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Bioproduction of 2-phenylethanol and 2-phenethyl acetate by *Kluyveromyces marxianus* through the solid-state fermentation of sugarcane bagasse

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Abstract

2-phenylethanol (2-PE) and 2-phenethyl acetate (2-PEA) are important aroma compounds widely used in food and cosmetic industries due to their rose-like odor. Nowadays, due to the growing demand for natural products, the development of bioprocesses for obtaining value-added compounds has become of great significance. 2-PE and 2-PEA can be produced through the biotransformation of L-phenylalanine using the generally recognized as safe strain *Kluyveromyces marxianus*. L-phenylalanine bioconversion systems have been typically focused on submerged fermentation processes (SmF), but there is no information about other alternative productive approaches. Here, the solid-state fermentation (SSF) of sugarcane bagasse supplemented with L-phenylalanine was investigated as a sustainable alternative for producing 2-PE and 2-PEA in a residue-based system using *Kluyveromyces marxianus* as inoculum. An initial screening of the operational variables indicated that air supply, temperature and initial moisture content significantly affect the products yield. Besides, it was found that the feeding strategy also affects the production and the efficiency of the process. While a basic batch system produced 16 $mg_{products}$ per gram of residue (dry basis), by using split feeding strategies (fed-batch) of only sugarcane bagasse, a maximum of 18.4 $mg_{Products}$ $g^{-1}_{residue}$ were achieved. Increase in product yield was also accompanied by an increase in the consumption efficiency of nutrients and precursor. The suggested system results as effective as other more complex SmF systems to obtain 2-PE and 2-PEA, showing the feasibility of SSF as an alternative for producing these compounds through the valorization of an agro-industrial residue.

Keywords

Solid-state fermentation, *Kluyveromyces marxianus*, Aroma compounds, Rose-like compounds, Waste to product.

Introduction

2-phenylethanol (2-PE) is a higher alcohol characterized by its rose-like odor. This condition has made 2-PE one of the most used components in fragrance, cosmetic and food industries as an organoleptic enhancer of final products (Hua and Xu 2011). 2-PE is also employed as precursor for the production of other valuable compounds like 2-phenethyl acetate (2-PEA), which also has a floral and rose-like odor and it is used for similar purposes than 2-PE (Carlquist et al. 2015). Both 2-PE and 2-PEA constitute the rose-like compounds, and most of their world's production is obtained by chemical synthesis (Morrisey et al. 2015). However, these processes tend to generate undesirable by-products which impart off-odors modifying the organoleptic profiles of the products (Etschmann et al. 2002). Natural 2-PE and 2-PEA are commonly extracted from the essential oils contained in some flowers and plants such as hyacinth or jasmine, but mainly from rose petals; however, their low concentration makes the recovery complex and expensive (Sendovski et al. 2010; Carlquist et al. 2015). Also, it is becoming more common that consumers favor ''natural'' components, driving the market demand towards naturally produced aromas (Hua et al. 2010). Considering that the current European and American legislation have determined 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), bioprocesses appear as promising alternatives for producing natural aroma compounds.

Biosynthesis of 2-PE and 2-PEA is performed by fungi like A*spergillus oryzae* (Masuo et al. 2015), bacteria as *Enterobacter sp* (Zhang et al. 2014), but mainly by yeasts like *Saccharomyces cerevisiae* (Eshkol et al. 2009) and *Kluyverom*yces *marxianus* (Gao and Daugulis 2009; Conde-Báez et al. 2017), Among these, *K. marxianus* has proved to be one of the most effective 2-PE producers (Carlquist et al. 2015; Morrisey et al. 2015). The most efficient biotechnological route to obtain 2-PE and 2-PEA is the bioconversion of L-phenylalanine (L-Phe) via the Ehrlich pathway (Etschmann et al. 2002). In this mechanism, L-Phe is converted into phenylpyruvate, an intermediate which is later decarboxylated to phenylacetaldehyde. Then, it is reduced to 2-PE by dehydrogenation, and this latter can be transesterified to 2-PEA. The biosynthesis of 2-PE and 2-PEA can also be achieved by *de novo* synthesis starting from simple sugars through the shikimate pathway. In this case, the glycolysis contributes with phosphoenol pyruvate and erythrose-4phosphate which are condensed to chorismate, leading to phenylpyruvate which is later converted into 2-PE and 2-PEA (Gientka and Duszkiewicz-Reinhard 2009). Even that, the

efficiency of this path is not high enough to be used as a productive alternative by itself (Carlquist et al. 2015).

The most common way to biotransform L-Phe into 2-PE and 2-PEA is by means of submerged fermentation (SmF). However, the main drawback of this technique is the 2-PE inhibition on the cells, limiting the final product concentrations and space-time yields (Wang et al. 2011a). To overcome this restraint, strategies like strain development, culture optimization, cells immobilization and in situ product removal techniques are commonly used (Hua and Xu 2011). Among these, the most studied systems include: two-phase liquid extraction systems (Gao and Daugulis 2009; Sendovski et al. 2010), in situ adsorption processes (Hua et al. 2010; Wang et al. 2011b), solvent immobilization (Stark et al. 2003a), organophilic evaporation (Etschmann et al. 2005), perfusion and pertraction (Červeňanský et al. 2017) and extraction with ionic liquids (Okuniewska et al. 2017). Nonetheless, most of these technologies have been carried out using synthetic media as substrates, and, in general, complex reaction systems with high consumption of resources.

Since the sustainability and efficiency of biotechnological processes are a current concern, by using low-priced residues as raw material, together with simple reaction systems, have become a valuable asset to be exploited (Laufenberg et al. 2003; de Oliveira Felipe et al. 2017). In this sense, solid-state fermentation (SSF) has proven its efficiency as an environmentally friendly and economical tool for the valorization of several organic solid wastes (Longo and Sanroman 2006; Yazid et al. 2017). In particular, valuable aroma compounds have been previously obtained through the SSF of agroindustrial residues (Medeiros et al. 2001; Martínez et al. 2017) but, to our knowledge, no attempts to produce 2-PE and 2-PEA via SSF have been reported yet.

In general, SSF presents some attractive characteristics for producing aroma compounds that make it a feasible alternative to SmF processes. For instance, SSF commonly presents high production rates and yields (Barrios-González 2012), and it can be run with low-priced raw materials (Soccol et al. 2017). If continuous air supply is used, it would also serve for stripping out a given fraction of the volatile and semi-volatile compounds from the solid matrix (Martínez et al. 2017), favoring the further aroma compounds production. In this sense, 2-PE and 2-PEA could be produced in simple reaction systems starting with organic wastes as raw material, applying the current thought “from trash to treasure” (Berger 2015).

This study aimed to assess the feasibility of the SSF of sugarcane bagasse (SCB) supplemented with L-Phe for obtaining 2-PE and 2-PEA in a residue-based system. With this purpose, the generally recognized as safe strain *K. marxianus* was used in a 0.5 L continuously air supplied reaction system. First, a screening of some operational variables was performed to identify those more relevant to the productive process. Then, we have focused on the development of some operational strategies to improve the production of these compounds as well as the global efficiency of the process.

Material and methods

Microorganism

Kluyveromyces marxianus (ATCC 10022) was obtained from Colección Española de Cultivos Tipo (CECT, Valencia, Spain). It was grown on agar slants containing 40 g L^{-1} glucose, 5 g L^{-1} soy peptone, 5 g L^{-1} yeast extract and 20 g L^{-1} agar at 30°C for 20 h. The strain was maintained at -80°C in cryovials with impregnated pearls with *K. marxianus.* The inoculum was prepared by adding one pearl to 100 mL of the liquid medium consisting of 40 g L^{-1} glucose, 5 g L^{-1} soy peptone and 5 g L^{-1} yeast extract. Then, the culture was incubated on a rotary shaker at 30°C and 180 rpm for 20 h. All media and materials were previously sterilized by autoclaving at 121°C for 20 min.

Substrate treatment

First, sugarcane bagasse was dried at 60°C in an air oven during 24 h. The dried substrate was ground in a granulator mill obtaining a particle size distribution between 0.5 and 32 mm (60% retained in sieves with a mean aperture diameter of 1.6 mm). It was stored at -20°C until its use. Preparation consisted of adjusting the moisture content, pH, precursor load and molasses content. This process was performed using a 1:1 (v:v) mixture of a phosphate buffer pH 7 (0.1M) and a nutrient solution containing 1.5 g L^{-1} $Fe(NO_3)_3.9H_2O$, 0.8 g L^{-1} $ZnSO_4.7H_2O$, 0.4 g L^{-1} $MnSO_4.4H_20$ and 3.0 g L^{-1} $MgSO_4.7H_2O$. Both, L-Phe and molasses were added and dissolved in this mixture until the final amount corresponded to the experiment requirements. Once substrate was impregnated, it was autoclaved at 121°C for 20 min and after cooling it was inoculated using a final concentration of 10^8 colony forming units (CFU) per gram of initial total solids content of substrate (g_{TS}).

SSF experiments

SSF was carried out in 0.5 L glass containers. The system consisted of a triplicate (up to 96 g of prepared substrate) placed in a temperature-controlled water bath and connected to a mass flow controller (Bronkhorst Hitec) that continuously supplied humidified air to each flask as detailed elsewhere (Ponsá et al. 2010). Gas streams were led to an oxygen sensor (αLphase Ltd.) in series with an IR CO_2 sensor (Sensotran IR), both connected to an online system recording O_2 and CO_2 concentrations every 5 minutes. The respirometric analysis consisted in computing the oxygen uptake rate (OUR), the carbon dioxide production rate (CO_2^P) and the respiration quotient (RQ: CO_2^P/OUR*(32/44)) as stated by (Medeiros et al. 2001). Experiments were followed during 72 h. When fed-batch approaches were tested, the reactor content was manually mixed with the fresh material into a 1 L glass beaker using a spatula and then, quantitatively loaded back into the reactor, always keeping the sterile conditions of the media (the procedure was performed in a laminar flow chamber).

Analytical methods

Determination of volatile compounds in the gas phase

The composition of the exhaust gases of the fermented substrate was determined by thermal desorption gas chromatography mass spectrometry (TD-GC-MS) as described in (Martínez et al. 2017) (See details in supplementary material, section 1).

Determination of L-Phe, 2-PE and 2-PEA in the solid phase

L-Phe, 2-PE and 2-PEA were quantified by HPLC (high-performance liquid chromatography) (Ultimate 3000, ThermoFisher) using a reverse phase Supelcosil LC-18 column (250mm length, 4.6mm diameter, 5µm particle size). A gradient method with constant flow rate at 1.0 mL min^{-1} comprising water/methanol was used as follows: 0-4 min 70/30, then concentration was increased until 50/50 at 7 min and hold for 1.5 min, and at this point, a 30/70 ratio was kept for 5 min. The temperature was set at 35°C, and detection was performed at 258 nm. L-Phe was quantified from 2 consecutive extracts obtained after a solid-liquid extraction using distilled water in a 1:7 (w/v) ratio at 50°C during 30 min. The supernatant was filtered through a 0.45 µm membrane filter and preserved at 4°C before the analysis. 2-PE and 2-PEA were extracted from the solid phase using

two successive extractions with methanol in a 1:7 (w/v) ratio at 30°C during 30 min. Similarly, the supernatant was filtered through a 0.45 μm membrane and preserved in vials at 4°C before their analysis. In both cases, the recovery efficiency after the consecutive extractions was 97.5% for L-Phe and 97.1% for 2-PE and 2-PEA. As standards, analytical grade reagents were used for 2-PE and 2-PEA, and reagent grade for L-phenylalanine (Sigma-Aldrich). Concentrations were calculated from external standard calibration curves, analyzed under identical conditions.

Sugar content

Reducing sugars of the fermented substrate were measured using the DNS method (Miller 1959) in the liquid phase obtained after a solid-liquid extraction using distilled water in a 1:7 (w/v) ratio at 50°C during 30 min. The supernatant was filtered through a 0.45 μm membrane filter and properly diluted before the measurement in the spectrophotometer.

K. marxianus cells counting

10 g of sample were introduced into a bottle with 100 mL of a NaCl 9 g L^{-1} solution. The mixture was shaken for 15 min at 180 rpm in an orbital shaker at room temperature. The supernatant was used to prepare dilutions in NaCl 9 g L^{-1} which were plated on Petri dishes (triplicates) containing the same media employed for the growing of the pure strain. Cultures were incubated for 20 h at 30°C, and the population was determined by counting the units. Results were expressed in CFU per gram of total solids (g_{TS}).

pH and moisture content

Moisture content (MC), total solids (TS), volatile solids (VS) and pH were determined according to the standard procedures (The US Department of Agriculture and The US Composting Council 2001).

Statistical analysis

Full factorial designs with three replicate in the center point were used to assess the influence of the operational variables on the production of 2-PE and 2-PEA. 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 3 replicates. Data were statistically analyzed with Minitab 16 (Minitab Inc.).

Results

SSF processes are influenced by biological and physicochemical factors affecting the strain growth and the metabolization of the flavor compounds (Rodriguez-Leon et al. 2008). The main factors include the carbon and nitrogen sources, the particle size of the substrate, the moisture and water activity, temperature and pH of the media as well as the aeration strategy (Pandey 2003). These factors are prone to be used as monitoring variables, thereby, a proper understanding of their effects becomes crucial (Chen 2013). To analyze the process, some performance indices have been used: Total cumulative production (P_{sub}) [$mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS}] (calculated as accumulated 2-PE and 2-PEA production divided the initial amount of substrate), molar yield (Y_{mol}) [% $mol_{2\text{-}PE+2\text{-}PEA}$ $mol^{-1}_{L\text{-}Phe0}$] (the cumulative 2-PE and 2-PEA production in moles divided the initial moles of L-Phe) and space-time yield ($Y_{s\text{-}t}$) [$mg_{2\text{-}PE+2\text{-}PEA}$ L^{-1} h^{-1}] (computed as the sum of the 2-PE and 2-PEA concentrations, referred to the reactor volume divided the time required to obtain those concentrations). In all cases, results express the contribution of 2-PE and 2-PEA found both in the solid-liquid interface and in the gas phase.

Influence of operational variables on P_{sub}

Initial experiments consisted of the evaluation of the effects of 6 of the main operational variables on the performance index P_{sub}. By doing this, it can be identified the conditions favoring the enhancement of both 2-PE and 2-PEA production, and not just one of the products. First, a 2^4 factorial design (design N°1) was used to assess the effects of temperature (T), specific air flow rate (S_{AFR}), L-Phe dose (P_d) and sugar beet molasses dose (M_d) (Table S1). pH, MC and inoculum load (I_L) were kept at 5.3, 68% and 10^8 CFU g^{-1}_{TS} respectively, based on our previous results (Martínez et al. 2017). T and P_d were valued between 30-37°C and 1.55 and 3.1% (dry basis), respectively, considering the results of former studies (Etschmann et al. 2003; Garavaglia et al. 2007; Gao and Daugulis 2009), while S_{AFR} and M_d were changed in the intervals 0.05-0.10 L h^{-1} g^{-1}_{TS} and 0-20% (dry basis) according to our preliminary results (Martínez et al. 2017). From Fig. 1(a) it is evident how 3 out of 4 variables significantly influence the combined 2-PE and 2-PEA production. In Fig. 1(b) it can be seen that operating at 30°C coincides with the

highest production while temperature increase only induces a reduction in P_{sub}. Similarly, when molasses were added, an apparent adverse effect is produced in the P_{sub} trend (Fig. 1(c)). Furthermore, from Fig. 1(d) it is presented the effect of the air supply in the production. For the evaluated interval, an increase in S_{AFR} is directly related to a higher production (Table S1).

Fig. 1

A second set of experiments (design N°2) was developed to evaluate the variables MC, pH and a further increase in P_d using a 2^3 factorial design (Table S2). Here, T, S_{AFR}, M_d and I_L were set at 30°C, 0.10 L h^{-1} g^{-1}_{TS}, 5% and 10^8 CFU g^{-1}_{TS} respectively, based on the previous results. In this case, pH was assessed between 4.8-6.0, according to previous studies (Etschmann and Schrader 2006; Garavaglia et al. 2007), MC from 60-76% (based on the range previously established by (Martínez et al. 2017)), and P_d was set between 3 and 6 % (dry basis) as a manner of supplying an excess of precursor. As shown in Fig. 1(e), MC is the only variable of the group with a significant influence on P_{sub}. It can also be seen in Fig. 1(f) and 1(g) how pH and P_d contour plots remain almost vertical lines, with a limited impact on P_{sub}, indicating a limited effect on the bioproduction of 2-PE and 2-PEA.

Based on this initial screening of the variables, a reference scenario (E_1) was defined employing the following set of conditions: 30°C, 0.10 L h^{-1} g^{-1}_{TS}, 66% MC, pH 4.8, 10^8 CFU g^{-1}_{TS}, 2% L-Phe, no molasses addition. As detailed in Fig. 2(a), under these conditions, the production rapidly grows the first 24-25 h of processing, coinciding with the maximum OUR and CO_2^P as well as the with the highest cell counts (Fig. 2(b)). At that point, the productivity suddenly decreases due to the depletion of the sugar content with the corresponding slowdown in the L-Phe conversion ($X_{L\text{-}Phe}$), reaching a P_{sub} of 16 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} after 72 h. Given the marked change in the production after the 24-25 h, the maximum $Y_{s\text{-}t}$ also agrees with this inflection point. Since L-Phe transformation coincides with the growth phase, the end of this stage could serve as a reference point for making changes in the process.

Fig. 2

Enhancement of P_{sub} based on operating conditions and glucose addition

Table 1 summarizes the characteristics of the strategies based on operating conditions and glucose addition (E_2 to E_5^*) to enhance the 2-PE and 2-PEA production. These strategies focused on the effect of S_{AFR}, T and the addition of glucose as complementary carbon source. The process has been divided into two stages based on the point *K. marxianus* reaches the maximum activity (measured as OUR and CO_2^P). Having this in mind, changes during the growth stage involved an increase of 40% in S_{AFR} (E_2) respect to E_1. On the other hand, variations in the subsequent stage (post maximum activity changes) include first, the addition of glucose as fresh carbon source to extend the *K. marxianus* activity (E_i^* strategies), and second, favoring of the anoxic condition of the media through increases in T (E_3) and reduction of S_{AFR} (E_4). By using these last strategies, it is expected to favor the selectivity to the fusel alcohols produced in the Ehrlich pathway instead of the fusel acids produced in an aerobic and glucose-limited environment.

Table 1

Fig. 3(a) shows the main performance parameters computed for strategies E_1 to E_5^*. As seen, the reference scenario (E_1) has a Y_{s-t} close to 19 $mg_{2\text{-}PE+2\text{-}PEA}$ L^{-1} h^{-1}, but once the S_{AFR0} is increased (E_2), P_{sub} and Y_{s-t} are improved in 16% and 71% respectively. The positive effect due to the air supply strategy also agrees with the higher growth of *K. marxianus* in the solid substrate (up to 2.2 10^8 CFU $g^{-1}{}_{TS}$). Besides, when the process is modified at the end of the maximum activity levels, it is clear that there are no substantial fluctuations on the maximum P_{sub} and Y_{s-t} neither when rising T (E_3) nor by reducing S_{AFR} in 1/3 (E_4). These latter strategies modify the O_2 transfer, increasing the respiration quotient far from a pure aerobic process toward an anoxic environment (RQ > 1.9). However, at the moment changes are made, the reducing sugars content is low enough (<8%) to be further exploited by the strain. Thus, the process might be considered ended after 36-40 h of fermentation when the carbon source is almost depleted, making impossible any further 2-PE and 2-PEA production, independently of the operational conditions.

Fig. 3

Taking this in mind, one of the SSF constraints for producing the rose-like compounds seems to be the way the available sugars are consumed and its correlation with the L-Phe

biotransformation. That is how using glucose additions at the end of the growth stage (as fresh sugar source) was also estimated to identify alternatives to increase the combined 2-PE and 2-PEA production. In E_1^* and E_2^* the addition of 85% of initial sugar content keeping the same operating conditions than the initial time has been assessed. Here, the maximum P_{sub} increased by 11% and 24% respectively until 19.9 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} in scenario E_2^*. Then, a further rise in production is obtained when adding glucose at the same time than favoring an anoxic atmosphere (E_3^*, E_4^*). Here, a change of almost 26% in P_{sub} relative to E_1 was obtained. In general, it can be stated that the process works in an anoxic environment from 25 to 48 h (RQ > 1.85), moment in which the available sugars are near depleted.

Based on the results of the strategies above, an extended strategy (E_5^*) was evaluated by adding glucose at two points of the process, after 25 and 48 h. This time, glucose additions corresponded to 100% of the initial sugar content and the process was followed till 100 h. As observed in Fig. 3(a), in E_5^* there are no significant changes in the maximum production compared to the previous scenarios where glucose was added. Moreover, as it was found before, Y_{mol} is around the average for the strategies using glucose (109 %) with similar $X_{L\text{-}Phe}$ values. Therefore, it is predictable that *K. marxianus* is limited to use the residual L-Phe even if the media is plenty of sugars. Fig. 2(c) and 2(d) show the time profile of the SSF for the strategy E_5^*. As detailed, the point in which maximum *K. marxianus* activity was achieved is similar to E_1. At this point, the microbial activity coincides with the highest cell counts for the strain (1.2 10^9 CFU g^{-1}_{TS}) (Fig. 2(d)). Nevertheless, differently than E_1, when glucose is supplied, an additional increase in activity is found, but it does not coincide with *K. marxianus* growth; instead, cells count remains at the same level during the time there are enough sugars to be consumed. Therefore, the effect on P_{sub}, $X_{L\text{-}Phe}$ and $Y_{s\text{-}t}$ is an extra increase lasting up to 36 h of fermentation (Fig. 2(c)), At this point, the activity starts falling and the performance parameters have only subtle changes, even when at 48 h a new glucose addition was performed. In that case, there are no significant improvements regarding the 2-PE and 2-PEA production, and the maximum P_{sub} is obtained after 72 h of processing when sugars are entirely exhausted.

Enhancement of P_{sub} based on the feeding strategy

Table 2 summarizes the strategies used for enhancing the 2-PE and 2-PEA production based on the feeding approach (E_6 to E_8). Here, the aim was to replicate the high yeast activity found in

those strategies where glucose was added, but replacing the glucose with partial additions of the same residue. Thus, the total substrate load was split as a manner of a fed-batch operation, and therefore, SCB has become the only carbon source for the development of the process. Since the reference scenario corresponds to a batch, splits were referred to the amount typically used in this scenario, e.g., a 50% split indicates only half of the SCB load (48 g) is loaded at the beginning of the process and the remaining half at the selected point. Also, a 33% split specifies a third part of the load (32 g) is fed at time zero and the remaining two-thirds later in the corresponding feeding points.

Table 2

Fig. 3(b) shows the performance of the strategies E_6 to E_8 compared to E_1. As it is observed, splitting the residue load increases the 2-PE and 2-PEA production, mostly when the load is divided into 3 or 4 fractions (E_7 and E_8). The trend shows how splits help to increase P_{sub} until a maximum of 18.4 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} in $E_{8,}$ mainly enhancing the 2-PEA production. This suggests the fed-batch strategy succeeds replacing the glucose additions presented in the previous section to increase the 2-PE and 2-PEA production. In general, by doing this, $Y_{s\text{-}t}$ is also improved reaching mean values around 29 $mg_{2\text{-}PE+2\text{-}PEA}$ L^{-1} h^{-1}.

Discussion

Operational variables screening

As detailed before, one of the most significant variables affecting the process is the temperature. In fact, 2-PE and 2-PEA production is strongly influenced by T, but in the opposite direction to the results exposed in preceding studies in SmF (Garavaglia et al. 2007). Although T contributes to the in situ removal step in SmF, its influence on the production has always been related to the strain growth rate. Since the biotransformation of L-Phe in yeasts is characteristically growth-dependent (Etschmann et al. 2002), it is expected the highest production will be achieved at the optimum growth T for the strain (as found here). Similarly, the S_{AFR} appears as one of the most critical variables for the process development. While for 2-PE production via SmF the air supply is mostly fixed to allow a specific dissolved oxygen concentration (Chreptowicz et al. 2016), in SSF it plays a role much more substantial since it not only provides the oxygen for running the

fermentation but it is also part of the heat and mass transport phenomena occurring in the heterogeneous system (Rodriguez-Leon et al. 2008). Actually, a proper air supply is indispensable for the strain growth since it defines in a high degree the aerobic/anoxic condition of the media (Chen 2013). Also, the use of high S_{AFR} agrees with results found for another SSF process for producing aroma compounds (Medeiros et al. 2006). From the variable screening, the third significant parameter for the process is the MC. In this case, data indicates that the higher the MC, the higher the production (Fig. 1(f) and 1(g)), reaching the maximum values when working near the water holding capacity of the substrate (WHC) at 76%. It is well known that MC plays a major role in SSF influencing other parameters like the substrate porosity and humidification/drying of the media, and these, in turn, affect the aerobic/anoxic condition of the fermentation (Martínez et al. 2017). While a high MC would contribute to the formation of anoxic zones (favoring the alcohol species), a low MC would lead to a slower fermentation due to the water availability; but at the same time, with a higher porosity, it would promote an aerobic environment ideal for the *K. marxianus* growth. Hence, selection of MC becomes a tradeoff to maximize productivity, avoiding problems like exceeding the WHC with the consequent leachate generation, or the excessive ethanol production and its synergistic inhibition of the strain. Having this in mind, using an intermediate MC is a useful approach to balance the dissimilar effects produced by this variable. In this case, it was set at around 66-68%, at the mid-point of the evaluated interval.

Some other operational variables have resulted less significant for the productive process. Using sugar beet molasses in SSF as a useful sugars complement supplier have been previously confirmed (Jiménez-Peñalver et al. 2016). Unfortunately, they also contribute with other nitrogen sources than amino acids, promoting different metabolization routes than the Ehrlich pathway, and therefore, hindering the 2-PE and 2-PEA biotransformation. In fact, the used molasses contain up to 1.9 % of nitrogen (dry basis), and it is estimated around 68% of these are not amino acid-N based (Olbrich H 2006), leading to contents of other sources above 10% of the total available nitrogen. Also, although initial pH is set up based on the strain needs, some microorganisms like *K. marxianus,* are more flexible to pH changes (Lane and Morrissey 2010). That is how P_{sub} is expected to be less affected by pH fluctuations, as found in other studies (Fabre et al. 1998; Etschmann and Schrader 2006; Garavaglia et al. 2007). Finally, the L-Phe dose plays a critical role in the process, since it is the primary source for the biotransformation.

However, it is clear that there is no a direct relationship between the L-Phe availability and the 2-PE and 2-PEA production. That is evident from the design N°2, where the further increase in P_d was valued to identify if the production could be enhanced due to a higher precursor availability. As observed in Fig. 1(g), P_{sub} reaches values up to 13.2 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS}, representing an increase of 19.7% respect to the maximum value found in design N°1. However, this increase does not correspond with the L-Phe consumption. While the average $X_{L\text{-}Phe}$ (for all the set of data in design N°2) only reaches 25%, in design N°1 it was around 43% (Tables S1 and S2). This data indicates *K. marxianus* is not using the L-Phe surplus efficiently for producing 2-PE and –PEA. Consequently, the subtle increase in production when using a higher L-Phe load may not compensate the waste of unreacted precursor. Under this scenario, different alternatives to increase the efficiency of *K. marxianus* for metabolizing the precursor have to be evaluated. In practice, contents below 3% seem to be adequate for running the process without wasting significant amounts of L-Phe.

P_{sub} Enhancement based on operating conditions and glucose addition

Once the process is divided in the point of maximum activity for *K marxianus*, two different aspects can be analyzed. First, for the evaluated interval, the higher S_{AFR} has promoted a higher growth of the strain, and as expected, this change has come with the corresponding increase in production. Then, the use of glucose to provide additional nutrients to the medium, and therefore, promoting a further strain activity was successful for enhancing the production. In this sense, the given improvement could be seen from a slightly higher $X_{L\text{-}Phe}$ (*K. marxianus* is still metabolizing the amino acid) but also in the intensification of the *de novo* synthesis when glucose is available in the media. This is evident from Y_{mol} which reaches values beyond 100% at the point of maximum production. These values imply that different sources than the provided L-Phe are being converted into 2-PE and 2-PEA. In this sense, some authors (Masuo et al. 2015) have encountered that *de novo* synthesis contribution is limited in liquid cultures, but other studies (Wang et al. 2013; Pan et al. 2014) show how different yeasts can produce significant amounts of 2-PE in SmF without L-Phe, coinciding with the use of residue-based culture media. Also for *K. marxianus,* yields beyond 100% due to *de novo* synthesis have been reported (Chreptowicz et al. 2016), and the ability of some yeasts to act as a catalyst in the transesterification of 2-PE and ethyl acetate (other product presented in significant amounts in the medium) into 2-PEA due to

their lipolytic activity have been studied (Białecka-Florjańczyk et al. 2012). Although *K. marxianus* is not typically lipase active, prior investigations (Deive et al. 2003) have proven the capability of this strain to produce extracellular lipolytic activity. Consequently, it is hard to identify the specific paths (beyond Ehrlich pathway) implicated in the biotransformations occurred in the substrate. The remarkable aspect lies in the global effect on the 2-PE and 2-PEA production, mainly the surplus obtained when the process is extended by means of glucose additions. Furthermore, when looking at the Y_{mol} values, this effect is presented in both cases, when there is an aerobic and an anoxic environment, since Y_{mol} is beyond 100% for these scenarios (Fig. 3(a))

Another aspect to highlight relates to the 2-PE inhibition effect. It is well known L-phenylalanine biotransformation with *K. marxianus* via SmF is constrained by this factor (Stark et al. 2003b), but there is no reference to this effect in SSF. However, analyzing the 2-PE levels found along the different strategies, it could be stated *K. marxianus* is able to keep active and consuming L-Phe at higher 2-PE concentrations than in SmF. As seen in Fig. 4, the batch process reaches equivalent 2-PE liquid concentrations higher (beyond 60% higher) than the most common inhibitory limit found in SmF of almost 4 g/L (Stark et al. 2003b; Etschmann and Schrader 2006; Wang et al. 2011a). The same occurs when the strategy is changed to the addition of glucose remaining at higher equivalent 2-PE liquid concentrations during more than 40 h of processing without any adverse effect in the *K. marxianus* activity. This data suggest that the further biotransformation of the precursor could be constrained by other physical phenomena like the ability of *K. marxianus* to access to L-Phe, or the intricate interactions existing in the solid media, affecting the development of the Ehrlich pathway (given the complexity of the solid media and the differences of porosity along the residue). Also, under the conditions above, the fraction of products efficiently stripped to the gas phase was a function of time but always limited to values below 5%, reinforcing the idea that the 2-PE inhibition effect is less marked in SSF than in SmF.

Fig. 4

P_{sub} Enhancement based on the feeding strategy

The time profile of the performance indices for the strategy E_7 is shown in Fig. 5(a). Here, 2-PE and 2-PEA production rise during the first 24 h of processing, the moment when the first SCB

addition is made. Due to the dilution effect of the fresh material, a substantial reduction in P_{sub} is presented, but within the next 6 h, this performance index recovers the values previously obtained. At 36 h the second SCB addition was made, and as expected, 2-PE and 2-PEA production have increased reaching the highest total P_{sub} at 48 h. Here, a subtle reduction in P_{sub} is presented until the end of the fermentation, suggesting there is a further transformation of 2-PE and 2-PEA to other species once the available sugars are depleted. It can also be observed that $X_{L\text{-}Phe}$ continuously grows, but the considerable changes occur when fresh SCB is added in the media, coinciding with the *K. marxianus* growth. From Fig. 5(b) it can be observed the direct relationship between cells count and the strain activity. It is evident the difference of these when compared to E_5^* (Fig. 2(d)). Here again, each SCB addition makes the cells counts to fall due to the dilution effect, making the strain to keep growing while there are available sugars. In fact, when sugar content is almost depleted (around 48 h) the process starts decaying, and no considerable L-Phe consumption is presented.

Fig. 5

By using a SSF fed-batch of four splits (25 % load each), the global behavior is similar than E_7, and in this case, the first 24 h of fermentation are analogous among them. In general, each addition of fresh SCB comes with the reinforcement of P_{sub} and a higher $X_{L\text{-}Phe}$ (Fig. 5(c)) as occurred in E_7. The main difference lies in the time *K. marxianus* remain active, which is a direct function of the sugars availability (Fig. 5(d)). As seen, in E_8 the maximum combined 2-PE and 2-PEA production is reached close to 54 h of processing. At that point, L-Phe consumption almost ceases due to the depletion of the available sugars and P_{sub} and $Y_{s\text{-}t}$ start falling as occurred in E_7.
In these fed-batch strategies, despite that the higher the split, the higher the final $X_{L\text{-}Phe}$, the extra L-Phe consumption is not efficiently used for 2-PE and 2-PEA production. In this case, Y_{mol} shows a trend in which only a fraction between 62 and 73% of the precursor is converted into these compounds. This put in evidence that *de novo* synthesis is not promoted in the same way as in strategies E_i^* due to the glucose effect. That might occurred, given that part of the available amino acid could also be metabolized through the cinnamate pathway, which is precisely favored under glucose absence (Etschmann et al. 2002) and it can contribute to the breakdown (catabolism) of more than 22% of the L-Phe, depending on the conditions of the media

(Wittmann et al. 2002). Besides, as detailed in the previous strategies, for the SSF fed-batch operation, the 2-PE inhibition effect seems to be also negligible at the evaluated conditions. In this case, the same dilution effect once supplying fresh solid material allows the 2-PE equivalent liquid concentration remain below the threshold of inhibition in SmF during most of the fermentation process (Fig 4). Intrinsically, this operating mode avoids high products concentrations during most of the process limiting the effect of the 2-PE on *K. marxianus* cells.

The evaluated SSF fed-batch strategies show an evident improvement compared to a SSF batch (up to >15%) regarding the combined 2-PE and 2-PEA production, but they also represent improvements compared to some SmF techniques. As detailed in Table 3, most of the typical SmF processes have a low L-Phe conversion to 2-PE and 2-PEA ($Y_{L\text{-}Phe}$). This indicates they required more L-Phe to produce the same amount of products than in a SSF process while keeping a high rate of unreacted precursor. Moreover, regarding the sugar consumption efficiency (Y_{Glu}), SSF processes result as good as most of the SmF techniques. The only exception corresponds to the most sophisticated and synthetic media-based SmF systems. On the contrary, residue-based SmF processes have a limited conversion effectiveness; thus, a critical advantage of the SSF approach for producing 2-PE and 2-PEA is the ability to use organic residues as nutrient source, as well as the efficiency to use them in the biotransformation.

Table 3

In general, the global performance of the processes might be analyzed using the space-time yield ($Y_{s\text{-}t}$). In this aspect, SSF approaches are as good as some of the simplest SmF techniques (Batch) not including those using in situ product removal, and it could be considered better compared to those SmF using residues as substrate. However, SSF is still behind the most sophisticated SmF processes involving the product removal with more complex systems and intensive use of resources.

As concluding remarks, it can be stated that the selected *K. marxianus* has used the agroindustrial residue sugarcane bagasse as sole carbon source for the biotransformation of L-phenylalanine into 2-PE and 2-PEA through SSF. The growth-associated biotransformation of L-Phe was corroborated in the solid media, and although the product stripping was limited, 2-PE inhibition

effect seems to be less significant than in SmF. In general, using SSF fed-batch processes have resulted in a simple and reliable way to increase the 2-PE and 2-PEA production. Moreover, the proposed SSF strategies can be considered less intensive in the use of resources than most of the evaluated SmF processes, becoming as efficient as some of these technologies but using a residue-based system. This suggests the principle of "from waste to product" might be applied for obtaining these valuable aroma compounds in a more sustainable and environmentally friendly way.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures Caption

Fig. 1 Pareto chart and variables effects for design N°1. (a), (b), (c), (d) and N°2 (e), (f), (g) on the cumulative production of 2-PE and 2-PEA for the solid-state fermentation of sugarcane bagasse with *K. marxianus.* (b) Temperature effect, (c) molasses load effect, (d) specific air flow rate effect. Initial pH: 5.3, MC: 68%. (f) pH and moisture content effects, (g) L-Phe dose and moisture content effects. T: 30°C, S_{AFR}: 0.10 L h^{-1} g^{-1}_{TS}, 5% molasses (dry basis).

Fig. 2 Time course of the solid-state fermentation of sugarcane bagasse for producing 2-PE and 2-PEA. (a), (b) reference scenario E_1, (c), (d) strategy E_5*. (a), (c) P_{sub}, Y_{s-t} and X_{L-Phe}; (b), (d) OUR, CO_2^p, cells count and reducing sugars profiles.

Fig. 3 Comparison of the performance indices in the strategies for improving the 2-PE and 2-PEA production. (a) Strategies based on operating conditions and glucose addition. (b) Strategies based on the substrate feeding. P_{sub}: 2-PE and 2-PEA cumulative production, Y_{mol}: Molar yield, Y_{s-t}: Space-time yield. P_{sub} and Y_{mol} are the maximum values in time while Y_{s-t} is the maximum value after the growth stage. Different capital letters indicate main values are significantly different ($P<0.05$) in the evaluated group.

Fig. 4 Equivalent 2-phenylethanol concentration in the liquid interface of the solid-state fermentation of sugarcane bagasse with *K. marxianus*. 2-PE: 2-phenylethanol; SmF: Submerged fermentation; Glu: Glucose addition. 2-PE^L computed based on the moisture content of the solid media.

Fig. 5 Time course of the solid-state fermentation of sugarcane bagasse for producing 2-PE and 2-PEA with fed-batch operation. (a), (b) Strategy E_7 (c), (d) Strategy E_8. (a), (c) P_{sub}, Y_{s-t} and X_{L-Phe}; (b), (d) OUR, CO_2^p, cells count and reducing sugars profiles.

Table 1. Characteristics of the strategies based on operating conditions and glucose addition.

Strategy	Growth stage	Changes after 25 h (post-maximum growth)			Objective	P_{sub} (mg_{Prod} g^{-1}_{TS})		
	S_{AFR} (L h^{-1} g^{-1}_{TS})	Glucose added	S_{AFR} (L h^{-1} g^{-1}_{TS})	T (°C)		2-PE	2-PEA	Total
E_1	0.10	-	N.C.	30	Base scenario	12.08	3.94	16.03
E_1^*	0.10	85% R. S. at t_o	N.C.	30	Glu after G	13.05	4.78	17.83
E_2	0.14	-	N.C.	30	S_{AFR} in G	15.49	3.16	18.65
E_2^*	0.14	85% R. S. at t_o	N.C.	30	Glu after G	13.57	6.29	19.86
E_3	0.10	-	N.C.	40	Anoxic T	8.50	6.98	15.48
E_3^*	0.10	85% R. S. at t_o	N.C.	40	Anoxic T & Glu	13.75	5.42	19.17
E_4	0.10	-	1/3 t_0	30	Anoxic S_{AFR}	11.34	4.39	15.73
E_4^*	0.10	85% R. S. at t_o	1/3 t_0	30	Anoxic S_{AFR} & Glu	12.85	7.35	20.20
E_5^*	0.10	100% R. S. at t_o[a]	N.C.	30	Long-term Glu	16.22	4.86	21.08

T_0:30°C, pH_0:4.8, MC_0:66%, Inoculum: 10^8CFU g^{-1}_{TS}, L-Phe_0:2% (dry basis), 2.5% for E_5^*. [a]A second addition of glucose was made at 48 h with the same characteristics. G: Growth stage; NC: No changes; S_{AFR}: Specific air flow rate; MC: Moisture content; L-Phe: L-phenylalanine; 2-PE: 2-phenylethanol; 2-PEA: 2-phenethyl acetate Glu: Glucose; R. S.: Reducing sugars content; $P_{sub:}$ maximum cumulative production

Table 2. Characteristics of the strategies based on the feeding of sugarcane bagasse

Strategy	Main characteristics	Residue & L-Phe loads[a]	P_{sub} (mg_{Prod} g^{-1}_{TS})		
			2-PE	2-PEA	Total
E_6	SCB Feeding is split into two parts	50% at t_0 50% at 24h	8.50	7.74	16.23
E_7	SCB feeding is split into three parts	33% at t_0 33% at 24h 33% at 36h	9.00	7.86	16.86
E_8	SCB feeding is split into four parts	25% at t_0 25% at 24h 25% at 36h 25% at 48h	10.21	8.20	18.41

T_0:30°C, pH_0:4.8, MC_0:68%, Inoculum: 10^8CFU g^{-1}_{TS}, S_{AFR}: 0.10 L h^{-1} g^{-1}_{TS}. L-Phe_0: 3% (dry basis)
S_{AFR}: Specific air flow rate; MC: Moisture content; L-Phe: L-phenylalanine; 2-PE: 2-phenylethanol; 2-PEA: 2-phenethyl acetate; SCB: Sugarcane bagasse; P_{sub}: maximum cumulative production. [a]The feeding percentage is referred to the total load of a batch fermentation, equivalent to 96g of prepared substrate per replicate.

Table 3. Comparative of some systems for 2-PE and 2-PEA bioproduction.

Process	Substrate	Configuration	V_R (L)	$Y_{L\text{-}Phe}$[a] (g_{Prod} $g^{-1}_{L\text{-}Phe0}$)	Y_{Glu}[b] (mg_{Prod} g^{-1}_{Glu0})	$Y_{s\text{-}t}$[c] (mg_{Prod} L^{-1} h^{-1})	Reference
Submerged fermentation	Synthetic media	Batch	0.25	0.35	28.6	28.6	(Fabre et al. 1998)
		Batch	5.0	0.09	27.7	57.4	(Gao and Daugulis 2009)
		Batch	0.5	0.15	13.1	17.5	(Yin et al. 2015)
		Batch	6.2	0.72	156.5	50.0	(Chreptowicz et al. 2016)
		Batch + adsorbents	0.25	0.51	51.4	171.4	(Mei et al. 2009)
		Batch + BILS	0.25	0.75	150.0	41.7	(Sendovski et al. 2010)
		Fed-batch	3.6	0.32	13.8	86.4	(Stark et al. 2002)
		Fed-batch	2.0	0.25	122.5	43.8	(Serp et al. 2003)
		Fed-batch Imm.	2.0	0.19	190.0	65.5	
		Liq-liq ILAL	15.0	0.44	24.3	156.3	(Mihal et al. 2012)

	Grape must	Batch	2.0	0.23	3.8	9.6	(Garavaglia et al. 2007)
	Tobacco waste	Batch	0.5	-	31.6	17.6	(Wang et al. 2013)
	Whey cheese	Batch	0.1	-	-	13.3	(Conde-Báez et al. 2017)
	Cassava wastewater	Batch	0.25	0.24	66.5	18.5	(Oliveira et al. 2015)
	Molasses	Batch + adsorbents	0.5	0.60	66.0	169.2	(Hua et al. 2010)
	Molasses + Glu	Batch OP	2.4	0.65	97.5	234.0	(Etschmann et al. 2005)
Solid-state fermentation	Sugarcane bagasse	Batch	0.5	0.76	51.6	19.73	This study
		Batch + Glu	0.5	0.78	38.6	22.90	This study
		Fed-batch	0.5	0.73	73.3	22.65	This study

V_R: Reactor volume; $Y_{L\text{-}Phe}$: Mass precursor yield; Y_{Glu}: Glucose yield; $Y_{s\text{-}t}$: Space-time yield; BILS: biphasic ionic liquid system; Imm: Immobilized; ILAL: Internal loop air-lift; OP: Organic pervaporation; Glu: Glucose; L-Phe: L-phenylalanine. [a]computed based on the total initial L-Phe content. [b]computed based on the total glucose or sugars used during the fermentation. [c]Computed based on the reaction volume and total process time. For SSF processes, the value used to compute $Y_{s\text{-}t}$ was 0.45 L