production, Y_{s-t}: Space-time yield, OUR: Oxygen uptake rate, Red Sug: Reducing
666 sugars. Arrows indicate the addition of fresh substrate in the fed-batch strategy.

667 **Figure 4.** Time course of the solid-state fermentation of sugarcane bagasse/sugar beet
668 molasses for producing fruit-like compounds in the near-isolated 4.5 L reactor. (a)
669 Static-batch, (b) Fed-batch 33%. P_{Vol}^{Acc}: Total cumulative volatile production, P_{Est}^{Acc}:
670 Total cumulative ester production, Y_{s-t}: Space-time yield, OUR: Oxygen uptake rate,
671 Red Sug: Reducing sugars, T: temperature. Continuous arrows indicate the addition of
672 fresh substrate in the fed-batch strategy, while dash arrows indicate the mixing points.

673 **Figure 5.** Time course of the solid-state fermentation of sugarcane bagasse/sugar beet
674 molasses for producing fruit-like compounds in the non-isolated 22 L reactor. (a) Static-
675 batch, (b) Fed-batch 33%. P_{Vol}^{Acc}: Total cumulative volatile production, P_{Est}^{Acc}: Total
676 cumulative ester production, Y_{s-t}: Space-time yield, OUR: Oxygen uptake rate, Red

677 Sug: Reducing sugars, T: temperature. Continuous arrows indicate the addition of fresh
678 substrate in the fed-batch strategy, while dash arrows indicate the mixing points.
679 **Figure 6.** Scaling-up effects on the volatile production of the solid-state fermentation of
680 sugarcane bagasse/sugar beet molasses. P_{Vol}^{Acc}: Total cumulative volatile production,
681 P_{Est}^{Acc}: Total cumulative ester production, T_{Avg}: Average temperature of the set of
682 fermentations at each evaluated scale. Different capital letters indicate significant
683 differences between the evaluated groups ($p<0.05$).
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Figure 1.

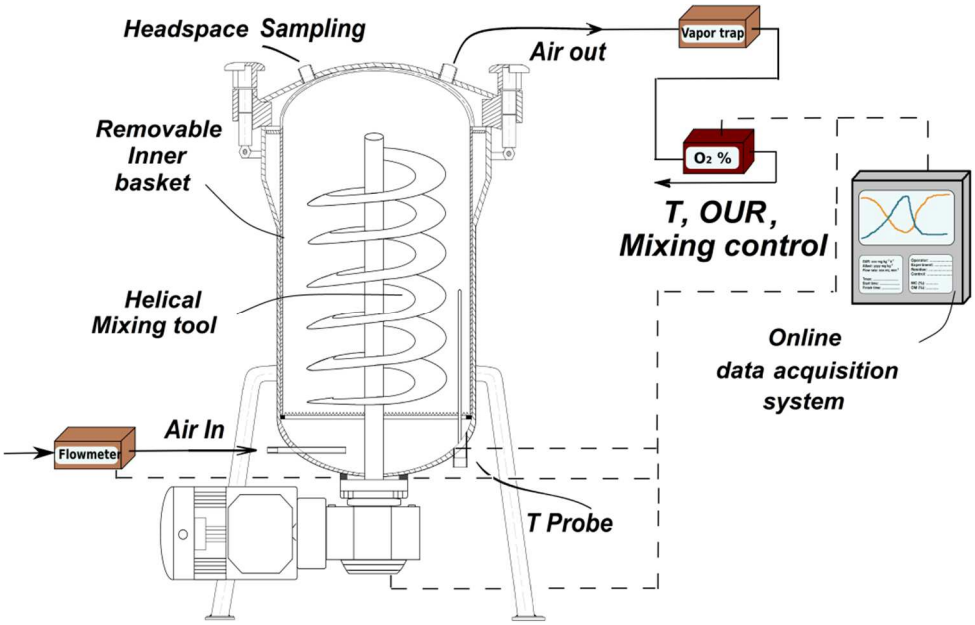

Headspace Sampling
Air out
Vapor trap
Removable Inner basket
O2 %
T, OUR, Mixing control
Helical Mixing tool
Online data acquisition system
Air In
Flowmeter
T Probe

Figure 2.

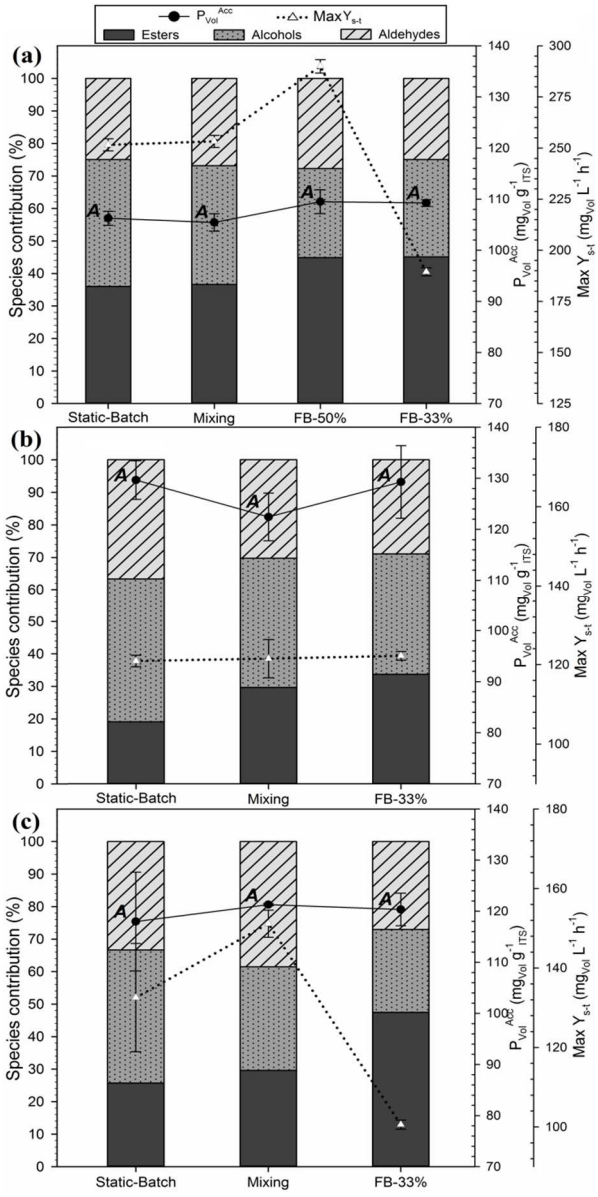

P_{Vol}^{Acc}
Max Y_{s-t}
Esters
Alcohols
Aldehydes
(a)
(b)
(c)
Species contribution (%)
P_{Vol}^{Acc} (mg_{Vol} g^{-1}_{ITS})
Max Y_{s-t} (mg_{Vol} L^{-1} h^{-1})
A
Static-Batch
Mixing
FB-50%
FB-33%

Figure 3.

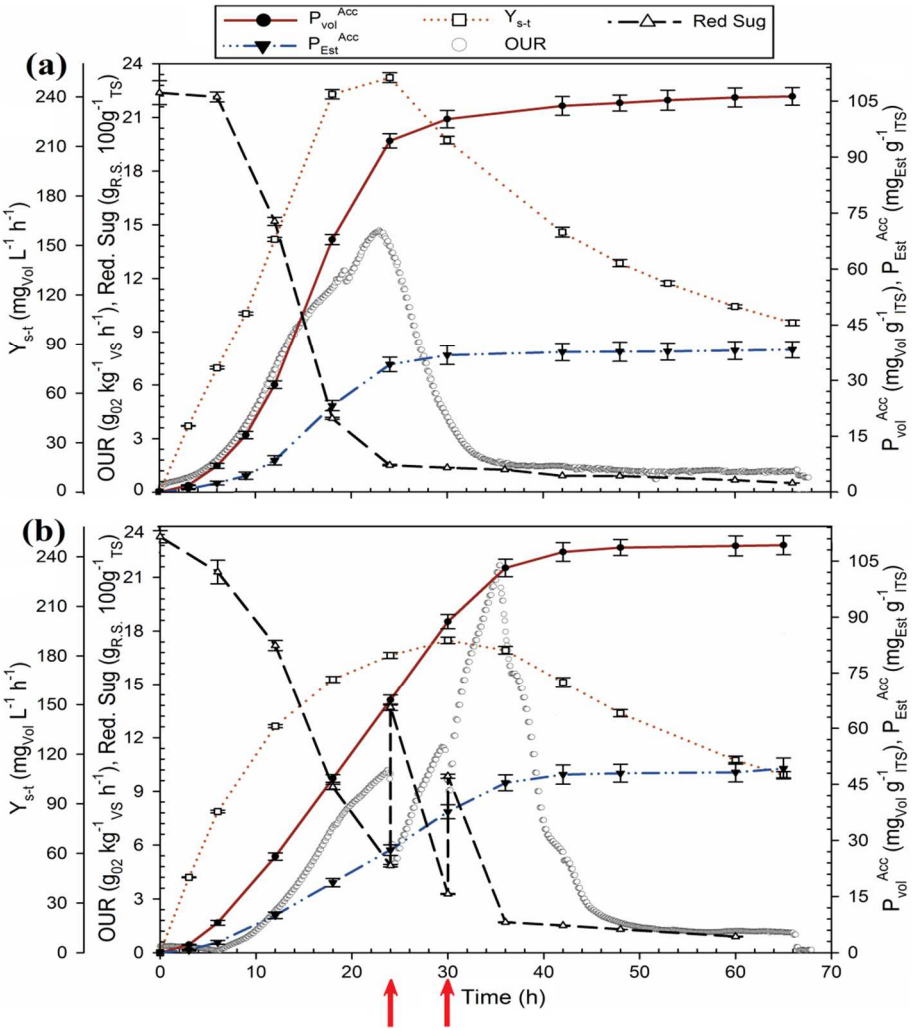

(a)
(b)
P_{vol}^{Acc}
P_{Est}^{Acc}
Y_{s-t}
OUR
Red Sug
Y_{s-t} (mg_{Vol} L^{-1} h^{-1})
OUR (g_{02} kg^{-1}_{VS} h^{-1}), Red. Sug ($g_{R.S.}$ $100g^{-1}_{TS}$)
P_{vol}^{Acc} (mg_{Vol} g^{-1}_{ITS}), P_{Est}^{Acc} (mg_{Est} g^{-1}_{ITS})
Time (h)

Figure 4.

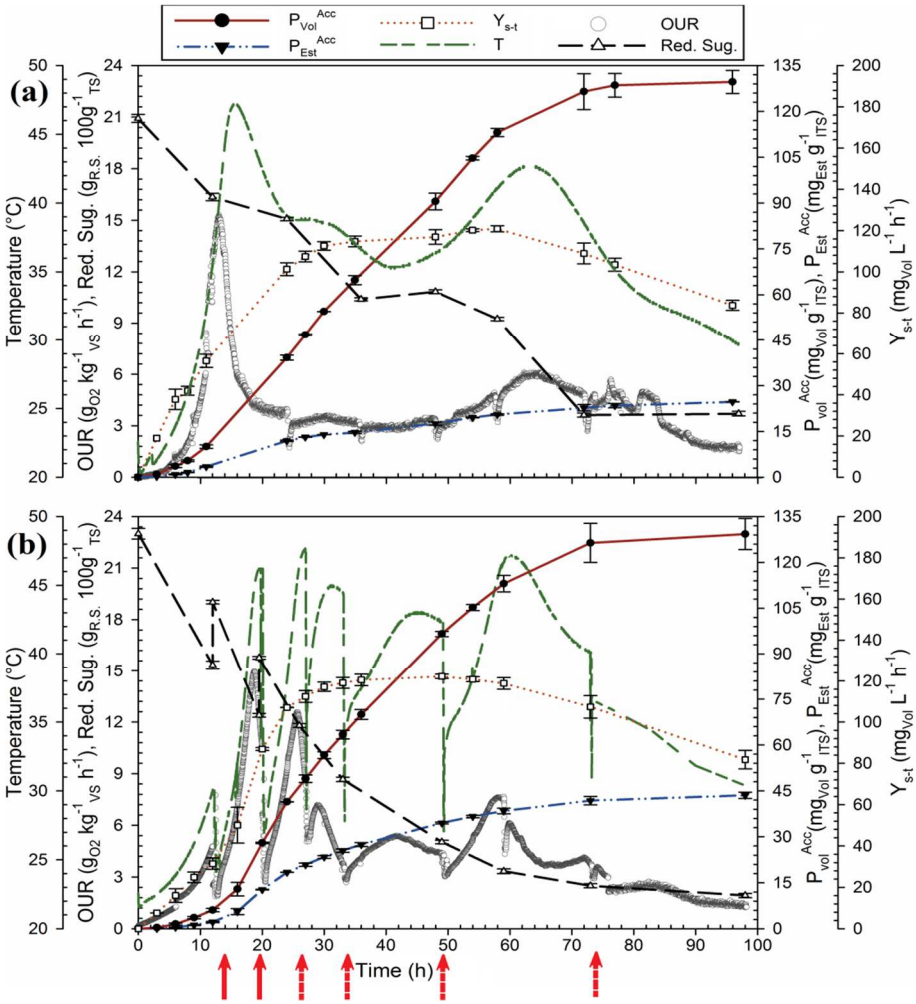

(a)
(b)
Temperature (°C)
OUR (g_{O2} kg^{-1}_{VS} h^{-1}), Red. Sug. ($g_{R.S.}$ $100g^{-1}_{TS}$)
P_{vol}^{Acc} (mg_{Vol} g^{-1}_{ITS}), P_{Est}^{Acc} (mg_{Est} g^{-1}_{ITS})
Y_{s-t} (mg_{Vol} L^{-1} h^{-1})
Time (h)
P_{Vol}^{Acc}
P_{Est}^{Acc}
Y_{s-t}
T
OUR
Red. Sug.

Figure 5.

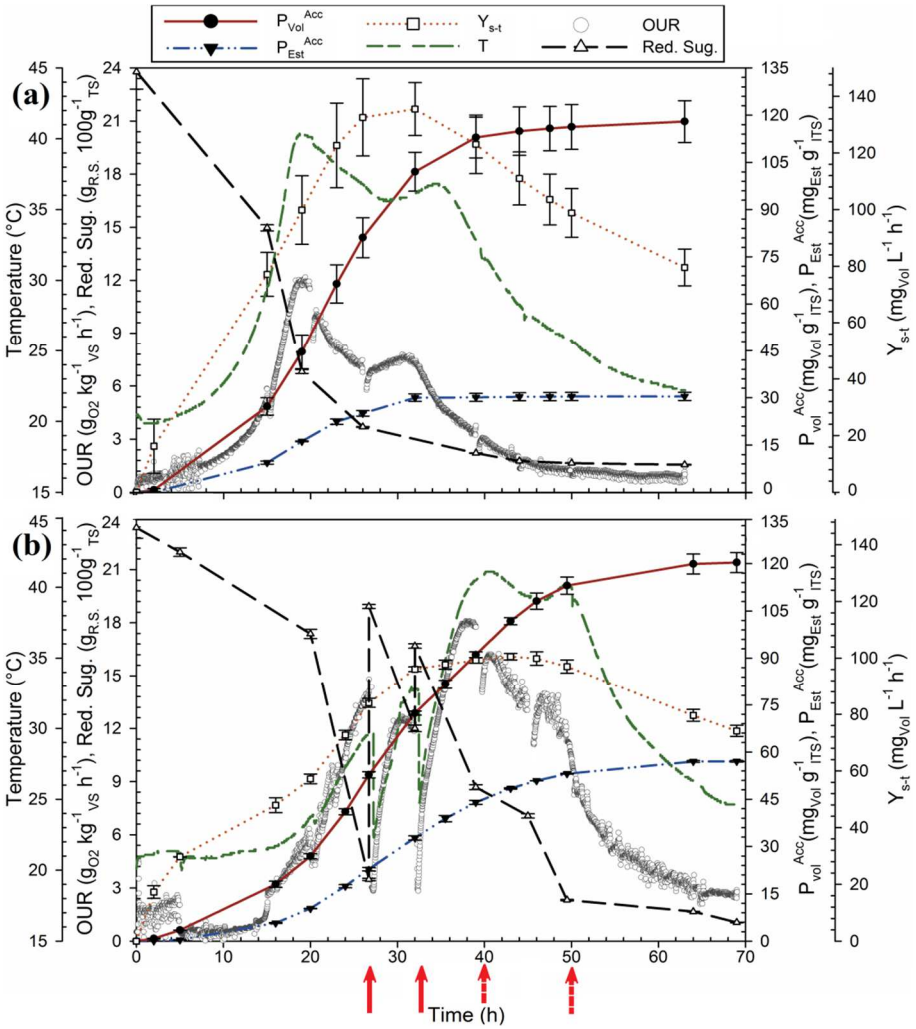
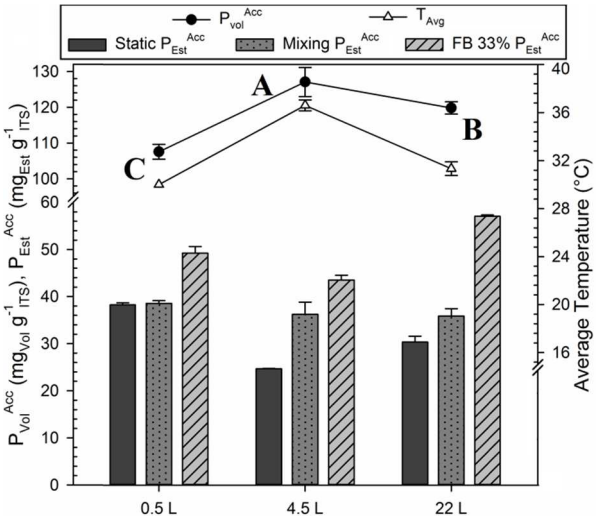

(a)
(b)
Temperature (°C)
OUR (g_{O2} kg^{-1}_{VS} h^{-1}), Red. Sug. ($g_{R.S.}$ $100g^{-1}_{TS}$)
P_{vol}^{Acc} (mg_{Vol} g^{-1}_{ITS}), P_{Est}^{Acc} (mg_{Est} g^{-1}_{ITS})
Y_{s-t} (mg_{Vol} L^{-1} h^{-1})
P_{Vol}^{Acc}
P_{Est}^{Acc}
Y_{s-t}
T
OUR
Red. Sug.
Time (h)

Figure 6.


P_{vol}^{Acc}
T_{Avg}
Static P_{Est}^{Acc}
Mixing P_{Est}^{Acc}
FB 33% P_{Est}^{Acc}
A
B
C
P_{Vol}^{Acc} (mg_{Vol} g_{TTS}^{-1}), P_{Est}^{Acc} (mg_{Est} g_{TTS}^{-1})
Average Temperature (°C)
0.5 L
4.5 L
22 L