Universitat Autònoma de Barcelona

Fruit-like and rose-like aroma production via Solid-State Fermentation of sugarcane bagasse: process optimization and production strategies

Oscar Mauricio Martínez Avila

ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons: http://cat.creativecommons.org/?page_id=184

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons: http://es.creativecommons.org/blog/licencias/

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license: https://creativecommons.org/licenses/?lang=en

Universitat Autònoma de Barcelona

Departament d'Enginyeria Química, Biològica i Ambiental

Escola d'Enginyeria

GICOM (Composting Research Group)

Fruit-like and rose-like aroma production via Solid-State Fermentation of sugarcane bagasse: process optimization and production strategies

PhD Thesis

Oscar Mauricio Martínez Avila

Supervised by:

Xavier Font

Raquel Barrena

Bellaterra, Barcelona

2018

Universitat Autònoma de Barcelona

Xavier Font, professor agregat i Raquel Barrena, investigadora postdoctoral del Departament d' Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

Certifiquen:

Que l'enginyer químic i màster en enginyeria química Oscar Mauricio Martínez Avila ha realitzat sota la nostra direcció el treball amb títol "Fruit-like and rose-like aroma production via Solid-State Fermentation of sugarcane bagasse: process optimization and production strategies", que es presenta en aquesta memòria i que constitueix la seva tesi per optar al Grau de Doctor en Ciència i Tecnologia Ambientals per la Universitat Autònoma de Barcelona.
I perquè en prengueu coneixement i consti als efectes oportuns, es presenta a l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona l'esmentada tesi, signant el present certificat.

Xavier Font

Raquel Barrena

Bellaterra, Maig 2018

A mi familia,

En especial a mi mamá y mi papá

Por su apoyo, cariño y comprensión

A Nancy,

Por ser el motor que mueve mi mundo

Acknowledgments

In the first place, I would like to thanks to my supervisors Raquel and Xavi who have adopted me through these years. Thanks for your support, your encouragement, but especially for your time and patience with me. I consider you more than my supervisors, a model to be followed.

Also, to all GICOM group in the head of Toni Sánchez. Thank you for allowing me to be one more of the composting family. Starting a project knowing anything about SSF was very difficult, but your help, support and advice were essential for me to succeed. This is something I really appreciate it. I hope I have contributed to GICOM a piece of what GICOM has contributed to me.

I would like to especially thank to my colleagues in the composting group. To those who are here in this moment (Ale, Paula, Laura, Maria, Alejandra, Eva, Dani and Arnau), and those who are now spread around the world (Cindy, Nora, Lucía, Ahmad and Pedro). Time has flown, but spending it with you has been an unforgettable experience.

To Rosi, Pili, Manuel and the administrative staff of the DEQBA for their valuable help through these years. Also to Juan and Mónica who helped me in the elaboration of many analytics during their practice stay in GICOM.

Similarly, thanks to many great people in the DEQBA I had the pleasure to meet. Particularly, to Gaby, Chan, Ale and Carlos. Thank you for your friendship, your help, and obviously, for waiting for me to take lunch so many times.

A mi familia, a quienes les debo mucho más de lo que podría mencionar en unas pocas palabras. Gracias por el continuo apoyo y comprensión. Sin su cariño todo hubiese sido más difícil. No olviden que siguen siendo fuente constante de mi inspiración y del deseo por dar todo lo mejor que llevo dentro.

Finally, the most sincere and deep gratitude to Nancy, who has helped me through all these years without expecting anything in return. I could not do anything without you. Thank you for your patience, for your love, your suggestions, and last but not least, for so many Saturdays and Sundays of hard work milling tons of sugarcane bagasse. This part of our nice trip is ending, but independently of what the future holds, I would like to share the next one with you (Como nunca, como a nadie y para siempre).

Overview of the thesis

This thesis focuses on the use of solid-state fermentation (SSF) as an alternative approach to the bioproduction of value-added aroma compounds through the valorization of selected agro-industrial residues. This study is developed in the framework of the project *Estrategias de optimización de procesos de obtención de bioproductos a partir de residuos orgánicos mediante fermentación en estado sólido* (BIOPRO, CTM2015-69513-R) and corresponds to the first work focused on the production of aroma compounds in the research group. Specifically, two groups of objective aroma compounds have been studied. First, the fruit-like compounds, among which could be included a set of volatile scented species such as aldehydes, ketones, alcohols and esters. From these, the straight-chain esters could be catalogued as those more appreciated due to their high fruit-like aroma profile. The second group of species studied here are the semi-volatile compounds known as rose-like compounds, which are constituted by 2-phenethyl alcohol (2-PE) and 2-phenethyl acetate (2-PEA), two value-added species widely used as additives due to the rose-like odor they provide. In both cases, the aim was to develop residue-based bioprocesses using as raw materials the agro-industrial residue sugarcane bagasse (SCB) and the industry by-product sugar beet molasses (SBM). With this aim, the Generally Recognized As Safe (GRAS) yeast *Kluyveromyces marxianus* was used in the study.

Developments related to the bioproduction of fruit-like compounds are exposed in the first section of the thesis. They include the initial substrate screening and an initial assessment of the operational variables affecting the process (Chapter 4). Then, the optimization of the fruit-like compounds production in a batch SSF at 0.5 L system is developed (Chapter 5). In that sense, it was found that the bioproduction is significantly affected by operational variables like the temperature and the air flow rate. While the maximum volatile production (including all the quantified species) was 161 mg_{Vol} per gram of dry substrate (g^{-1}_{TS}) at 40°C, 0.14 L h^{-1} g^{-1}_{TS} and 35% SBM, the ester species were maximized at 30°C, 0.11 L h^{-1} g^{-1}_{TS} and 25% SBM up to 47 mg_{Est} g^{-1}_{TS}. Finally, based on the optimization of the batch SSF at lab scale, the evaluation of the process at bench-scale was performed (4.5 and 22 L scales) (Chapter 6). In this case, the analysis has been focused on the effects of some operational strategies named intermittent mixing and fed-batch SSF in the global performance of the bioprocess. Fed-batch has shown interesting characteristics affecting the selectivity of the ester species and also improving some of the evaluated performance indices.

Bioproduction of the rose-like compounds via SSF is the core of the second section of this thesis. In this case, the first part is devoted to the evaluation of the feasibility of the

process under sterile conditions in a batch SSF 0.5 L system (Chapter 7). Here, the performed screening has served as starting point to develop different alternatives for running the SSF process. It was found that extending the *K. marxianus* activity is a key factor to increase the 2-PE and 2-PEA production. In this sense, by adding supplementary carbon sources to the media or splitting the substrate load (as a manner of a fed-batch mode) resulted in efficient and consistent ways to promote the biotransformation of these compounds. Based on the results at 0.5 L, the process was further evaluated at bench-scale (1.6 and 22 L scales) by releasing some of the constraints found at lab-scale such as the temperature control and the sterilization of the substrates (Chapter 8). Again, the analysis was focused on the assessment of the operational strategies fed-batch and sequential-batch as alternative approaches to enhance the global behavior of the process. Here, strategies have succeeded increasing the 2-PE and 2-PEA production from 17 $mg_{2\text{-}PE+2\text{-}PEA}$ per gram of dry substrate (g^{-1}_{TS}) in a batch scenario, up to 19.2 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} by using a fed-batch approach, and until 21 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} through a sequential-batch.

Overall, the results exposed in this thesis represent a step forward in the development of SSF as an alternative approach for producing valuable aroma compounds from agro-industrial wastes.

Resumen

Esta tesis se centra en la evaluación de la fermentación en estado sólido (FES) de residuos agroindustriales seleccionados, como enfoque alternativo para la producción de aromas de valor añadido. Este estudio se ha desarrollado en el marco del proyecto *Estrategias de optimización de procesos de obtención de bioproductos a partir de residuos orgánicos mediante fermentación en estado sólido* (BIOPRO, CTM2015-69513-R) y corresponde al primer trabajo orientado a la producción de aromas en el grupo de investigación. Concretamente, dos grupos de compuestos han sido considerados como objetivos a estudiar. En primer lugar, los aromas de fruta, entre los cuales se pueden incluir algunos compuestos volátiles como aldehídos, cetonas, alcoholes y ésteres de bajo peso molecular. De estos, los ésteres alifáticos se pueden considerar aquellos con más valor añadido dado el particular olor a frutas que proveen. El segundo grupo de compuestos son los denominados aromas de rosas, conformados por el 2-fenil etil alcohol (2-FA) y el 2-fenil etil éster (2-FE); compuestos de gran valor añadido empleados principalmente como aditivos dado el olor a rosas que proveen. En ambos casos, el objetivo ha sido el desarrollo de bioprocesos basados en el uso de residuos, empleando como materias primas el bagazo de caña y las melazas de remolacha, ambos, subproductos provenientes de la industria azucarera. Con este objetivo, se ha utilizado a lo largo del estudio la levadura Generalmente Reconocida como Segura *Kluyveromyces marxianus.*

Los resultados de la bioproducción de los aromas de fruta se exponen en la primera sección de la tesis. Esta incluye la selección del sustrato por medio de un barrido inicial, así como la evaluación preliminar de algunas de las variables de proceso que afectan el desarrollo de la fermentación (Capítulo 4). Una vez seleccionado el sustrato, la producción de estos compuestos volátiles ha sido evaluada a escala laboratorio en bioreactores de 0.5 L operados por lotes (Capítulo 5). De este apartado se concluye que el proceso se ve significativamente afectado por variables como la temperatura y el caudal de aire. En el sistema valorado se encontró que la máxima producción de volátiles (incluidas todas las especies identificadas y cuantificadas) fue de 161 mg_{Vol} por cada gramo de sustrato inicial en base seca (g_{ST}), siendo alcanzado a 40°C, 0.14 L h^{-1} g^{-1}_{ST} y adicionando un 35% de melazas (base seca). Por otra parte, la máxima producción de ésteres fue promovida a 30°C, 0.11 L h^{-1} g^{-1}_{ST} y adicionando un 25% de melazas, alcanzando 47 mg_{Est} g^{-1}_{ST}. Por último, partiendo de la optimización del proceso por lotes a escala laboratorio, la producción de estos compuestos fue evaluada en escalas superiores (4.5 y 22 L) (Capítulo 6). En este caso, la evaluación se ha enfocado en el efecto que tienen las estrategias de operación sobre el desempeño global del proceso. Así, la

FES por lotes se ha comparado ante el proceso empleando mezclado intermitente y operando por lotes alimentados. Esta última estrategia ha resultado ser una herramienta que afecta positivamente la selectividad hacia los ésteres, mejorando al mismo tiempo algunos de los índices de desempeño que describen el proceso.

La bioproducción de aromas de rosa por FES es el objetivo de la segunda sección de este documento. Así, la primera parte de la sección ha sido dedicada a la evaluación de la FES en condiciones estériles en bioreactores de 0.5 L (Capítulo 7). En este caso, se realizó un barrido inicial sobre las variables de operación que afectan el proceso, así como de diversas alternativas para mejorar la producción de estos compuestos vía FES. De dicho estudio se encontró que la producción de 2-FA y 2-FE está directamente asociada con la actividad microbiológica del *K. marxianus*, con lo cual extender dicha actividad es un factor que resulta decisivo para mejorar la producción de estos compuestos. En este sentido, la adición de glucosa como fuente adicional de carbono, o el uso de una alimentación fraccionada (usando una alimentación por lotes) han resultado ser alternativas consistentes y eficientes para mejorar la producción de 2-FA y 2-FE. A continuación, y con base a los resultados de la evaluación a escala laboratorio, el proceso fue valorado a escalas mayores (1.6 y 22 L) eliminando algunas de las limitaciones asociadas al trabajo a dicha escala, como el control de temperatura y la esterilización de los sustratos (Capítulo 8). Del mismo modo que para los aromas de fruta, el análisis de esta parte se enfocó en el uso de estrategias de operación como herramientas para mejorar el desempeño global del proceso. En esta sección, la fermentación por lotes ha sido contrastada ante la FES por lotes alimentados y empleando lotes secuenciales. En este caso, la producción alcanzada en el proceso por lotes de 17 $mg_{2\text{-}FA+2\text{-}FE}$ por gramo de sustrato inicial seco fue mejorada al implementar las estrategias alternativas, alcanzando 19.2 $mg_{2\text{-}FA+2\text{-}FE}\ g^{-1}_{ST}$ en el caso de lotes alimentados y hasta 21 $mg_{2\text{-}FA+2\text{-}FE}\ g^{-1}_{ST}$ en los lotes secuenciales.

De modo global, los resultados presentados en esta tesis constituyen un paso adelante en el desarrollo de la FES como alternativa para producir estos compuestos de valor añadido a partir de residuos agroindustriales.

Resum

Aquesta tesi es centra en l'avaluació de la fermentació en estat sòlid (FES) de residus agroindustrials seleccionats, com un enfocament alternatiu per a la producció d'aromes de valor afegit. Aquest estudi s'ha desenvolupat en el marc del projecte *Estrategias de optimización de procesos de obtención de bioproductos a partir de residuos orgánicos mediante fermentación en estado sólido* (BIOPRO, CTM2015-69513-R) i correspon al primer treball orientat a la producció d'aromes en el grup de recerca. Concretament, dos grups de compostos han estat considerats com a objectius a estudiar. En primer lloc, aromes de fruita, entre els quals es poden incloure alguns compostos volàtils com aldehids, cetones, alcohols i èsters de baix pes molecular. D'aquests, els èsters alifàtics es poden considerar aquells amb més valor afegit donat el seu particular olor a fruites. El segon grup de compostos són els anomenats aromes de roses, constituïts pel 2-fenil etil alcohol (2-FA) i el 2-fenil etil èster (2-FE); compostos de gran valor afegit, utilitzats principalment com a additius gràcies al seu olor a roses. En tots dos casos, l'objectiu ha estat el desenvolupament de bioprocessos basats en l'ús de residus, emprant com a matèries primeres el bagàs de canya i les melasses de remolatxa, tots dos, residus provinents de la indústria sucrera. Amb aquest objectiu, s'ha utilitzat al llarg de l'estudi el llevat Generalment Reconeguda com Segur *Kluyveromyces marxianus.*

Els resultats associats amb la bioproducció de les aromes de fruita s'exposen a la primera secció de la tesi. Aquesta inclou la selecció del substrat per mitjà d'un escombrat inicial, així com l'avaluació preliminar d'algunes de les variables de procés que afecten el desenvolupament de la fermentació (Capítol 4). Un cop seleccionat el substrat, la producció d'aquests compostos volàtils ha estat avaluada a escala laboratori en bioreactors de 0.5 L operats en discontinu (Capítol 5). D'aquest apartat es pot indicar que el procés es veu significativament afectat per variables com la temperatura i el cabal d'aire. En el sistema valorat es va trobar que la màxima producció de volàtils (incloses totes les espècies identificades i quantificades) va ser de 161 mg_{Vol} per gram de substrat inicial en base seca (g_{ST}), que es va aconseguir a 40°C, 0.14 L h^{-1} g^{-1}_{ST} i addicionant un 35% de melasses (base seca). D'altra banda, la màxima producció d'èsters va ser obtinguda a 30°C, 0.11 L h^{-1} g^{-1}_{ST} i addicionant un 25% de melasses, aconseguint-se 47 mg_{Est} g^{-1}_{ST}. Finalment, partint de l'optimització del procés en discontinu a escala laboratori, la producció d'aquests compostos va ser avaluada a escales superiors (4.5 i 22 L) (Capítol 6). En aquest cas, l'estudi s'ha enfocat en l'efecte que tenen les estratègies d'operació sobre l'acompliment global del procés. Així, la FES en discontinu s'ha comparat davant el procés emprant barrejat intermitent i operant en

fed-batch. Aquesta última estratègia ha resultat ser una eina que afecta positivament la selectivitat cap als èsters, millorant al mateix temps alguns dels índexs de rendiment que descriuen el procés.

La bioproducció d'aromes de rosa per FES és el objectiu de la segona secció d'aquest document. La primera part d'aquesta secció ha estat dedicada a l'avaluació de la FES en condicions estèrils en bioreactors de 0.5 L (Capítol 7). En aquest cas, es va desenvolupar un estudi inicial sobre les variables d'operació que afecten el procés, així com de diverses alternatives per millorar la producció d'aquests compostos via FES. D'aquest estudi es va trobar que la producció de 2-FA i 2-FE està directament associada amb l'activitat microbiològica del *K. marxianus*, amb la qual cosa allargar aquesta activitat resulta en un factor decisiu per millorar la producció d'aquests compostos. En aquest sentit, l'addició de glucosa com a font addicional de carboni, o l'ús d'una alimentació fraccionada (usant una *fed-batch*) han resultat ser alternatives consistents i eficients per millorar la producció de 2-FA i 2-FE. Amb base en als resultats de l'avaluació a escala laboratori, el procés va ser estudiat a escales més grans (1.6 i 22 L) retirant algunes de les limitacions associades als processos a aquesta escala, com el control de temperatura i l'esterilització dels substrats (Capítol 8). De la mateixa manera que per les aromes de fruita, l'anàlisi d'aquesta part s'ha enfocat a l'ús d'estratègies d'operació com a eines per millorar l'acompliment global del procés. Així, la fermentació en discontinu ha estat contrastada davant la FES per *fed-batch* i la discontinua seqüencial. En aquest cas, la producció assolida en el procés en discontinu (17 $mg_{2\text{-}FA + 2\text{-}FE}$ per gram de substrat inicial sec) va ser millorada a l'implementar les estratègies alternatives, aconseguint-se 19.2 $mg_{2\text{-}FA + 2\text{-}FE}$ g^{-1}_{ST} al cas del *fed-batch.*

De manera global, els resultats presentats en aquesta tesi constitueixen un pas endavant en el desenvolupament de la FES com a alternativa per produir aromes com a compostos de valor afegit a partir de residus agroindustrials.

Table of contents

List of abbreviations

Abbreviation	Definition	Units
2-PE	2-phenethyl alcohol	
2-PE^{L}	Equivalent 2-PE concentration in the liquid interface	$g_{2\text{-PE}}\ L^{-1}$
2-PEA	2-phenethyl acetate	
AFP	Air filled porosity	%
AP	Apple pomace	
At_4	Cumulative index after four days	$g_{O2}\ kg^{-1}{}_{TS}$
BD_w	Wet bulk density	$kg\ L^{-1}$
C_{Air}	Total air consumption	$NL_{Air}\ g^{-1}{}_{PTS}$
C_i	Concentration of species *i*	$mg\ L^{-1}$
CFU	Colony forming units	
CH	Coffee husk	
$CO_2{}^{P}$	Carbon dioxide production rate	$g_{CO2}\ kg^{-1}{}_{VS}\ h^{-1}$
COC	Cumulative oxygen consumption	$g_{O2}\ kg^{-1}{}_{TS}$
COE	Council of Europe	
DRI_{24h}	Dynamic respiration index	$g_{O2}\ kg^{-1}{}_{VS}\ h^{-1}$
FB	Fed-batch mode	
FDA	Food and Drug Administration	
FEMA	Flavor and Extract Manufacturers' Association	
F&F	Fragrance and flavor	
GRAS	Generally recognized as safe	
HPLC	High-performance liquid chromatography	
Ino_C	Inoculum concentration	$CFU\ mL^{-1}{}_{Ino}$
Ino_L	Inoculum load	$\%\ (mL_{ino}\ g^{-1}{}_{subs})$
Ino_R	Inoculum ratio	$CFU\ g^{-1}{}_{TS}$
Ino_{Req}	Inoculum requirement	$CFU\ g^{-1}{}_{PTS}$
ISPR	In situ product removal	
JECFA	Joint Expert Committee on Food Additives	
L-Phe	L-Phenylalanine	
MC	Moisture content	$\%\ (g_{H2O}\ g^{-1}{}_{substrate})$
M_d	Sugar beet molasses dose	$\%\ (g_{TSmol}\ g^{-1}{}_{TSsub})$
OP	Orange peel	

Abbreviation	Definition	Units
OUR	Oxygen uptake rate	$g_{O2}\ kg^{-1}_{VS}\ h^{-1}$
P_d	L-phenylalanine dose	% ($g_{L\text{-}Phe}\ g^{-1}_{TSsub}$)
PTS	Processed total solids	
P_{vol}^{t}	Total volatile productivity (Fruit-like)	$mg_{Vol}\ g^{-1}_{TS}\ h^{-1}$
P_{Est}^{t}	Ester species productivity (Fruit-like)	$mg_{Est}\ g^{-1}_{TS}\ h^{-1}$
$P_{2\text{-}PE}^{t}$	2-phenethyl alcohol productivity	$mg_{2\text{-}PE}\ g^{-1}_{TS}\ h^{-1}$
$P_{2\text{-}PEA}^{t}$	2-phenethyl acetate productivity	$mg_{2\text{-}PEA}\ g^{-1}_{TS}\ h^{-1}$
P_{Vol}^{Acc}	Total volatile cumulative production (Fruit-like)	$mg_{Vol}\ g^{-1}_{TS}$
P_{Est}^{Acc}	Total ester cumulative production (Fruit-like)	$mg_{Est}\ g^{-1}_{TS}$
P_{sub}^{Tot}	Total cumulative production (Rose-like)	$mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{TS}$
$P_{sub}^{2\text{-}PE}$	2-phenethyl alcohol cumulative production	$mg_{2\text{-}PE}\ g^{-1}_{TS}$
P_t	Average maximum productivity (Rose-like)	$mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{TS}\ h^{-1}$
QPS	Qualified Presumption of Safety	
Red Sug	Reducing sugars content	$g_{R.S.}\ 100g^{-1}_{TS}$
RQ	Respiration quotient	
S_{AFR}	Specific air flow rate	$L\ h^{-1}\ g^{-1}_{TS}$
SB	Sequential-batch mode	
SBM	Sugar beet molasses	
SCB	Sugarcane bagasse	
SCG	Spent coffee grounds	
SmF	Submerged fermentation	
SSF	Solid-state fermentation	
TD-GC	Thermal desorption gas chromatography	
TS	Total solids content	
VS	Volatile solids content	
WHC	Water holding capacity	$g_{H2O}\ g^{-1}_{material}$
$X_{L\text{-}Phe}$	L-Phenylalanine conversion	$mol_{L\text{-}Pher}\ mol^{-1}_{L\text{-}Phe0}$
$Y_{L\text{-}Phe}$	Mass yield (Rose-like)	$g_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{L\text{-}Phe0}$
Y_{Glu}	Sugar consumption efficiency	$mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{Sug}$
$Y_{s\text{-}t}$	Space-time yield (Fruit-like)	$mg_{Vol}\ L^{-1}\ h^{-1}$
$Y_{s\text{-}t}$	Space-time yield (Rose-like)	$mg_{2\text{-}PE+2\text{-}PEA}\ L^{-1}\ h^{-1}$
Y_{mol}	Molar yield (Rose-like)	$mol_{2\text{-}PE+2PEA}\ mol^{-1}_{L\text{-}Phe0}$

Chapter 1

Introduction

1.1 Aroma compounds

Aroma compounds are constituted by several volatile and nonvolatile components with specific physicochemical properties such that they produce odor perceptions in our brain. They are perceived by the odor receptor sites of the smell organ, reaching the receptors when drawn in through the nose (orthonasal detection) and also via the throat after being released by chewing (retronasal detection) (Belitz et al., 2009). Aroma compounds are typically constituted by several volatile and nonvolatile components with a great variety of chemical and physicochemical properties arranged inside complex matrices. The variability of the media in which aroma compounds are present is such that they can be found from food, spices, essential oils, and wine until perfumes or fragrances, flowers, plants, among others (Dastager, 2009). A vast collection of compounds are responsible for the odor of the matrices above, such as alcohols, aldehydes, esters, dicarbonyls, short to medium-chain free fatty acids, methyl ketones, lactones, phenolic compounds and sulphur compounds (Gatfield, 1988).

Aroma compounds are typically analyzed as part of the fragrance and flavor sector (F&F), but these compounds have also become crucial for many other industries. Cosmetic, chemical and pharmaceutical industries also use aroma compounds as additives due to the effect on the products, enhancing their organoleptic properties, and therefore, positively influencing the final consumer's perception and acceptance (Ziegler, 2007). As seen in Figure 1.1, annual fragrance and flavor market has continuously grown in the last decades, reaching almost US$ 24.5 billion in 2016 with a permanent increase above 4% each year (The Freedonia group, 2016). This represents over a quarter of the world market for food additives (Dubal et al., 2008) and for aroma compounds, it implies a share near to 16% of the F&F market, reaching US$ 4.1 billion in 2016 (Stratistics consulting, 2016).

1.1.1 Aroma compounds production

As it can be seen in Figure 1.2, aroma compounds are obtained by different processes with particular characteristics. Thus, natural aromas are typically extracted from the matrices containing these compounds, but the low concentration of these compounds within the matrices makes their recovery inefficient and consequently costly (Longo and Sanroman, 2006). Furthermore, since most of the matrices containing aroma compounds correspond to classical agricultural sources, the recovery of these compounds also depend on factors

difficult to control such as weather conditions and plant diseases, or the type of bonding form they have inside the matrix (Ben Akacha and Gargouri, 2015). Evidently, all those aspects affect the amount and quality of the produced aromas, causing that natural sources become insufficient to cover the increasing demand of the market.

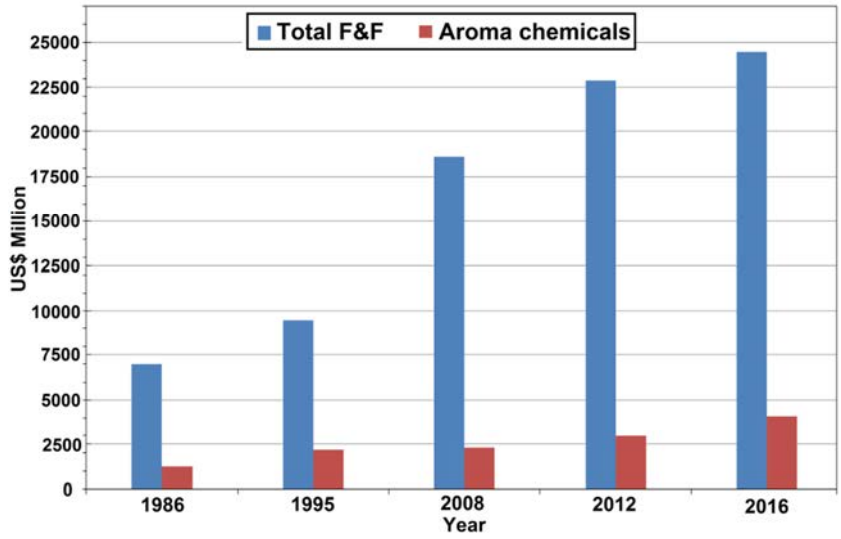


Figure 1.1. World fragrance and flavors market sales. Data from Armstrong and Yamazaki, (1986); Leffingwell & Associates, (2015); Stratistics consulting, (2016); The Freedonia group, (2016); Ziegler, (2007)

Hence, after the elucidation of the chemical structure of aroma compounds, synthetic flavors have been produced by chemical synthesis. Although these routes are, by far, less expensive than aroma extraction, through these paths, it is possible to only partially reproduce an aroma (Ben Akacha and Gargouri, 2015). For instance, chemically synthetized vanillin has a price around US$ 12 per kg, while natural vanillin could have a price as high as US$ 1200–4000 per kg (Sindhwani et al., 2012). Moreover, chemical synthesis results in a questionable way of obtaining aroma compounds. These paths are under question, due to severe drawbacks such as poor substrate selectivity leading to undesirable side reactions, low yields, high manufacturing efforts, and scarce environmentally friendly techniques (Longo and Sanroman, 2006). Moreover, the formation of undesirable racemic mixtures is frequently found in chemical synthesis, generating undesirable by-products imparting off-odors that change the organoleptic profiles of the aroma (Belitz et al., 2009).

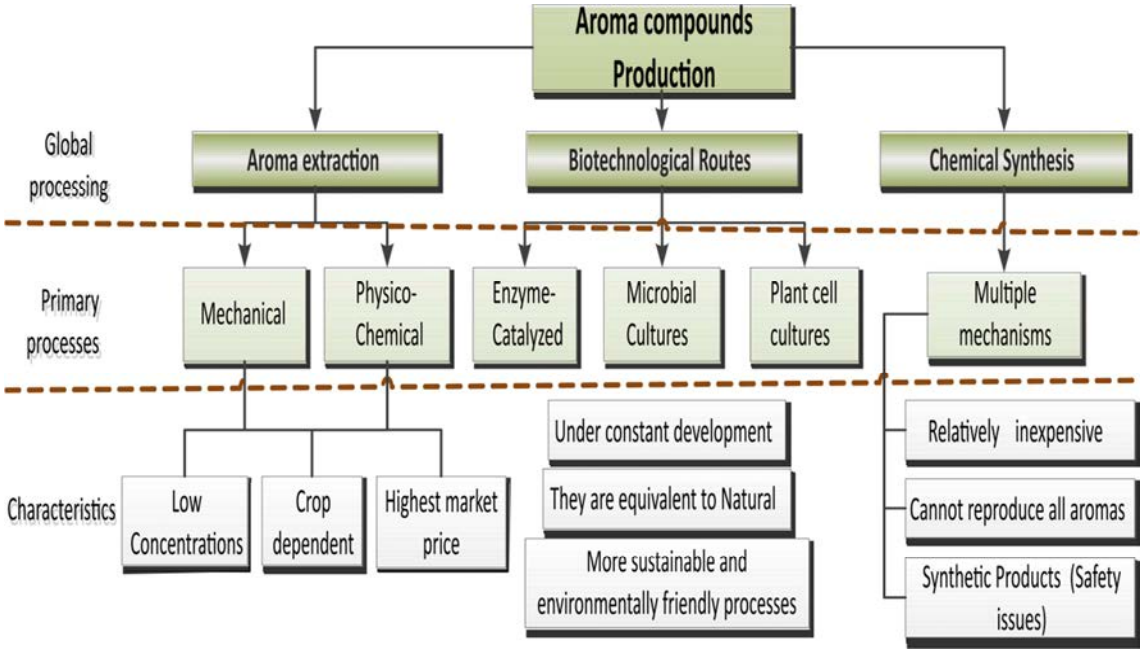


Figure 1.2. Conventional productive technologies for producing aroma compounds.

At the same time, another relevant aspect influencing the aroma compounds sector is the current trend of consumers towards natural products. While in food and home products industries a generalized "chemophobia-attitude" has been developed by a high number of consumers (Vandamme and Wim, 2002), in many others, buyers tend to favor natural compounds because of the global idea, that these compounds are healthier and environmentally friendly (Carlquist et al., 2015). As a consequence, even that synthetic aromas are cheaper than their natural counterparts, the characteristics of these products are perceived as negative compared to those considered natural (Dastager, 2009). Thus, the rising demand of consumers for natural products poses in evidence the need for alternative processes to obtain non-artificial aroma compounds.

According to this, a promising alternative route for aroma production is based on microbial biosynthesis or bioconversion (Aguedo et al., 2004; Ben Akacha and Gargouri, 2015; Berger, 2015; de Oliveira Felipe et al., 2017; Krings and Berger, 1998; Vandamme and Wim, 2002). These bioprocesses based on microorganisms (bacteria, fungi, yeasts) and their enzymes are framed within white biotechnology. In this context, biotechnological routes appear as promising substitutes given the ability of some microorganisms to transform sugars and amino acids of some raw materials into aroma compounds (Longo and Sanroman, 2006). Furthermore, these processes are encouraged by the current European and American legislation due to the qualification of Generally Recognized As Safe (GRAS). This qualification determines 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; Vandamme and Wim, 2002). At the same time, bioconversion for producing aroma compounds is in accordance with the aim of exploiting renewable sources and agro-industrial waste to promote clean production, less energy intensive systems and more economical processes (Laufenberg et al., 2003). Among the benefits of using biotechnological routes for producing aroma compounds, some authors (Berger, 2015; de Oliveira Felipe et al., 2017) highlight as the most remarkable aspects, the ability to undertake the productive process throughout the year without any seasonal interference, the capability to use less stringent resources, saving energy and reducing the disposal of harmful reagents to the environment, and the possibility to optimize the processes. Accordingly, biotechnological approaches for obtaining aroma compounds meet two of the main demands of the modern society. First, supplying natural products that fulfill the consumers' expectations, and second, providing a more sustainable alternative that promotes the environment preservation at the same time that contributes with the producer credibility (de Oliveira Felipe et al., 2017).

1.1.1.1 Biotechnological approaches for producing aroma compounds

As observed in Figure 1.3, biotechnological approaches for producing aromas can be divided into three classes. In the first place, it can be found the enzymatic paths in which many of the problems found in chemical synthesis methods are bypassed due to the substrate specificity, regio and enantioselectivity of these biocatalysts (Longo and Sanroman, 2006). Also, a significant amount of enzymes are able to directly produce flavor molecules by hydrolysis of larger precursors with higher productivities than direct extraction from plants (Ben Akacha and Gargouri, 2015). However, enzymatic paths have some disadvantages such as the need of long and complicated steps for enzyme isolation and purification. Similarly, the differences of solubility between species become a problem since enzymatic processes are usually performed in aqueous phase, while many aroma precursors and products are not soluble in water (Sarma et al., 2014).

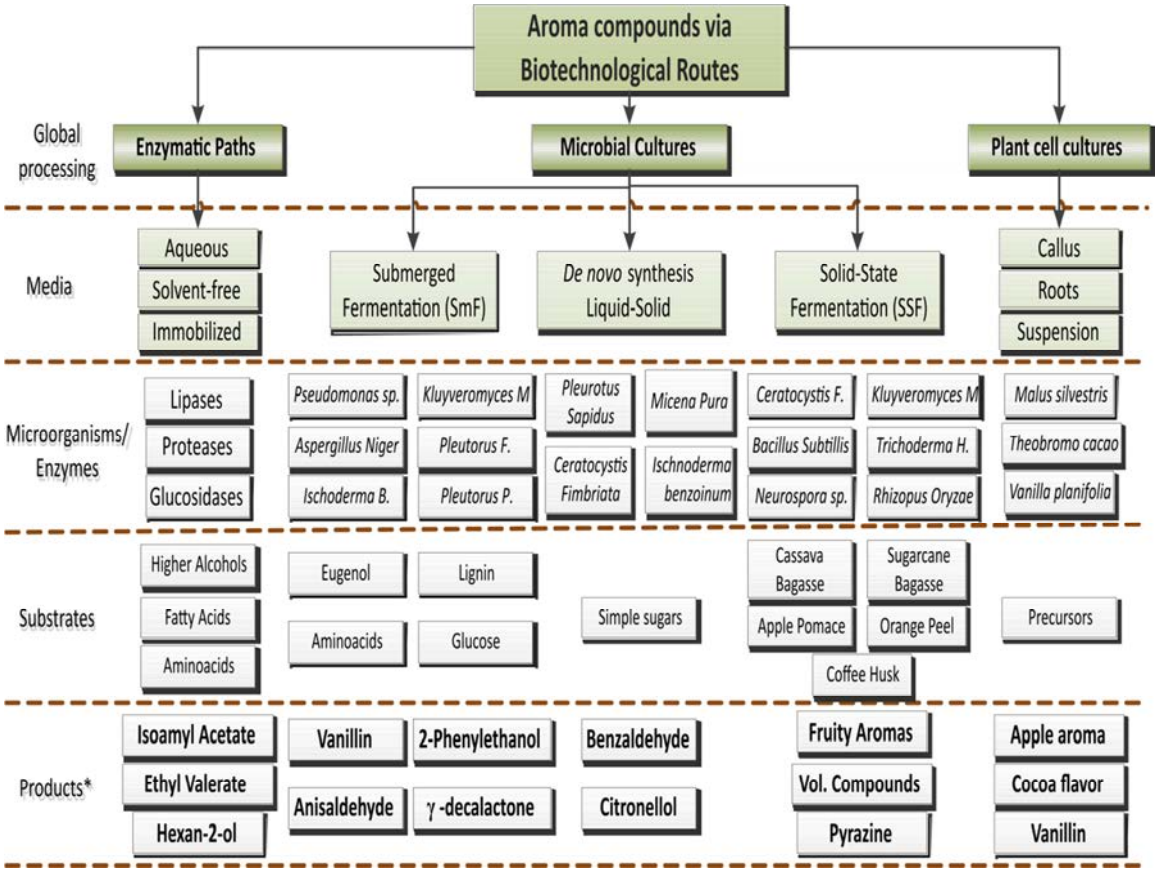


Figure 1.3. Biotechnological routes for producing aroma compounds and some of the typical products obtained through these approaches.*Obtained at investigation level.

Another biotechnological route used to produce aroma compounds is the plant cell cultures. In general, aroma compounds are the result of many biotransformations inside the plant cells, and they could be considered as terminal metabolites containing abundant biochemical information (Medeiros et al., 2010). In this approach, the genetic and totipotency capacity of a particular plant is exploited to increase the amount of chemical components constituting the natural flavors by feeding precursors into the biosynthetic pathways (Dastager, 2009). Nevertheless, several challenges are still unsolved to use plant cell cultures at large-scale. For instance, the relatively long growth cycles, low yields, emission of rare sub products or the sensitivity to shear stress (Longo and Sanroman, 2010).

The last group of biotechnological routes for producing aromas is known as microbial cultures or biotransformation. Perhaps this path is more recognized than the previous ones since the use of some microorganisms in the elaboration of flavor components of many foods is spread over the world. Thus, different strains are involved in the production of wine, vinegar, beer or milk, but many microorganisms may also be used for large-scale biotransformation of precursors to natural flavors for different applications (Ben Akacha and

Gargouri, 2015). As detailed in Figure 1.3, the technologies involving microbial cultures have been developed mainly on submerged fermentation (SmF) and solid-state fermentation (SSF). In general, some microorganisms are able of synthesizing aroma compounds through their grown on liquid or solid culture media. These microorganisms have the ability to perform biotransformations of a wide variety of nutrients, requiring multiple chemical steps in the cells (Medeiros et al., 2010). To obtain a specific aroma compound, the biocatalytic conversion of a structurally related precursor is often a more suitable strategy allowing the bioproduction of the desired compound (Dubal et al., 2008). However, the "*de novo*" synthesis appears in the middle as a potential alternative to the use of precursors. In this case, complex species can be formed starting from simple molecules such as sugars or amino acids, as secondary metabolites produced in different steps of the cell metabolism. Compared to biotransformation, *de novo* synthesis produces a mixture of several aroma compounds by using the whole metabolic system of the microorganism instead of producing a major constituent (Ben Akacha and Gargouri, 2015; Vandamme and Wim, 2002).

1.1.2 Aroma compounds of industrial interest produced by microbiological routes

Aroma compounds are commonly classified according to their chemical structures, physicochemical properties, or sensorial characteristics, but the most convenient categorization is based on the chemical family of the substrate. On this basis, lipid-derived aroma compounds are one of the most important families, including lactones, volatile fatty acids, esters, aldehydes, ketones and alcohols (Longo and Sanroman, 2010). As seen in Table 1.1, many valuable aroma compounds belonging to this group could be listed. Some of these compounds are highly appreciated in industry, and their demand is continuously growing, so they have been studied for possible commercial production by bioprocess technologies (Sarma et al., 2014).

In this sense, compounds like vanillin or benzaldehyde have received much attention due to their spread use in many industries, as well as by the growing demand for natural components in the food industry (Longo and Sanroman, 2010). Furthermore, lactones such as the γ-decalactone has become a desirable biotechnological product because of its oily-peach aroma is widely used for flavor applications (Braga and Belo, 2016).

Simultaneously, some other aroma compounds are gaining relevance in the industry mainly due to the increasing demand for additives. In this group, the alcohols, mainly the rose-like scented 2-phenethyl alcohol and the so-called fruit-like compounds (mainly straight-

chain esters) have been studied because of their potential to become productive biotechnological processes (Medeiros et al., 2010; Sindhwani et al., 2012).

Table 1.1. Some important aroma compounds produced through biotechnological approaches.

Aroma Compound	Odor perception	Precursor	Microorganisms	Reference
Lactones				
6-pentyl-α-pyrone	Coconut-like	Oleic acids	Fungi/Yeasts	(Fadel et al., 2015)
γ-decalactone	Peach-like	Ricinoleic acid	Yeasts/Enzymes	(Braga et al., 2015)
Alcohols				
(Z)-3-hexen-1-ol ('leaf alcohol')	Grassy-green	Linoleic acid	Yeasts	(Buchhaupt et al., 2012)
2-phenethyl alcohol	Rose-like	L-phenylalanine	Yeasts	(Chreptowicz et al., 2016)
Esters				
Ethyl acetate	Sweet	Ethanol	Yeast/Fungi/ Enzymes	(Löser et al., 2014)
Isoamyl acetate	Banana	Fusel alcohols	Yeasts/Enzymes	(Torres et al., 2010)
2-phenethyl acetate	Flower-like	Fusel alcohols	Yeasts/Enzymes	(Białecka-Florjańczyk et al., 2012)
Aldehydes & Ketones				
Vanillin	Vanilla	Ferulic acid	Streptomyces	(Hua et al., 2007)
Benzaldehyde	Almond-like	Phenylalanine	Bacteria/Enzymes	(Okrasa et al., 2004)
Diacetyl	Butter-like	-	Lactic acid bacteria	(Alvandi et al., 2008)

1.1.2.1 Fruit-like aroma compounds

Fruit-like aroma compounds are mainly composed of short-chain esters, very appreciated because of the fruity aromas they provide. They are used as additive in fruit-flavored products (*e.g.* beverages, jellies, candies and jams), wines or dairy products (*e.g.* yogurt, sour cream, butter or cheese) (Amaral et al., 2010). From these, acetate esters constitute the main fruit-like aromas. Ethyl acetate, isoamyl acetate and ethyl butyrate are examples of the most recognized esters employed in the flavoring of wine and other grape-derived alcoholic beverages (Longo and Sanroman, 2006). Table 1.2 summarizes some of the most important fruit-like compounds and their odor characteristics.

Table 1.2. Main ester species producing fruit-like aromas

Compound	Odor	Vapor pressure (mm_{Hg} at 25°C)
Methyl Acetate	Sweet-fruity	368.35
Ethyl Acetate	Sweet	111.7
Ethyl propionate	Pineapple	35.9
Propyl Acetate	Pears	35.2
Ethyl butyrate	Pineapple	12.8
Ethyl Isobutyrate	Fruity-alcoholic	25.4
Isobutyl Acetate	Raspberry-pears	17.8
Isopropyl Acetate	Sweet-banana	60.7
Butyl Acetate	Banana-apple	11.5
Isoamyl Acetate	Banana	5.6
Ethyl Butanoate	Pineapple	12.8
Isoamyl Butyrate	Pears-banana	0.95
Hexyl acetate	Banana-apple	1.39

Ester formation can be accomplished by the alcoholysis of acyl-CoA compounds or by the direct esterification of an organic acid with an alcohol. The enzyme catalyzing this reaction is an alcohol acetyltransferase (AATase), which is a common enzyme produced by yeasts like *Saccharomyces cerevisiae* or *Kluyveromyces marxianus* (Morrisey et al., 2015). Besides, ester biotransformation is also performed by hydrolytic enzymes like lipases, helping in the biocatalysis of esterification and transesterification reactions (Ben Akacha and Gargouri, 2015).

Beyond the inherent fruity odor perception given by these compounds, the other relevant characteristic they share is their high volatility. As seen in Table 1.2, the vapor pressure of the most representative fruity ester compounds is relatively high at 25°C, which represents some problems for the recovery of products when the process are undertaken in liquid media. Thus, this phase transfer has to be taken into account by the implementation of integrated product recovery techniques (Löser et al., 2014). On the contrary, this intrinsic characteristic seems to be an asset when the biotechnological approach is conducted via SSF. As some authors state (Medeiros et al., 2006), fruit-like aromas can be recovered directly in the gas phase, by using more efficient processes like adsorption techniques.

At the same time, given the ability of yeast to metabolize different carbohydrate sources (*i.e.* different sugars), biosynthesis of fruit-like compounds can be carried out starting with different substrates of diverse origin (Sarma et al., 2014). This implies that bioprocesses for producing fruit-like compounds are prone to be run with low-cost raw materials like agro-

industrial residues rich in carbohydrates. By doing this, a remarkable save of resources might be obtained regarding energy if comparing the biotechnological processes against the traditional extraction from fruits, where intensive evaporation-distillation steps are required to concentrate the aroma compounds (Diban Gómez, 2008). In this sense, to obtain a concentrated fraction able to be further processed for the recovery of the fruit-like compounds, a typical extraction-concentration method requires a concentration factor (folds) ranging the 100-200, that would imply the use of 300 to 600 kg of fruits to achieve 1 kg of concentrated solution (Mushtaq, 2018).

1.1.2.2 2-Phenethyl alcohol and 2-phenethyl acetate

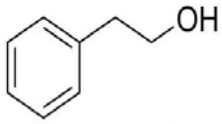
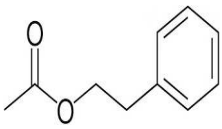
2-Phenethyl alcohol or 2-phenylethanol (2-PE) is a higher alcohol characterized by its pleasant rose-like odor. This condition has made 2-PE one of the most used components in fragrance, cosmetic and food industries as an organoleptic enhancer of final products (Chreptowicz et al., 2016; Hua and Xu, 2011). However, 2-PE has also shown other interesting characteristics like its antifungal and antibacterial properties, making of this compound an appreciated additive in disinfectant, pest control, cleaning and personal care products (Etschmann et al., 2002; Sendovski et al., 2010). 2-PE is also employed as a 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) as detailed in Table 1.3. 2-PEA is typically used in decorative cosmetics, fine fragrances, toilet soaps, shampoos, and non-cosmetic products such as household cleaners and detergents (McGinty et al., 2012).

While 2-PE is found naturally in the essential oils of many flowers and plants, such as roses, hyacinths, jasmine or lilies (Etschmann et al., 2002), 2-PEA is present in nature in significant amounts in plants like evergreen trees (*Cinnamomum* species) and cloves (*eugenia caryo-phyllata thunberg*) (Kim and Park, 2017). Furthermore, these compounds are found in many fermented food products, like cocoa, bread, wine, beer, cheese or soy sauce (Burdock, 2010). However, the recovery process from these sources is commonly complex due to the need of multiple separation steps and the relative low content of 2-PE and 2-PEA in the matrices containing them (Białecka-Florjańczyk et al., 2012; Chreptowicz et al., 2016; Kuo et al., 2014b; Mei et al., 2009).

Both compounds have been approved as flavoring agents in food by many worldwide organizations like the Flavor and Extract Manufacturers' Association (FEMA), the Food and

Drug Administration (FDA), the Joint Expert Committee on Food Additives (JECFA) or the Council of Europe (COE), considering 2-PE and 2-PEA as generally recognized as safe (GRAS) flavoring agents, making them high value-added aroma compounds (Burdock, 2010; McGinty et al., 2012; Scognamiglio et al., 2012).

Table 1.3. Main characteristics of 2-PE and 2-PEA.

	Chemical Structure	**Odor characteristics**	**Vapor pressure (mm_{Hg} at 25°C)**	**Application of food, flavor, fragrance**	**Natural occurrence in fruits/plants**
2-PE		Sweet, floral rose, fresh	0.087	Perfumery, Soaps, cigarettes, soft drinks, deodorants	Berries, peach, cinnamon, roses, jasmine, hyacinth.
2-PEA		Sweet, honey, floral rosy, green nectar fruity	0.056	Apricot, peach vanilla, tutti-frutti, shampoos, soaps, soft drinks	Apple, grapes, tomato, *freesia magnolia, Hyacinth reseda*.

1.2 *K. marxianus* as aroma compounds producer

As detailed in section 1.1.1.1, in the biotechnological routes involving biotransformations, yeasts seem to be one of the most significant microorganisms producing aroma compounds. Their ability to produce a wide variety of volatile compounds has been intensely studied for many applications (Carlquist et al., 2015). One of the most studied strains for producing aroma compounds is the *Kluyveromyces marxianus* (Wittmann et al., 2002). It is described as homothallic and hemiascomycetous yeast, sharing, with its better-known sister *Kluyveromyces lactis*, the ability to assimilate lactose to use it as a sole carbon source. Commonly, this trait has been used in their isolation process using dairy products like fermented milk, yogurt and cheeses as source of *Kluyveromyces* genus (Fonseca et al., 2008). The direct association of these yeasts with food products has favored their qualification as GRAS, and QPS (Qualified Presumption of Safety) strains in the United States and European Union, respectively (Lane and Morrissey, 2010).

While *K. lactis* has been typically used as a model for non-conventional yeasts, *K. marxianus* has been progressively implemented by industry, mainly because it possesses traits that are desired for biotechnological applications (Morrisey et al., 2015). Some of those characteristics include fast growth, thermotolerance, broad substrate spectrum, limited fermentation at sugar excess, and secretion of extracellular glycolytic enzymes (Gombert et al., 2016). Also, *K. marxianus* can grow on glucose, fructose, xylose, galactose and inulin as the sole carbon sources, and therefore, it is expected that it can also grow in wastes containing significant amounts of these carbon sources like in forestry or sugar industry residues (Pentjuss et al., 2017).

Although many yeasts have been reported for the production of aroma compounds, only a few of these are considered GRAS, making more feasible their use in industrial applications (Medeiros et al., 2000, 2001). In this sense, *K. marxianus* has been reported as suitable strain in the production of aroma compounds such as fruit esters, carboxylic acids, ketones, furans or a wide variety of alcohols (Fonseca et al., 2008). However, the most studied biotechnological processes for producing aroma compounds using *K. marxianus* have been focused on the production of valuable higher alcohols such as 2-PE and acetate esters such as 2-PEA and the fruit-like compounds (Morrisey et al., 2015). As previously stated, yeasts are potential catalysts for the production of these compounds, either via *de novo* synthesis or by biotransformation through one or multistep whole-cell biocatalysis from specific precursors (Carlquist et al., 2015).

As Figure 1.4 depicts, inside the *K. marxianus* cell, two main metabolic routes are responsible for the production of these group of compounds. On the one hand, the cell assimilates sugars to obtain energy incorporating the pyruvate produced through the glycolysis process in the TCA cycle, but at the same time, the pyruvate can be converted into aldehydes, alcohols and esters due to enzymatic activity promoted by specific conditions. On the contrary, when an amino acid source is the primary nitrogen source, this nutrient can be assimilated by the cell in different ways, but mainly through the Ehrlich pathway. In this mechanism, the amino acid is converted into an α-keto acid, an intermediate which is later decarboxylated to a fusel aldehyde (Etschmann et al., 2002). Then, it is reduced to the correspondent fusel alcohol by dehydrogenation, and this latter can be transesterified to the fusel ester. By this mechanism 2-PE, 2-PEA and other fusel products are typically bioproduced.

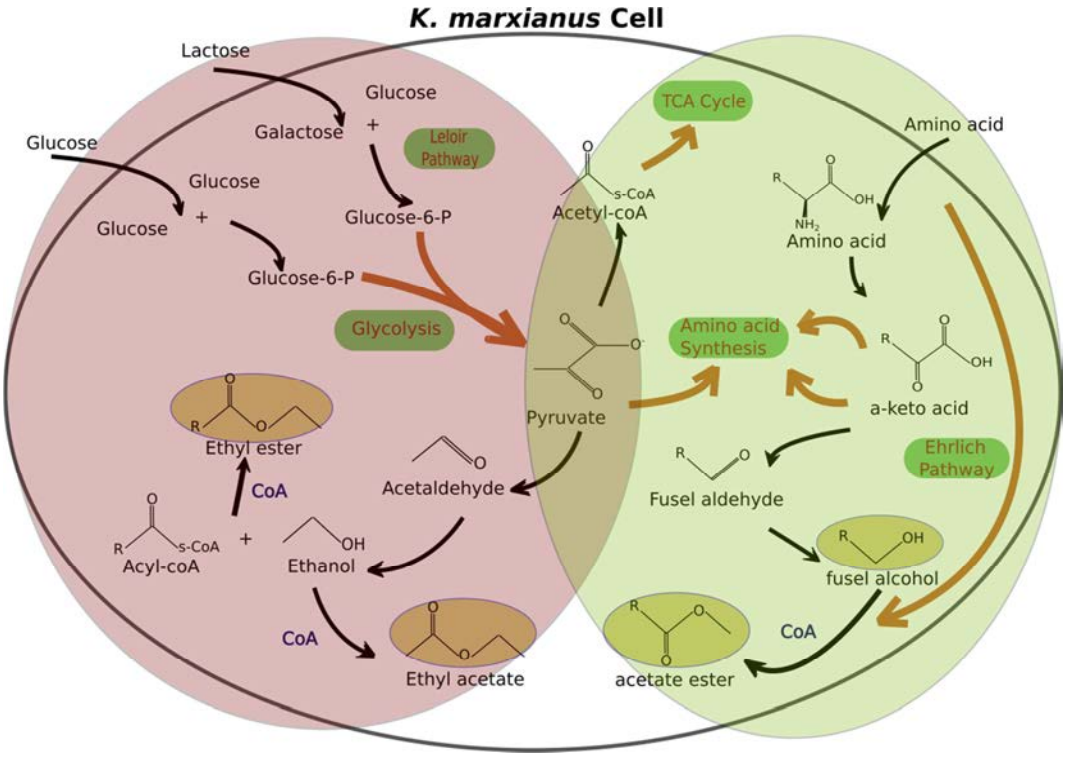


Figure 1.4. Metabolic pathways for synthesis of aroma compounds using *K. marxianus*. Red shading indicates the routes involved in the ester production, while green shading indicates those for producing fusel alcohols. Adapted from Morrisey et al., (2015)

Simultaneously, the formation of these fusel derivatives can also be conducted via *de novo* synthesis. 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).

Another relevant characteristic of *K. marxianus* is its ability to produce different kind of enzymes. Among the reported ones, inulinase and β-galactosidases are the most common (Manera et al., 2008; Mazutti et al., 2010), but it also produces native enzymes such as pectinases (Piemolini-Barreto et al., 2015), polygalacturonases (Serrat et al., 2004), lipases (Deive et al., 2003) and more recently it has been used as host for the production of heterologous proteins (Gombert et al., 2016). This ability results in an asset to *K. marxianus* to deal with a wide variety of substrates, using these enzymes in the production of intermediates compounds of easier assimilation. All the factors previously mentioned characterizing *K. marxianus* suggest that this yeast could be a potential alternative to be used in the production of aroma compounds through the valorization of organic residues, mainly, exploiting the availability of nutrients in different agro-industrial residues produced in diverse applications.

1.3 Solid-state fermentation as a biotechnological approach

As stated in section 1.1.1.1, solid-state fermentation is one of the biotechnological approaches potentially used in the production of aroma compounds. Although most of the investigation in the biotechnological production of aroma compounds have been focused on SmF technologies and the use of enzymes as biocatalysts, SSF present intrinsic characteristics making of this approach an interesting player in the bioproduction of aroma compounds.

1.3.1 General SSF characteristics

SSF is a three-phase system constituted by a gas phase in contact with a solid phase containing a thin layer of moisture. In general, SSF implies the growth of the microorganism on this layer of moisture in the absence or near absence of free water (Chen, 2013; Thomas et al., 2013). The solid phase is generally insoluble, so it acts as a support for the microorganisms, and depending on the origin of the substrate, it can also provide part of the nutrients needed in the fermentation process (Costa et al., 2018). However, the solid-liquid interface has to possess enough moisture to support the microorganisms' growth (Singhania et al., 2009). At the same time, the gas phase (commonly air) is responsible for supplying the oxygen required for running the microbe metabolization of the available nutrients.

It could be stated that SSF tends to reproduce the natural microbiological environment found in other processes such as composting and ensiling. In this sense, SSF provides an environment similar to those where microbes usually are and from where they are isolated. This seems to constitute a decisive factor to achieve higher product yields when compared with SmF carried out at optimal conditions for microorganisms growth (Costa et al., 2018; Thomas et al., 2013).

As seen in Table 1.4, SSF possess some remarkable advantages compared to the conventional SmF processes. In general, the SSF technology is more effective in numerous aspects including lower production costs (when using organic residues as raw materials), lower energy and lower water demand, but also higher fermentation productivity, extended stability of the products and reduced production of liquid wastes (Rodríguez and Sanromán, 2006; Singhania et al., 2009; Soccol et al., 2017).

On the other hand, SSF is at clear disadvantage with the well-known SmF technology, considering the difficulties in the online monitoring of process variables such as pH, moisture and nutrient availability. Furthermore, problems in the scaling-up are frequently related to heat mass transfer limitations inherent to the nature of the used solid substrates (Mitchell et

al., 2006; Pandey, 2003; Soccol et al., 2017). As a consequence, the use of SSF in large-scale applications is still limited due to the challenges and limitations concerning monitorization, control and scaling-up of the process (Salihu et al., 2012; Singhania et al., 2010).

Table 1.4. Comparison of the main characteristics of SSF and SmF. Adapted from Chen, (2013); El-Bakry et al., (2015)

Characteristic	SSF	SmF
Substrates	Commonly low-cost (*e.g.* organic residues)	Commonly synthetic media
Water content	No free water	Liquid media required
Oxygen availability	Directly from the gas phase	Oxygen needed to be dissolved
Process control (T, pH)	Difficult	Easy
Agitation	Difficult	Easy
Nutrient availability	Diffusion (Concentration gradients)	High (No concentration gradients
Products concentration	Typically high	Typically low
Downstream processing	Process dependent	Commonly difficult and expensive
Scale-up	Difficult, *ad-hoc* designs required	Easy, commercial technology available
Investment	Commonly low-cost equipment	Commonly high-cost equipment
Liquid wastes	Reduced quantities	High quantities

Nevertheless, SSF has been proved as a prominent alternative for the processing of several residues to perform biological transformation of those wastes into value-added products of different characteristics. SSF is a versatile process able to produce such variety of products like enzymes (Abraham et al., 2013; Biz et al., 2016; Cerda et al., 2016; Oliveira et al., 2017), biopesticides (Ballardo et al., 2017, 2016), biosurfactants (Jiménez-Peñalver et al., 2016; Kiran et al., 2010), biofuels (Li et al., 2013; Rodríguez et al., 2010), biopolymers (Castilho et al., 2009; Sindhu et al., 2015), and other secondary metabolites including the aroma compounds (Soccol et al., 2017; Thomas et al., 2013). In many of these applications, SSF constitutes the state-of-the-art technology given the advantages that present regards other processes like SmF (Ben Akacha and Gargouri, 2015).

As detailed in Table 1.5, SSF performance is affected by several operating conditions influencing the behavior of the process in different ways. Optimization of these conditions is crucial for the feasibility of the SSF processes (Chen, 2013).

One of the choices of major significance in the development of any SSF is the substrate selection (Pandey, 2003). This choice depends upon a number of aspects like the availability and cost, but it is also relevant the convenience of a given substrate to produce a specific product (Ben Akacha and Gargouri, 2015). Typically, substrates used in SSF technology are a naturally occurring solid substrate such as agricultural crops or agro-industrial residues, but they can also be inert supports (Barrios-González, 2012). In this sense, food and agro-industrial waste become an ideal source of substrates for SSF considering that they are produced all over the world (availability), most of the time those residues are generated in huge amounts, their cost is significantly low, and they are rich in carbohydrates and other nutrients prone to be further exploited (Mussato et al., 2012; Rodríguez and Sanromán, 2006; Yazid et al., 2017).

Table 1.5. Main factors affecting the SSF behavior.

Factor	Main affected condition
Substrate selection	Nutrient availability/ Microorganism growth
Particle size distribution	Porosity/ nutrients diffusion
C: N ratio	Microorganism growth/ Selectivity products
Inoculum size	Microorganism growth
Air supply strategy	O_2 availability/ Heat and products removal*
pH of the media	Microorganism growth/ Nutrient availability
Initial moisture content	Microorganism growth/ Nutrient availability/ Porosity
Temperature	Microorganism growth/ Selectivity products
Agitation	Nutrient availability/ Heat removal
Feeding strategy	Nutrient availability/ Heat removal/ Selectivity products
Downstream process	Products quality

*Air supply strategy could influence the product removal when products are volatiles, and the air is continuously supplied.

In this context, by using low-cost organic residues could provide several advantages. For instance, reduced production costs, enhanced energy efficiency, reutilization/treatment of the waste, and as a global outcome, a more sustainable process. Furthermore, it is evident that there is a growing need for the development of more sustainable waste management alternatives, not just to reduce the environmental impacts of these residues, but also to minimize costs (Maina et al., 2017).

Accordingly, nowadays a beneficial waste management could be considered that one based on the Circular Economy, encouraging the reduction of waste generation, its valorization, and the exploiting of the renewable resources (Allesch and Brunner, 2014).

Perhaps, one of the most used alternatives in this framework is the biorefinery concept, which is defined as the sustainable conversion of biomass into a range of marketable bio-based products (food, feed, biomaterials, chemicals, etc.) and/or bioenergy (fuels, power, heat, etc.) (Jacquet et al., 2015; Nizami et al., 2017). Consequently, in the context of Circular Economy, SSF seems to fulfill with these principles, emerging as a potential strategy for the biorefinery of value-added feedstock and bioproducts, through the valorization of diverse solid organic residues (Abdul Manan and Webb, 2017).

1.3.2 Challenges and perspectives of SSF in the aroma compounds bioproduction

In general, aroma compounds production through SSF has been developed at lab-scale, and it has been limited, in many of the reported studies, to the feasibility assessment of the process. This scenario possesses in evidence a significant gap in the development of the SSF approach towards the implementation as a large-scale application. In this sense, several challenges must be addressed. Some of them include the use of only residues as substrates (minimizing the use of precursors), using not-sterilized substrates and the implementation of operational strategies improving the global performance of the SFF processes.

On the other hand, some conventional constraints inherent to SSF technology have to be also considered. The scale-up effect is a typical disadvantage of SSF process due to the heat and mass transfer phenomena presented in the solid-liquid-gas interphases (Cerda et al., 2017b; Soccol et al., 2017), hampering the development of the technology at industrial scale. Since the behavior of the fermentation changes because of these effects, productivity, selectivity and global efficiency are prone to be affected as well, so the implementation of operating approaches able to minimize these adverse effects (Astolfi et al., 2011) becomes of major importance.

Regarding the first constraints, it is expected that they could be solved through the systematic study of each issue. For instance, the improvement in the substrate selection could be enhanced by finding suitable substitutes for the precursors (*e.g.*, glucose could be substituted by sugar-rich sources like molasses) and/or by pondering the potential of combined substrates constituted by agro-industrial residues with complementary properties (*e.g.*, using a high porosity material like sugarcane bagasse acting as support with apple pomace as a source of sugars).

On the other hand, it is evident that the bioproduction of aroma compounds via SSF needs to be adressed at higher scales. Besides, as described by some authors (de Oliveira

Felipe et al., 2017; Sánchez et al., 2015), by integrating more efficient strategies in SSF, it is also expected to contribute in the improvement of the process sustainability, as well as in the industrial development of the technology in the framework of the Circular Economy. From that point of view, an interesting approach is the analysis of the SSF processes at bench and pilot plant scales without limiting the assessment to the production or productivity indices, but also involving indices related to the efficiency and sustainability of the SSF, such as the consumption of resources (energy, time, etc.) or the environmental effects of the technology (*e.g.*, using a life cycle assessment approach).

As seen in Figure 1.5(a), the investigation in the field of aroma compounds production via SSF has been very limited. However, in the last years, the number of published papers dealing with this technology has increased significantly compared to the last decades. In fact, in this area, 14 papers have been published during the last four years, (four of them within the last year) which equals the scientific production in the area from 2000 to 2013. This trend also shows a change in the type of microorganisms used for producing the aroma compounds. As Figure 1.5(b) details, in general, fungi have been predominant in the reported studies, but considering only the studies performed in the last five years, almost 80% of the studies have focused on the use of yeasts for obtaining a wide variety of compounds (Figure 1.5(c)).

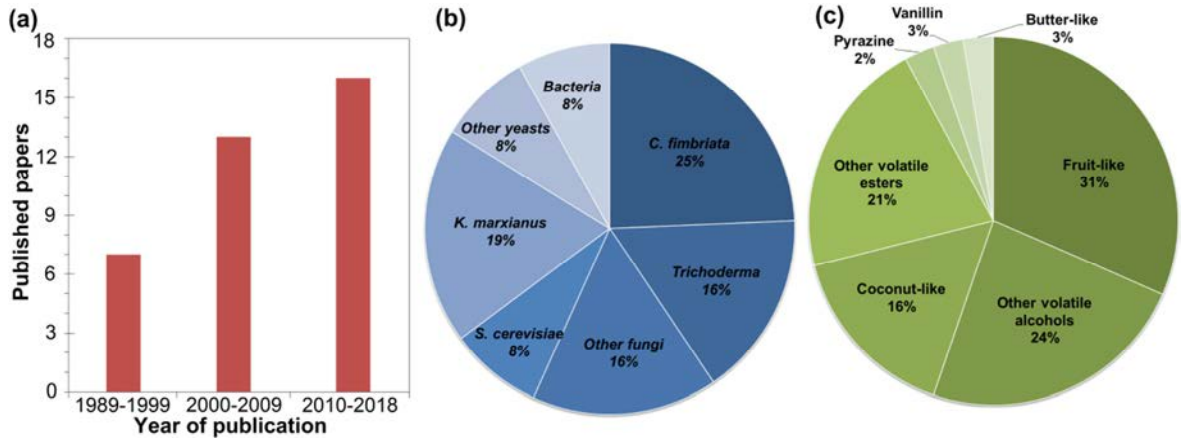


Figure 1.5. Statistics of the research in aroma production via SSF. (a) Published papers; (b) microorganisms used in the processes; (c) Target products of the SSF studies. Data collected until May 2018 using the database of Web of science (http://apps.webofknowledge.com)

Based on the above scenario, the use of SSF as an alternative for producing aroma compounds could be considered in an early phase of development. Nevertheless, the inherent potential of this technology and the boom for implementing more sustainable productive processes could act as driving force. Thus, promoting the required synergies that can reduce

the gap for making of this approach, a feasible alternative for producing value-added aroma compounds.

References

Abdul Manan, M., Webb, C., 2017. Modern microbial solid state fermentation technology for future biorefineries for the production of added-value products. Biofuel Res. J. 4, 730–740.

Abraham, J., Gea, T., Sánchez, A., 2013. Potential of the SSF of soy fibers residues by native microbial populations for bench-scale alkaline protease production. Biochem. Eng. J. 74, 15–19.

Aguedo, M., Ly, M.H., Belo, I., Teixeira, J.A., Belin, J.M., Waché, Y., 2004. The use of enzymes and microorganisms for the production of aroma compounds from lipids. Food Technol. Biotechnol. 42, 327–336.

Allesch, A., Brunner, P.H., 2014. Assessment methods for solid waste management: A literature review. Waste Manag. Res. 32, 461–473.

Alvandi, H., Mahin, A., Alsadati, S., 2008. Diacetyl production in batch fermentation process by lactic starter culture. Iran. J. Food Sci. Technol. 5, 27–39.

Amaral, P.F.., da Rocha Leão, M.H.M., Coelho, M.A.Z., 2010. Bioconversion of flavors, in: Hui, Y.H. (Ed.), Handbook of Fruit and Vegetable Flavors. Wiley & Sons, Inc., New Jersey, pp. 115–128.

Armstrong, D., Yamazaki, H., 1986. Natural flavours production: a biotechnological approach. Trends Biotechnol. 4, 264–268.

Astolfi, V., Joris, J., Verlindo, R., Oliveira, V., Maugeri, F., Mazutti, M., De Oliveira, D., Treichel, H., 2011. Operation of fixed-bed bioreactor in batch and fed-batch modes for production of inulinase by solid-state fermentation. Biochem. Eng. J. 58–59, 39–49.

Ballardo, C., Abraham, J., Barrena, R., Artola, A., Gea, T., Sánchez, A., 2016. Valorization of soy waste through SSF for the production of compost enriched with *Bacillus thuringensis* with biopesticide properties. J. Environ. Manage. 169, 126–131.

Ballardo, C., Barrena, R., Artola, A., Sánchez, A., 2017. A novel strategy for producing compost with enhanced biopesticide properties through solid-state fermentation of biowaste and inoculation with *Bacillus thuringiensis*. Waste Manag. 70, 53–58.

Barrios-González, J., 2012. Solid-state fermentation: Physiology of solid medium, its molecular basis and applications. Process Biochem. 47, 175–185.

Belitz, H.-D., Grosch, W., Schieberle, P., 2009. Aroma Compounds, in: Food Chemistry. Springer Berlin Heidelberg, Berlin, pp. 340–402.

Ben Akacha, N., Gargouri, M., 2015. Microbial and enzymatic technologies used for the production of natural aroma compounds: Synthesis, recovery modelling, and bioprocesses. Food Bioprod. Process. 94, 675–706.

Berger, R.G., 2015. Biotechnology as a source of natural volatile flavours. Curr. Opin. Food Sci. 1, 38–43.

Białecka-Florjańczyk, E., Krzyczkowska, J., Stolarzewicz, I., Kapturowska, A., 2012. Synthesis of 2-phenylethyl acetate in the presence of *Yarrowia lipolytica* KKP 379

biomass. J. Mol. Catal. B Enzym. 74, 241–245.

Biz, A., Finkler, A.T., Oliveira, L., Schweitzer, B., Krieger, N., Mitchell, D.A., 2016. Production of pectinases by solid-state fermentation of a mixture of citrus waste and sugarcane bagasse in a pilot-scale packed-bed bioreactor. Biochem. Eng. J. 111, 54–62.

Braga, A., Belo, I., 2016. Biotechnological production of γ-decalactone, a peach like aroma, by *Yarrowia lipolytica*. World J. Microbiol. Biotechnol. 32, 1–8.

Braga, A., Mesquita, D.P., Amaral, A.L., Ferreira, E.C., Belo, I., 2015. Aroma production by *Yarrowia lipolytica* in airlift and stirred tank bioreactors: Differences in yeast metabolism and morphology. Biochem. Eng. J. 93, 55–62.

Buchhaupt, M., Guder, J.C., Etschmann, M.M.W., Schrader, J., 2012. Synthesis of green note aroma compounds by biotransformation of fatty acids using yeast cells coexpressing lipoxygenase and hydroperoxide lyase. Appl. Microbiol. Biotechnol. 93, 159–168.

Burdock, G.A., 2010. Fenaroli's handbook of flavor ingredients, 6th ed, Taylor & Francis Group. Boca Raton, FL.

Carlquist, M., Gibson, B., Yuceer, Y.K., Paraskevopoulou, A., Sandell, M., Angelov, A.I., Gotcheva, V., Angelov, A.D., Etschmann, M.M.W., 2015. Process engineering for bioflavour production with metabolically active yeast - a mini-review. Yeast 32, 123–143.

Castilho, L., Mitchell, D.A., Freire, D., 2009. Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. Bioresour. Technol. 100, 5996–6009.

Cerda, A., El-Bakry, M., Gea, T., Sánchez, A., 2016. Long term enhanced solid-state fermentation: Inoculation strategies for amylase production from soy and bread wastes by *Thermomyces sp*. in a sequential batch operation. J. Environ. Chem. Eng. 4, 2394–2401.

Cerda, A., Mejías, L., Gea, T., Sánchez, A., 2017. Cellulase and xylanase production at pilot scale by solid-state fermentation from coffee husk using specialized consortia: The consistency of the process and the microbial communities involved. Bioresour. Technol. 243, 1059–1068.

Chen, H., 2013. Modern Solid State Fermentation, First Edition. Ed. Springer, New York.

Chreptowicz, K., Wielechowska, M., Główczyk-Zubek, J., Rybak, E., Mierzejewska, J., 2016. Production of natural 2-phenylethanol: From biotransformation to purified product. Food Bioprod. Process. 100, 275–281.

Costa, J.A.V., Treichel, H., Kumar, V., Pandey, A., 2018. Advances in Solid-State Fermentation, in: Pandey, A., Larroche, C., Soccol, C.R. (Eds.), Current Developments in Biotechnology and Bioengineering: Current Advances in Solid-State Fermentation. Elsevier B.V., Amsterdam, Netherlands, pp. 1–17.

Dastager, S., 2009. Aroma compounds, in: Sinhg nee' Nigam, P., Pandey, A. (Eds.), Biotechnology for Agro-Industrial Residues Utilization. Springer Books, Netherlands, pp. 105–127.

de Oliveira Felipe, L., de Oliveira, A.M., Lemos, J., 2017. Bioaromas- Perspectives for sustainable development. Trends Food Sci. Technol. 62, 141–153.

Deive, F.J., Costas, M., Longo, M.A., 2003. Production of a thermostable extracellular lipase by *Kluyveromyces marxianus*. Biotechnol. Lett. 25, 1403–1406.

Diban Gómez, N., 2008. Separación de aromas en etapas del procesado de zumos de frutas y bebidas. PhD thesis. Universidad de Cantabria.

Dubal, S.A., Tilkari, Y.P., Momin, S.A., Borkar, I. V, 2008. Biotechnological routes in flavour industries. Adv. Biotech 14, 20–31.

El-Bakry, E., Abraham, J., Cerda, A., Barrena, R., Ponsá, S., Gea, T., Sánchez, A., 2015. From Wastes to High Value Added Products: Novel Aspects of SSF in the Production of Enzymes. Crit. Rev. Environ. Sci. technology 42, 1999–2042.

Etschmann, M.M.W., Bluemke, W., Sell, D., Schrader, J., 2002. Biotechnological production of 2-phenylethanol. Appl. Microbiol. Biotechnol. 59, 1–8.

Fadel, H.H.M., Mahmoud, M.G., Asker, M.M.S., Lotfy, S.N., 2015. Characterization and evaluation of coconut aroma produced by *Trichoderma viride* EMCC-107 in solid state fermentation on sugarcane bagasse. Electron. J. Biotechnol. 18, 5–9.

Fonseca, G.G., Heinzle, E., Wittmann, C., Gombert, A.K., 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl. Microbiol. Biotechnol. 79, 339–354.

Gatfield, I.L., 1988. Production of Flavor and Aroma Compounds by Biotechnology. Food Technol. 42, 110–122.

Gombert, A.K., Madeira, J.V., Cerdán, M.-E., González-Siso, M.-I., 2016. *Kluyveromyces marxianus* as a host for heterologous protein synthesis. Appl. Microbiol. Biotechnol. 100, 6193–6208.

Hua, D., Ma, C., Song, L., Lin, S., Zhang, Z., Deng, Z., Xu, P., 2007. Enhanced vanillin production from ferulic acid using adsorbent resin. Appl. Microbiol. Biotechnol. 74, 783–790.

Hua, D., Xu, P., 2011. Recent advances in biotechnological production of 2-phenylethanol. Biotechnol. Adv. 29, 654–660.

Jacquet, N., Haubruge, E., Richel, A., 2015. Production of biofuels and biomolecules in the framework of circular economy: A regional case study. Waste Manag. Res. 33, 1121–1126.

Jiménez-Peñalver, P., Gea, T., Sánchez, A., Font, X., 2016. Production of sophorolipids from winterization oil cake by Solid-state fermentation: Optimization, monitoring and effect of mixing. Biochem. Eng. J. 115, 93–100.

Kim, H., Park, C., 2017. Enzymatic synthesis of phenethyl ester from phenethyl alcohol with acyl donors. Enzyme Microb. Technol. 100, 37–44.

Kiran, G.S., Thomas, T.A., Selvin, J., 2010. Production of a new glycolipid biosurfactant from marine *Nocardiopsis lucentensis* MSA04 in solid-state cultivation. Colloids Surfaces B Biointerfaces 78, 8–16.

Krings, U., Berger, R.G., 1998. Biotechnological production of flavours and fragrances. Appl. Microbiol. Biotechnol. 49, 1–8.

Kuo, C.H., Liu, T.A., Chen, J.H., Chang, C.M.J., Shieh, C.J., 2014. Response surface methodology and artificial neural network optimized synthesis of enzymatic 2-phenylethyl acetate in a solvent-free system. Biocatal. Agric. Biotechnol. 3, 1–6.

Lane, M.M., Morrissey, J.P., 2010. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. Fungal Biol. Rev. 24, 17–26.

Laufenberg, G., Kunz, B., Nystroem, M., 2003. Transformation of vegetable waste into value added products : (A) the upgrading concept; (B) practical implementations. Bioresour.

Technol. 87, 167–198.

Leffingwell & Associates, 2015. Flavor & Fragrance Top Companies. Canton, USA.

Li, S., Li, G., Zhang, L., Zhou, Z., Han, B., Hou, W., Wang, J., Li, T., 2013. A demonstration study of ethanol production from sweet sorghum stems with advanced solid state fermentation technology. Appl. Energy 102, 260–265.

Longo, M.A., Sanroman, M.A., 2006. Production of food aroma compounds: Microbial and enzymatic methodologies. Food Technol. Biotechnol. 44, 335–353.

Longo, M.A., Sanroman, M.A., 2010. Vegetable flavors from cell culture, in: Hui, Y.H. (Ed.), Handbook of Fruit and Vegetable Flavors. Wiley & Sons, Inc., New Jersey, pp. 663–680.

Löser, C., Urit, T., Bley, T., 2014. Perspectives for the biotechnological production of ethyl acetate by yeasts. Appl. Microbiol. Biotechnol.

Maina, S., Kachrimanidou, V., Koutinas, A., 2017. A roadmap towards a circular and sustainable bioeconomy through waste valorization. Curr. Opin. Green Sustain. Chem. 8, 18–23.

Manera, A.P., Da Costa Ores, J., Ribeiro, V.A., André, C., Burkert, V., Kalil, S.J., 2008. Optimization of the culture medium for the production of β-galactosidase from *Kluyveromyces marxianus* CCT 7082. Food Technol. Biotechnol. 46, 66–72.

Mazutti, M.A., Zabot, G., Boni, G., Skovronski, A., de Oliveira, D., Di Luccio, M., Rodrigues, M.I., Treichel, H., Maugeri, F., 2010. Kinetics of inulinase production by solid-state fermentation in a packed-bed bioreactor. Food Chem. 120, 163–173.

McGinty, D., Vitale, D., Letizia, C.S., Api, A.M., 2012. Fragrance material review on phenethyl acetate. Food Chem. Toxicol. 50, S491–S497.

Medeiros, A., Pandey, A., Freitas, R., Christen, P., Soccol, C.R., 2000. Optimization of the production of aroma compounds by *Kluyveromyces marxianus* in solid-state fermentation using factorial design and response surface methodology. Biochem. Eng. J. 6, 33–39.

Medeiros, A.B.P., Pandey, A., Christen, P., Fontoura, P.S.G., de Freitas, R.J.S., Soccol, C.R., 2001. Aroma compounds produced by *Kluyveromyces marxianus* in solid state fermentation on a packed bed column bioreactor. World J. Microbiol. Biotechnol. 17, 767–771.

Medeiros, A.B.P., Pandey, A., Vandenberghe, L.P.S., Pastorel, G.M., Soccol, C.R., 2006. Production and recovery of aroma compounds produced by solid-state fermentation using different adsorbents. Food Technol. Biotechnol. 44, 47–52.

Medeiros, A.B.P., Rossi, S.C., Soccol, C.R., 2010. Cell culture for flavour production, in: Hui, Y.H. (Ed.), Handbook of Fruit and Vegetable Flavors. Wiley & Sons, Inc., New Jersey, pp. 95–100.

Mei, J., Min, H., Lü, Z., 2009. Enhanced biotransformation of L-Phenylalanine to 2-Phenylethanol using an in situ product adsorption technique. Process Biochem. 44, 886–890.

Mitchell, D.A., Berovič, M., Krieger, N., 2006. Solid-State Fermentation Bioreactor Fundamentals: Introduction and Overview, in: Mitchell, D.A., Berovič, M., Krieger, N. (Eds.), Solid-State Fermentation Bioreactors: Fundamentals of Design and Operation. Springer Berlin Heidelberg, New Jersey, pp. 1–12.

Morrisey, J.P., Etschmann, M.M.W., Schrader, J., Billerbeck, G.M., 2015. Cell factory

applications of the yeast *Kluyveromyces marxianus* for the biotechnological production of natural flavour and fragrance molecules. Yeast 32, 3–16.

Mushtaq, M., 2018. Extraction of fruit juice: An overview, in: Rajauria, G., Tiwari, B.K. (Eds.), Fruit Juices: Extraction, Composition, Quality and Analysis. Academic Press, Massachusetts, USA, pp. 131–159.

Mussato, S.I., Ballesteros, L.F., Martins, S., Teixeira, J.A., 2012. Use of Agro-Industrial Wastes in Solid-State Fermentation Processes, in: Show, K.W., Guo, X. (Eds.), Industrial Waste. InTech, pp. 121–141.

Nizami, A.-S., Rehan, M., Waqas, M., Naqvi, M., Ouda, O.K.M., Shahzad, K., Miandad, R., Khan, M.Z., Syamsiro, M., Ismail, I.M.I., Pant, D., 2017. Waste biorefineries: Enabling circular economies in developing countries. Bioresour. Technol. 241, 1101–1117.

Okrasa, K., Guibé-Jampel, E., Plenkiewicz, J., Therisod, M., 2004. In vitro bi-enzymatic synthesis of benzaldehyde from phenylalanine: Practical and mechanistic studies. J. Mol. Catal. B Enzym. 31, 97–101.

Oliveira, F., Salgado, J.M., Abrunhosa, L., Pérez-Rodríguez, N., Domínguez, J.M., Venâncio, A., Belo, I., 2017. Optimization of lipase production by solid-state fermentation of olive pomace: from flask to laboratory-scale packed-bed bioreactor. Bioprocess Biosyst. Eng. 40, 1123–1132.

Pandey, A., 2003. Solid - state fermentation. Biochem. Eng. J. 13, 81–84.

Pentjuss, A., Stalidzans, E., Liepins, J., Kokina, A., Martynova, J., Zikmanis, P., Mozga, I., Scherbaka, R., Hartman, H., Poolman, M.G., Fell, D.A., Vigants, A., 2017. Model-based biotechnological potential analysis of *Kluyveromyces marxianus* central metabolism. J. Ind. Microbiol. Biotechnol. 44, 1177–1190.

Piemolini-Barreto, L.T., Antônio, R.V., Echeverrigaray, S., 2015. Comparison of a pectinolytic extract of *Kluyveromyces marxianus* and a commercial enzyme preparation in the production of Ives (*Vitis labrusca*) grape juice. World J. Microbiol. Biotechnol. 31, 755–762.

Rodríguez, L.A., Toro, M.E., Vazquez, F., Correa-Daneri, M.L., Gouiric, S.C., Vallejo, M.D., 2010. Bioethanol production from grape and sugar beet pomaces by solid-state fermentation. Int. J. Hydrogen Energy 35, 5914–5917.

Rodríguez, S., Sanromán, M.Á., 2006. Application of solid-state fermentation to food industry-A review. J. Food Eng. 76, 291–302.

Salihu, A., Alam, M.Z., AbdulKarim, M.I., Salleh, H.M., 2012. Lipase production: An insight in the utilization of renewable agricultural residues. Resour. Conserv. Recycl. 58, 36–44.

Sánchez, A., Artola, A., Gea, T., Barrena, R., Font, X., 2015. A new paradigm for waste management of organic materials. Waste Manag. 42, 1–2.

Sarma, S.J., Dhillon, G.S., Hedge, K., Brar, S.K., Verma, M., 2014. Utilization of Agro-industrial wastes for the production of aroma compounds and fragrances, in: Brar, S.K., Dhillon, G.S., Fernandes, M. (Eds.), Biotransformation of Waste Biomass into High Value Biochemicals. Springer Books, New York, pp. 99–115.

Scognamiglio, J., Jones, L., Letizia, C.S., Api, A.M., 2012. Fragrance material review on phenylethyl alcohol. Food Chem. Toxicol. 50, S224–S239.

Sendovski, M., Nir, N., Fishman, A., 2010. Bioproduction of 2-Phenylethanol in a Biphasic Ionic liquid Aqueous system. J. Agric. Food Chem. 58, 2260–2265.

Serrat, M., Bermúdez, R., Villa, T., 2004. Polygalacturonase and ethanol production in *Kluyveromyces marxianus*. Appl. Biochem. Biotechnol. 117, 49–64.

Sindhu, R., Pandey, A., Binod, P., 2015. Solid-state Fermentation for the Production of Poly(hydroxyalkanoates). Chem. Biochem. Eng. Q. 29, 173–181.

Sindhwani, G., Uk, I., Aeri, V., 2012. Microbial transformation of eugenol to vanillin. J. Microbiol. Biotechnol. Res. 2, 313–318.

Singhania, R.R., Patel, A.K., Soccol, C.R., Pandey, A., 2009. Recent advances in solid-state fermentation. Biochem. Eng. J. 44, 13–18.

Singhania, R.R., Sukumaran, R.K., Patel, A.K., Larroche, C., Pandey, A., 2010. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microb. Technol. 46, 541–549.

Soccol, C.R., Costa, E.S.F. da, Letti, L.A.J., Karp, S.G., Woiciechowski, A.L., Vandenberghe, L.P. de S., 2017. Recent developments and innovations in solid state fermentation. Biotechnol. Res. Innov. 1, 52–71.

Stratistics consulting, 2016. Aroma chemicals, global market outlook (2017-2023). Gaithersburg, USA.

The Freedonia group, 2016. World Flavors & Fragrances: 2017-2020, Industry study with forecast for 2017-2020. Cleveland.

Thomas, L., Larroche, C., Pandey, A., 2013. Current developments in solid-state fermentation. Biochem. Eng. J. 81, 146–161.

Torres, S., Pandey, A., Castro, G.R., 2010. Banana flavor: Insights into isoamyl acetate production, in: Cohen, A. (Ed.), Bananas: Nutrition, Diseases and Trade Issues. Nova Publishers, New York, pp. 227–245.

Vandamme, E., Wim, S., 2002. Bioflavours and fragrances via fermentation and biocatalysis. J. Chem. Technol. Biotechnol. 77, 1323–1332.

Wittmann, C., Hans, M., Bluemke, W., 2002. Metabolic physiology of aroma-producing *Kluyveromyces marxianus*. Yeast 19, 1351–1363.

Yazid, N.A., Barrena, R., Komilis, D., Sánchez, A., 2017. Solid-state fermentation as a novel paradigm for organic waste valorization: A review. Sustainability. 9, 1–28.

Ziegler, H., 2007. Flavourings: Production, composition, Applications, Regulations, Second edition. Ed, Flavourings. Wiley-VCH, Weinheim.

Chapter 2

Research Objectives

The main objective of this research was to study the bioproduction of volatile value-added compounds via solid-state fermentation using the yeast *Kluyveromyces marxianus,* as an alternative to the valorization of agro-industrial residues. As target compounds, fruit-like aroma and rose-like aroma were selected to be scaled from 0.5L to 22L reactors.

To achieve this main goal, the following specific objectives have been developed:

- To identify the potential of several agro-industrial residues for producing aroma compounds using *K. marxianus* in solid-state fermentation.
- Once identified the residue to be valorized, specific objectives are divided into two main blocks:

  Fruit-like compounds production:
  - To assess the feasibility of producing fruit-like compounds in a lab-scale batch system using the selected residue.
  - To study the effects of the main operational variables in the production of fruit-like compounds at lab scale and sterile conditions.
  - To assess the impact of some operational strategies in the production and selectivity of fruit-like compounds at different scales with the non-sterile residue.

  Rose-like compounds production:
  - To evaluate at lab-scale the feasibility of producing 2-phenethyl alcohol and 2-phenethyl acetate via solid-state fermentation in a residue-based system using *K. marxianus*.
  - To develop alternative operational strategies to enhance the production and efficiency of the solid-state fermentation for producing 2-phenethyl alcohol and 2-phenethyl acetate.
  - To evaluate the scaling effect on the selected strategies for producing 2-phenethyl alcohol and 2-phenethyl acetate.

Chapter 3

Materials and methods

3.1 Materials

3.1.1 Microorganism

Kluyveromyces marxianus (ATCC 10022) was obtained from Colección Española de Cultivos Tipo (CECT, Valencia, Spain). When arrived, 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 following the procedure described in Figure 3.1.

3.1.2 Inoculum

Preparation of the inoculum consisted of adding one pearl into a 250 mL Erlenmeyer flask filled with 100 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. Figure 3.2 contains the *K. marxianus* growth kinetics in the aforementioned media, at the established conditions.

Every time the inoculum was prepared, its concentration was measured by serial dilutions in NaCl (9 g L^{-1}) which were plated on Petri dishes (in triplicate) containing the same media mentioned before. Cultures were incubated at 30°C for 20 h, and the *K. marxianus* population was determined by counting the units on each replicate. Results were expressed as the main value, in Colony forming units of *K. marxianus* (CFU) per mL of solution.

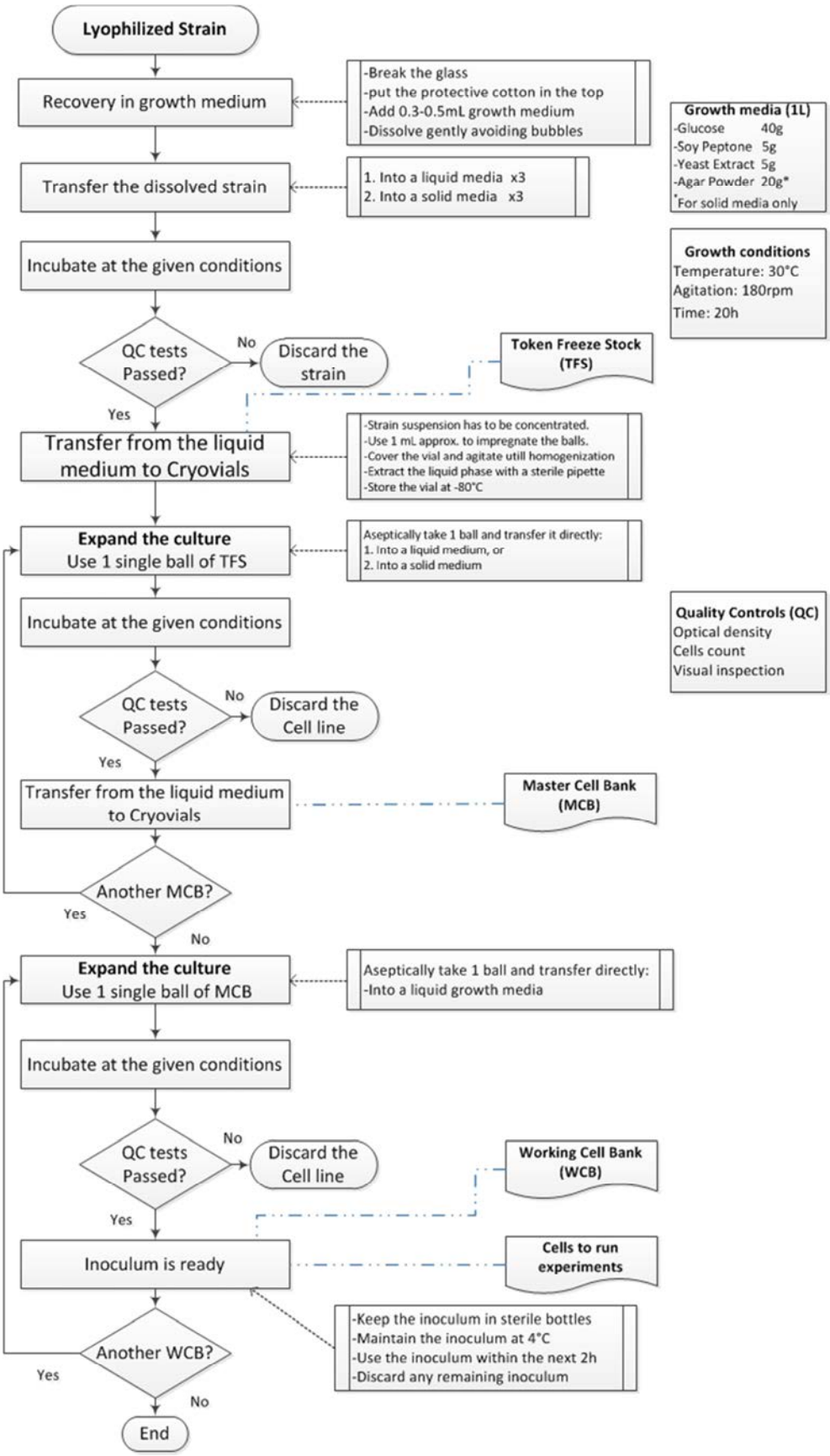


Figure 3.1. *K. marxianus* handling and storage procedures.

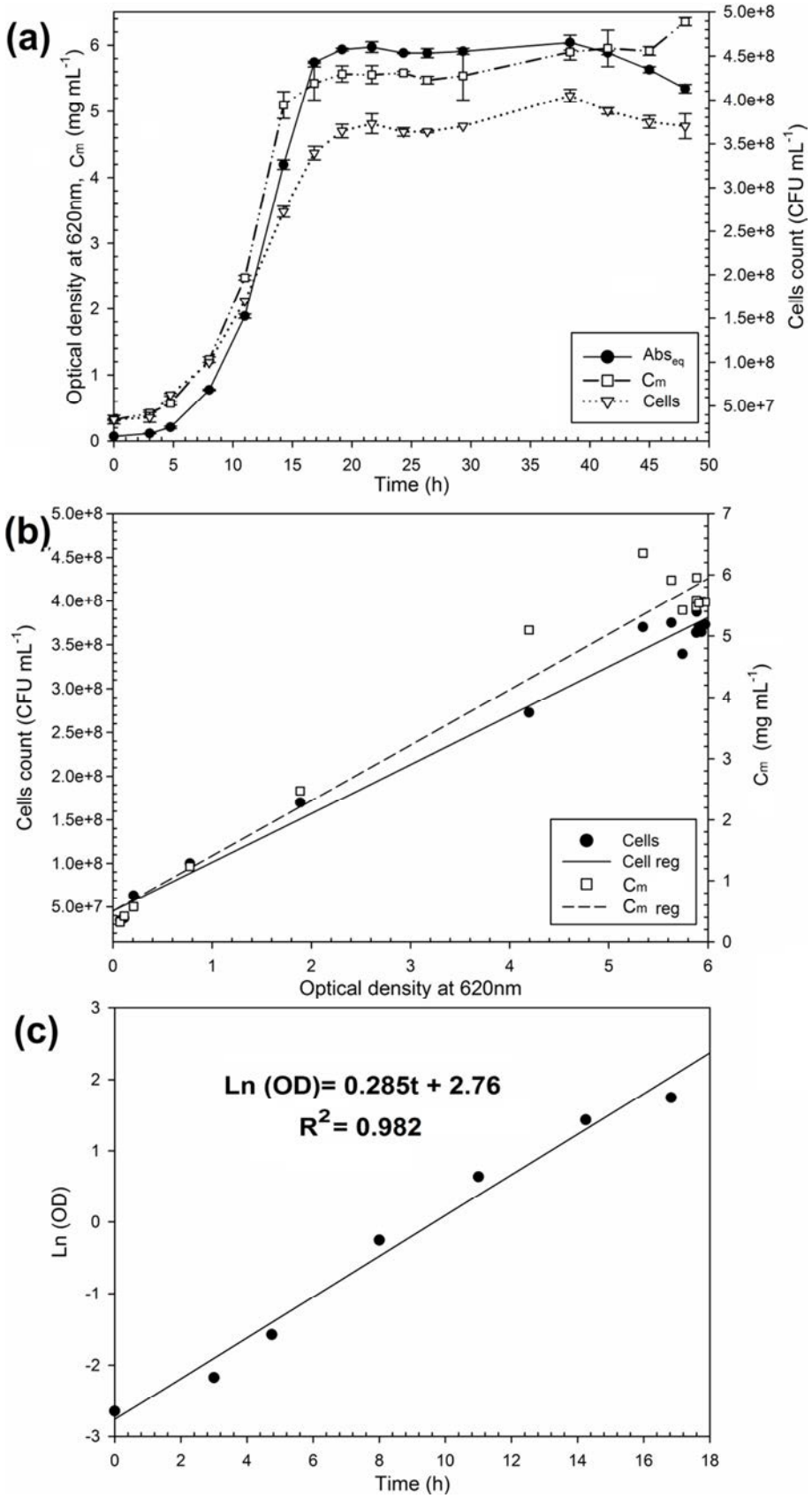


Figure 3.2. Growth kinetics for *K. marxianus* in liquid growth media. (a) Growth curve, (b) Optical density equivalences, (c) Specific growth rate. T: 30°C, 180 rpm. C_m: mass concentration; reg: linear regression; CFU: Colony forming units of *K. marxianus*.

3.2 Substrates

Sugarcane bagasse (SCB) (Figure 3.3(a)) was supplied by Ingenio Ntra. Sra. del Carmen (Málaga, Spain). First, the improper fraction was removed (to withdraw mainly stones and remains) before a drying process at 60°C in an air oven during 24 h. Then, SCB was ground to achieve a particle size distribution in the range 0.5-32 mm (Figure 3.4) by means of a granulator mill. The dried and grounded SCB was stored at -20°C until it was used. On the other hand, sugar beet molasses (SBM) (Figure 3.3(b)) were provided by the sugar company AB Azucarera Iberia S.A. (Madrid, Spain) and they were maintained in hermetic containers at 4°C until they were used.

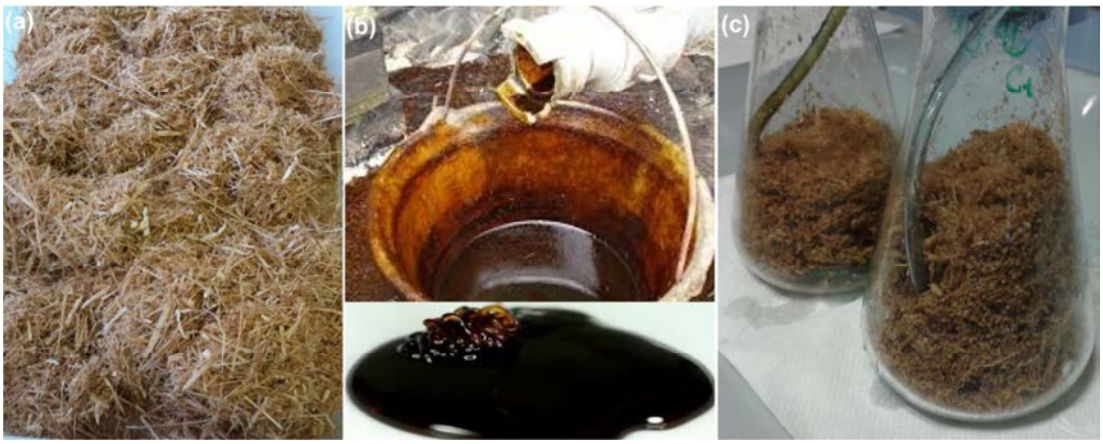


Figure 3.3. (a) Sugarcane bagasse appearance, (b) sugar beet molasses appearance, (c) residue mixture after fermentation.

For the production of fruit-like compounds, preparation of the substrate consisted of adjusting the pH, moisture content, and SBM content. This process was performed using a 1:1 (v:v) mixture of a phosphate buffer pH 7 (0.1 M) 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} $MnSO_4.4H_20$, 3.0 g L^{-1} $MgSO_4.7H_2O$ and 1.9 g L^{-1} $(NH_4)_2SO_4$. The SBM content was added to the liquid mixture above, and once they were dissolved, the mixture was incorporated into the dried SCB.

For the production of rose-like compounds, preparation additionally consisted of adjusting the precursor load. Similarly, this process was performed using the same phosphate buffer pH 7 solution mixed with the nutrient solution containing the same components, except the $(NH_4)_2SO_4$. In this case, both, L-phenylalanine (the precursor) and SBM were added and dissolved in this mixture until the final amount corresponded to the experiment requirements.

In both cases, once substrate was impregnated, it was either, autoclaved at 121°C for 20 min and inoculated using the prepared inoculum of section 3.1.2, or directly inoculated without sterilization, depending on the experiment requirements.

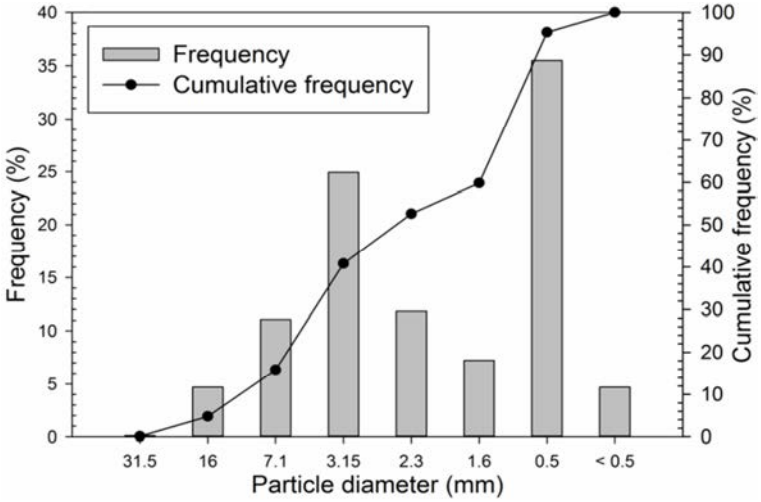


Figure 3.4. Particle size distribution of the used sugarcane bagasse.

In the preliminary experiments focused on the substrate screening, some additional substrates have been used. These residues were orange peels (OP) (Figure 3.5), apple pomace (AP) (Figure 3.6), coffee husk (CH) (Figure 3.7) and spent coffee grounds (SCG) (Figure 3.8). These substrates were obtained from local industries in Barcelona. Particularly, coffee husk as the main waste generated by the coffee production was kindly supplied by Marcilla®, Mollet del Vallès, Barcelona, Spain. Apple pomace was supplied by Sidrería MOOMA, Mas SAULOT, Girona, Spain, while orange peels and spent coffee capsules were supplied by a local restaurant and the SCG were collected manually. In all the cases, substrates were maintained at -20°C until they were used. Preparation of these substrates included the addition of wood chips as bulking agent (to increase the substrate porosity) in a ratio 1:1 (v:v).

For these experiments (substrate screening), the used substrates were conditioned regarding moisture content by adding the above nutrients solution according to the specific requirement of each material. pH was adjusted with NaOH or HCl 0.1M depending on the substrate needs. Once substrates were conditioned, they were autoclaved at 121°C for 20 min, and after cooling at room temperature, they were inoculated using the prepared inoculum of section 3.1.2.

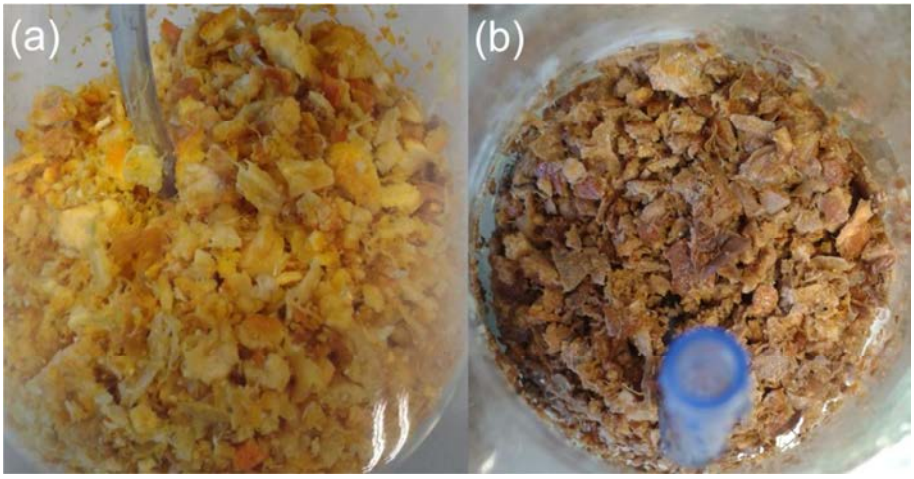


Figure 3.5. Orange peels. (a) residue before fermentation; (b) residue after fermentation.

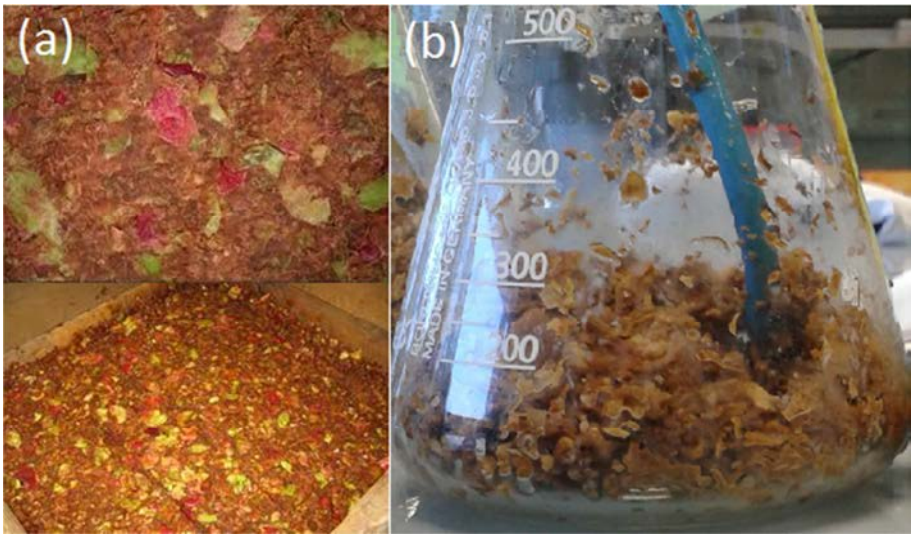


Figure 3.6. Apple pomace. (a) residue before fermentation; (b) residue after fermentation.

Figure 3.7. Coffee husk. (a) residue before fermentation; (b) residue after fermentation.

Figure 3.8. Spent coffee grounds. (a) residue before fermentation; (b) residue after fermentation.

3.3 Solid-state fermentation systems

3.3.1 Reaction system 1 (0.5 L bioreactors)

Reaction system 1 has been designed and built according to Barrena, (2006). Briefly, the system consisted of 0.5 L reactors (Erlenmeyer flasks) submerged in a temperature-controlled water bath. Each reactor was connected to a mass flow controller (Bronkhorst Hitec) that supplied continuously humidified air to each flask across the lid to the bottom such that the air is forced to flow through the substrate until it reaches the top of the reactor as detailed by Pognani, (2011) (Figure 3.9). Gas streams were independently led to an oxygen sensor (αLphase Ltd.) in series with an IR CO_2 sensor (Sensotran IR), both connected to an on-line self-made data acquisition system (Arduino®-based), that recorded O_2 and CO_2 concentrations.

This system allowed working with a maximum load of 96g of the prepared substrate. However, when intermittent mixing and fed-batch approaches were tested, the reactor content was manually mixed (with the fresh material in the fed-batch case) into a 1 L glass beaker using a spatula and then, quantitatively loaded back into the reactor. When the experiments required sterile conditions, all materials were autoclaved at 121°C for 20 min, and the procedure was performed in a laminar flow chamber.

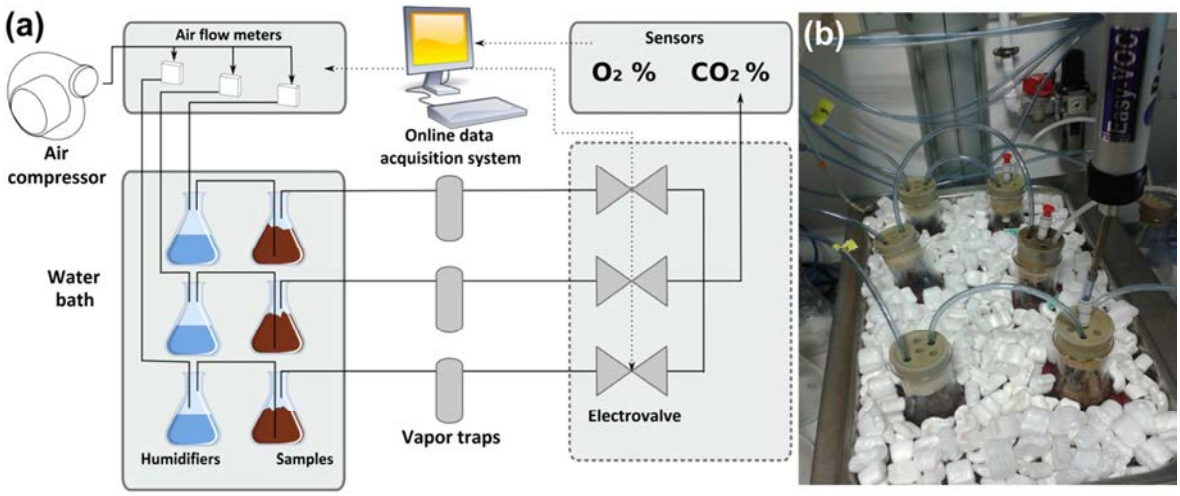


Figure 3.9. 0.5 L reaction system. (a) Experimental set-up. Source: Pognani, (2011), (b) bioreactors appearance.

3.3.2 Reaction system 2 (1.6 L bioreactors)

Reaction system 2 consisted of polyvinyl chloride (PVC) cylindrical bioreactors of 1.6 L working volume (200 mm height, 101mm internal diameter). Reactors were provided with an internal net in the bottom, so the solid media was held on it. As Figure 3.10 shows, air enters in the bottom by means of a couple of rapid connectors, and it goes through the solid bed until it reaches the top. The system was adapted to monitor the temperature of the solid media in the midpoint of the bed (Pt-100 sensors, Sensotrans), and the exhausted gases were conducted to an oxygen sensor (αLphase Ltd.) connected to a similar data acquisition system (Arduino®-based) as detailed in section 3.3.1.

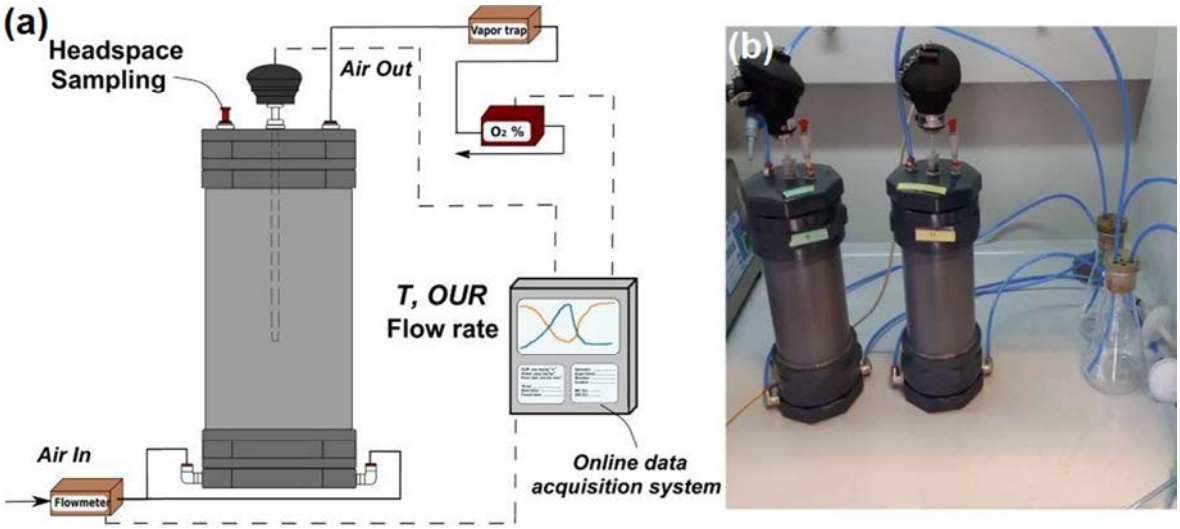


Figure 3.10. 1.6 L reaction system. (a) Experimental set-up, (b) bioreactors appearance.

This system was loaded with a maximum of 300g of the prepared substrate. When fed-batch approaches were tested, the reactor was emptied, and the content was manually mixed with the fresh material in 5 L plastic trays and then, quantitatively loaded back into the reactor. For the sequential-batch tests, replacement of the reactor content was performed by putting the content into 5 L plastic trays, and then, manually mixing the predefined fractions of fermented material and fresh substrate in a second 5 L plastic tray. Finally, the mixed substrate was loaded back into the reactor.

3.3.3 Reaction system 3 (4.5 L bioreactors)

This system consisted of 4.5 L air-tight packed-bed reactors (adapted Dewar® vessels) as described by Abraham et al. (2013). These static reactors are thermally isolated, so they provide near-adiabatic conditions with negligible heat exchange with the surroundings. Thus, the process can proceed with insignificant heat losses. As detailed in Figure 3.11, the temperature of the solid media (measured at the midpoint of the bed) (Pt-100 sensors, Sensotrans) and the exhausted gases oxygen concentration (αLphase Ltd.) were monitored online using a similar data acquisition system (Arduino®-based), as detailed in section 3.3.1.

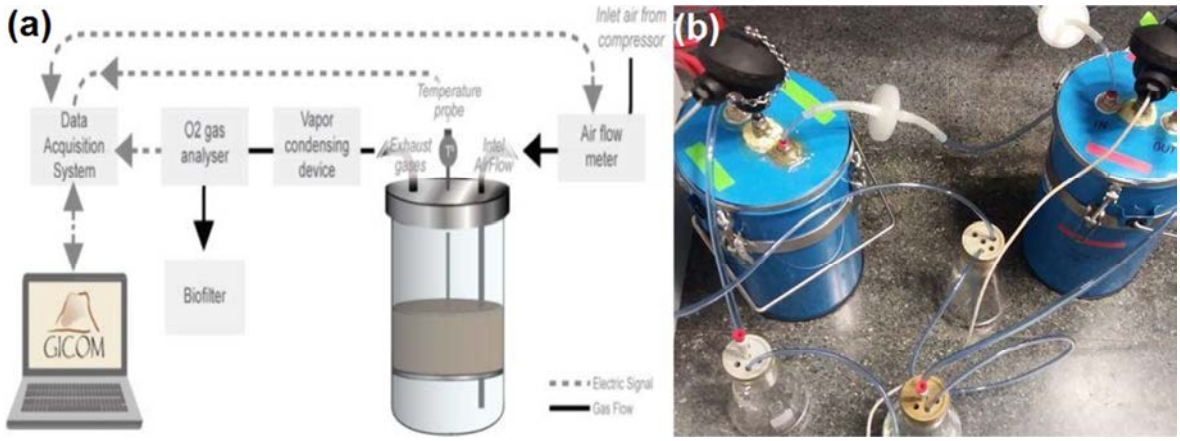


Figure 3.11. 4.5 L reaction system. (a) Experimental set-up. Source: Cerda, (2017), (b) appearance of the bioreactors.

This system has allowed working with a maximum of 860g of the prepared substrate. For intermittent mixing and fed-batch tests, the reactor was emptied into a 5 L plastic tray, and the content was manually mixed (with the fresh material in the fed-batch case) and then, quantitatively loaded back into the reactor.

3.3.4 Reaction system 4 (22 L bioreactor)

Reaction system 4 consisted of a cylindrical stainless steel reactor with a total volume of 22 L. Reactor was provided with an automatic helical ribbon mixer and a removable inner basket where the substrate is placed (Figure 3.12). The system was filled with a maximum of 2.5 kg of the prepared substrate (90% capacity), and it was monitored similarly than reactors described in section 3.3.2 and 3.3.3. In this case, the temperature probe for monitoring the solid media was located adjacent to the helical ribbon mixer, in the space among the mixer and basket wall (Figure 3.12).

Intermittent mixing was automatically set using 14 rpm during 5 min at predefined intervals. For the fed-batch tests, the fresh material was added directly into the reactor basket, and the content was mixed at 14 rpm for 5 min. For the sequential-batch tests, replacement of the reactor content was performed by taking out the removable basket and leaving all the content into a 25 L tray. Then, after repositioning the removable basket into the reactor, the predefined fractions of fermented material and fresh substrate were loaded back into the reactor´s basket. Once the reactor was loaded, the content was mixed at 14 rpm for 5 min.

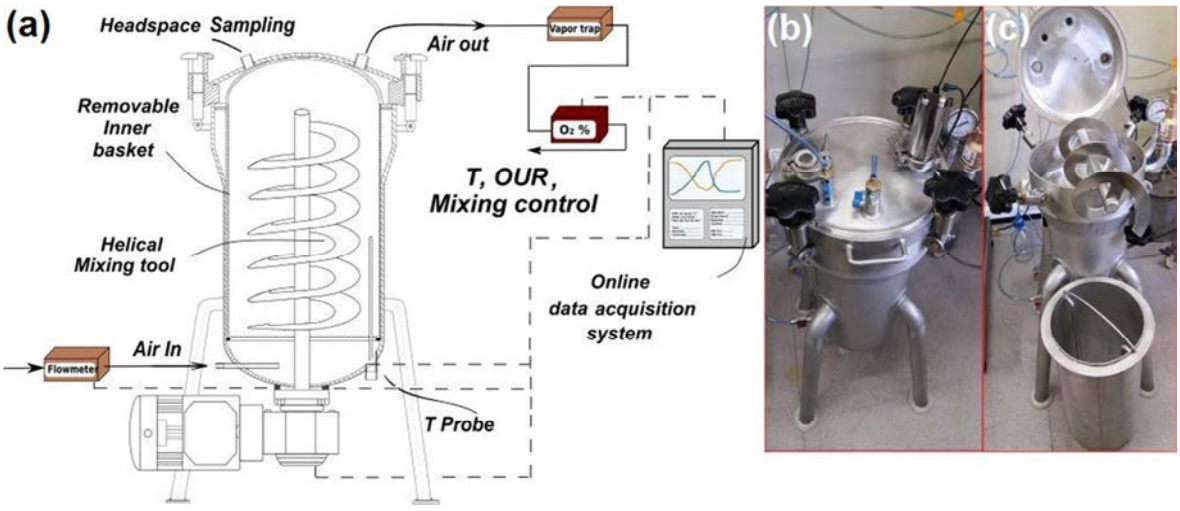


Figure 3.12. 22 L reaction system. (a) Experimental set-up, (b) and (c) bioreactor appearance.

3.4 Analytical methods

3.4.1 Respiration indices

The oxygen uptake rate (OUR), carbon dioxide production rate (CO_2^p) and the cumulative oxygen consumption (COC) have been used as an indirect measure of the

biological activity. These indices can be computed from the oxygen and CO_2 concentrations and the air flow rate based on a steady state balance (Ponsá, 2010). Thus, OUR as a function of time and CO_2^p were calculated as:

$$OUR = F\left(O_{2,In} - O_{2,Out}\right)\frac{PMW_{O_2}}{RTm_{sub}TS_{sub}VS_{sub}} \quad (1)$$

$$CO_2^p = F * CO_{2,Out}\frac{PMW_{CO_2}}{RTm_{sub}TS_{sub}VS_{sub}} \quad (2)$$

Where

OUR: Specific oxygen uptake rate (g_{O2} kg^{-1}_{VS} h^{-1})

CO_2^p: Carbon dioxide production rate (g_{CO2} kg^{-1}_{VS} h^{-1})

F: Air flow rate supplied to the system (L h^{-1})

$O_{2,In}$, $O_{2,Out}$: Oxygen concentration in the air flow at the inlet and outlet of the system (molar fraction)

$CO_{2,Out}$: Carbon dioxide concentration in the air flow at the outlet of the system (molar fraction)

P: Pressure of the system (Assumed constant at 101.3 kPa)

MW_{O2}: Oxygen molecular weight (31.9 g_{O2} mol_{O2}^{-1})

MW_{CO2}: Carbon dioxide molecular weight (44 g_{CO2} mol_{CO2}^{-1})

R: Ideal gas constant (8.31 kPa L K^{-1} mol^{-1})

T: Temperature at which F is measured (K)

m_{sub}: Loaded mass of prepared material (g_{sub})

TS_{sub}: Total solid content of the prepared material (g_{TS} g^{-1}_{sub})

VS_{sub}: Volatile solid content of the dried material (g_{VS} g^{-1}_{TS})

Additionally, the COC can be determined as the area below OUR (computed through the numerical integration in time), and it is expressed as g_{O2} kg^{-1}_{VS}.

3.4.2 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). For this purpose, a set of metallic sampling tubes containing 380 mg of Texan TA 35/60, Carbograph 1TD 40/60 and Carboxen 1003 40/60 (Markes Int.) were used to capture the volatile compounds

after grabbing a 100 mL of headspace (outlet of the reaction system) with an easy-VOC system (Markes Int.) (Figure 3.13).

Samples were stored capped at ambient temperature for a maximum of 12 h until their analysis. TD-GC-MS was performed in an Agilent 7820A coupled to 5975MSD and TD Unity2 (Markes Int.). Thermal desorption of sampling tubes was carried out in a two-step mode. First, volatile compounds were desorbed at 295°C during 6 min and a flow rate of 80 mL min^{-1} of He to be driven to a graphitized carbon trap at 2°C. Afterwards, the trap was desorbed in a second step at 310°C during 3 min using fast heating (100°C s^{-1}). For the fruit-like compounds determination, the capillary transfer line to the GC was kept at 170°C, and for the rose-like compounds, it was kept at 210°C. Specific split flows were set to adjust the resolution of the chromatogram and to avoid saturation of the column. All sampling tubes were conditioned at 330°C for 35 min after each measurement to avoid interferences.

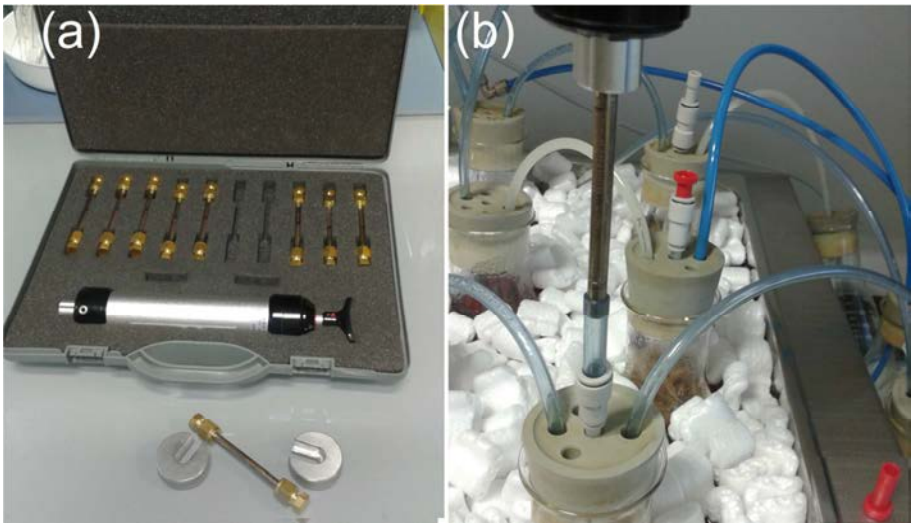


Figure 3.13. Sampling system used to capture the volatile compounds in the headspace. (a) Sampling tubes, (b) headspace sampling in reaction system 1.

Separation of the volatile compounds corresponding to the fruit-like compounds (Table 3.1) was performed in a capillary column DB-624ms 60mx0.25mmx1.4µm. Injection port was kept at 220°C while oven temperature was held at 40°C for 5 min, then raised till 90°C by 5°C min^{-1}, followed by an increase until 200°C by 20°C min^{-1} and concluding with a rise till 240°C at 35°C min^{-1}.

Separation of 2-phenethyl alcohol and 2-phenethyl acetate was performed in a capillary column SPB-5ms 30mx0.25mmx0.25µm. Injection port was kept at 220°C while oven temperature was held at 40°C for 7 min, then raised till 245°C by 26°C min^{-1} and hold for 1 min.

In both cases, electron impact mass spectra were recorded at 70eV ionization energy in the 15–300 m/z mass range. Ion trap and GC/MSD interface temperatures were 150 and 230°C, respectively. The identification was carried out by comparing the retention times and mass spectra of the evaluated compounds to those of the selected analytical standards by mass spectra obtained from the wiley275 library. Quantification of the individual components was performed using calibration curves (external standard) using analytical standards (Sigma-Aldrich) and the calibration solution loading rig (CSLR) of the Unity system.

Total volatile productivity for the fruit-like compounds (P_{vol}^{t}) and ester species productivity (P_{Est}^{t}) at time t, were computed using equation 3 and 4 respectively:

$$P_{vol}^{t}\left[\frac{mg_{Vol}}{h\ g_{TS}}\right] = \sum_{i=1}^{n} S_{AFR}\ C_i \quad (3)$$

$$P_{Est}^{t}\left[\frac{mg_{Est}}{h\ g_{TS}}\right] = \sum_{j=1}^{m} S_{AFR}\ C_j^{Est} \quad (4)$$

Where

S_{AFR}: Specific air flow rate supplied to the reactor at time t (L h^{-1} g^{-1}_{TS})

C_i,C_j^{Est}: Headspace concentrations of the compound *i* and ester species *j* ($mg_{i,j}$ L^{-1})

n: Total amount of quantified volatile compounds (Table 3.1)

m: Total ester species quantified (Table 3.1)

Similarly, 2-phenethyl alcohol ($P_{2\text{-}PE}^{t}$) and 2-phenetyl acetate ($P_{2\text{-}PEA}^{t}$) productivity in the gas phase at time t were computed as:

$$P_{2-PE}^{t}\left[\frac{mg_{2-PE}}{h\ g_{TS}}\right] = S_{AFR} C_{2-PE} \quad (5)$$

$$P_{2-PEA}^{t}\left[\frac{mg_{2-PEA}}{h\ g_{TS}}\right] = S_{AFR} C_{2-PEA} \quad (6)$$

Where

$C_{2\text{-}PE}$, $C_{2\text{-}PEA}$: Headspace concentrations of 2-phenethyl alcohol and 2-phenetyl acetate, respectively at time t (mg L^{-1})

On the other hand, the total cumulative production in the gas phase of the different group of species was calculated through the numerical integration in time of the correspondent productivity as detailed in equation 7.

$$P_i^{Acc}\left[\frac{\mathrm{mg}_i}{\mathrm{g_{TS}}}\right] = \int_0^{t_f} P_i^t \,\mathrm{dt} \approx \frac{1}{2}\sum_{\mathrm{t=0}}^{t_f}(P_i^t + P_{i+1}^t)\Delta\mathrm{t} \qquad (7)$$

Where

P_i^{Acc}: Total cumulative production of the compounds group *i* (*i.e.* total volatile, total ester, total 2-phenethyl alcohol or total 2-phenetyl acetate)

t_f: Total fermentation time (h)

P_i^t: Productivity of the compounds group *i* at time *t*, computed with equations (3) to (6)

Table 3.1. Volatile compounds identified and quantified during the solid-state fermentation of sugarcane bagasse-sugar/beet molasses with *K. marxianus* for fruit-like production.

Compound	Odor	Compound	Odor
Aldehydes		Esters	
Acetaldehyde	Ripe fruits	Methyl Acetate	Fruity
Crotonaldehyde*	Pungent	Ethyl Acetate	Sweet
Alcohols		Ethyl propionate	Pineapple
Ethanol	-	Propyl Acetate	Pears
2-Propanol	-	Ethyl Isobutyrate	Sweet-Pineapple
Isobutyl Alcohol	Sweet-Musty	Isobutyl Acetate	Raspberry-Pears
Isoamyl Alcohol	Pungent	Isopropyl Acetate	Fruits-Sweet
2-Methyl-1Butanol	Black Truffle	Butyl Acetate	Banana-Apple
2-Phenethyl alcohol*	Rose	Isoamyl Acetate	Banana
Ketones		Ethyl Butanoate*	Pineapple
Acetone*	Sweet	Isoamyl Butyrate*	Pears-Banana
Diacetyl*	Butter	Amyl formate*	Fruity-Banana
2-Butanone*	Ethereal	2-Phenethyl acetate*	Flower-Rose

*Compounds identified but not quantified

3.4.3 Semivolatile compounds in the solid phase

L-phenylalanine (L-Phe), 2-phenethyl alcohol (2-PE) and 2-phenetyl acetate (2-PEA) content in the solid media 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) following an adapted method from (Wang et al., 2011a; Yin et al., 2015). A gradient method with a 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 set at 216 nm.

L-Phe was quantified from two 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-Phe (Sigma-Aldrich). Concentrations in the liquid extract were calculated from external standard calibration curves, analyzed under identical conditions. L-Phe, 2-PE and 2-PEA concentration in the solid substrate were computed as:

$$C_{sub}^{i}\left[\frac{mg_i}{g_{TS}}\right] = C_L^i V_{ext}\frac{1}{W_{sam}TS_{sam}} \quad (8)$$

Where

C_{sub}^{i}: Concentration of the species *i* (*i.e.* L-Phe, 2-PE or 2-PEA) referred to the solid fermented substrate

C_L^i: Concentration of the species *i* in the liquid extract ($mg_i\ L^{-1}$)

V_{ext}: Final volume of the liquid extract (recovered after the extraction) (L)

W_{sam}: Weight of the fermented substrate aliquot used in the extraction (g_{sample})

TS_{sam}: Total solids content of the fermented substrate used in the extraction ($g_{TS}\ g^{-1}_{substrate}$)

3.4.4 *K. marxianus* cells population

K. marxianus population in the solid substrate was measured using a modified method from Ballardo et al. (2016) adding 10 g of sample into a bottle containing 100 mL of a NaCl 9 g L^{-1} solution. The mixture was shaken for 20 min at 200 rpm in an orbital shaker at 25°C. The supernatant was used to prepare dilutions in NaCl 9 g L^{-1} which were plated on Petri dishes (four replicate) 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 in Colony forming units (CFU) of *K. marxianus* per gram of total solids (g_{TS}).

3.4.5 Microbial identification

Identification of yeast was performed from the cultures obtained, following the procedure explained in section 3.4.4, isolating the objective colonies in Petri dishes. This analysis was carried out in the Instrumental techniques laboratory (Nucleic acids analysis area) of Universidad de Leon. Briefly, the PCR (quantitative polymerase chain reaction) was performed using a thermal cycler (GeneAmp PCR 2700 (Applied Biosystems)). PCR products were purified using a NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) kits, and they were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Identification of the yeast was performed by comparison of the sequences with the data bank GenBank NCBI (National Center for Biotechnology Information, using the software BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

3.4.6 Sugar content

3.4.6.1 Reducing sugars

Reducing sugars content of the fermented substrate were quantified using the DNS method (Miller, 1959). The analysis was performed using the supernatant obtained 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, when needed, it was properly diluted before the measurement. Briefly, the method consisted of adding 3 mL of DNS reagent to 1 mL of filtered supernatant in a 25 mL glass test tube.

Then, the mixture was placed in a boiling water bath for 5 min. After cooling, 20 mL of distilled water were added, and the sample was shaken. For the blank, one mL of distilled water, instead of the sample was used. The absorbance of the prepared sample was measured at 540 nm in a spectrophotometer (Cary 50 Bio, UV-Visible Spectrophotometer). Analyses were performed in triplicate. A calibration curve was prepared using glucose with concentrations ranging from 0 to 2 g L^{-1}. The reducing sugar content was expressed as grams of glucose-equivalent ($g_{R.S.}$) per gram of total solids (g_{TS}).

3.4.6.2 Total Sugars

The total sugar content of the fermented substrate was estimated using the anthrone method (Scott and Melvin, 1953). The analysis was performed using the same extract obtained in section 3.4.6.1. Anthrone reagent was prepared by dissolving 200 mg anthrone into 100 mL of 95% H_2SO_4 previously cooled. This reagent was prepared each time the measurement was performed. Briefly, one mL of diluted and filtered supernatant was placed in a 25 mL glass test tube, and 4 mL of anthrone reagent were added. Then, the mixture was placed in a boiling water bath for 8 min and rapidly cooled by using an ice bath. The absorbance of the diluted aliquot was measured at 630 nm in a spectrophotometer (Cary 50 Bio, UV-Visible Spectrophotometer). For the blank, one mL of distilled water was used instead of 1 mL of diluted and filtered supernatant. Analyses have been performed in triplicate. A calibration curve was prepared using glucose with concentrations ranging from 0 to 0.1 g L^{-1}. Total sugars content ($g_{Tot.\ S.}$) was expressed as gram of glucose-equivalent per gram of total solids (g_{TS}).

3.4.7 Water holding capacity

Water holding capacity (WHC) is defined as the ability of a solid material to retain water. It was determined following the procedure exposed by Jiménez-Peñalver, (2017) starting with the dried material (65°C for 24 h). First, approximately 5 g of the dried material were placed in a cylindrical tube containing a net at the bottom (to retain all the solid material), and it was saturated by adding distilled water. The material was allowed to drain for 30 min. The saturated material was weighted to determine the amount of retained water (procedure was performed in duplicate). WHC was computed as:

$$WHC\ \left[\frac{g_{H_2O}}{g_{d,material}}\right] = \frac{W_f - W_{d,mat}}{W_{d,mat}} \qquad (9)$$

Where:

W_f: Weight of the saturated material (g_{sub})

$W_{d,mat}$: Weight of the dried material ($g_{d,mat}$)

3.4.8 Air filled porosity

Air filled porosity (AFP), also known as free air space (FAS) refers to the ratio of gas-filled pores volume of the sample (V_g) to total volume of sample (V_s) (Haug, 1993). In

this study, it has been estimated by using the empirical correlation shown in equation (10) (Agnew and Leonard, 2002) associated with straw, a material of similar physical characteristics than the sugarcane bagasse.

$$AFP\ [\%] = -0.0891 BD_W + 93.53 \qquad (10)$$

Where,

BD_w: Wet bulk density (kg L^{-1})

Similarly, BD_W is defined as the weight per unit of volume of wet sample. It has been computed using the equation (11).

$$BD_w\ \left[\frac{kg}{L}\right] = \frac{W_w}{V_w} \qquad (11)$$

Where,

W_w: Weight of a given sample of wet material (kg)

V_w: Occupied volume by the given weight of the wet sample (L)

3.4.9 Standard methods

Routine methods were determined according to standard procedures included in the "Test Methods for the Examination of Composting and Compost" (The US Department of Agriculture and The US Composting Council, 2001). Results have been expressed as the mean value of duplicate (triplicate in the case of elemental analysis).

3.4.9.1 pH

pH was determined by mixing a ratio of 1:5 w/v of sample into distilled water. The sample was shaken at room temperature during 30 min to allow the salts to solubilize into the liquid phase. Then, the supernatant was used to measure the pH in an electronic pH-meter (Crison®, micro CM2100).

3.4.9.2 Moisture content

Moisture content (MC) and total solids (TS) determination were performed by gravimetric analysis. First, a given sample of the fermented substrate (among 5-10 g) was

placed in a previously weighted dried crucible, and the set was left in an air oven at 105ºC for at least 24 h to dry the sample. After cooling, the set was weighted, and MC and TS were computed as:

$$MC\ \left[\%\ \frac{g_{H2O}}{g_{substrate}}\right] = 100\frac{W_i - W_f}{W_i - W_o} \quad (12)$$

$$TS\ \left[\%\frac{g_{TS}}{g_{substrate}}\right] = 100 - MC \quad (13)$$

Where

W_o: Weight of the empty crucible (g)

W_i: Initial weight of sample and crucible (g)

W_f: Final weight of the dried sample and crucible (g)

3.4.9.3 Organic matter

Determination of volatile solids (VS) (equivalent to the organic matter content) was performed by gravimetric analysis. Here the dried sample obtained in section 3.4.9.2 was ignited at 550°C for 3 h in excess of air. After cooling, remaining ashes were weighted, and VS were computed as:

$$VS\ \left[\%\ \frac{g_{VS}}{g_{TS}}\right] = 100\frac{W_f - W_{ash}}{W_f - W_o} \quad (14)$$

Where

W_o: Weight of the empty crucible (g)

W_f: Final weight of the dried sample and crucible (g)

W_{ash}: Final weight of the ignited sample and crucible (g)

3.4.9.4 Elemental analysis

C, N and H elemental determination was performed through the servei d'Anàlisi Química-UAB. Briefly, the analysis was carried out using a CHNS elemental analyzer Flash 2000 (Thermo Scientific). Samples were combusted at 1200°C with air excess and quantification was performed by using gas chromatography.

References

Abraham, J., Gea, T., Sánchez, A., 2013. Potential of the SSF of soy fibers residues by native microbial populations for bench-scale alkaline protease production. Biochem. Eng. J. 74, 15–19.

Agnew, J.M., Leonard, J.J., 2002. Using a modified pycnometer to determine free air space and bulk density of compost mixture while simulating compressive loading, in: Michel, F.C., Rynk, R.., Hoiting, H.A.J. (Eds.), International Symposium Composting and Compost Utilization. Columbus, OH, USA, pp. 183–204.

Ballardo, C., Abraham, J., Barrena, R., Artola, A., Gea, T., Sánchez, A., 2016. Valorization of soy waste through SSF for the production of compost enriched with *Bacillus thuringensis* with biopesticide properties. J. Environ. Manage. 169, 126–131.

Barrena, R., 2006. Compostaje de residuos sólidos orgánicos. Aplicación de técnicas respirométricas en el seguimiento del proceso. PhD thesis. Universitat Autonòma de Barcelona.

Cerda, A., 2017. Sustainable carbohydrase production using organic wastes through solid-state fermentation:Operational strategies and microbial communities assessment. PhD thesis. Universitat Autònoma de Barcelona.

Haug, R.T., 1993. The Practical Handbook of Compost Engineering, First edition. Ed. CRC Press, Boca Raton, FL, USA.

Jiménez-Peñalver, P., 2017. Sophorolipids production by solid-state fermentation: from lab-scale to pilot plant. PhD thesis. Universitat Autònoma de Barcelona.

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 31, 426–428.

Pognani, M., 2011. Organic matter evolution in biological solid-waste treatment plants. Raw waste and final product characterization. PhD thesis. Universitat Autonòma de Barcelona.

Ponsá, S., 2010. Different indices to express biodegradability in organic solid wastes. Application to full scale waste treatment plants. PhD thesis. Universitat Autonòma de Barcelona.

Scott, T.A., Melvin, E.H., 1953. Determination of Dextran with Anthrone. Anal. Chem. 25, 1656–1661.

The US Department of Agriculture, The US Composting Council, 2001. Test Methods for the Examination of Composting and Compost, First edition. Ed. Edaphos international, Houston, Texas.

Wang, H., Dong, Q., Guan, A., Meng, C., Shi, X., Guo, Y., 2011. Synergistic inhibition effect of 2-phenylethanol and ethanol on bioproduction of natural 2-phenylethanol by *Saccharomyces cerevisiae* and process enhancement. J. Biosci. Bioeng. 112, 26–31.

Yin, S., Zhou, H., Xiao, X., Lang, T., Liang, J., Wang, C., 2015. Improving 2-Phenylethanol production via Ehrlich pathway using genetic engineered *Saccharomyces cerevisiae* strains. Curr. Microbiol. 70, 762–767.

Section 1. Fruit-like aroma compounds

This section includes Chapters 4, 5 and 6. Through these chapters, developments focused on the bioproduction of fruit-like compounds via solid-state fermentation are presented.

Introduction

As detailed in Chapter 1, fruit-like compounds are one of the important groups of aroma additives used in many applications. While the biotechnological production of these species has been typically focused on the use of SmF, the role played by SSF has been secondary. However, the intrinsic characteristics of SSF processes possess in evidence the opportunity to take advantage of these to valorize organic residues through the bioproduction of value-added products such as aroma compounds.

In this context, one of the firsts reports regarding the use of SSF as a biotechnological approach for producing aroma compounds was presented by Yamauchi et al. (1989) using polished rice as substrate. However, it was until the late 90s when the integration of agro-industrial residues with SSF was considered as an alternative for producing volatile compounds. At that moment, studies were focused on the evaluation of several agro-industrial residues such as sugarcane bagasse, cassava bagasse, apple pomace, soybeans or coffee husk as main substrates for running the SSF.

While most of these studies have focused on the production of fruit-like compounds (Bramorski et al., 1998b; Christen et al., 1997; Medeiros et al., 2000; Soares et al., 2000b), only few of them were devoted to obtain other volatiles products like pyrazine (Besson et al., 1997) or coconut-like aroma (De Aráujo et al., 2002). In that stage, reported studies had dealt mainly with the evaluation of diffusive static systems (*i.e.* without forced air supply) analyzing the effects of a series of variables affecting the production of the aroma compounds. Thus, in some cases the evaluation of additional carbon and nitrogen sources was predominant (Bramorski et al., 1998b; Christen et al., 1997; Soares et al., 2000a), while some others have addressed the process through the optimization of the typical operational variables involved in the process (e.*g.*, temperature, pH, C:N ratio, etc.) (Besson et al., 1997; Medeiros et al., 2000).

Continuously air supplied systems for fruit-like aroma production was first used by Medeiros et al. (2001) and (2006). They found that in a packed-bed lab-scale bioreactor (20g capacity), *C. fimbriata* was able to transform coffee husk enriched with glucose into a series of alcohols, aldehydes and esters with pleasant fruity odor, obtaining a maximum productivity of 0.251 µmol L^{-1} g^{-1} h^{-1}. Also, by changing the configuration to a horizontal drum bioreactor (1.5 kg capacity), the maximum productivity was increased until 1.52 µmol L^{-1} g^{-1} h^{-1} after 72 h. The improvement in the interaction between strain and nutrients,

provided by an intermittent mixing was considered the main factor affecting the product yield.

Although continuously air supplied SSF systems present significant advantages compared to those diffusive-based (Medeiros et al., 2006), the study of aroma compounds production has been typically limited to these latter systems, simulating at lab-scale, the industrial operation of other SSF technologies like the tray bioreactors. A classic example of this trend is the bioproduction of fruit-like compounds. Aside from the above studies, reported systems are composed by diffusive-static bioreactors. However, as mentioned before, this technology has evolved regarding the agro-industrial residues used as substrate using various organic fractions such as cassava bagasse, sugarcane bagasse, coffee husk or orange peels, and also regarding the microorganisms used as inoculum source.

While the most frequently used strain has been the fungi *C. fimbriata*, some authors have employed yeasts due to their capability to metabolize different carbon sources (Carlquist et al., 2015). Hence, authors like Aggelopoulos et al. (2014) have used a mixture of *S. cerevisiae* and *K .marxianus* to obtain a wide range of volatile esters, alcohols and organic acids starting with a mixture of agro-industrial residues in a biorefinery concept. Also, Rodríguez et al., (2015) have used a mixture of indigenous cider yeasts (*Saccharomyces cerevisiae*, *Hanseniaspora valbyensis* and *Hanseniaspora uvarum*) in the SSF of apple pomace, compiling a detailed list of volatile compounds produced during the different phases of 21 days fermentation. Besides, in the study developed by Mantzouridou et al. (2015), it can be seen how *S. cerevisiae* strain uses the available nutrients of orange peels for producing fruity aromas in a biorefinery concept. More recently, Try et al. (2018) have shown the ability of *Y. lipolytica* for producing though SSF γ-decalactones similar to those found in fruits and fermented foods.

Some other authors (da Penha et al., 2012; De Souza et al., 2008; Fadel et al., 2015; Ladeira et al., 2010) have focused on the production of 6-pentyl-α-pyrone (6-PP) through SSF due to the coconut-like odor this compound provides. In these studies, *Trichoderma* species have been used in the biotransformation of simple sugars (mainly glucose) to obtain the coconut-like aroma. For this purpose, substrates such as sugarcane bagasse or green coir powder have been used as support of the microorganisms. While De Souza et al. (2008) obtained up to 5 mg 6-PP per gram of dry matter (g_{DM}), Fadel et al. (2015) reached up to 3.62 mg 6-PP g^{-1}_{DM} without needing the addition of precursors in the solid substrate.

Finally, dos Santos Barbosa et al. (2008) have proposed using green coconut husk as support for *Phanerochaete chrysosporium* in the biotransformation of sugars (mainly glucose) into vanillin, obtaining a maximum of 44.4 $\mu g_{vanillin}$ per gram of dry support.

References

Aggelopoulos, T., Katsieris, K., Bekatorou, A., Pandey, A., Banat, I.M., Koutinas, A.A., 2014. Solid state fermentation of food waste mixtures for single cell protein, aroma volatiles and fat production. Food Chem. 145, 710–716.

Besson, I., Creuly, C., Gros, J.B., Larroche, C., 1997. Pyrazine production by *Bacillus subtilis* in solid-state fermentation on soybeans. Appl. Microbiol. Biotechnol. 47, 489–495.

Bramorski, A., Soccol, C.R., Christen, P., Revah, S., 1998. Fruity Aroma production by *Ceratocystis Fimbriata* in solid cultures from agro-industrial wastes. Rev. Microbiol. 29, 3–11.

Carlquist, M., Gibson, B., Yuceer, Y.K., Paraskevopoulou, A., Sandell, M., Angelov, A.I., Gotcheva, V., Angelov, A.D., Etschmann, M.M.W., 2015. Process engineering for bioflavour production with metabolically active yeast - a mini-review. Yeast 32, 123–143.

Christen, P., Meza, J.C., Revah, S., 1997. Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*: influence of the substrate type and the presence of precursors. Mycol. Res. 101, 911–919.

da Penha, M.P., da Rocha Leão, M.H.M., Leite, S.G.F., 2012. Sugarcane bagasse as support for the production of coconut aroma by solid state fermentation (SSF). BioResources 7, 2366–2375.

De Aráujo, Á., Pastore, G.M., Berger, R.G., 2002. Production of coconut aroma by fungi cultivation in solid-state fermentation. Appl. Biochem. Biotechnol. 98–100, 747–751.

de Souza, A., Fiaux, S.B., Ferreira, S., 2008. Production of 6-pentyl-alpha-pyrone by *Trichoderma harzianum* in solid-state fermentation. Brazilian J. Microbiol. 39, 712–717.

dos Santos, E., Perrone, D., do Amaral, A.L., Ferreira, S.G., 2008. Vanillin production by *Phanerochaete chrysosporium* grown on green coconut agroindustrial husk in solid state fermentation. BioResources 3, 1042–1050.

Fadel, H.H.M., Mahmoud, M.G., Asker, M.M.S., Lotfy, S.N., 2015. Characterization and evaluation of coconut aroma produced by *Trichoderma viride* EMCC-107 in solid state fermentation on sugarcane bagasse. Electron. J. Biotechnol. 18, 5–9.

Ladeira, N., Peixoto, V.J., Penha, M.P., Barros, E.B., Gomes Ferreira, S., 2010. Optimization of 6-pentyl-a-pyrone production by solid state fermentation using sugarcane bagasse as residue. BioResources 5, 2297–2306.

Mantzouridou, F.T., Paraskevopoulou, A., Lalou, S., 2015. Yeast flavour production by solid state fermentation of orange peel waste. Biochem. Eng. J. 101, 1–8.

Medeiros, A., Pandey, A., Freitas, R., Christen, P., Soccol, C.R., 2000. Optimization of the production of aroma compounds by *Kluyveromyces marxianus* in solid-state fermentation using factorial design and response surface methodology. Biochem. Eng. J. 6, 33–39.

Medeiros, A.B.P., Pandey, A., Christen, P., Fontoura, P.S.G., de Freitas, R.J.S., Soccol, C.R., 2001. Aroma compounds produced by *Kluyveromyces marxianus* in solid state fermentation on a packed bed column bioreactor. World J. Microbiol. Biotechnol. 17, 767–771.

Medeiros, A.B.P., Pandey, A., Vandenberghe, L.P.S., Pastorel, G.M., Soccol, C.R., 2006. Production and recovery of aroma compounds produced by solid-state fermentation using different adsorbents. Food Technol. Biotechnol. 44, 47–52.

Rodríguez, R., Pando, R., Suárez, B., 2015. Production and characterization of aroma compounds from apple pomace by solid-state fermentation with selected yeasts. LWT - Food Sci. Technol. 64, 1342–1353.

Soares, M., Christen, P., Pandey, A., Raimbault, M., Soccol, C.R., 2000a. A novel approach for the production of natural aroma compounds using agro-industrial residue. Bioprocess Eng. 23, 695–699.

Soares, M., Christen, P., Pandey, A., Soccol, C.R., 2000b. Fruity flavour production by *Ceratocystis fimbriata* grown on coffee husk in solid-state fermentation. Process Biochem. 35, 857–861.

Try, S., De-Coninck, J., Voilley, A., Chunhieng, T., Waché, Y., 2018. Solid state fermentation for the production of γ-decalactones by *Yarrowia lipolytica*. Process Biochem. 64, 9–15.

Yamauchi, H., Akita, O., Obata, T., Amachi, T., Hara, S., Yoshizawa, K., 1989. Production and Application of a Fruity Odor in a Solid-State Culture of *Neurospora sp*. Using Pregelatinized Polished Rice. Agric. Biol. Chem. 53, 2881–2886.

Chapter 4

Substrate screening and preliminary experiments

4.1 Overview

In this chapter, the selection of a solid organic residue as raw material for the bioproduction of aroma compounds via SSF has been detailed. Selection of this residue has focused on the use of agro-industrial residues, and it has been performed simultaneously with the assessment of three of the operational variables affecting the SSF process. Once the substrate was chosen, a preliminary screening of some additional operational variables was performed to obtain a starting point for subsequent experimental designs.

4.2 Results

4.2.1 Substrate screening

Preliminary experiments consisted of the evaluation of some solid organic substrates as a potential carbon source for the growing of *K. marxianus*. Since the substrate characteristics directly affect the behavior of the strain in the aroma production process via SSF (Christen et al., 1997), it is essential to identify the effects of growing the strain using different substrates. In this case, the strain activity (measured as the oxygen uptake rate (OUR) and the cumulative oxygen consumption (COC) served as a first indirect index of the aroma compounds production, having in mind these compounds are obtained as secondary metabolites in the growing stage of the microorganisms (Medeiros et al., 2003). Some authors have possessed in evidence the relationship among microbial activity and products for several SSF applications (Carvalho et al., 2006; Jiménez-Peñalver et al., 2016; Robinson et al., 2001), indicating it would be a useful and easy way to evaluate the potential of the substrates.

Substrate screening was performed using the five agro-industrial residues shown in Table 4.1, and it was undertaken in 0.5 L bioreactors at sterile conditions. Selection of these substrates was based on their potential to produce aroma volatiles via SSF as previously indicated in the literature (Bramorski et al., 1998b; Christen et al., 1997; Medeiros et al., 2003; Rossi et al., 2009; Soares et al., 2000b). Initially, spent coffee grounds (SCG), sugarcane bagasse (SCB) and orange peel (OP) were also assessed regarding the importance of air flow, inoculum ratio and inoculum concentration on the strain growth. From the results obtained on these substrates, apple pomace (AP) and coffee husk (CH) experiments were further defined.

Table 4.1. Characterization of the evaluated substrates.

Substrate	%C	%N	C:N	Red Sug (% dry basis)	VS (% dry basis)	WHC (g_{H2O} g^{-1}_{subs})	pH
Spent coffee ground	50.9	2.0	25.5	0.48-0.53	98.1	1.5 ± 0.1	5.9
Sugarcane bagasse	47.0	0.2	204.4	15.0-20.1	93.9	3.2 ± 0.2	3.2
Orange Peel	46.2	0.9	53.1	29.6-35.6	95.6	1.6 ± 0.1	4.2
Apple Pomace	45.8	0.5	88.1	45.1-53.4	97.5	3.4 ± 0.1	4.5
Coffee Husk	45.0	3.4	13.3	0.75-1.20	90.3	1.8 ± 0.2	6.5

C: Total carbon content; N: Total nitrogen content; C:N: ratio carbon to nitrogen; Red Sug: Reducing sugars content; VS: Volatile solids content; WHC: Water holding capacity.

As detailed in Table 4.2, assessed variables included the aeration strategy (measured as the specific air flow rate (S_{AFR})), the inoculum ratio (Ino_R) (fraction of inoculum added per mass of substrate) and the inoculum concentration (Ino_C). Also, these variables were evaluated at two conditions (level 1 and level 2). As commented before, for these set of experiments, the maximum oxygen uptake rate (OUR_{Max}) and the cumulative oxygen consumption have been used as response variables. For these experiments, temperature was set at 30°C (referred as the optimum for the *K. marxianus* growth), initial pH at 5 (a suitable pH for the *K. marxianus* growth) and the MC was fixed in the interval 60-75 % (in order to start the fermentation at the water holding capacity (WHC) of each substrate) according to the results shown by Bramorski et al. (1998), Medeiros et al. (2000) and Soares et al. (2000b). All the substrates, except SCB where mixed with 1-3 cm wood sticks acting as bulking agent in a 1:1 (v:v) ratio to promote the porosity of these materials. Fermentations were performed in duplicate and followed until 90 h. To obtain the highest inoculum concentration, it was centrifuged for 10 minutes at 4200 rpm in sterilized vessels and collected until obtaining the required volume for each experiment. The concentration showed in Table 4.2 for this level corresponded to the mean value of the centrifuged inoculum. The lowest concentration corresponds to the inoculum without centrifugation.

Figure 4.1 presents the time course of the SSF of the spent coffee grounds. As observed in Figure 4.1(a), when using 10^8 Colony forming units of *K. marxianus* (CFU) per mL, there is a rapid growth in the first hours of processing until the maximum activity is achieved around 4 to 5 h. Then, activity starts falling, and no further significant activity is detected. A similar scenario is found when increasing the

inoculum concentration to 10^9 CFU mL^{-1} (Figure 4.1(b)), but this time the OUR_{Max} is achieved near 3 h of processing. It can be observed that the main differences seem to be related to the S_{AFR} effect. The trend shows that using 0.08 L h^{-1} g^{-1}_{TS} allows a higher activity than 0.04 L h^{-1} g^{-1}_{TS} independently of the inoculum addition.

Table 4.2. Operational variables included in the initial assessment.

Factor	Level 1	Level 2
Specific air Flow rate (L h^{-1} g^{-1}_{TS})	0.04	0.08
Inoculum ratio (%)(mL_{ino} g^{-1}_{subs})	10	20
Inoculum Concentration (CFU mL^{-1}_{ino})	$2.7x10^8$	$6.0x10^9$

TS: Initial total solids content; CFU: Colony forming units of *K. marxianus*.

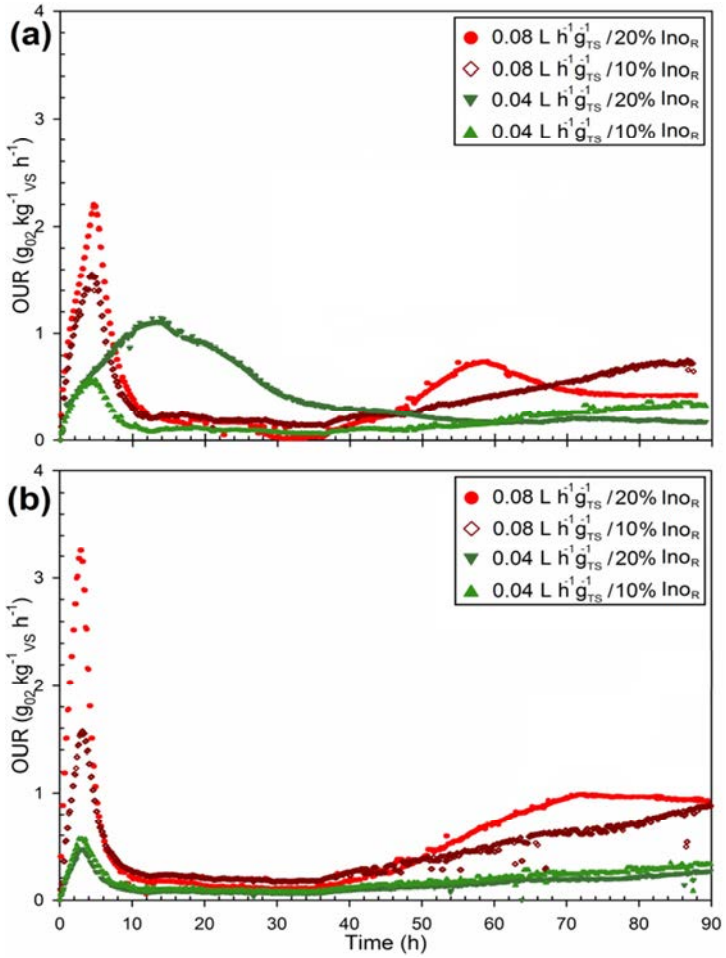


Figure 4.1. *K. marxianus* activity during the solid-state fermentation of spent coffee grounds. (a) Level 1 (2.7 10^8 CFU mL^{-1}) and (b) level 2 (6.0 10^9 CFU mL^{-1}). OUR: Oxygen uptake rate; Ino_R: Inoculum rate.

As it is shown in Figure 4.2(a), the Pareto chart for the evaluated variables indicates that when using the OUR_{Max} as the response variable, none of them

significantly affect the *K. marxianus* activity. On the contrary, using COC (Figure 4.2(c)) suggests that S_{AFR} and Inoculum ratio are relevant for the growing of the strain in the SCG. In general, when using SCG the trend of both response variables indicates that *K. marxianus* grows better in the level 2 for S_{AFR} (0.08 L h^{-1} g^{-1}_{TS}) and using an inoculum ratio of 20% (Figure 4.2(b) and (d)).

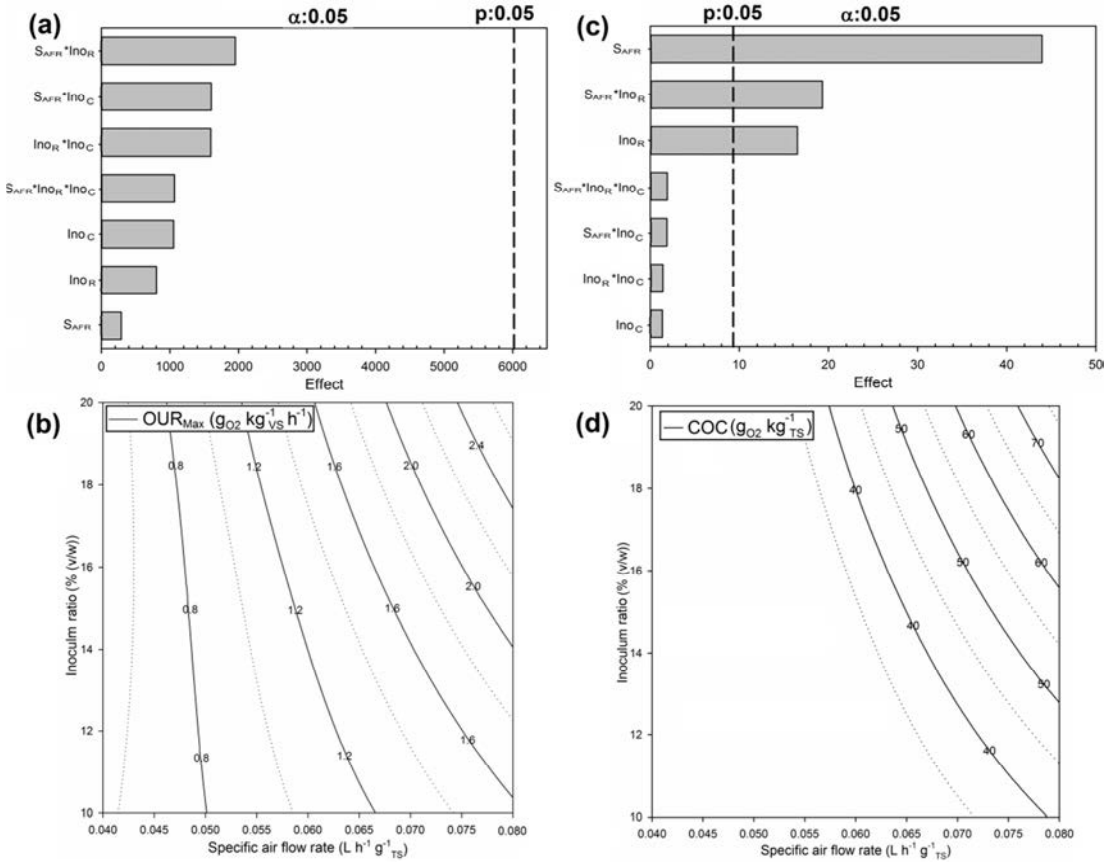


Figure 4.2. Pareto chart and contour plots for the evaluation of spent coffee ground. (a) Pareto chart for OUR_{Max}; (b) Contour plot for OUR_{Max}. (c) Pareto chart for COC; (d) Contour plot for COC. OUR_{Max}: Maximum oxygen uptake rate; COC: Cumulative oxygen consumption.

The second evaluated substrate was the sugarcane bagasse. As seen in Figure 4.3(a), when the low inoculum concentration was used, fermentation has started rapidly almost without lag phase, and the activity has grown during the first 14-15 h, reaching the OUR_{Max} in this interval. Similarly, using the high Ino_C (Figure 4.3(b)), the trend shows how the activity increases until the OUR_{Max} is achieved around 14 h of processing. In both cases, the process could be considered finished after 50 h, when activity was almost null.

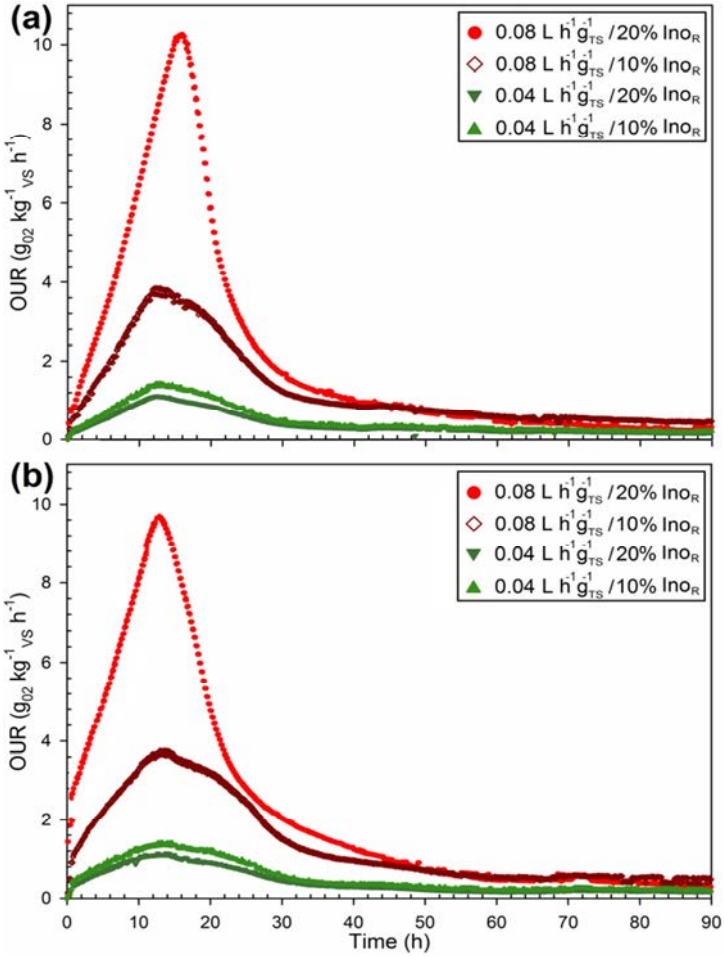


Figure 4.3. *K. marxianus* activity during the solid-state fermentation of sugarcane bagasse. (a) Level 1 (2.7 10^8 CFU mL^{-1}) and (b) level 2 (6.0 10^9 CFU mL^{-1}). OUR: Oxygen uptake rate; Ino_R: Inoculum rate.

As occurred with SCG, S_{AFR} seems to play an essential role for the *K. marxianus* growth for the evaluated interval. In this case, S_{AFR} and Ino_R significantly influence both, the OUR_{Max} and the COC as suggested by the Pareto charts in Figure 4.4(a) and (c). In fact, the highest OUR_{Max} was achieved when both S_{AFR} and Ino_R were in level 2 (Figure 4.4(c)). The same result was found when the COC was used as response variable (Figure 4.4(d)).

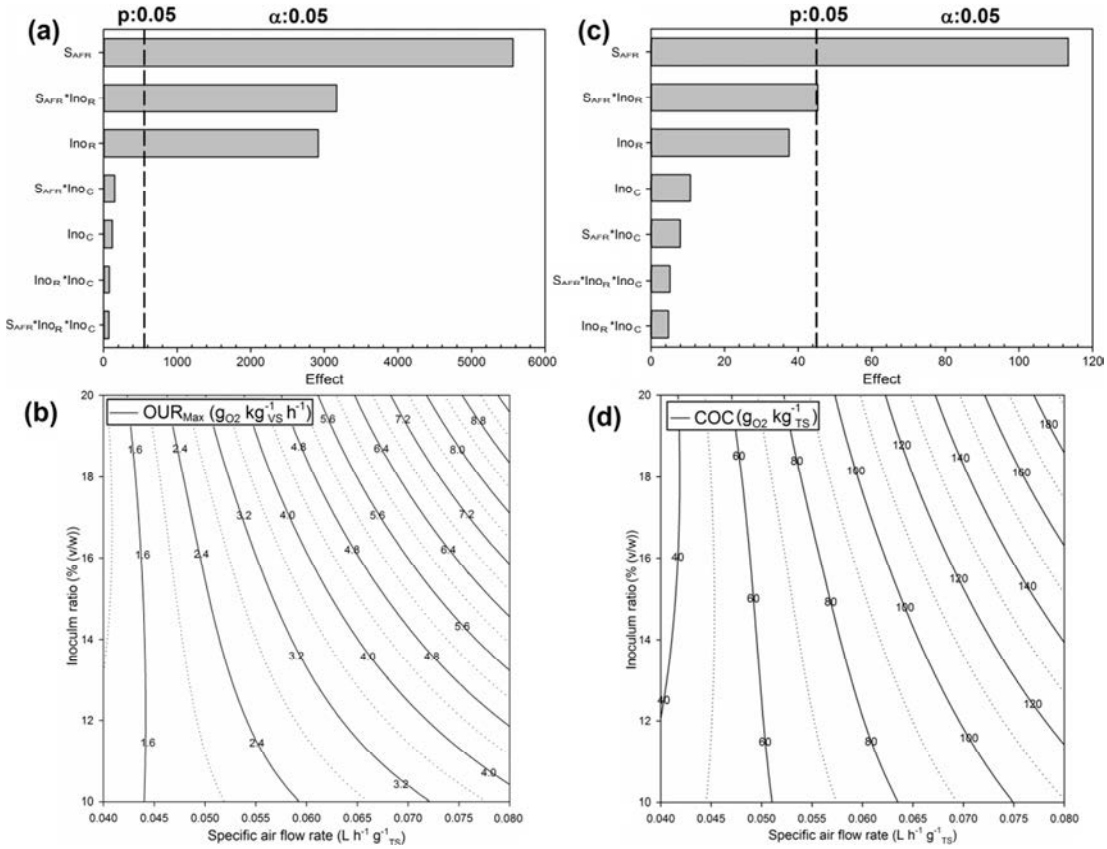


Figure 4.4. Pareto chart and contour plots for the evaluation of sugarcane bagasse. (a) Pareto chart for OUR_{Max}; (b) Contour plot for OUR_{Max}. (c) Pareto chart for COC; (d) Contour plot for COC. OUR_{Max}: Maximum oxygen uptake rate; COC: Cumulative oxygen consumption.

Finally, when using orange peels as substrate, the OUR profile presents two different behavior respect to the inoculum concentration. As seen in Figure 4.5(a), working at the low Ino_C allows the *K. marxianus* to increase the activity until the OUR_{Max} is achieved in the interval 40-54 h. Here, the duration of the lag phase was almost 8 h, and as occurred with the previous substrates, OUR profile seems to be favored at high S_{AFR}. When the Ino_C was increased (Figure 4.5(b)), the trend has changed, and this time the OUR_{Max} was achieved between 24-32 h of fermentation. This change in the fermentation behavior was accompanied by a reduction in the Ino_R effect.

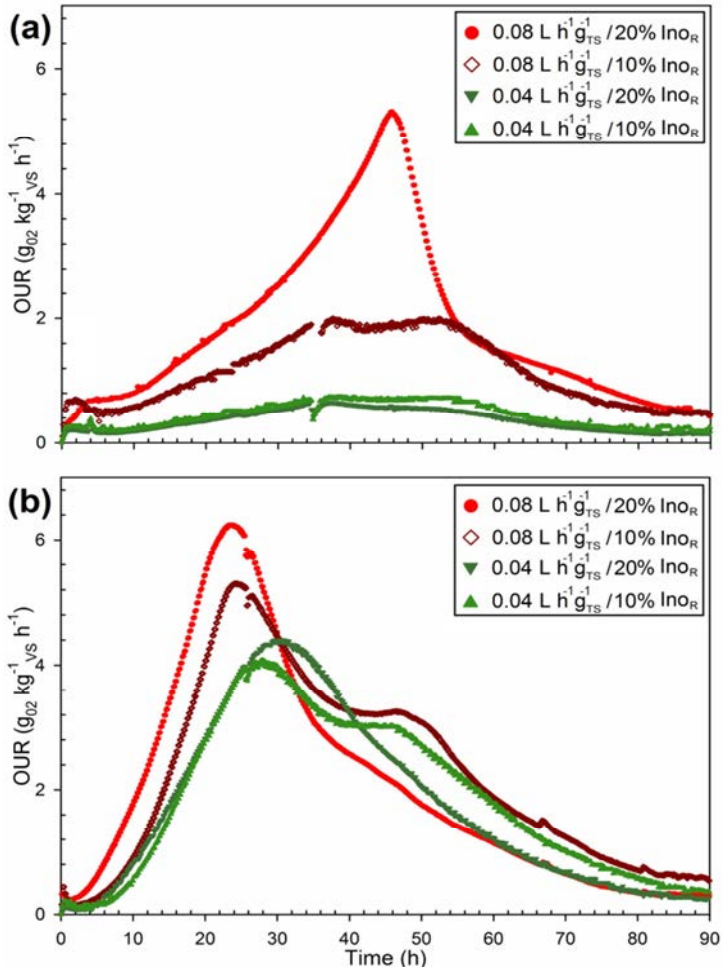


Figure 4.5. *K. marxianus* activity during the solid-state fermentation of orange peels. (a) Level 1 (2.7 10^8 CFU mL^{-1}) and (b) level 2 (6.0 10^9 CFU mL^{-1}). OUR: Oxygen uptake rate; Ino_R: Inoculum rate.

In this scenario, the Pareto charts indicate that the assessed variables have a limited role in the *K. marxianus* activity (Figure 4.6(a) and (c)). The results also show that the higher OUR_{Max} and COC were found at level 2 (Figure 4.6(b) and (d)), but in the case of this latter response variable, a definite influence of S_{AFR} was observed.

Considering the effect of the evaluated variables on OUR_{Max} and COC for the three first substrates, it is clear that only S_{AFR} and Ino_R seem to affect significantly the *K. marxianus* activity. Based on these results, the evaluation of the remaining two substrates was limited to the effect of S_{AFR} and Ino_R, keeping constant the Ino_C at the lowest level (2.7 10^8 CFU mL^{-1}) in Table 4.2. Thus, reducing the number of experiments and saving important amounts of materials, particularly inoculum.

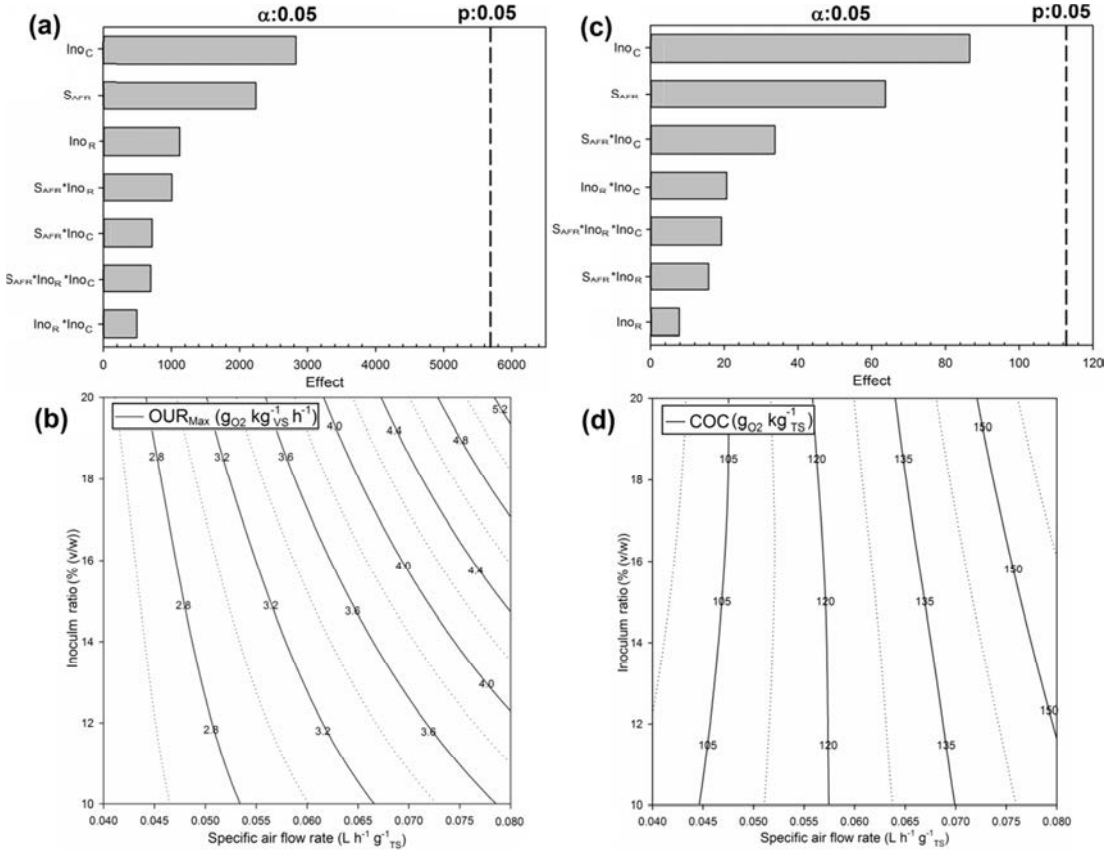


Figure 4.6. Pareto chart and contour plots for the evaluation of orange peels. (a) Pareto chart for OUR_{Max}; (b) Contour plot for OUR_{Max}. (c) Pareto chart for COC; (d) Contour plot for COC. OUR_{Max}: Maximum oxygen uptake rate; COC: Cumulative oxygen consumption.

Figure 4.7 presents the OUR profiles for apple pomace (a) and coffee husk (b) for the evaluated intervals. As observed, AP show a rapid increase in the activity reaching a peak after 10 h, moment when the activity falls until almost a 40% of the maximum previously achieved. From that point, OUR levels remain almost unchanged until 88 h, when the process was finished. On the contrary, the OUR profile for CH presents an initial increase during the first 6 h, when the OUR_{Max} is achieved. At that point, the activity suddenly falls almost to zero after 30 h. Then, a further, but lower increase after 50 h of processing is presented, lasting the remaining 40 h of fermentation.

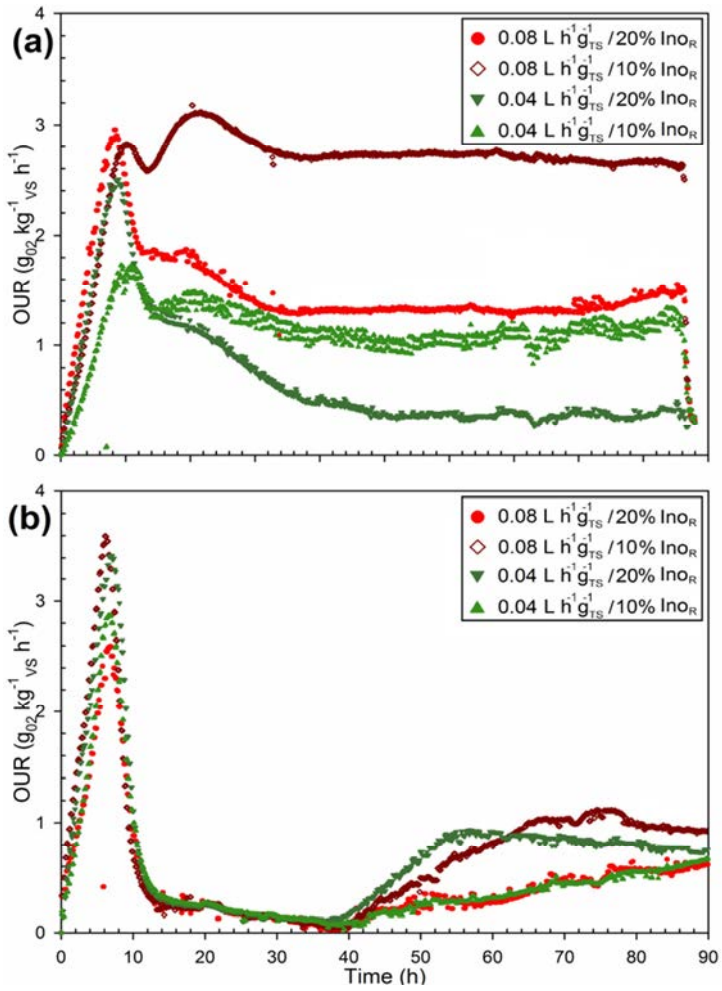


Figure 4.7. *K. marxianus* activity during the solid-state fermentation of (a) apple pomace and (b) coffee husk. OUR: Oxygen uptake rate; Ino_R: Inoculum rate. (Ino_c: 2.7 10^8 CFU mL^{-1}).

In the AP case, the evaluated variables have shown a different trend than the previous substrates. As seen in Figure 4.8(a) and (c) OUR_{Max} seems to be only affected by the S_{AFR} since the trend indicates that the contour lines behave as vertical lines. For COC, the highest value was found at the highest S_{AFR} and 10% Ino_R. Differently, for CH the contour plots show a saddle point in the midpoint of the evaluated interval (Figure 4.8(b) and (d)) and the highest values for OUR_{Max} and COC were obtained at the highest S_{AFR} and the lowest Ino_R.

Table 4.3 summarizes the OUR_{Max} and COC obtained for the set of assessed substrates in the evaluated interval. When comparing the OUR_{Max} achieved for the substrates, it is clear the difference of SCB over the remaining substrates. While SCB has reached almost 10 g_{O2} kg^{-1}_{VS} h^{-1}, the next best value was a 47% lower (5.3 g_{O2} kg^{-1}_{VS} h^{-1}), and it was obtained using OP. At the same conditions, SCB, CH and AP were

able to reach their best OUR_{Max} between 2-3.6 g_{O2} kg^{-1}_{VS} h^{-1}. On the other hand, analyzing the COC, the highest value was obtained when using AP (247.1 g_{O2} kg^{-1}_{TS}), but it seems that it was an outlier when it is compared to the trend of the remaining data. Thus, without that point, the trend is the same found for OUR_{Max}, the highest values are obtained using SCB, followed by the OP, AP, CH and SCG, these last very close each other. Based on these results, SCB appears to be the most suitable substrate among the assessed residues for the growing of *K. marxianus*, and therefore, it is expected that it would favor the production of aroma compounds.

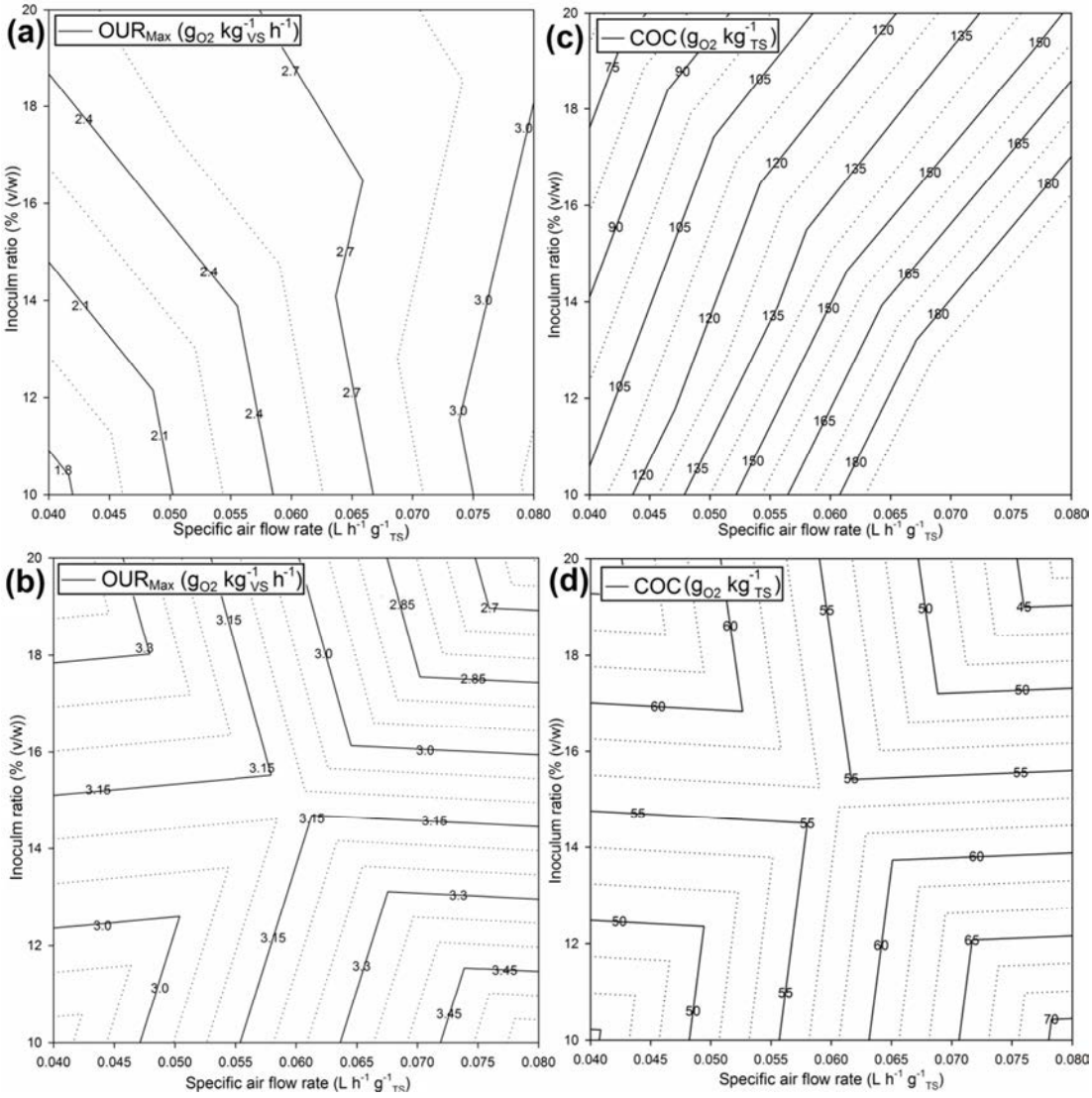


Figure 4.8. Contour plots for the evaluation of apple pomace (a) and (c) and coffee husk (b) and (d). (a), (b) OUR_{Max}; (c), (d) COC. OUR_{Max}: Maximum oxygen uptake rate; COC: Cumulative oxygen consumption.

Table 4.3. Main results obtained after the substrate screening.

Substrates	S_{AFR} ($L\ h^{-1}\ g^{-1}_{TS}$)	Inoculum rate ($mL_{ino}\ g^{-1}_{subs}$) 10%		20%	
		OUR_{Max} ($g_{O2}\ kg^{-1}_{VS}\ h^{-1}$)	COC ($g_{O2}\ kg^{-1}_{TS}$)	OUR_{Max} ($g_{O2}\ kg^{-1}_{VS}\ h^{-1}$)	COC ($g_{O2}\ kg^{-1}_{TS}$)
SCG	0.08	2.2	80.0	1.6	40.8
	0.04	0.5	12.9	0.6	16.2
SCB	0.08	10.0	178.1	3.8	105.2
	0.04	1.1	32.5	1.4	39.9
OP	0.08	5.3	164.7	2.0	101.2
	0.04	0.7	32.2	0.7	38.7
AP	0.08	3.0	151.5	3.2	247.1
	0.04	2.5	64.8	1.7	107.5
CH	0.08	2.6	42.2	3.6	71.3
	0.04	3.4	66.6	2.9	44.5

S_{AFR}: specific air flow rate; OUR_{Max}: maximum oxygen uptake rate; COC: cumulative oxygen consumption; SCB: sugarcane bagasse; SCB: spent coffee grounds; OP: orange peels; AP: apple pomace; CH: coffee husk.

The evident differences found for SCB might be associated with the synergy of some factors. First, even that the elemental composition indicates that the evaluated substrates have similar carbon content and also a similar organic fraction, the main difference in their composition lies in the available sugars contained in each of them. Thus, the substrates coming from the coffee industry (SCG and CH) have the most reduced content (less than 2% of reducing sugars) making more difficult the *K. marxianus* to grow in there (Table 4.1). On the contrary, sugars availability seems to be an asset for the other three substrates, particularly for AP since almost half of its mass contains sugars.

This aspect is not directly mentioned in previous studies as a significant factor for the aroma production, but these results suggest that there is a strong relationship between sugar availability and the performance of the fermentation process. However, despite the higher sugar availability of substrates like OP and AP, SCB performed better to reach higher microbial activities. This could be due to the inherent macro-physical structure of these substrates. While SCB behaves like a combination of small particles and bigger stick shape pieces (Figure 3.3), OP and AP contained a quite higher fraction

of fine material needing the addition of a bulking agent to improve the porosity of the substrate. This characteristic makes the SCB a suitable substrate for SSF since it acts as a nutrient provider, but also as a support for the process without needing any bulking agent. Since the mass transfer phenomena involved in the solid media are typically complex (Barrios-González, 2012), micro and macro porosity plays an essential role in its development, and it seems the particle size distribution obtained for SCB helps to induce a high porosity although the process is run at the water holding capacity of the substrate.

On the contrary, AP and OP behave more like a mud once they are moisture and mixed with the bulking agent, making more difficult to *K. marxianus* to access the available sugars due to their lower porosity. As a consequence, the SSF processes run with AP and OP are slower than in SCB, and optimization of the bulking agent would be needed to exploit the potential of AP and OP. Having these factors in mind, The SCB was selected as substrate to deep in the bioproduction of aroma compounds.

The second relevant aspect to remark here is the effect of the selected operational variables on the microbial activity. The results suggest that S_{AFR} is a significant variable for the process considering the changes obtained working at the two selected levels. In general, the highest S_{AFR} level has promoted higher OUR_{Max} as well as the highest COC.

In the case of the Ino_R, the trend was not as clear as for S_{AFR}. Even when in most of the cases the higher the Ino_R, the higher the OUR_{Max} and the COC, for some substrates the trend was different, indicating both the opposite (for CH), or suggesting there is a limited effect in the response variable (AP and OP). At the same time, the preliminary evaluation of Ino_C suggests that the use of a concentrated inoculum (centrifuged one) is not significantly different compared to a non-centrifuged inoculum, and therefore, there is no need of this additional step in the preparation of the inoculum.

However, since Ino_R and Ino_C are related each other, it is necessary to further evaluate its effect by using a more explicit variable, representing the applied inoculum load. This is why it was decided to set as the variable for future experiments the inoculum load (Ino_L), expressed as the colony forming units of *K. marxianus* loaded in the reactor per gram of initial solid substrate (g^{-1}_{TS}).

4.2.2 Variables screening

Once the SCB was selected as substrate, the next step was the evaluation of some of the most critical variables affecting the SSF process. In this section, the response variable was the accumulated volatile production (P_{Vol}^{Acc}) of the fruit-like compounds obtained at the end of the fermentation, which represents the sum of all the quantified compounds, analyzed in the exhausted gases of the system (Table 3.1).

4.2.2.1 Inoculum load

As detailed above, the inoculum load is an essential parameter for the SSF since it defines the initial population of *K. marxianus* in the solid media. In most of the previous studies (Bramorski et al., 1998b; Mantzouridou et al., 2015; Medeiros et al., 2001; Rossi et al., 2009) this is a fixed parameter and no much attention is focused on it, using a predetermined value of 1 10^7 CFU per gram of dry substrate (g^{-1}_{Sub}). Here, the evaluation consisted of the direct comparison of the SSF starting with three different Ino_L levels corresponding to 5.2 10^7, 1.0 10^8 and 1.5 10^8 CFU g^{-1}_{TS}. The temperature was set at 30°C, initial pH at 5, MC was fixed at 76 % (WHC of the SCB) and S_{AFR} in 0.08 L h^{-1} g^{-1}_{TS} according to the previous results. Fermentations were performed in duplicate and followed until 60 h.

Figure 4.9(a) shows the OUR profile for the three Ino_L evaluated levels. As detailed, the first hours of fermentation are analogous among the tested scenarios with a rapid increase in OUR until 10-12 h, when the slope suddenly changes, and the increase becomes slower in the period from 12 to 32-36 h. At that point, the OUR start falling until the process is ended after 60 h of processing. As seen, these profiles are substantially different compared to those found in section 4.2.1. The OUR_{Max} was lower, but with an extended period in which activity is maintained. In this sense, some changes have induced this new behavior. First, the implementation of an improved inoculation procedure (improving the mix inoculum-SCB), and secondly, the pH control by using a buffer. In this sense, in the experiments of section 4.2.1 with SCB, initial pH was adjusted using a diluted NaOH solution, but the pH measured at the end of the fermentation was too low (below 4) indicating that the use of this base was not such appropriate. On the contrary, changing to a buffer, final pH levels were considerably higher (around 5) suggesting that pH drops are less marked, and therefore, allowing *K.*

marxianus to be active for more time. Thus, it seems these changes have positively affected the global behavior of the process.

The OUR profiles also show slight differences among the evaluated scenarios regarding the OUR_{Max}. The highest value was obtained starting with 1.0 10^8 CFU g^{-1}_{TS} (7.2 g_{O2} kg^{-1}_{VS} h^{-1}), followed by the highest Ino_L (6.9 g_{O2} kg^{-1}_{VS} h^{-1}) and the intermediate scenario (6.2 g_{O2} kg^{-1}_{VS} h^{-1}). However, as seen in Figure 4.9(b), the volatile compounds production has had a different trend. The highest P_{Vol}^{Acc} corresponded to the highest Ino_L (1.5 10^8 CFU g^{-1}_{TS}), then 1.0 10^8 CFU g^{-1}_{TS} and finally when the lowest Ino_L was used starting at 5.2 10^7 CFU g^{-1}_{TS} (Figure 9).

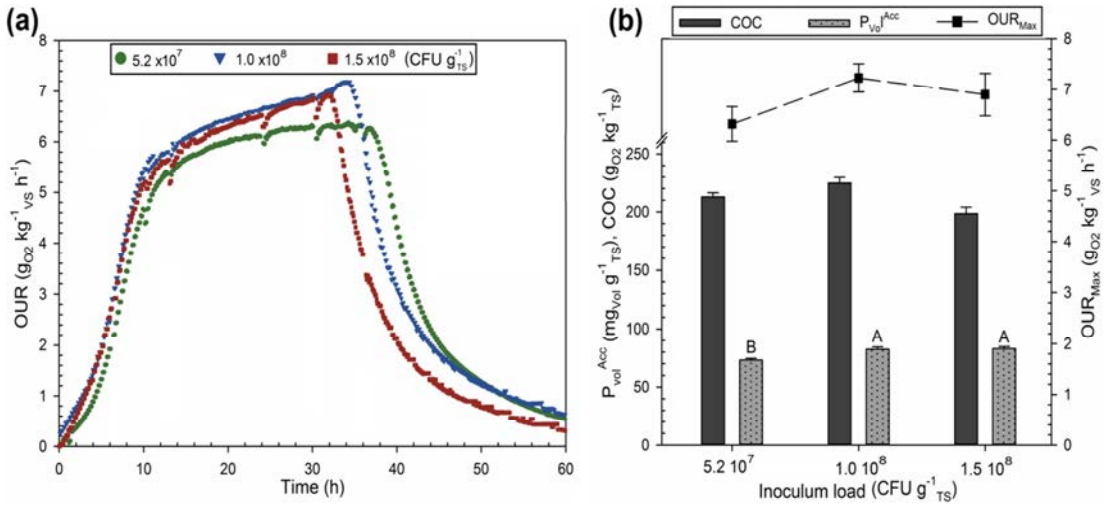


Figure 4.9. Inoculum load effects on the solid-state fermentation of sugarcane bagasse. (a) OUR profiles, (b) Respirometric indices and accumulated volatile production. OUR: oxygen uptake rate; COC: cumulative oxygen consumption; P_{Vol}^{Acc}: total volatile cumulative production. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$).

This data suggest that the relationship between OUR and the volatile production is not directly linked, as it has been shown in other SFF applications (Jiménez-Peñalver et al., 2016). In other words, OUR could be used as an indirect measure of the *K. marxianus* activity, but it is not a direct index of the total volatile accumulated production. Based on the previous results, it was decided to use as reference Ino_L a dose of 1.0 10^8 CFU g^{-1}_{TS} since it promotes the same P_{Vol}^{Acc} than using 1.5 10^8 CFU g^{-1}_{TS} (no significant differences (p 0.101)) but using a lower amount of inoculum. Evidently, needing less inoculum for running the fermentation is an asset, since it influences the economy of the global process.

4.2.2.2 Glucose addition

Since the sugar availability has shown to be of significant importance in the development of SSF process for fruity aromas production (Christen et al., 1997; Rossi et al., 2009; Soares et al., 2000b), it was decided to assess the effect of adding glucose as additional carbon source. Glucose has been typically used as precursor in many studies (Medeiros et al., 2000; Soares et al., 2000b) to enhance the aroma compounds bioproduction via SSF. Since the reported processes are quite different among them and included the use of a wide range of solid substrates, it was decided to analyze the glucose effect on the system used in this study with the particular characteristics of the used SCB. Experiments consisted of the direct comparison of the fermentation starting with three different analytical grade glucose addition levels corresponding to 0% (no addition), 5 and 10% (dry basis). The temperature was set at 30°C, initial pH at 5, MC was fixed at 76 %, S_{AFR} in 0.08 L h^{-1} g^{-1}_{TS} and Ino_L in 1.0 10^8 CFU g^{-1}_{TS}. Fermentations were performed in duplicate and followed until 78 h

Figure 4.10(a) shows the OUR profile for the evaluated scenarios. As occurred in the Ino_L evaluation experiments, the fermentation has shown a very similar trend during the first 10-12 h of processing. After this rapid growth phase, the OUR has slowed down, and the OUR_{Max} was reached after 30-38 h of fermentation. In this case, significant differences are presented when the glucose addition was 10%; as observed in Figure 4.10(b), the other two scenarios are equivalent reaching an OUR_{Max} of 6.6 and 6.7 g_{O2} kg^{-1}_{VS} h^{-1} at 31 h, while using 10% of glucose promoted a similar OUR_{Max} of 6.8 g_{O2} kg^{-1}_{VS} h^{-1} but it is reached at 37 h.

Regarding the volatile production, no significant differences were found for 0 and 5% additions reaching 83.5 and 86.7 mg_{Vol} g^{-1}_{TS}, whereas a significant increase when adding 10% was observed with a total cumulative production of 128.2 mg_{Vol} g^{-1}_{TS}. Hence, results show the positive effect of the supplementary carbon source in the aroma production, coinciding with previous findings (Soares et al., 2000a, 2000b). However, since the use of pure reagents is not strictly coherent with the idea of valorizing a residue, substitutes of glucose, coming from other industrial residues, will be tested to produce a similar effect on the volatile production. This evaluation will be addressed in Chapter 5.

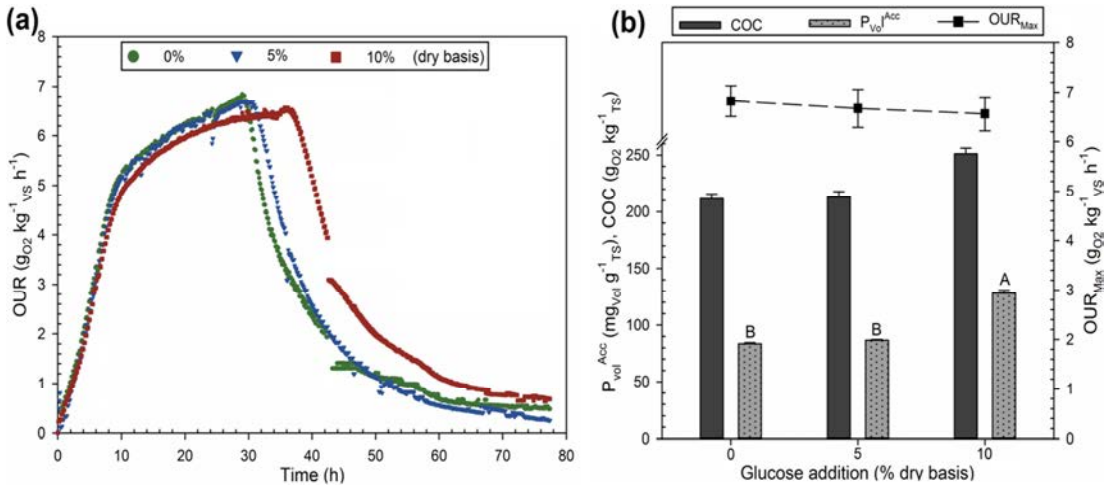


Figure 4.10. Glucose addition effect on the solid-state fermentation of sugarcane bagasse. (a) OUR profiles, (b) Respirometric indices and accumulated volatile production. OUR: oxygen uptake rate; COC: cumulative oxygen consumption; P_{Vol}^{Acc}: total volatile cumulative production. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$).

4.2.2.3 Temperature

The last assessed variable was the temperature effect. Even that most of the reported studies determined the working temperature considering only its influence in the growth of the used strain (Bramorski et al., 1998b; Medeiros et al., 2000, 2006), it is expected the temperature to play a most crucial role in the development of the process. In fact, the temperature is not just related to the growth rate of the microorganisms, but it also influences the mass transfer phenomena involved in the solid media (Barrios-González, 2012; Rodriguez-Leon et al., 2008). Thus, the temperature could influence the stripping of the produced compounds (due to the continuous stream of air), by increasing the volatility of these at higher temperatures. As a result, an increase in temperature would favor the product removal, and this, in turn, the further biotransformation of products in the solid-liquid interface.

Temperature evaluation consisted of the direct comparison of the fermentation performed at four different temperature levels, corresponding to 20, 25, 30 and 35°C. Initial pH was set at 5, MC was fixed at 76 %, S_{AFR} was 0.08 L h^{-1} $g^{-1}_{TS,}$ Ino_L at 1.0 10^8 CFU g^{-1}_{TS} and no glucose was added. Fermentations were performed in duplicate and followed until 110 h.

Figure 4.11(a) presents the OUR profiles of the SSF at the evaluated temperatures. As expected, the change in temperature has influenced the activity rate

during the first hours of fermentation. Thus, the highest slope is achieved working at 35°C while the lowest is found at 20°C. At the lowest temperature, OUR has reached a maximum of 4.7 g_{O2} kg^{-1}_{VS} h^{-1} after 44 h. At 25°C and 30°C this maximum was reached almost simultaneously after 34 h (6.6 and 6.4 g_{O2} kg^{-1}_{VS} h^{-1} respectively), and at 35°C OUR_{Max} was 5.3 g_{O2} kg^{-1}_{VS} h^{-1} after only 10 of processing.

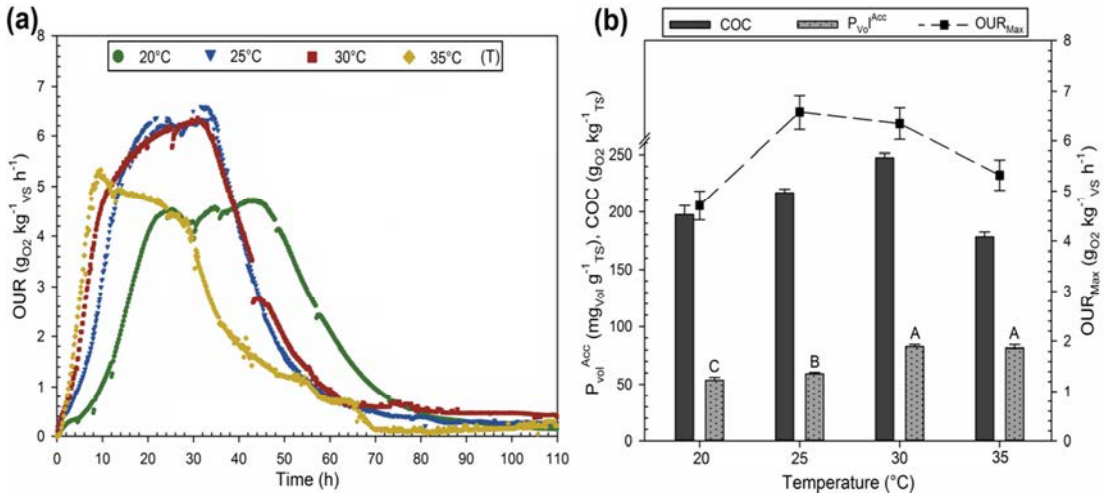


Figure 4.11. Temperature effect on the solid-state fermentation of sugarcane bagasse (a) OUR profile, (b) Respirometric indices and accumulated volatile production. OUR: oxygen uptake rate; COC: cumulative oxygen consumption; P_{Vol}^{Acc}: total volatile cumulative production. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$).

When comparing the P_{Vol}^{Acc} obtained at the different levels (Figure 4.11(b)), it is found that the lowest production was achieved at 20°C (53.7 mg_{Vol} g^{-1}_{TS}) not so far of the performance at 25°C (59. 2 mg_{Vol} g^{-1}_{TS}). On the contrary, a sudden increase was observed at 30°C and 35°C reaching 82.5 and 81.5 mg_{Vol} g^{-1}_{TS} respectively. Again, the volatile production seems not to be directly linked with the OUR, and the small differences when operating at 20 and 25°C, and those found between 30 and 35°C possess in evidence a much more complicated influence of temperature on the volatile production. In general, these changes suggest that *K. marxianus* grows differently depending on the temperature (as expected), but it could be thought it uses the available nutrients differently according to the temperature of the media. Since there is no additional information about the temperature effect in the aroma compounds production, further analysis is needed to understand the role this variable plays. This evaluation will be conducted in chapter 5.

Final remarks

Sugarcane bagasse has shown very interesting characteristics, making of this agro-industrial residue a potential alternative for the production of aroma compounds through the solid-state fermentation. Also, the performed screening on the selected operational variables, suggests that the air flow strategy, temperature and sugar availability are key factors in the development of the process. These results are part of the base for the evaluation of the production of the aroma compounds in the subsequent chapters.

References

Barrios-González, J., 2012. Solid-state fermentation: Physiology of solid medium, its molecular basis and applications. Process Biochem. 47, 175–185.

Bramorski, A., Soccol, C.R., Christen, P., Revah, S., 1998. Fruity Aroma production by *Ceratocystis Fimbriata* in solid cultures from agro-industrial wastes. Rev. Microbiol. 29, 3–11.

Carvalho, J., Pandey, A., Oishi, B., Brand, D., Rodriguez-Leon, J., Soccol, C.R., 2006. Relation between growth, respirometric analysis and biopigments production from *Monascus* by solid-state fermentation. Biochem. Eng. J. 29, 262–269.

Christen, P., Meza, J.C., Revah, S., 1997. Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*: influence of the substrate type and the presence of precursors. Mycol. Res. 101, 911–919.

Jiménez-Peñalver, P., Gea, T., Sánchez, A., Font, X., 2016. Production of sophorolipids from winterization oil cake by Solid-state fermentation: Optimization, monitoring and effect of mixing. Biochem. Eng. J. 115, 93–100.

Mantzouridou, F.T., Paraskevopoulou, A., Lalou, S., 2015. Yeast flavour production by solid state fermentation of orange peel waste. Biochem. Eng. J. 101, 1–8.

Medeiros, A., Christen, P., Roussos, S., Gern, J.C., Soccol, C.R., 2003. Coffee residues as substrates for aroma production by *Ceratocystis fimbriata* in solid state fermentation. Brazilian J. Microbiol. 34, 245–248.

Medeiros, A., Pandey, A., Freitas, R., Christen, P., Soccol, C.R., 2000. Optimization of the production of aroma compounds by *Kluyveromyces marxianus* in solid-state fermentation using factorial design and response surface methodology. Biochem. Eng. J. 6, 33–39.

Medeiros, A.B.P., Pandey, A., Christen, P., Fontoura, P.S.G., de Freitas, R.J.S., Soccol, C.R., 2001. Aroma compounds produced by *Kluyveromyces marxianus* in solid state fermentation on a packed bed column bioreactor. World J. Microbiol. Biotechnol. 17, 767–771.

Medeiros, A.B.P., Pandey, A., Vandenberghe, L.P.S., Pastorel, G.M., Soccol, C.R., 2006. Production and recovery of aroma compounds produced by solid-state fermentation using different adsorbents. Food Technol. Biotechnol. 44, 47–52.

Robinson, T., Singh, D., Nigam, P., 2001. Solid-state fermentation: a promising microbial technology for secondary metabolite production. Appl. Microbiol. Biotechnol. 55, 284–289.

Rodriguez-Leon, J., Soccol, C.R., Pandey, A., Rodriguez, D., 2008. Factors affecting solid-state fermentation, in: Pandey, A., Soccol, C.R., Larroche, C. (Eds.), Current Developments in Solid-State Fermentation. Springer, New Delhi, p. 521.

Rossi, S.C., Vandenberghe, L.P.S., Pereira, B.M.P., Gago, F.D., Rizzolo, J.A., Pandey, A., Soccol, C.R., Medeiros, A.B.P., 2009. Improving fruity aroma production by fungi in SSF using citric pulp. Food Res. Int. 42, 484–486.

Soares, M., Christen, P., Pandey, A., Raimbault, M., Soccol, C.R., 2000a. A novel approach for the production of natural aroma compounds using agro-industrial residue. Bioprocess Eng. 23, 695–699.

Soares, M., Christen, P., Pandey, A., Soccol, C.R., 2000b. Fruity flavour production by *Ceratocystis fimbriata* grown on coffee husk in solid-state fermentation. Process Biochem. 35, 857–861.

Chapter 5

Optimization of the batch SSF of sugarcane bagasse/sugar beet molasses for producing fruit-like compounds

Part of this chapter has been published in: Martínez, O., Sánchez, A., Font, X., Barrena, R. 2017. Valorization of sugarcane bagasse and sugar beet molasses using *Kluyveromyces marxianus* for producing value-added aroma compounds via solid-state fermentation. Journal of Cleaner production. 158, 8-17.

5.1 Overview

In this chapter, the study of the operational conditions affecting the bioproduction of fruit-like compounds in a batch SSF has been addressed. Although most of the reported studies in this area (Besson et al., 1997; Bramorski et al., 1998b; Christen et al., 1997; De Aráujo et al., 2002; Soares et al., 2000a) have shown the feasibility of producing fruit-like compounds via solid-state fermentation by means of several strains and residues, there are some challenges that must be addressed to use them in large-scale processes. Some of them include the use of only residues as substrates, understanding the behavior of the process on a larger scale (including the particle size distribution and aeration scheme) and comprehending the effect of the operating parameters on the type of produced volatiles (selectivity to fruit-like compounds) as well as their total production. Since these aspects are key points in the development of the bioprocess, the most relevant factors influencing the process will deepen along this chapter.

Thus, the aim of this chapter was the optimization of the SSF process for producing fruit-like compounds using a sterilized mixture of sugarcane bagasse with the industrial by-product sugar beet molasses (SBM) after inoculation of the GRAS strain *K. marxianus*. With this purpose, the continuous air supplied 0.5 L reaction system (operated in batch mode) was used. The specific objectives include: (i) to optimize the production of total volatiles (P_{Vol}^{Acc}) and ester species (P_{Est}^{Acc}) in terms of the substrate composition, temperature and aeration rate; (ii) to determine the effect of MC and C:N ratio on the selectivity and productivity of the volatile compounds in the mixture used as substrate; (iii) to go more deeply into the cause-effect relationship between selectivity of volatiles and the monitoring variables.

5.2 Results

5.2.1 Supplementary substrate effects

As it was identified in Chapter 4, the addition of available sugars as a supplementary carbon source for the SCB has enhanced the volatile production in significant levels. Although previous works (Medeiros et al., 2000) have suggested SCB is not by itself a suitable substrate for aroma production, the preliminary screening of some agro-industrial residues (Chapter 4) has shown that SCB contains the required

characteristics for running a SSF process. As a consequence, it could be considered a reasonable raw material for the production of the fruit-like compounds. Besides, this result is in accordance with some other author findings (Christen et al., 1997).

In this part, the aim was to identify a residue-based substitute for the glucose, such that it could improve the production of the volatile compounds, keeping the system under the original concept of waste valorization. With this purpose, sugar beet molasses were used as alternative low-price carbon source. This by-product has shown suitable characteristics for other SSF processes (Jiménez-Peñalver et al., 2016). Also, since its disposal is of major concern, its use as a supplement of SCB would be an alternative for valorizing the residue. SBM is the syrup leftover obtained after the final sugar crystallization process, and it is characterized by its high sugar content. Although the main component of this substrate is sucrose, it contains significant amounts of glucose and fructose (Razmovski and Vučurović, 2012), making of this substrate a good alternative for substituting glucose. Table 5.1 summarizes the main characteristics of the selected substrates for running the SSF process.

Table 5.1. Main characteristics of the used substrates.

	Sugarcane bagasse	**Sugar beet molasses**
pH	3.20 ± 0.1	6.81 ± 0.1
Organic matter (%)	93.9 ± 1.0	88.1 ± 0.5
Glucose (%)	1.98 ± 0.1	0.23 ± 0.02
Reducing sugars (%)	17.9 ± 2.1	5.16 ± 0.1
Total sugars (%)	n.a.	78.0 ± 1.5
Nitrogen (%)	0.23 ± 0.02	1.92 ± 0.1

n.a.: Not analyzed. Percentages referred to a dry basis. Data presented as mean ± standard deviation

Experiments consisted of the evaluation of glucose and SBM as co-substrates of the SCB for the production of the mixture of fruit-like compounds listed in Table 3.1. Temperature, initial pH, MC and inoculum load were kept at 30°C, 5.3, 76% and 10^8 CFU g^{-1}_{TS} respectively, based on the results of (Medeiros et al., 2000). Specific air flow rate (S_{AFR}) was set to 0.06 L h^{-1} g^{-1}_{TS} as suggested by (Medeiros et al., 2001) and air filled porosity (AFP) of the substrate was in the range 73-78%. Experiments were monitored for 75 h. Figure 5.1 shows the OUR profile and total cumulative volatile production (P_{Vol}^{Acc}) of the SSF process when SCB was used alone and after the addition of 10% (dry basis) of glucose and SBM, respectively.

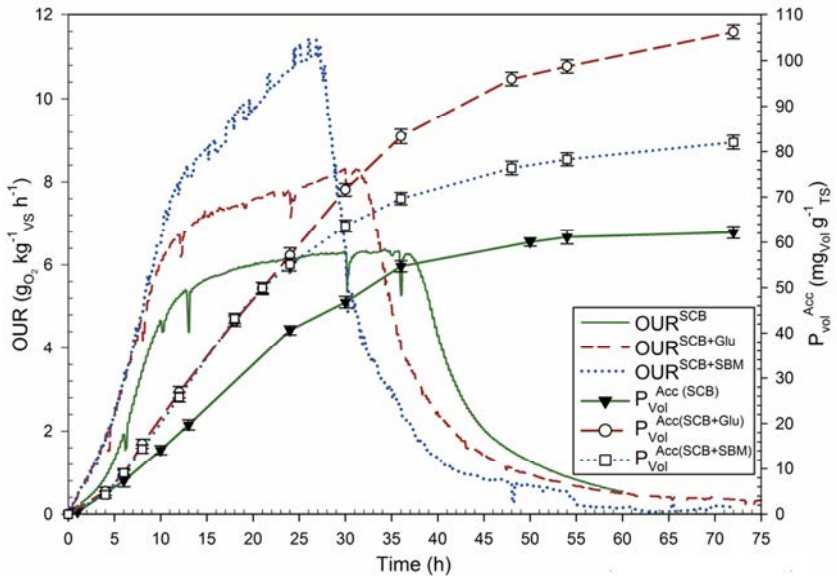


Figure 5.1. Effects of the supplementary substrate (Glu: Glucose; SBM: Sugar beet molasses) in the solid-state fermentation of sugarcane bagasse (SCB) with *K. marxianus.* P_{Vol}^{Acc}: total cumulative volatile production; OUR: oxygen uptake rate.

As it can be observed, the total cumulative volatile production was influenced by the dose of the co-substrates. In the reference case, using just SCB as substrate, P_{Vol}^{Acc} have reached 62.2 mg_{Vol} g^{-1}_{TS} while the maximum oxygen uptake rate (approximately 6.0 g_{O2} kg^{-1}_{VS} h^{-1}) took place in a period of 24 h. This result was achieved starting with an initial reducing sugars content close to 18% (Table 5.1). When SCB was supplemented with glucose, initial reducing sugars content reached 28.1%, and this supplementary carbon source increased 70.1% the volatiles production until 106.2 mg_{Vol} g^{-1}_{TS}. This result is in accordance with the findings of some other authors (Christen et al., 1997; Medeiros et al., 2000; Soares et al., 2000a) who have encountered that the presence of glucose in the medium is an advantageous condition for promoting the biosynthesis of esters and aldehyde compounds, the primary responsible for fruity odor. When SBM was added to the SCB, the P_{Vol}^{Acc} was also enhanced compared to the reference scenario (a 32% respect to SCB alone) reaching 82.1 mg_{Vol} g^{-1}_{TS}, starting with an equivalent of 18.5% of reducing sugars.

These results suggest that the main contribution of SBM is not reducing sugars, but other carbohydrates that seem to be also used by *K. marxianus* during the fermentation process. As stated by Rossi et al. (2009), molasses, in general, also show good characteristics as co-substrate thanks to the diversity of carbon sources that contains. This helps the final substrate to mimic the natural environment better for the

microorganism, and therefore, favoring the development of the strains. It was also observed that the process had been developed faster when using SBM, reaching the OUR_{max} (around 11.0 g_{O2} kg^{-1}_{VS} h^{-1}) in less than 30 h. Besides, the process behavior regarding the OUR indicates that there are no significant differences among adding glucose or SBM in the first 10 h. More importantly, comparing the P_{Vol}^{Acc} profiles, it could be stated the differences are practically null for the first 25 h of processing, just a couple of hours before the process using SBM reached the maximum activity.

Having these results in mind, it could be deduced that *K. marxianus* is using with similar efficiency the provided sugars to produce the fruit-like mixture independently of the source of the nutrients (only comparing glucose and SBM). At least, it could be perceived that SBM provides a sugar content, as available as the one provided by glucose, but the first is spend faster since there is a considerable difference among the glucose and SBM contents. Although glucose presents a better performance, the use of a pure reagent makes no such attractive the process considering its cost, whereby it is preferable the use of a supplementary carbon source based on by-products like the sugar beet molasses. Since the performed comparison offers promising results when using SBM, the use of SBM as co-substrate will be included in the subsequent sections.

5.2.2 Moisture content and air flow rate effects

The oxygen availability has been proven to be relevant for the biosynthesis of volatile compounds using different strains (Ito et al., 1990; Medeiros et al., 2001). While a lack of oxygen promotes the production of alcohols and aldehydes through anaerobic routes, a rich oxygen environment makes more accessible the production of ester compounds, main responsible of fruity aromas. Taking this in mind, the air supply and the moisture content become critical parameters in the bioproduction of the fruit-like aromas. To identify the influence of the initial moisture content and the supplied air flow rate, a set of experiments based on a 3^2 factorial design were carried out using as substrate a mixture 1:9 (w/w dry basis) of sugar beet molasses and sugarcane bagasse. For these experiments, the remaining operational conditions were kept identical to those explained in section 5.2.1 while MC and S_{AFR} have been evaluated from 58% to 77%, (corresponding to a low MC for a typical SSF process and the water holding capacity of

the substrate) and 0.04 and 0.08 L h^{-1} g^{-1}_{TS}, considering the results obtained in variable screening tests.

Under the experimental conditions assayed, results based on P_{Vol}^{Acc}, show that MC had no significant effects on the aroma production (p 0.142) while S_{AFR} (p 0.003) became a relevant factor in the development of the process. This can be observed in Figure 5.2(a), where it is clear that the higher the S_{AFR}, the higher the production of volatile compounds regardless of the initial moisture content. There was only a trend of higher production in the proximity of MC around 66-68% that can play a significant role considering the influence of this variable on other parameters like the substrate porosity. On the other hand, when results are analyzed from the perspective of the ester species production (P_{Est}^{Acc}), it was found that MC was again not significant (p 0.575) with a slight positive slope in the contour plots, suggesting the use of a lower MC, could increase the ester species production (Figure 5.2(b)).

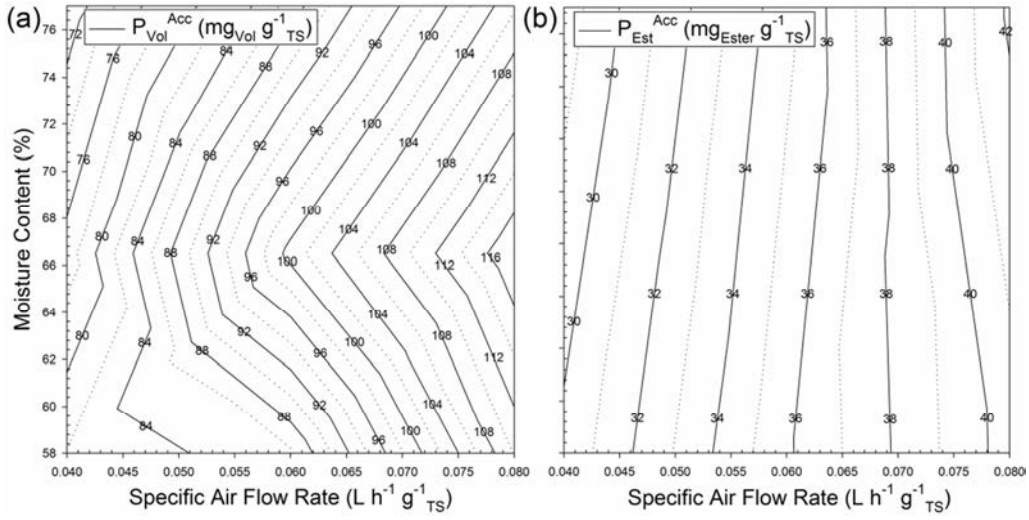


Figure 5.2. Moisture content and air flow rate effects (a) on the total cumulative volatile production (P_{Vol}^{Acc}), and (b) on the cumulative ester species production (P_{Est}^{Acc}) for the solid-state fermentation of sugarcane bagasse/sugar beet molasses with *K. marxianus.*

Beyond the experimental results, it has to be considered that working under a low MC would lead to a slower development of the process due to the lower water availability. At the same time, it would imply a higher porosity, promoting an aerobic environment needed for the *K. marxianus* growth. On the other side, working at the WHC (77%) would contribute to the formation of anaerobic zones (due to the lower porosity of the solid media), which would favor the alcohols production. Additionally,

the system would be prone to go beyond this absorption capacity given the expected increase in the moisture content due to the mass loss during the fermentation. Considering all these factors, an intermediate point close to 68% of MC seems to be adequate for weighting these effects on the synthesis of the ester species, while favoring the production of total volatile compounds.

5.2.3 Optimization of the process

Based on the information compiled before, the total cumulative volatile production was optimized through the evaluation of three operational parameters. First, the air supply, since S_{AFR} plays a significant role in the production of the fruity aromas as shown in section 5.2.2, and from these results is evident that this parameter can be further increased since there are no dilution effects in the upper limit in which it was tested. Furthermore, the temperature was selected considering its importance not only in the *K. marxianus* kinetics but also in the complex mass transfer phenomena involved in the reaction system as detailed in Chapter 4. Moreover, the molasses load was considered as a third factor for the optimization, since it is essential to identify how much co-substrate is needed for enhancing the production as well as the selectivity of the ester species of the fermentation process. For the experiments, initial pH was fixed at 5.3, MC at 68%, and the inoculum load was 10^8 CFU g^{-1}_{TS}. Experiments were monitored for 72 h. Table 5.2 shows the levels used for the experimental design (Box-Behnken design), based on the preliminary experiments performed in chapter 4 as well as in section 5.2.1.

Table 5.2. Levels used in the design of experiments for the optimization process.

Factor	Levels		
S_{AFR} (L h^{-1} g^{-1}_{TS})	0.06	0.10	0.14
T (°C)	21	30	40
M_d (%, dry basis)	15	25	35

S_{AFR}: Specific air flow rate; M_d: Sugar beet molasses load

Below, the results found in the optimization of the process based on the variables P_{Vol}^{Acc} and P_{Est}^{Acc}.

5.2.3.1 Optimization based on the total cumulative volatile production

When P_{Vol}^{Acc} was used as the response variable of the optimization, it could be stated that the fermentation process was mainly governed by the temperature. From Figure 5.3(a) and (b) it is evident how an increase in temperature was directly linked to an increase in the total production of fruit-like compounds. In fact, the maximum P_{Vol}^{Acc} was found at the highest evaluated level at 40°C with 161 mg_{Vol} g^{-1}_{TS}. Also, it was evident the loss of significance of the other two evaluated variables when the temperature reached 30°C or higher, being almost vertical lines in the contour plots.

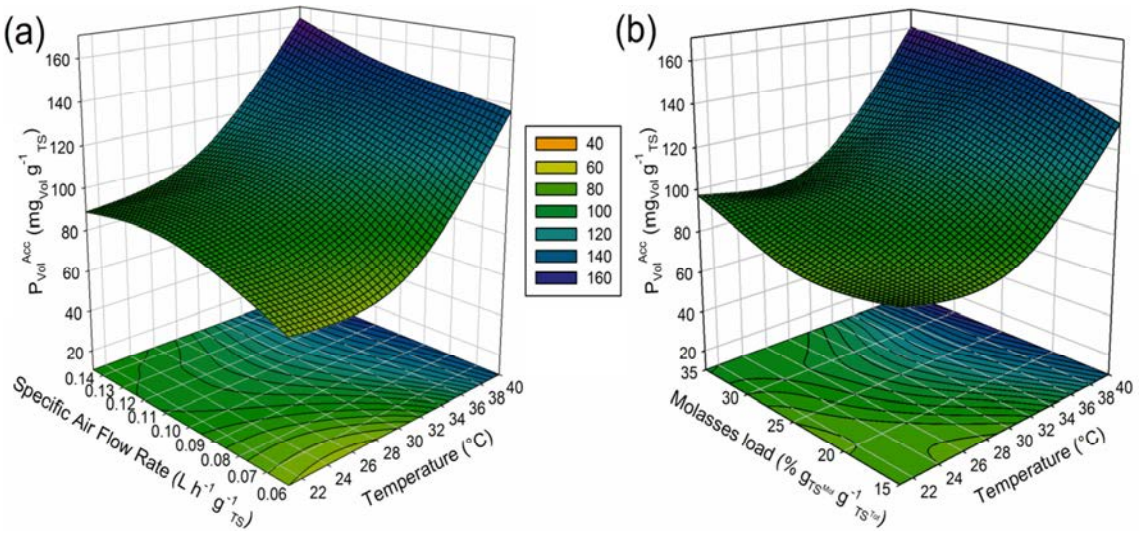


Figure 5.3. Effects on the total cumulative volatile production (P_{Vol}^{Acc}) of (a) air flow rate and (b) molasses content as a function of temperature during the solid-state fermentation of sugarcane bagasse/sugar beet molasses with *K. marxianus*.

Equation (15) represents the model for P_{Vol}^{Acc} as a function of the evaluated variables with R^2: 98.93%, R^2 (Pred): 83.17% and R^2 (Adj):97.01%.

$$P_{Vol}^{Acc} = 161.59 - 10.94T + 607.96S_{AFR} + 0.21M_d + 0.20T^2 - 5255.34S_{AFR}^2 - 0.06{M_d}^2 + 6.83TS_{AFR} + 0.04TM_d + 22.51S_{AFR}M_d \quad (15)$$

Working under the conditions maximizing P_{Vol}^{Acc} (40°C, S_{AFR} 0.14 L h^{-1} g^{-1}_{TS}, 35% molasses (dry basis)), the volatile compounds produced during the fermentation were mainly alcohols (43%) of which ethanol was the most abundant (37%), then acetaldehyde (39%) and the esters compounds with a percentage close to 18% (Figure

5.4). Given the high alcohol content, it is expected that *K. marxianus* was producing these species preferentially through an anaerobic route rather than the fruity odor components (ester species) via aerobic routes.

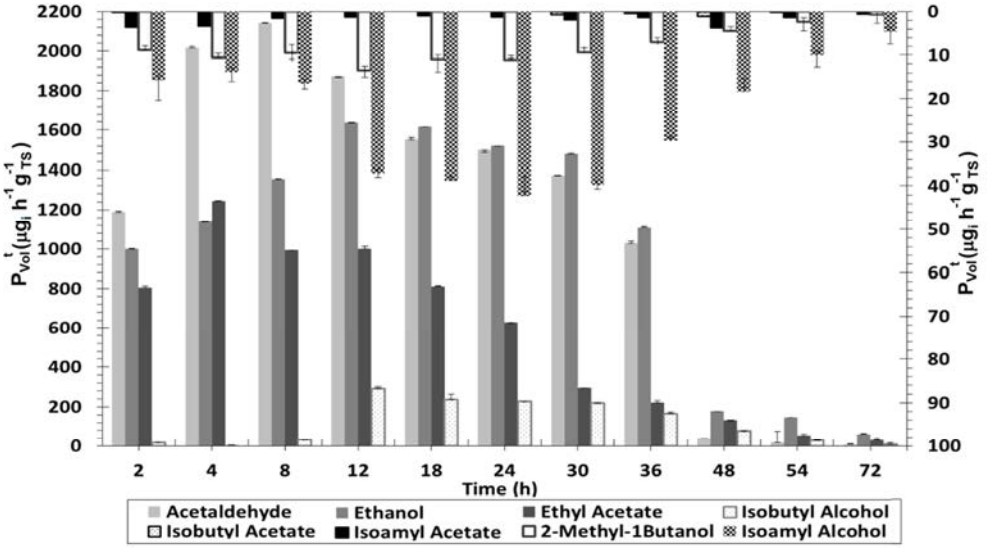


Figure 5.4. Total volatile productivity (P_{vol}^{t}) of the most relevant species (*i*) found during the solid-state fermentation of sugarcane bagasse/sugar beet molasses with *K. marxianus* at 40°C

In this scenario, some synergistic effects occur thanks to the high-temperature level. First, there is a fast stripping of the volatiles from the solid phase while the organic matter degradation induces a loss of weight. At the same time, this loss is accompanied with an increase in the MC (Figure 5.5(b)), which is reflected in the compression of the solid substrate reaching AFP between 60-63% much lower than the initial one. Figure 5.5(a) also shows how the anaerobic fermentation prevails over the aerobic routes when considering the evolution of the OUR and the CO_2^{p}.

From the respiration analysis, an apparent higher CO_2 production concerning the oxygen consumption is observed, particularly in the first 20 h where the respiration quotient ($RQ=CO_2^{p}/OUR*(32/44)$) reached values as high as 4 in the first 2 h of processing, followed by 11 h of RQ around 3. This suggests a significant deviation from a pure aerobic process (RQ around 1) as in a composting process (Gea et al., 2004). Furthermore, it is also remarkable the effect of temperature on the *K. marxianus* growth.

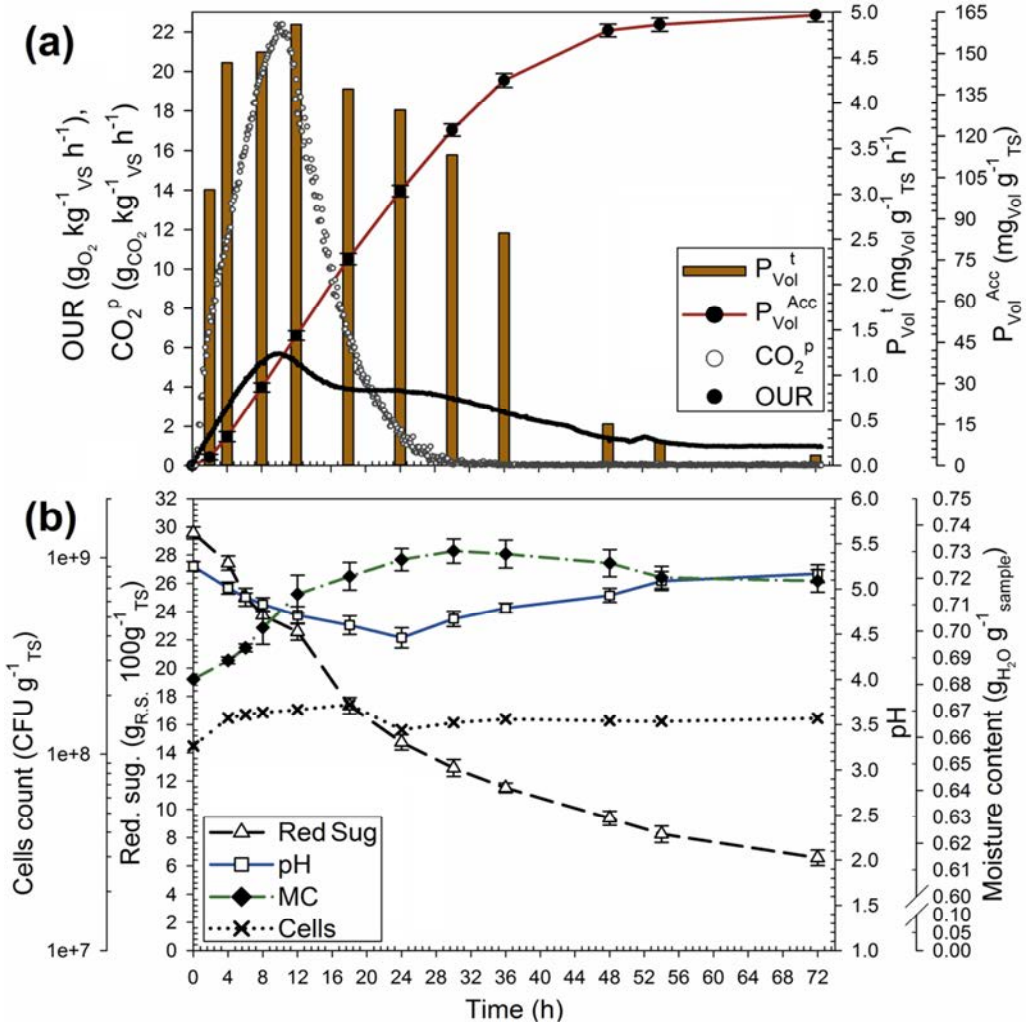


Figure 5.5. Time course of the solid-state fermentation for producing fruit-like aromas at 40°C: (a) OUR, CO_2^p and total cumulative volatile production; (b) pH, MC, cells count and reducing sugars.

As observed in Figure 5.5(b) there was just a subtle growth during the fermentation even when around 45% of the available reducing sugars were consumed until that point. This behavior might indicate that the yeast was under stress conditions, and it was forced to metabolize the carbon source into alcohols, instead of using it for its growth. It is during this period that *K. marxianus* seems to be more active, given the rate of consumption of reducing sugars (0.71 $g_{R.S}$ g^{-1}_{TS} h^{-1}) as well as the evolution of the pH, an indirect indicator of the formation of intermediate species during the degradation of the substrate.

It was precisely at 24 h when pH has reached its minimum value (4.5) and then a further recovery that coincides with the stabilization of other parameters like the MC, the *K. marxianus* growth and the reduced consumption of reducing sugars (0.14 $g_{R.S}$ g^{-}

$^{1}{}_{TS}$ h^{-1}). These results also suggest that RQ can be considered as an indirect parameter for monitoring the selectivity towards ester species, considering the significant effect of an aerobic environment in the promotion of the fruit-like compounds biosynthesis, or the positive effect of an anaerobic environment in the alcohol species bioproduction.

5.2.3.2 Optimization based on the total cumulative ester production

To identify the operational parameters that promote the production of the ester species, the accumulated sum of only these 9 compounds (Table 3.1) was also considered as the response variable. As it can be seen in Figure 5.6(a) and (b), all the evaluated variables have an optimum condition that maximizes the ester content. This optimum is located close to the mid-range of the variables, around 30°C, 25% M_d and 0.11 L h^{-1} $g^{-1}{}_{TS}$ producing a total cumulative ester production of 47.6 mg_{Ester} $g^{-1}{}_{TS}$ after 72 h of fermentation. The model representing the response for $P_{Est}{}^{Acc}$ is presented in equation 16 with an R^2: 99.04%, R^2 (Pred): 86.27% and R^2 (Adj):97.31%.

$$P_{Est}^{Acc} = -45.17 + 4.18T + 289.72S_{AFR} + 1.13M_d - 0.09T^2 - 3198.36S_{AFR}^2 + 0.04{M_d}^2 + 10.02TS_{AFR} + 1.8.10^{-4}TM_d + 5.98S_{AFR}M_d \quad (16)$$

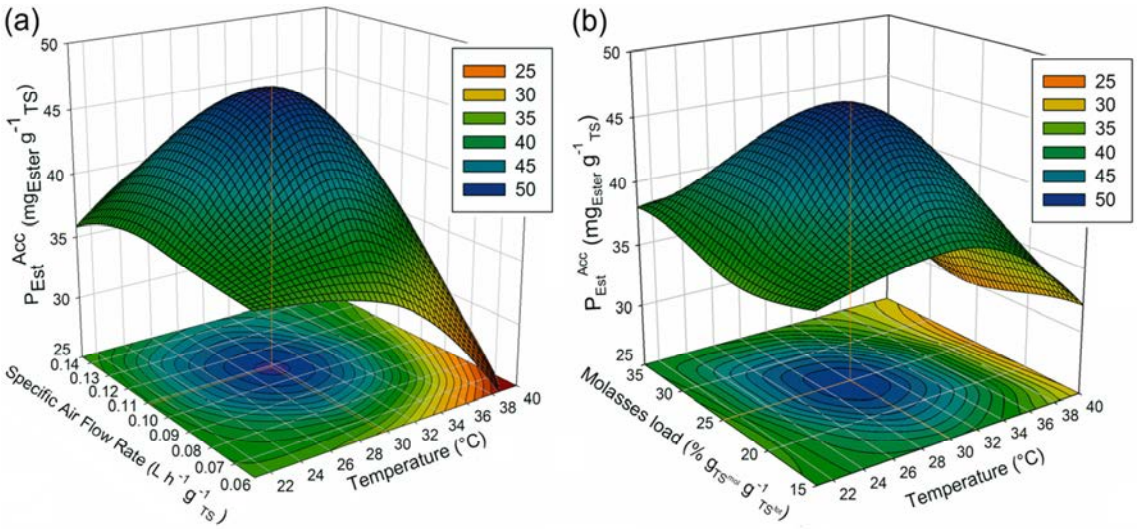


Figure 5.6. Effects on the total cumulative ester production ($P_{Est}{}^{Acc}$) of (a) air flow rate and (b) molasses content as a function of temperature during the solid-state fermentation of sugarcane bagasse/sugar beet molasses with *K. marxianus*.

The marked differences in this scenario respect to P_{Vol}^{Acc} can be better understood analyzing the evolution of the fermentation at these conditions. As Figure 5.7(a) states, working at the optimum for producing ester species, promotes an approaching of the OUR and CO_2^p profiles. Consequently, it is expected a lower RQ trend and therefore a less anaerobic environment during the fermentation. In fact, after the first 4 h of processing RQ stabilized around 2, to then continuously falls from 18 h to 35 h reaching a value close to 1 which was unchanged until the end of the fermentation.

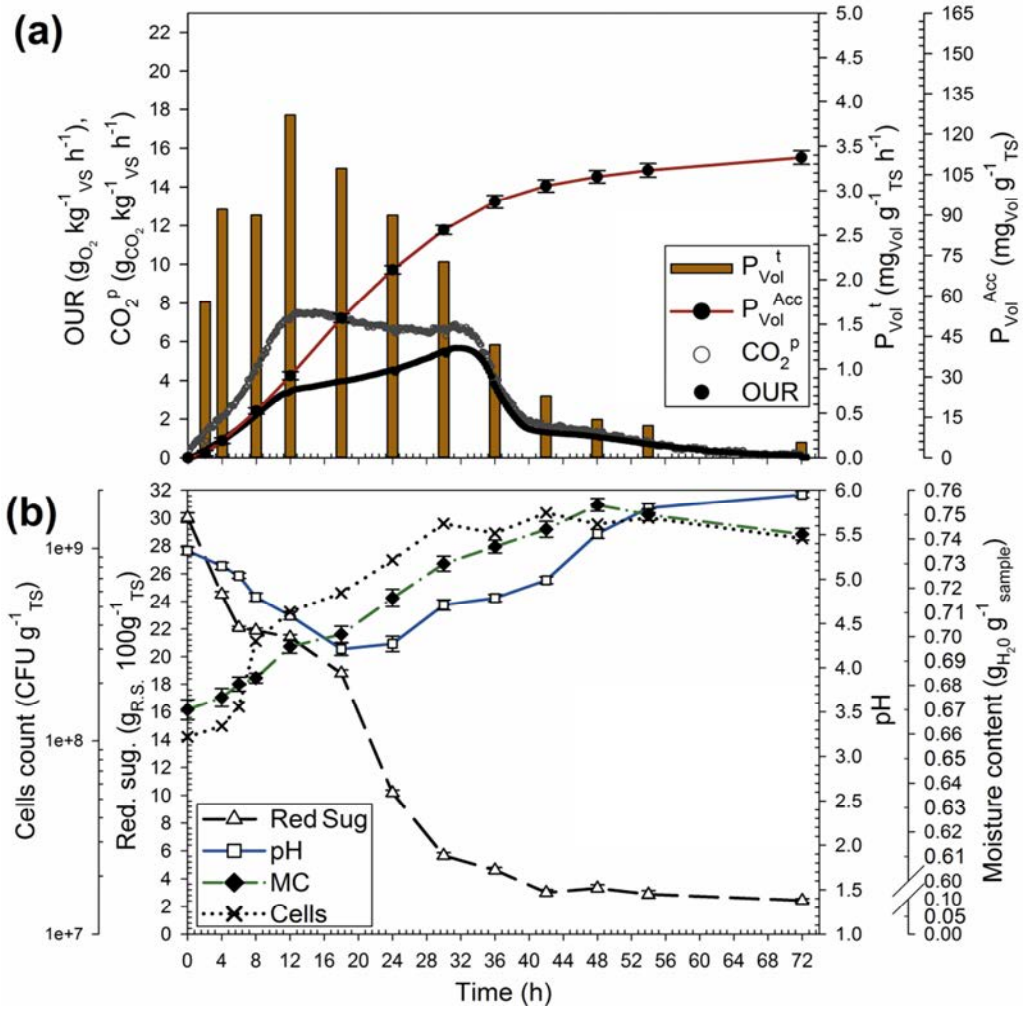


Figure 5.7. Time course of the solid-state fermentation for producing fruit-like aromas at 30°C: (a) OUR, CO_2^p and total cumulative volatile production; (b) pH, MC, cells count and reducing sugars.

As occurred in all the different runs, produced aroma was a strict function of time, but in this scenario, a pleasant fruity odor was detected. The odor became stronger

between the 16 and 30 h of processing, corresponding both to the maximum productivity of volatile compounds as well as the maximum oxygen consumption, similarly to found by other authors (Bramorski et al., 1998b).

This effect has occurred because the composition of the produced aroma is considerably different to that at 40°C. At 30°C, the total ester contribution was 35% (mainly ethyl acetate) followed by acetaldehyde (27%), ethanol (26%) and other alcohols (12%) (Figure 5.8). It was evident that even in this case there is a significant amount of alcohols in the final aroma, which suggests that parallel metabolization paths compete for the available sugar content.

Also, a different picture than the one obtained at 40°C was presented regarding the comportment of the *K. marxianus* in the media. As it can be observed in Figure 5.7(b), under these conditions, the strain was able to use part of the carbon sources for growing up to 1 order of magnitude, almost depleting the available reducing sugars. Similarly to the findings in section 5.2.3.1, pH has fallen at the beginning of the fermentation to a similar level (4.3), but in this case, this drop has matched with the starting of the maximum activity around 18 h. It is also noted that MC has grown at a lower rate than at 40°C, and its stabilization occurred, similarly, after the end of the maximum *K. marxianus* activity (42 h).

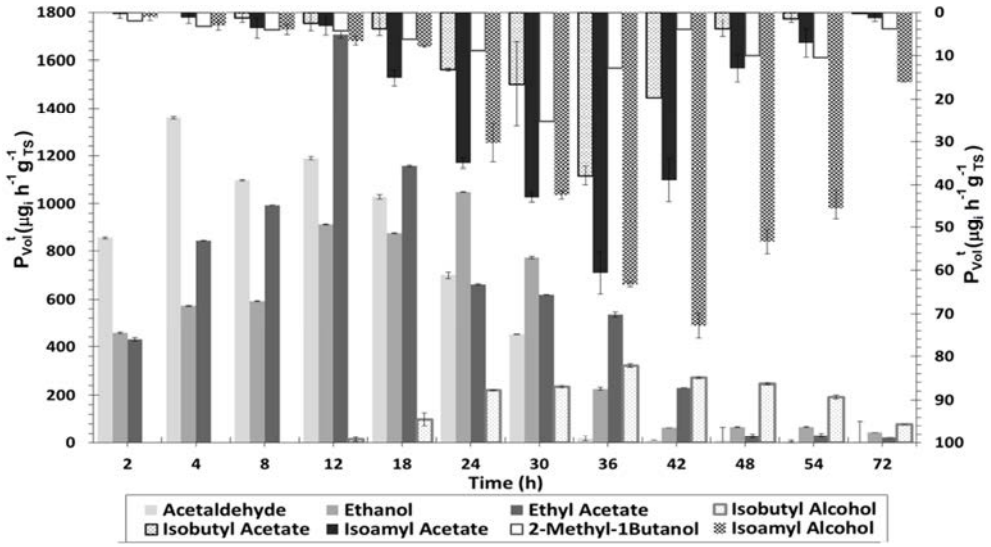


Figure 5.8. Total volatile productivity (P_{vol}^{t}) of the most relevant species (*i*) found during the solid-state fermentation of sugarcane bagasse/sugar beet molasses with *K. marxianus* at 30°C

5.2.4 Respirometric behavior

Since the volatile compounds bioproduction is an indirect consequence of the growing of the strain, in chapter 4 the metabolic activity of *K. marxianus* (measured as OUR) was used as an index of both, the *K. marxianus* growth and volatile compounds production. Since the preliminary results of the variable screening suggested that the assumed relationship was not such linear, in this section a brief description of the respirometric indices has been detailed. Figure 5.9 shows the surface plots for OUR_{Max} (a) and the COC (b) obtained by means of the data compiled for the optimization process, but using as response variables the OUR_{Max} and the COC respectively.

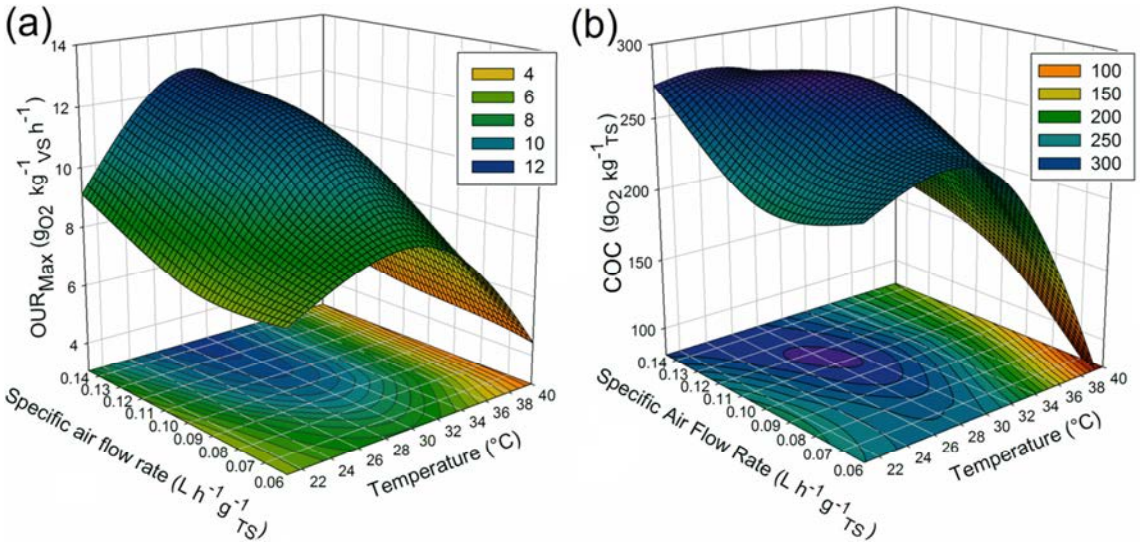


Figure 5.9. Effects of the air flow rate and temperature on (a) maximum oxygen uptake rate (OUR_{Max}) and (b) cumulative oxygen consumption (COC) during the solid-state fermentation of sugarcane bagasse/sugar beet molasses with *K. marxianus* for producing fruit-like aroma compounds.

As observed, there is a clear trend of high OUR_{Max} in the nearby of 30°C independently of the S_{AFR}, coinciding with the optimum temperature for the growing of *K. marxianus* as detailed in some prior investigations (Aggelopoulos et al., 2014; Medeiros et al., 2000, 2001). Besides, OUR_{Max} increases almost linearly with the increase of S_{AFR} until it reaches approx. 0.10 L h^{-1} g^{-1}_{TS}. At that point and beyond, OUR_{Max} remains almost unchanged reaching a mean value of 12.5 g_{O2} kg^{-1}_{VS} h^{-1}. These results suggest that the S_{AFR} does not influence the microbial activity once a minimum condition is achieved, similarly than occurred in another similar biological processes (Almeira et al., 2015; Mejías et al., 2017). In the same way, a similar trend has been

found for COC. In this case, the highest values are also obtained around 28-30°C, and the S_{AFR} only significantly affect this variable in the region from 0.06 and 0.105 L h^{-1} g^{-1}_{TS}. In the upper part of the interval, COC remains almost unaffected with values in the order of 270 g_{O2} kg^{-1}_{TS}.

The results corroborate that the respirometric indices have a different behavior compared the bioproduction of the volatile compounds, and therefore, they cannot be used to estimate the potential production of these compounds. However, they still provide significant information on the fermentation performance, used to understand the behavior of the global process.

5.2.5 C:N ratio effect

This set of experiments was performed to identify the influence of the nitrogen content in the substrate mixture on the production and selectivity of the volatile compounds. As stated in previous studies (Bramorski et al., 1998b; Soares et al., 2000a), C:N ratio directly affects the growth of the strain, and therefore it has a marked influence in the biosynthesis of these secondary metabolites. With this purpose, the C:N ratio was changed from the reference condition using the mixture SCB:SBM (7.5:2.5 w:w, C:N: 58), until a C:N of 13 supplementing the mixture with yeast extract as suggested by Gethins et al. (2015). These changes were assessed for both scenarios exposed in sections 5.2.3.1 and 5.2.3.2 keeping the same operational conditions, and results were expressed per gram of initial substrate added (g_{sub}) (Not including the mass of yeast extract added).

As observed in Figure 5.10, high nitrogen contents seem to be deleterious for the production of the fruit-like compounds. This trend is specially marked when working at 40°C, where alcohols were the most affected, getting a P_{Vol}^{Acc} just of 48% of the value achieved at the reference condition of C:N 58. At 30°C, P_{Vol}^{Acc} had no significant changes in the C:N interval from 13 to 39. Under these conditions, it is notable that some other fruity species appeared like ethyl butanoate, amyl formate or isoamyl butyrate, but accompanied with pungent compounds like 2-methyl pyrazine, crotonaldehyde, vinyl acetate and diacetyl.

As a consequence, the feeling of the produced aroma has evolved from a pleasant fruity odor to a strong one in the final hours of the fermentation. At the same time, the enhancement of other aroma compounds typically amino acid derived, like the

fusel alcohols 2-phenethyl alcohol and Isoamyl acetate was evident, indicating the presence of alternative metabolic routes like the Ehrlich pathway, were used by the *K. marxianus* to produce these valuable compounds (Gethins et al., 2015).

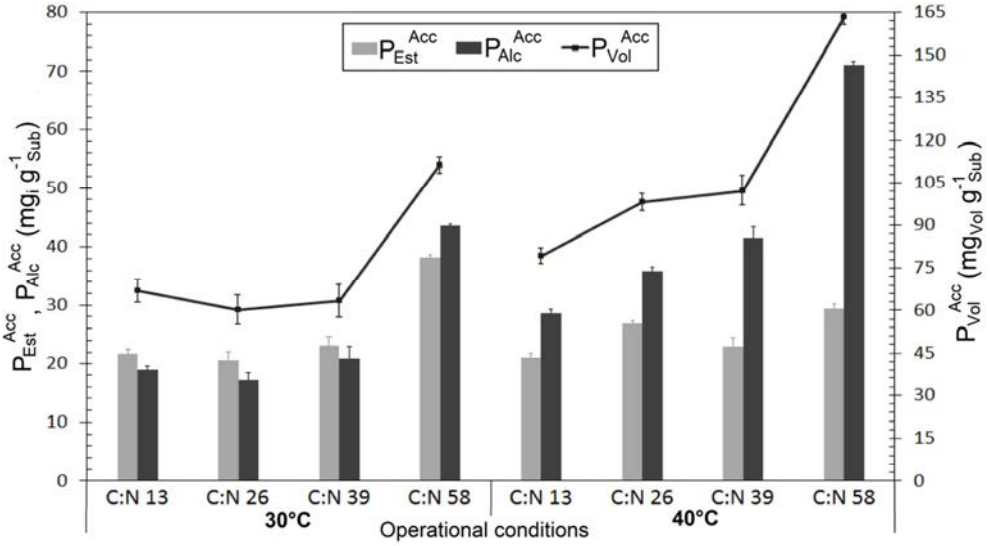


Figure 5.10. Effect of C:N ratio on the fruit-like compounds production. P_{Est}^{Acc}: total cumulative ester production; P_{Vol}^{Acc}: total cumulative volatile production; P_{Alc}^{Acc}: total cumulative alcohols production.

On the other hand, additional nitrogen has promoted a better growth of *K. marxianus*,both at 30°C and 40°C, reaching a maximum cell count 3.5 times higher than the obtained with the reference substrate, agreeing with some author findings (Bramorski et al., 1998a; Soares et al., 2000a). These results indicate that similar to the high temperatures effect, stressing conditions like the scarcity of a nutrient are key factors for promoting the production of the fruit-like compounds by *K. marxianus*.

Final remarks

K. marxianus has proven its efficiency for the biosynthesis of volatile compounds of fruit-like characteristics using only agro-industrial residues as sole substrate for the solid-state fermentation. The optimized batch SSF has shown promising prospects for the further development of the bioprocess. In fact, results here presented constitute the base for the developments shown in the subsequent chapters.

References

Aggelopoulos, T., Katsieris, K., Bekatorou, A., Pandey, A., Banat, I.M., Koutinas, A.A., 2014. Solid state fermentation of food waste mixtures for single cell protein, aroma volatiles and fat production. Food Chem. 145, 710–716.

Almeira, N., Komilis, D., Barrena, R., Gea, T., Sánchez, A., 2015. The importance of aeration mode and flow rate in the determination of the biological activity and stability of organic wastes by respiration indices. Bioresour. Technol. 196, 256–262.

Besson, I., Creuly, C., Gros, J.B., Larroche, C., 1997. Pyrazine production by *Bacillus subtilis* in solid-state fermentation on soybeans. Appl. Microbiol. Biotechnol. 47, 489–495.

Bramorski, A., Christen, P., Ramirez, M., Soccol, C.R., Revah, S., 1998a. Production of volatile compounds by the edible fungus *Rhizopus oryzae* during SSF on tropical agro-industrial substrates. Biotechnol. Lett. 20, 359–362.

Bramorski, A., Soccol, C.R., Christen, P., Revah, S., 1998b. Fruity Aroma production by *Ceratocystis Fimbriata* in solid cultures from agro-industrial wastes. Rev. Microbiol. 29, 3–11.

Christen, P., Meza, J.C., Revah, S., 1997. Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*: influence of the substrate type and the presence of precursors. Mycol. Res. 101, 911–919.

De Aráujo, Á., Pastore, G.M., Berger, R.G., 2002. Production of coconut aroma by fungi cultivation in solid-state fermentation. Appl. Biochem. Biotechnol. 98–100, 747–751.

Gea, T., Barrena, R., Artola, A., Sánchez, A., 2004. Monitoring the biological activity of the composting process: Oxygen uptake rate (OUR), respirometric index (RI), and respiratory quotient (RQ). Biotechnol. Bioeng. 88, 520–527.

Gethins, L., Guneser, O., Demirkol, A., Rea, M., Stanton, C., Ross, P., Yuceer, Y.K., Morrissey, J.P., 2015. Influence of carbon and nitrogen source on production of volatile fragrance and flavour metabolites by the yeast *Kluyveromyces Marxianus*. Yeast 32, 67–76.

Ito, K., Yoshida, K., Ishikawa, T., Shinya, K., 1990. Volatile compounds produced by the fungus *Aspergillus oryzae* in rice Koji and their changes during cultivation. J. Ferment. Bioeng. 70, 169–172.

Jiménez-Peñalver, P., Gea, T., Sánchez, A., Font, X., 2016. Production of sophorolipids from winterization oil cake by Solid-state fermentation: Optimization, monitoring and effect of mixing. Biochem. Eng. J. 115, 93–100.

Medeiros, A., Pandey, A., Freitas, R., Christen, P., Soccol, C.R., 2000. Optimization of the production of aroma compounds by *Kluyveromyces marxianus* in solid-state fermentation using factorial design and response surface methodology. Biochem. Eng. J. 6, 33–39.

Medeiros, A.B.P., Pandey, A., Christen, P., Fontoura, P.S.G., de Freitas, R.J.S., Soccol, C.R., 2001. Aroma compounds produced by *Kluyveromyces marxianus* in solid

state fermentation on a packed bed column bioreactor. World J. Microbiol. Biotechnol. 17, 767–771.

Mejías, L., Komilis, D., Gea, T., Sánchez, A., 2017. The effect of airflow rates and aeration mode on the respiration activity of four organic wastes: Implications on the composting process. Waste Manag. 65, 22–28.

Razmovski, R., Vučurović, V., 2012. Bioethanol production from sugar beet molasses and thick juice using *Saccharomyces cerevisiae* immobilized on maize stem ground tissue. Fuel 92, 1–8.

Rossi, S.C., Vandenberghe, L.P.S., Pereira, B.M.P., Gago, F.D., Rizzolo, J.A., Pandey, A., Soccol, C.R., Medeiros, A.B.P., 2009. Improving fruity aroma production by fungi in SSF using citric pulp. Food Res. Int. 42, 484–486.

Soares, M., Christen, P., Pandey, A., Raimbault, M., Soccol, C.R., 2000. A novel approach for the production of natural aroma compounds using agro-industrial residue. Bioprocess Eng. 23, 695–699.

Chapter 6

Enhancing the bioproduction of fruit-like compounds via SSF: Operational strategies and scaling-up of the process

Part of this chapter has been published in: Martínez, O., Sánchez, A., Font, X., Barrena, R. 2018. 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. Bioresource Technology. 263, 136-144.

6.1 Overview

As stated in previous chapters, the efforts made to verify the feasibility of SSF for producing aroma compounds have been 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; Carlquist et al., 2015). Also, in chapter 5 the importance of the operating conditions on production and selectivity of the volatile compounds has become clear for a batch process. However, there is no information about the effect of the operational strategies (operating modes) on the volatile compounds generation via SSF, and even less reported studied working at non-sterilized conditions.

In general, reported systems are limited to those in which the sterilized substrates are completely loaded at the beginning of the process and considering that the solid media remains static during the fermentation time (a static-batch). Since the availability of nutrients is also a crucial parameter to the products selectivity in SSF processes (Raimbault, 1998), the way the fermentation is performed, could serve as a tool to modify this availability along the process. Having this in mind, understanding the effects of the operating mode becomes essential for the future development of this bioprocess.

In the same way, the scale-up effect is a typical disadvantage of SSF process due to the heat and mass transfer phenomena presented in the solid-liquid-gas interphases (Cerda et al., 2017b; Soccol et al., 2017), hampering the development of the technology at industrial scale. Since the behavior of the fermentation changes because of these effects, production and selectivity are prone to be affected as well, making more relevant the implementation of alternative operating approaches able to minimize these adverse effects (Astolfi et al., 2011).

In this chapter, the assessment of some operational strategies such as static-batch, intermittent mixing and fed-batch was performed. Here, the aim was to enhance the production of the fruit-like compounds by using these alternative strategies. Also, it was detailed how these strategies affect the selectivity of the produced ester species. With this purpose, they 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. Table 6.1 contains the main description of the performed experiments.

Table 6.1. 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. 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 (FB) 50% was performed only at 0.5 L scale. [b]Mixing points were defined based on the temperature profile; once the maximum temperature was achieved a mixing was performed.

6.2 Results

Analysis of the process behavior was performed by means of the same performance indices used in chapter 5 (P_{vol}^{Acc} and P_{Est}^{Acc}), the space-time yield ($Y_{s\text{-}t}$) [$mg_{vol}\ L^{-1}\ h^{-1}$] (computed as the total productivity per reactor volume), the total air consumption (C_{air}) [$NL_{air}\ g^{-1}{}_{PTS}$] (the volume of air consumed per gram of processed substrate in dry basis (g_{PTS})) and the inoculum requirement (I_{Req}) [CFU $g^{-1}{}_{PTS}$] (Corresponding to the colony forming units of *K. marxianus* needed per gram of processed substrate in dry basis (g_{PTS})). Table 6.2 summarizes some of the main performance indices obtained for the different strategies at the selected scales.

Table 6.2. Main performance parameters of the solid-state fermentation 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}_{TS})	106.3	129.6	118.0
	P_{Est}^{Acc} (mg_{Ester} g^{-1}_{TS})	38.3	24.7	30.4
	C_{air} (NL_{air} g^{-1}_{PTS})	7.4	7.5	8.0
	COC (g_{O2} kg^{-1}_{TS})	254.8	238.0	217.8
Intermittent mixing	P_{vol}^{Acc} (mg_{Vol} g^{-1}_{TS})	105.4	122.4	121.3
	P_{Est}^{Acc} (mg_{Ester} g^{-1}_{TS})	38.6	36.3	35.9
	C_{air} (NL_{air} g^{-1}_{PTS})	7.4	7.5	8.0
	COC (g_{O2} kg^{-1}_{TS})	232.1	230.4	234.3
Fed-batch 33%	P_{vol}^{Acc} (mg_{Vol} g^{-1}_{TS})	109.3	129.3	120.3
	P_{Est}^{Acc} (mg_{Ester} g^{-1}_{TS})	49.3	43.5	57.1
	C_{air} (NL_{air} g^{-1}_{PTS})	5.5	6.7	5.6
	COC (g_{O2} kg^{-1}_{TS})	310.0	388.1	442.6

P_{vol}^{Acc}: Total cumulative volatile production, P_{Est}^{Acc}: total cumulative ester production, C_{air}: total air consumption of the strategy per gram of processed substrate, COC: cumulative oxygen consumption.

6.2.1 Process behavior at lab scale

These experiments consisted of evaluating the effect of the operational strategies shown in Table 6.1 on the P_{vol}^{Acc} as well as on the selectivity to the ester species (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}_{TS}, 20% (dry basis) and 10^8 CFU g^{-1}_{TS} respectively according to the results found in chapter 5.

Figure 6.1 summarizes some of the performance indices computed for the lab scale experiments. 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}_{TS}. 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 at a constant temperature, 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 6.1, when the production is split into the three major groups of obtained species (aldehydes, alcohols and esters) there is a change in the

contribution of these species to P_{vol}^{Acc} depending on the selected strategy. Thus, in the reference scenario (static-batch), the mean contribution comes from the alcohol species (39.0±0.1%), followed by the esters (36.0±0.1%) and finally the aldehydes (25.0±0.1%).

When the intermittent mixing is used, the contribution has the same pattern, but there is a subtle increase in the amount of aldehydes and esters with the corresponding lower alcohol production. Then, when a fed-batch strategy is used, the trend changes, and this time the primary contribution is due to the ester species, with similar amounts of alcohols and aldehydes (around 27% each). Based on these results it can be stated that splitting the substrate load is an efficient way to limit the available nutrients in the media, forcing *K. marxianus* to use them to metabolize the ester species preferentially.

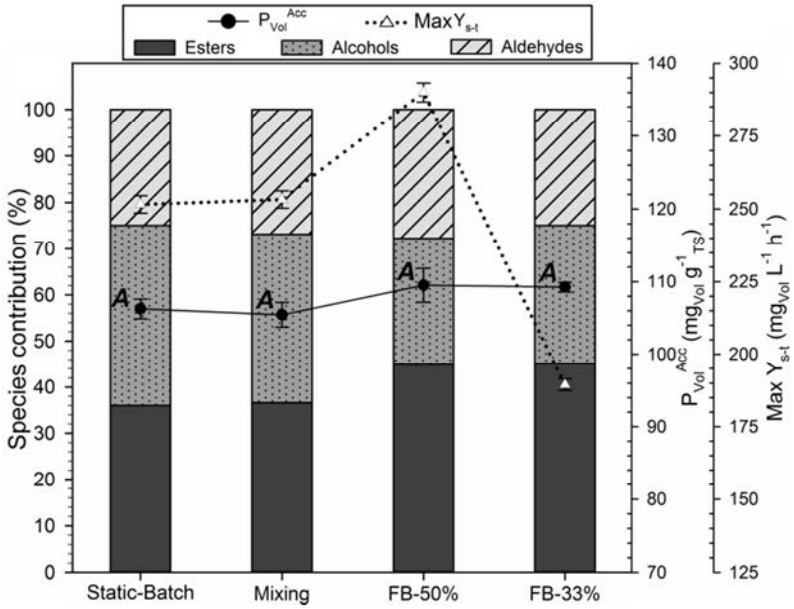


Figure 6.1. Performance indices of the evaluated operational strategies at 0.5 L and constant temperature. P_{Vol}^{Acc}: total cumulative volatile production, Max Y_{s-t}: maximum space-time yield, FB-50%: fed-batch operation with two splits, FB-33%: fed-batch operation with three splits. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$).

In Figure 6.2, the time course of the SSF at 0.5 L and constant temperature in static-batch (a), intermittent mixing (b) and fed-batch 33% (c) modes can be observed. The batch SSF shows a rapid increase of activity (measured as OUR) until 24 h of processing when the maximum activity is achieved, coinciding with the depletion of available sugars. At that point, volatile production almost ceases, so the maximum Y_{s-t}

is also achieved. A similar trend is found for the intermittent mixing, but this time, OUR_{Max} is reached at 20 h, when most of the available sugars have been consumed, and more than 90% of the total fruit-like compounds have been produced. This rapid development has allowed to these strategies reaching Y_{s-t} as high as 250 mg_{Vol} L^{-1} h^{-1}.

In the fed-batch strategy, the process starts similarly than in a static mode with a rapid growth until the maximum activity is achieved around 24 h. 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 seems to promote 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 and intermittent mixing modes is achieved. Therefore, a significant decrease in Y_{s-t} is observed (Figure 6.1 and Figure 6.2(c)).

As seen in Table 6.2, the intermittent mixing has produced negligible changes regarding P_{Vol}^{Acc} and P_{EstAcc} compared to the batch process. Although it is expected an improvement in the interaction of inoculum and nutrients when using mixing, it seems the enhancement is limited due to the scale. In this sense, as observed in Figure 6.2(a) and 2(b), the time course of both processes are quite similar, and it is possible that at this scale in the batch mode, *K. marxianus* was able to reach the available nutrients without any significant constraint.

On the other hand, 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 6.2(c), the maximum activity was achieved after the two successive additions of fresh material (21.3 g_{02} kg^{-1}_{VS} h^{-1}), 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 nutrients content 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) (The mean error for the *K. marxianus* population was less than 5% in the triplicate of each measure).

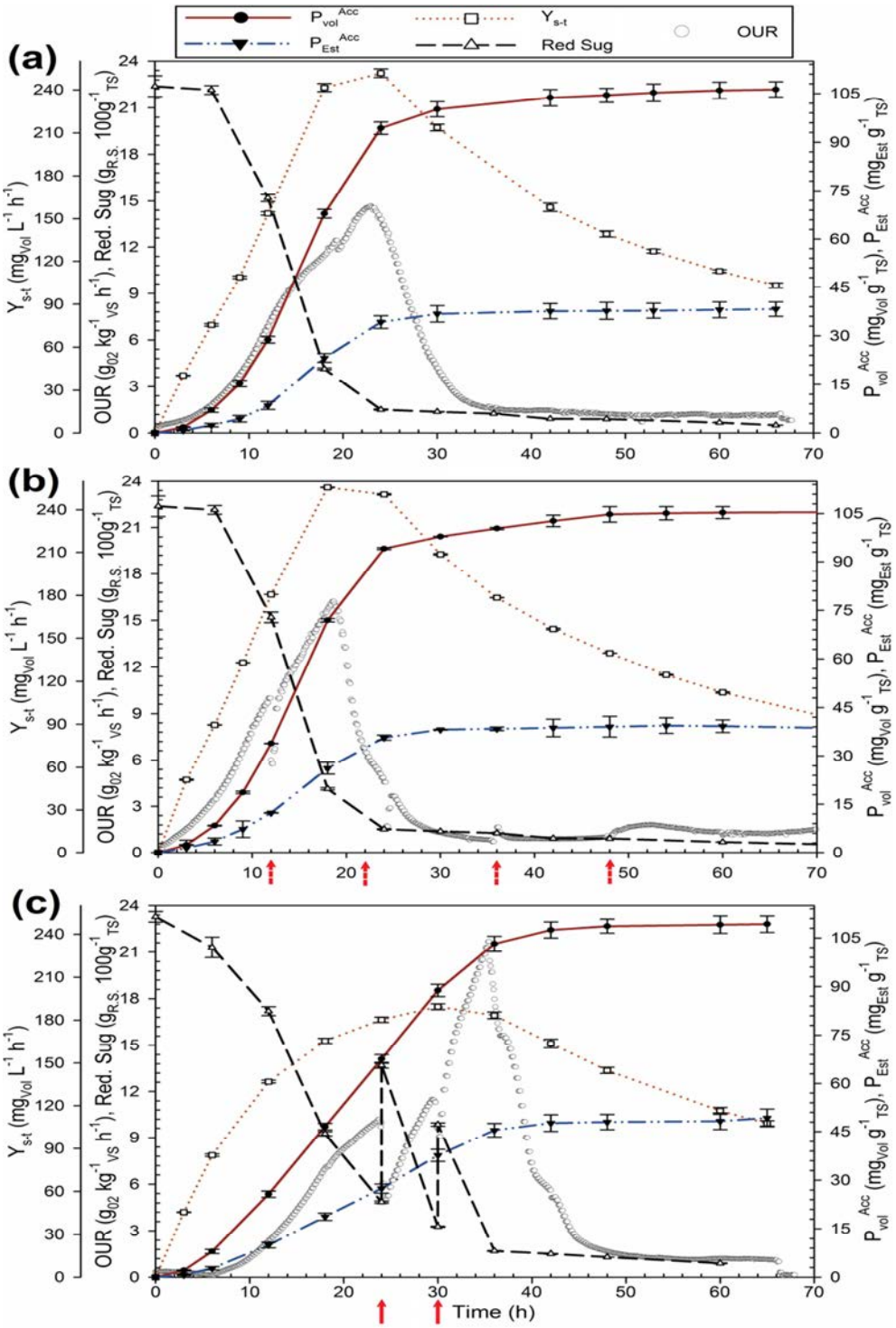


Figure 6.2. Time course of the solid-state fermentation of sugarcane bagasse/sugar beet molasses for producing fruit-like compounds at 0.5 L scale. (a) static-batch, (b) intermittent mixing (c) fed-batch 33%. Continuous arrows indicate the addition of fresh substrate in the fed-batch strategy, while dash arrows indicate the mixing points.

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 oxygen (as shown in chapter 5), and therefore, it would limit the biotransformation. When a fed-batch strategy is used, compression of the media is reduced compared to a batch mode since the partial feeding implies a mixing in which the bed is readjusted to its initial condition, and porosity of the bed is partially recovered.

Another aspect of the fermentation influenced by the strategy is the Crabtree effect. Because of this effect, in a static-batch SSF, the yeast can use the available sugars to produce ethanol even under aerobic conditions, spending a considerable amount of nutrients on it and limiting the ester production. In a fed-batch strategy, this aspect is compensated when the fresh substrate is added since the global effect is a dilution of the available nutrients (Carvalho et al., 2006). By doing this, similarly than in a submerged fermentation (SmF), the amount of sugars in the media is kept lower than in a static-batch SSF regards the number of cells growing in the media (Figure 6.3).

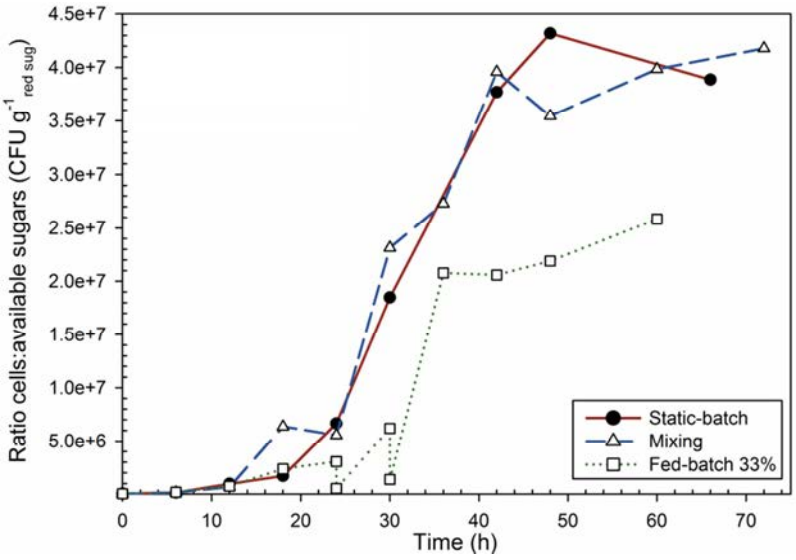


Figure 6.3. Ratio *K. marxianus* cells:Available sugars for the solid-state fermentation of sugarcane bagasse/sugar beet molasses at 0.5 L.

Therefore, *K. marxianus* becomes less prone to produce ethanol due to the Crabtree effect. Even when some authors suggest *K. marxianus* is Crabtree negative (Fonseca et al., 2008), many references indicate the opposite (Etschmann et al., 2003;

Garavaglia et al., 2007; Lane and Morrissey, 2010) and given the results of this study; it is clear that the studied strain is part of the group of Crabtree positive yeast.

6.2.2 Process in a 4.5 L near-isolated reactor

This set of experiments consisted of evaluating the effect of the operational strategies on the P_{vol}^{Acc} and P_{Est}^{Acc} of the SSF considering the effect of working with variable temperature in near-isolated reactors. By doing this, the process was assessed resembling a typical large-scale SSF or composting operation, in which the mass of 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) serves as one of the driving force for running the fermentation. Initial pH, MC, S_{AFR}, molasses load, and inoculum load were set at 5.3, 68%, 0.11 L h^{-1} g^{-1}_{TS}, 20% (dry basis) and 10^8 CFU g^{-1}_{TS} respectively as done in section 6.2.1.

Figure 6.4 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}_{TS} 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 than the one found in section 6.2.1. As observed, the static-batch operation promotes mainly the production of alcohols (44.3±0.1%) and just a 19.1±0.1% of esters. Once the intermittent mixing is integrated, the distribution changes, inducing an increase in ester accumulation (29.6±0.1%), with the corresponding reduction in alcohols species (40.1±0.1%). Nonetheless, the most significant changes occur when the process is operated in fed-batch mode. In that case, the percentage of ester species reaches 33.7±0.1% while alcohols are reduced until 37.4±0.1%.

In this scenario, the operational strategies affect the same factors aforementioned in section 6.2.1, but in addition, they also influence the temperature profile of the solid media, causing significant changes in the development of the fermentation. As seen in Figure 6.5(a), the time course 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^{-1}_{VS} h^{-1}) and

temperature (48°C) are achieved. Then, a second peak is presented at 64 h after a period of lower activity reaching up to 6 g_{02} kg^{-1}_{VS} h^{-1} and 42°C, moment in which the process starts decaying until its end.

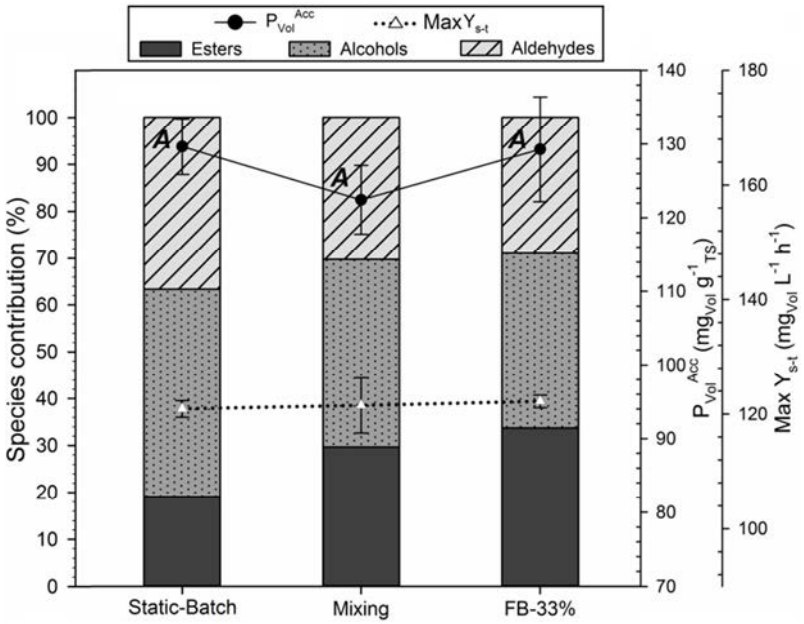


Figure 6.4. Performance indices of the evaluated operational strategies in the near-isolated 4.5 L reactor. P_{Vol}^{Acc}: total cumulative volatile production, Max Y_{s-t}: maximum space-time yield, FB-33%: fed-batch operation with three splits. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$).

In general, by comparing the batch process at constant temperature with the near-isolated system, evident differences appear. Perhaps the clearest is that in the latter, the ester selectivity suffers a vast change since it is a direct function of temperature as stated in chapter 5. In fact, since in the latter scenario the fermentation proceeds most of the time at temperatures above 38°C (during 40.7 h), *K. marxianus* is prone to produce alcohol species preferentially.

When using intermittent mixing, the first 18 h of processing are analogous to the batch process, reaching almost the same OUR_{Max} and temperature after 16 h. At that point, intermittent mixing has as global effect punctual reductions of temperature which allow *K. marxianus* remaining more active than in batch mode until 60 h. This higher activity has no significant consequences in the total volatile productivity (and therefore, in Y_{s-t}), but it has significant effects on the ester productivity (Figure 6.5(b)). In the end, using intermittent mixing enhances the ester content presumably, due to the higher *K. marxianus* activity, as it was exposed in section 6.2.1.

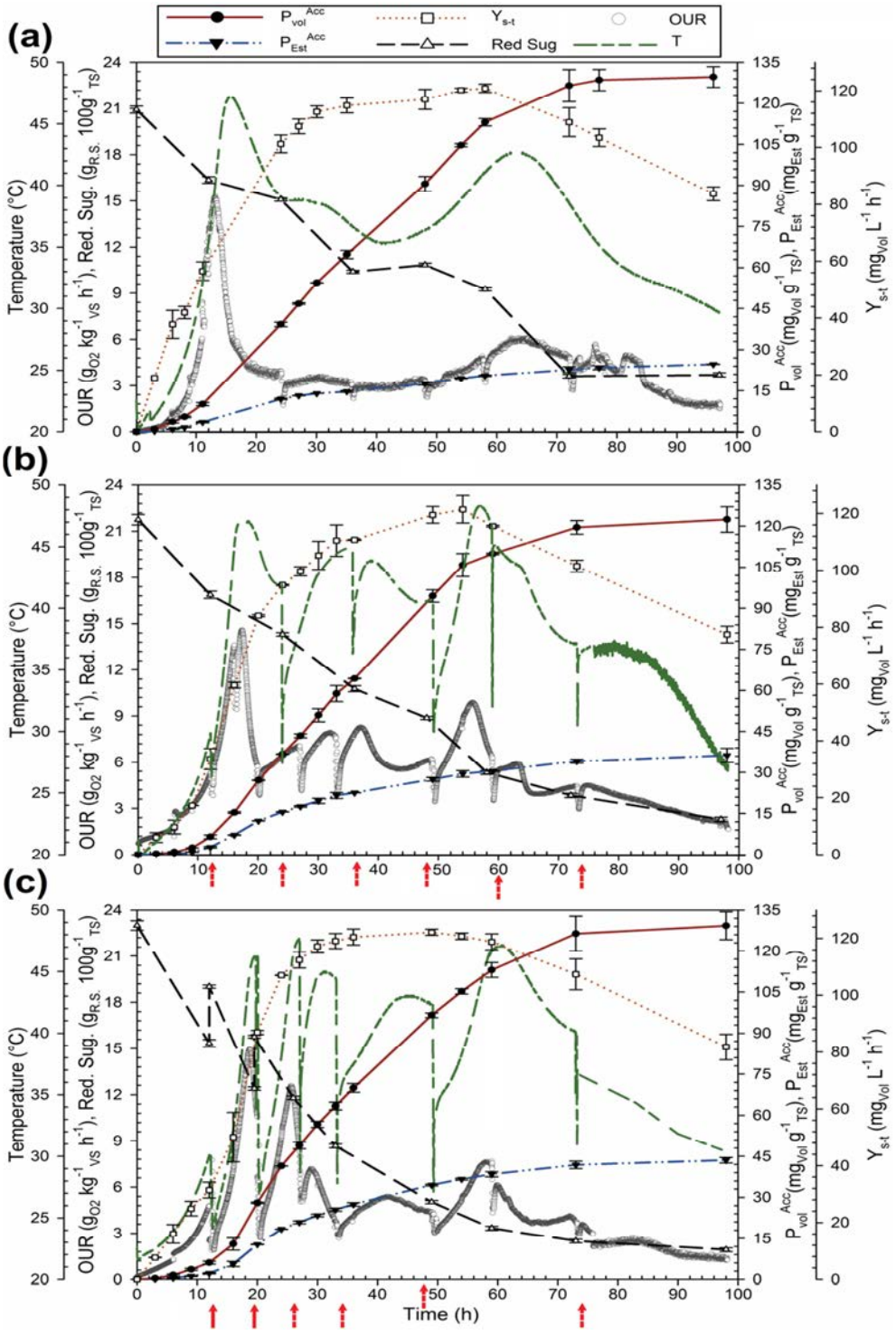


Figure 6.5. Time course of the solid-state fermentation of sugarcane bagasse/sugar beet molasses for producing aroma compounds at 4.5 L scale. (a) static-batch, (b) intermittent mixing (c) fed-batch 33%. Continuous arrows indicate the addition of fresh substrate in the fed-batch strategy, while dash arrows indicate the mixing points

In the same way, when the fed-batch strategy is used at the near-isolated conditions, the feeding points (at 12 and 19.5 h) help to reduce temperature in around

20°C, allowing *K. marxianus* to grow at temperature below 35°C for more extended periods of time than in a static mode (22 h against 14 h) (Figure 6.5(c)). Consequently, the accumulation of esters becomes not just a function of the substrate split as occurred at constant temperature, but mainly a function of the fermentation temperature. Thus, P_{Est}^{Acc} can be improved keeping temperature controlled along the fermentation. In this case, since two additions of fresh material are not enough to control this variable, successive mixing where carried out each time temperature has achieved a maximum (mixing without any substrate addition) to reduce the temperature of the media (Figure 6.5(c)).

By doing this, a higher activity for the *K. marxianus* and the consequent higher production of ester species was obtained. Since the operational strategy by itself is not good enough to control the main variable affecting the adiabatic process (temperature), a combination of operational strategy and reactor design could be another approach to consider for large-scale applications.

6.2.3 Process in a 22 L non-isolated reactor

Experiments in this section involved the evaluation of the operational strategies on P_{vol}^{Acc} and P_{Est}^{Acc} considering the effect of working with variable temperature in a non-isolated steel 22 L reactor with automated mixing. Initial pH, MC, S_{AFR}, molasses load, and inoculum load were set at 5.3, 66%, 0.11 L h^{-1} g^{-1}_{TS}, 20% (dry basis) and 10^8 CFU g^{-1}_{TS} respectively. Figure 6.6 shows the performance indices and the distribution of the produced species for the different operational strategies at 22 L scale. As seen, there are no significant differences among the P_{vol}^{Acc} for the assessed strategies, reaching a mean of 119.9 mg_{vol} g^{-1}_{TS}. Also, the distribution of aldehydes, alcohols and ester species follows a similar trend when it is compared among the strategies.

In the static-batch mode, the major components of the produced volatiles are alcohols (40.9±0.1%), then aldehydes (33.3±0.1%) and finally esters (25.8±0.1%). Using intermittent mixing the final distribution changes and, this time, aldehydes are the main components (38.5±0.1%), followed by alcohols (31.9±0.1%) and esters (29.6±0.1%). In the case of fed-batch operation, the trend suddenly changes, and 47.5±0.1% of P_{vol}^{Acc} become ester species, while alcohols and aldehydes are almost equally distributed with 25.5±0.1% and 27.0±0.1% respectively.

These results corroborate the trend found in the previous sections in which the introduction of more sophisticated operational strategies mainly improves the selectivity of the obtained volatiles, enhancing the production of the ester species, those with a higher value-added regarding the contribution to the fruity aroma profile. At this scale, a static-batch SSF presents a time course (Figure 6.7(a)) starting with 11 h lag phase, the time required to *K. marxianus* to increase the temperature of the media from 20°C to 24°C.

At that point, a rapid growth allows reaching the maximum activity and 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 activity, coinciding with the highest Y_{s-t} and the depletion of the available sugars.

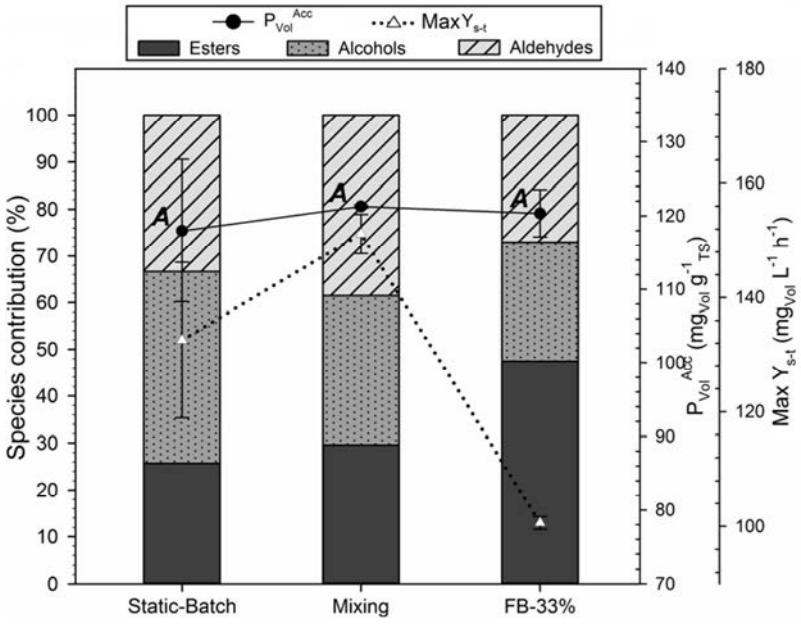


Figure 6.6. Performance indices of the evaluated operational strategies in the non-isolated 22 L reactor. P_{Vol}^{Acc}: total cumulative volatile production, Max Y_{s-t}: maximum space-time yield, FB-33%: fed-batch operation with three splits. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$).

In general, at 22 L scale, the intermittent mixing has a similar trend than in batch mode (Figure 6.7(b)). As observed, after a lag phase period, a rapid growth allows achieving the OUR_{Max} (14.4 g_{02} kg^{-1}_{VS} h^{-1}) and maximum temperature (38.6°C) around

20 h after the beginning of the fermentation. Then, a second peak of activity is found at 35 h, and a sudden fall of activity induces the end of the process after 50 h of processing. In this strategy, although the highest activity was reached later than in batch mode, the total volatile productivity has had a faster development, reaching the maximum $Y_{s\text{-}t}$ just a couple of hours after the first peak of activity (24 h). In the batch case, this maximum is found at 34 h, close to the second peak of activity. As a consequence, using intermittent mixing at these conditions helps to improve the *K. marxianus* activity, and it allows an increase in the ester production of 18.2 %.

In the fed-batch operation (Figure 6.7(c)), 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 is 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, have allowed the strain to remain active for another 7 h (until 39 h), point when the process arrives at the maximum activity (18 g_{02} kg^{-1}_{VS} h^{-1}) and to 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 sugars availability 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 nutrients are consumed, the process falls 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.

The same aspects affecting the selectivity of esters at constant temperature could be perceived in the non-isolated reactor. Notably, 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).

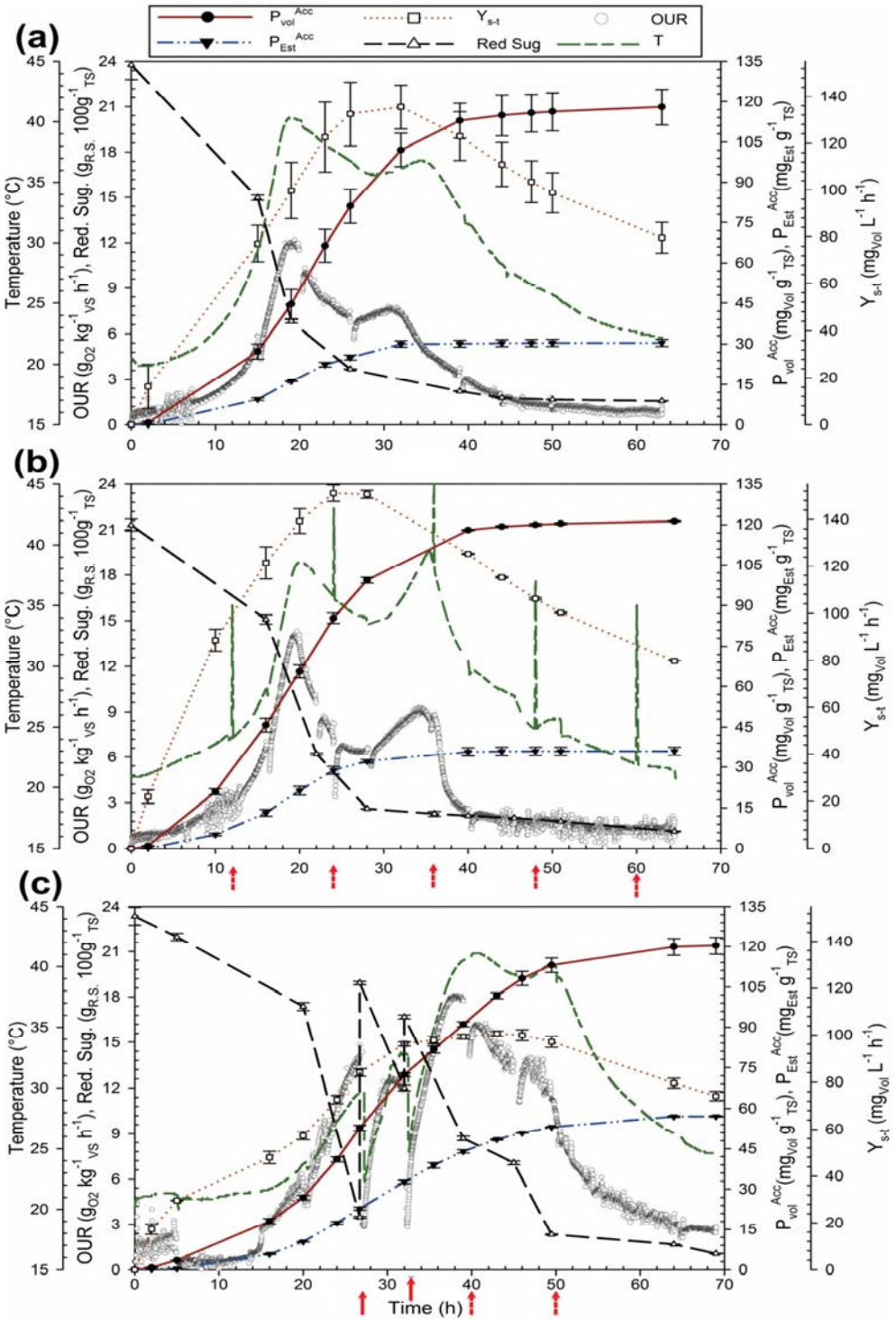


Figure 6.7. Time course of the solid-state fermentation of sugarcane bagasse/sugar beet molasses for producing aroma compounds at 22 L scale. (a) static-batch, (b) intermittent mixing (c) fed-batch 33%. Continuous arrows indicate the addition of fresh substrate in the fed-batch strategy, while dash arrows indicate the mixing points.

Also, using fed-batch operation seems to reduce the Crabtree effect (compared to the static-batch mode), since the available sugars regarding the number of cells growing in the media, resulted in just a fraction of the presented in the static-batch

(Figure 6.8). As seen, in the non-isolated scenario, the fed-batch strategy has positively affected the ester selectivity as occurred at lower scales, confirming its benefits on the process.

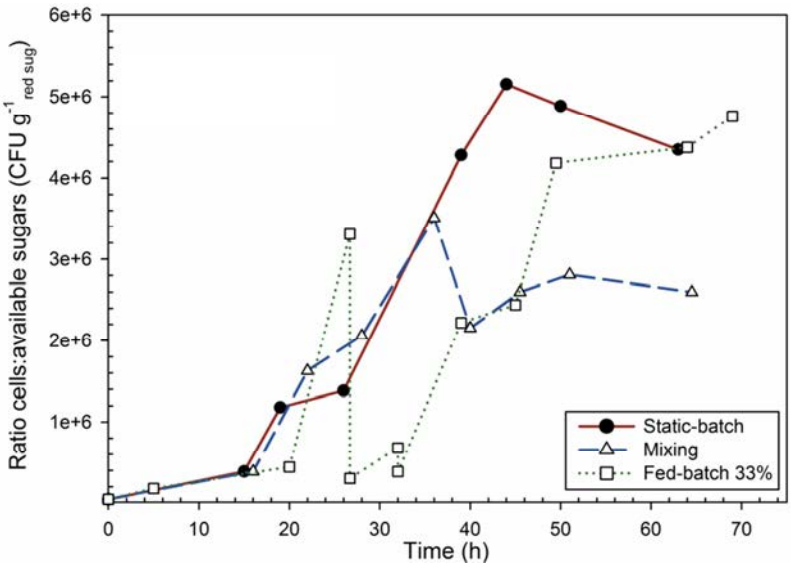


Figure 6.8. Ratio *K. marxianus* cells:Available sugars for the solid-state fermentation of sugarcane bagasse/sugar beet molasses at 22 L scale.

6.2.4 Scaling and operating strategy effects

As detailed in Figure 6.9, 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 in chapter 5. 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 the fed-batch mode shows a higher improvement than in the mixing strategy and, at 22 L it is able to reach a level above the one achieved at 0.5 L.

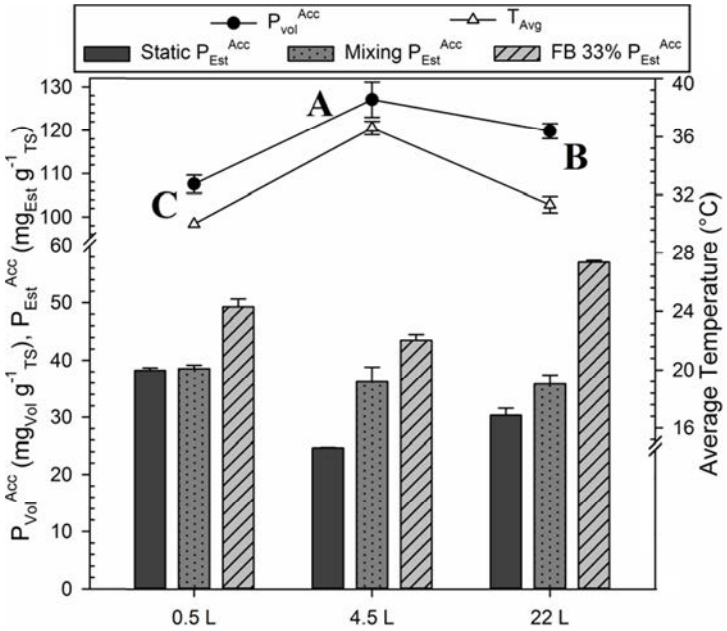


Figure 6.9. Scaling-up effects over the volatile production of the solid-state fermentation of sugarcane bagasse/sugar beet molasses. P_{Vol}^{Acc}: total cumulative volatile production, P_{Est}^{Acc}: total cumulative ester production, T_{Avg}: average temperature of the set of fermentations at each evaluated scale. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$).

Regarding the operating strategies effects, it could be stated that, in general, SSF processes are undertaken in batch mode, and the study of alternative SSF operational strategies has been typically limited. However, the positive results in this study, as well as some other SSF bench-scale applications (Astolfi et al., 2011; Cerda et al., 2017a) using alternative operating strategies, possess in evidence their potential to improve the SSF performance in a reliable and simple way. Particularly, considering the fed-batch strategy, the positive impacts are not limited to the production and selectivity aspects.

As detailed in Table 6.2, despite fed-batch strategy has the higher cumulative oxygen consumption, it has the lowest total air consumption with reductions ranging among 10-29 % compared to the static-batch scenarios. Furthermore, this strategy requires only a fraction of the inoculum needed in batch systems (I_{Req}) (*e.g.*, only 33% for a fed-batch 33%), representing a considerable save of resources which positively influence on economic and sustainability aspects of the process.

6.2.5 Advantages and weaknesses of the SSF approach

The fruit-like bioproduction via SSF assessed in chapter 5 and 6 represents an improvement regarding the state of the art in the fruity-aromas bioproduction using the SSF approach. As it can be seen in Table 6.3, previously reported static diffusive systems are not able to achieve the same productivity than the continuous forced air systems, although the presumably higher concentration of the volatile compounds in the headspace of these reactors. This is, systems with continuous air supply reach the same, or sometimes higher, concentration levels per hour than the total cumulative concentration of a diffusive system in the headspace.

In general, this happens mainly due to the phase equilibrium achieved in diffusive configurations that avoid the further transfer from the solid to the gas phase of the volatile compounds. On the contrary, a continuous air supplied system continuously displaces the equilibrium by stripping the compounds out the system, favoring the further production of these compounds.

In addition, compared to other dynamic systems, productions levels found in these sections are not such far from the best result obtained by Medeiros et al. (2006), where the system consisted of a 1.5 kg substrate rotary drum bioreactor. Furthermore, the approaches proposed here reach these productivities in a shorter time, saving valuable resources.

However, the remarkable aspect lies in the fact these results have been achieved using a substrate composed only of agro-industrial residues and industrial by-products without the need of additional precursors, in contrast to previous studies where glucose become an essential component for the metabolization. Also, the use of non-sterilized substrates is a step forward, showing that *K. marxianus* can work in such environment without significant performance losses

Even that the positive results show good prospects; further investigation is needed to make more efficient and sustainable the SSF approaches for producing fruit-like compounds. Perhaps, the most significant issue to be solved refers to the economic feasibility of the process.

Table 6.3. Main characteristics of several fruity-aroma bioprocesses using solid-state fermentation.

Substrate-precursor	Strain	Scale (g_{TS})/ System	Maximum Concentration-Productivity[a]	P_{Vol}^{Acc} (µmol L^{-1} g^{-1}_{TS})[b] (µmol g^{-1}_{TS})[c]	Time (h)	Reference
SCB-G	*C.F.*	7.5/D	6	307	140	(Christen et al., 1997)
CH-G	*C.F.*	15/D	20	2577	504	(Soares et al., 2000a)
CB-G	*K.M.*	10/D	30	3211	72	(Medeiros et al., 2000)
CB-G	*K.M.*	20/B	20	1030	136	(Medeiros et al., 2001)
CH-G	*C.F.*	20/B	23	1315	216	(Medeiros et al., 2006)
CH-G	*C.F.*	525/BM	144	4400	192	(Medeiros et al., 2006)
SCB-SBM*	*K.M.*	30/B	105	3494	72	This study
SCB-SBM+	*K.M.*	30/B	70	1808	72	This study
SCB-SBM	*K.M.*	30/FB	71	1796	69	This study
SCB-SBM	*K.M.*	275/FB	101	2806	98	This study
SCB-SBM	*K.M.*	800/FB	94	2332	69	This study

P_{Vol}^{Acc}: total cumulative volatile production; SCB: sugarcane bagasse; CH.: coffee husk; G: glucose; CB: cassava bagasse; SBM: sugar beet molasses; C.F.: *C. fimbriata*; K.M.: *K. marxianus*; D: diffusive static system; B: batch; FB: fed-batch; BM: batch with intermittent mixing in a drum reactor.
*at 40°C; +: at 30°C. [(a)]For diffusive systems concentration was expressed as µmol of ethanol equivalent L^{-1} g^{-1}_{TS}, for dynamic systems productivity is expressed as µmol of ethanol equivalent h^{-1} g^{-1}_{TS}. [(b)]For static systems; [(c)] for dynamic systems.

6.2.6 Final biological stability of the fermented substrates

One of the significant factors making SSF an interesting alternative for valorizing the agro-industrial residues is its ability to process them, obtaining as final residue a degraded organic fraction much closer to be stabilized. As seen in Table 6.4, the aerobic respiration indices for the residues obtained after the fermentation process using the different strategies at 22 L scale are presented. It can be observed that the initial mixture SCB/SBM possess a high potential to be aerobically degraded, and it could be classified as a residue with high biodegradability considering its dynamic respiration index (DRI) is higher than 5 g_{O2} kg^{-1}_{VS} h^{-1} (Barrena et al., 2011).

On the other hand, the final residues present respiration indices significantly lower than the initial mixture, with a clear trend in which the static-batch mode promotes the highest degradation, followed by the intermittent mixing and the fed-batch strategies. Although the final residues still present biodegradable organic activity that needs to be stabilized, the SSF approaches have reduced their biodegradability in 80, 65 and 54% respectively (based on DRI_{24h}), while obtaining value-added products.

Table 6.4. Aerobic respiration indices of the residues after the solid-state fermentation for producing fruit-like compounds.

Strategy	DRI_{24h} (g_{O2} kg^{-1}_{VS} h^{-1})	DRI_{24h} (g_{O2} kg^{-1}_{TS} h^{-1})	$OUR_{Max\text{-}1h}$ (g_{O2} kg^{-1}_{VS} h^{-1})	At_4 (g_{O2} kg^{-1}_{TS})
Initial residue mixture	5.5 ± 0.1	5.1 ± 0.1	7.3 ± 0.1	227.6 ± 11.5
Static-batch	1.1 ± 0.1	1.0 ± 0.1	2.0 ± 0.1	85.9 ± 4.6
Intermittent mixing	1.9 ± 0.1	1.8 ± 0.1	2.3 ± 0.1	134.2 ± 4.0
Fed-batch 33%	2.5 ± 0.2	2.3 ± 0.2	3.1 ± 0.1	173.0 ± 8.7

DRI: Dynamic respiration index; At_4: cumulative index after 4 days; $OUR_{Max\text{-}1h}$: 1 h average maximum oxygen uptake rate. Indices have been computed based on the dynamic respirometric analysis at 37°C.

Under this condition, the success of the batch mode for promoting a higher biodegradation could be a consequence of the time spent by the organic fraction inside the bioreactors. Hence, an alternative to increase the stability of the residues (if necessary) could be letting the fermentation to proceed beyond the point the volatiles compounds have been completely produced, given extra time for a further biodegradation.

6.2.7 *De novo* 2-phenethyl alcohol production

Another additional aspect considered in this section was the ability of *K. marxianus* for producing other volatile compounds in parallel to the fruit-like compounds. As stated in chapter 1, this yeast has proven its efficiency for bioproducing valuable compounds like 2-phenethyl alcohol (2-PE) and 2-phenethyl acetate (2-PEA) in submerged fermentations (Fabre et al., 1998; Morrisey et al., 2015). Although the biotransformation is possible mainly due to the use of L-phenylalanine as precursor, some studies have reported the production of these semi-volatiles compounds by *de novo* transformations in liquid systems (Chreptowicz et al., 2016; Pan et al., 2014).

However, this condition has not been evaluated in SSF systems yet. Considering the potential benefits of using SSF for obtaining these valuable compounds (chapter 1), in this section, it was studied the ability of *K. marxianus* to produce 2-PE and 2-PEA via *de novo* synthesis. With this aim, simultaneously to the determination of the fruit-like compounds, it was quantified the 2-PE and 2-PEA production for the set of experiments exposed in section 6.2.3.

Figure 6.10 shows the time profile of the total production (P_{sub}^{Tot}) and 2-PE production ($P_{sub}^{2\text{-}PE}$) of the different tested strategies at 22 L scale. As it can be seen in Figure 6.10(a), in the static-batch mode a maximum of 3.6 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{TS}$ were obtained starting with SCB and SBM as the only carbon sources in the media. Similarly, in the mixing strategy P_{sub}^{Tot} reached a maximum of 3.9 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{TS}$ (Figure 6.10(b)) while using fed-batch 33% it reached 3.8 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{TS}$ (Figure 6.10(c))(no significant differences among the strategies).

This suggests that 2-PEA is being transformed into other non-identified compounds while 2-PE is still produced by *K. marxianus*. These results are evidence of the *K. marxianus* ability for using the available sugars to produce 2-PE and 2-PEA without needing a precursor.

Notwithstanding, the obtained production is still too low to be considered as a productive option by itself, and it is expected that *de novo* synthesis would be hindered by the metabolization of the fruit-like compounds. This is, under these conditions, the strain is more prone to produce the fruit-like compounds than the semi-volatiles 2-PE and 2-PEA, due to the complex metabolic routes involving their bioproduction. As a consequence, a more detailed study of the ability of *K. marxianus* to produce these compounds by adding a precursor is essential to improve the feasibility of the process.

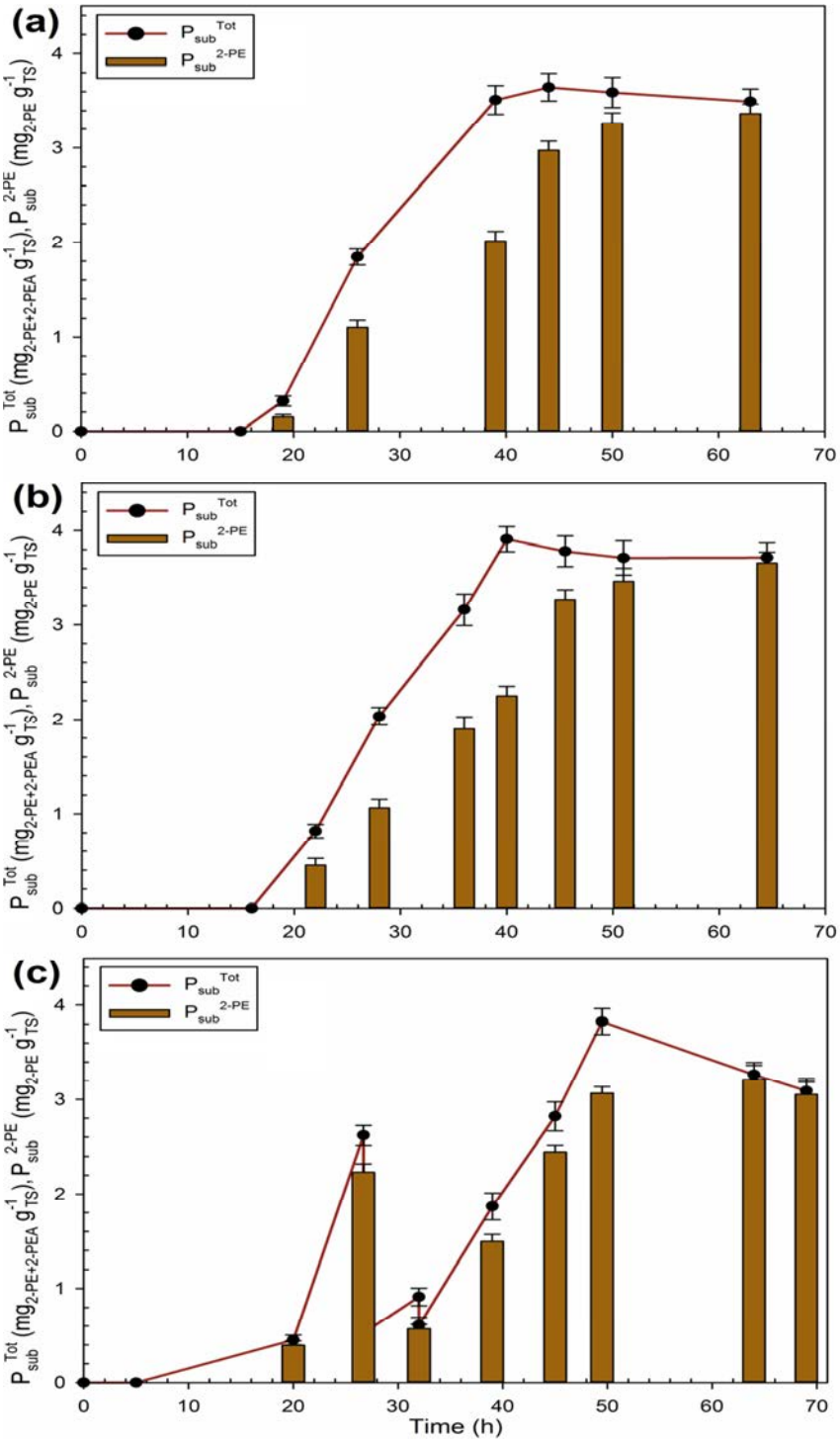


Figure 6.10. 2-phenethyl alcohol (2-PE) and 2-phenethyl acetate (2-PEA) production via *de novo* synthesis in the solid-state fermentation of sugarcane bagasse/sugar beet molasses in the 22 L system. (a) batch mode, (b) intermittent mixing, (c) fed-batch 33%.

Final remarks

The use of SSF fed-batch strategies resulted in a more productive way to obtain the value-added fruit-like compounds, enhancing the amount of ester species in the accumulated volatiles. The synergy among substrate availability, the ability to keep an aerobic environment and temperature control are the main responsible for the success of the fed-batch strategies for improving the ester species selectivity at the different evaluated scales.

The influence of the scaling-up is strategy-dependent. These effects are more evident when a static-batch mode is used, and it is slightly reduced by using intermittent mixing. On the contrary, by means of a fed-batch strategy, the scaling effects are minimized at the evaluated conditions.

At 22 L scale, it was also identified and quantified the *de novo* production of 2-phenethyl alcohol and 2-phenethyl acetate, proving that *K. marxianus* possess this ability when growing in a solid media.

References

Aggelopoulos, T., Katsieris, K., Bekatorou, A., Pandey, A., Banat, I.M., Koutinas, A.A., 2014. Solid state fermentation of food waste mixtures for single cell protein, aroma volatiles and fat production. Food Chem. 145, 710–716.

Astolfi, V., Joris, J., Verlindo, R., Oliveira, V., Maugeri, F., Mazutti, M., De Oliveira, D., Treichel, H., 2011. Operation of fixed-bed bioreactor in batch and fed-batch modes for production of inulinase by solid-state fermentation. Biochem. Eng. J. 58–59, 39–49.

Barrena, R., Canovas, C., Sánchez, A., 2006. Prediction of temperature and thermal inertia effect in the maturation stage and stockpiling of a large composting mass. Waste Manag. 26, 953–959.

Barrena, R., Gea, T., Ponsá, S., Ruggieri, L., Artola, A., Font, X., Sánchez, A., 2011. Categorizing Raw Organic Material Biodegradability Via Respiration Activity Measurement: A Review. Compost Sci. Util. 19, 105–113.

Barrios-González, J., 2012. Solid-state fermentation: Physiology of solid medium, its molecular basis and applications. Process Biochem. 47, 175–185.

Carlquist, M., Gibson, B., Yuceer, Y.K., Paraskevopoulou, A., Sandell, M., Angelov, A.I., Gotcheva, V., Angelov, A.D., Etschmann, M.M.W., 2015. Process engineering for bioflavour production with metabolically active yeast - a mini-review. Yeast 32, 123–143.

Carvalho, J., Pandey, A., Oishi, B., Brand, D., Rodriguez-Leon, J., Soccol, C.R., 2006. Relation between growth, respirometric analysis and biopigments production from *Monascus* by solid-state fermentation. Biochem. Eng. J. 29, 262–269.

Cerda, A., Gea, T., Vargas-García, M.C., Sánchez, A., 2017a. Towards a competitive solid state fermentation: Cellulases production from coffee husk by sequential batch operation and role of microbial diversity. Sci. Total Environ. 589, 56–65.

Cerda, A., Mejías, L., Gea, T., Sánchez, A., 2017b. Cellulase and xylanase production at pilot scale by solid-state fermentation from coffee husk using specialized consortia: The consistency of the process and the microbial communities involved. Bioresour. Technol. 243, 1059–1068.

Chreptowicz, K., Wielechowska, M., Główczyk-Zubek, J., Rybak, E., Mierzejewska, J., 2016. Production of natural 2-phenylethanol: From biotransformation to purified product. Food Bioprod. Process. 100, 275–281.

Christen, P., Meza, J.C., Revah, S., 1997. Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*: influence of the substrate type and the presence of precursors. Mycol. Res. 101, 911–919.

Etschmann, M.M.W., Sell, D., Schrader, J., 2003. Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. Biotechnol. Lett. 25, 531–536.

Fabre, C.E., Blanc, P.J., Goma, G., 1998. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus.* Biotechnol. Prog. 14, 270–274.

Fonseca, G.G., Heinzle, E., Wittmann, C., Gombert, A.K., 2008. The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl. Microbiol. Biotechnol. 79, 339–354.

Garavaglia, J., Flôres, S.H., Pizzolato, T.M., Peralba, M.D.C., Ayub, M.A.Z., 2007. Bioconversion of L-phenylalanine into 2-phenylethanol by *Kluyveromyces marxianus* in grape must cultures. World J. Microbiol. Biotechnol. 23, 1273–1279.

Lane, M.M., Morrissey, J.P., 2010. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. Fungal Biol. Rev. 24, 17–26.

Medeiros, A., Pandey, A., Freitas, R., Christen, P., Soccol, C.R., 2000. Optimization of the production of aroma compounds by *Kluyveromyces marxianus* in solid-state fermentation using factorial design and response surface methodology. Biochem. Eng. J. 6, 33–39.

Medeiros, A.B.P., Pandey, A., Christen, P., Fontoura, P.S.G., de Freitas, R.J.S., Soccol, C.R., 2001. Aroma compounds produced by *Kluyveromyces marxianus* in solid state fermentation on a packed bed column bioreactor. World J. Microbiol. Biotechnol. 17, 767–771.

Medeiros, A.B.P., Pandey, A., Vandenberghe, L.P.S., Pastorel, G.M., Soccol, C.R., 2006. Production and recovery of aroma compounds produced by solid-state fermentation using different adsorbents. Food Technol. Biotechnol. 44, 47–52.

Morrisey, J.P., Etschmann, M.M.W., Schrader, J., Billerbeck, G.M., 2015. Cell factory applications of the yeast *Kluyveromyces marxianus* for the biotechnological production of natural flavour and fragrance molecules. Yeast 32, 3–16.

Pan, X., Qi, H., Mu, L., Wen, J., Jia, X., 2014. Comparative metabolomic-based metabolic mechanism hypothesis for microbial mixed cultures utilizing cane molasses

wastewater for higher 2-Phenylethanol production. J. Agric. Food Chem. 62, 9927–9935.

Raimbault, M., 1998. General and microbiological aspects of solid substrate fermentation. Electron. J. Biotechnol. 1, 1–15.

Soares, M., Christen, P., Pandey, A., Raimbault, M., Soccol, C.R., 2000. A novel approach for the production of natural aroma compounds using agro-industrial residue. Bioprocess Eng. 23, 695–699.

Soccol, C.R., Costa, E.S.F. da, Letti, L.A.J., Karp, S.G., Woiciechowski, A.L., Vandenberghe, L.P. de S., 2017. Recent developments and innovations in solid state fermentation. Biotechnol. Res. Innov. 1, 52–71.

Section 2. Rose-like aroma compounds

This section includes Chapters 7 and 8. Through these chapters, developments focused on the bioproduction of rose-like compounds via solid-state fermentation are presented.

Introduction

As stated in chapter 1, one of the most interesting compounds *K. marxianus* can bioproduced is the rose-like odor 2-phenethyl alcohol (2-PE). Together with 2-PE, the also flower-like odor 2-phenethyl acetate (2-PEA) is obtained by biotechnological routes using yeasts (Carlquist et al., 2015). Although it is less known, and therefore, less used in industry, 2-PEA has similar characteristics than 2-PE that make of it a suitable additive in the same applications 2-PE is commonly used (Białecka-Florjańczyk et al., 2012; Wang et al., 2011a).

In general, the chemical production of 2-PE is based on three paths widely used at industrial scale: i) Friedel-Craft reaction of ethylene oxide with benzene, ii) Catalytic reduction of styrene oxide, iii) Oxidation of propylene with 2-phenylethyl hydroperoxide. As seen in Figure S2.1, the chemical paths for obtaining 2-PE involve the use of petrochemicals, some catalysts and sometimes high-temperature conditions, which impart green-gassy or metallic-chlorine off-odors in the final product (Etschmann et al., 2002). These undesirable by-products modify the organoleptic profiles of the 2-PE, requiring extensive use of purification steps to minimize their adverse effects. Thus, these routes are far to be ideal for the production of GRAS additives like 2-PE.

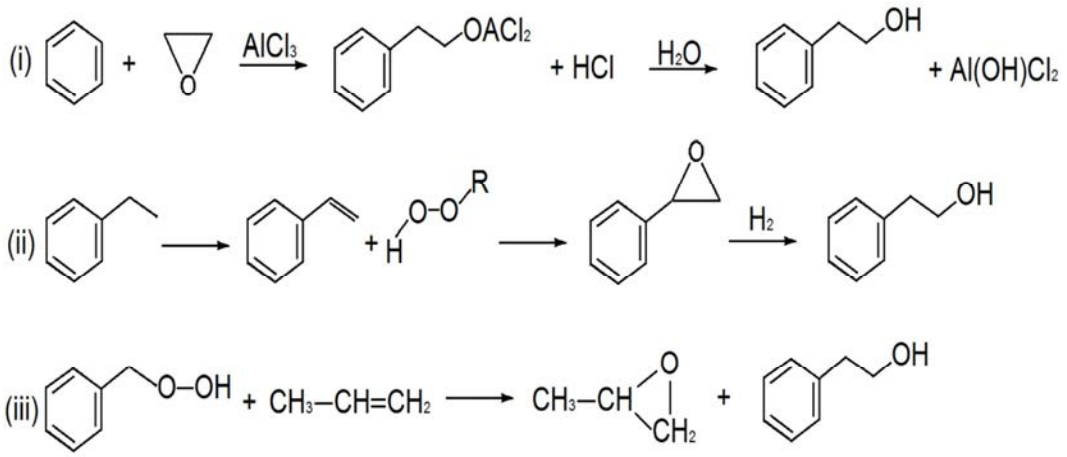


Figure S2.1. Chemical synthesis routes for producing 2-PE. (i) Friedel-Craft reaction, (ii) styrene oxide reduction, (iii) propene oxidation.

At the same time, the 2-PEA chemical production is derived from the availability of 2-PE which is its precursor. In general, 2-PEA can be obtained from the esterification of 2-PE with acetic acid and through the transesterification of 2-PE with

acetate esters (Figure S2.2). These processes require the use of acid catalysis (could also be basics for the transesterification) at high temperatures, and it can be run with low-cost precursors like ethyl acetate or vinyl acetate with high yields. However, as it occurs with 2-PE synthesis, the purification process is complex to limit the presence of undesirable by-products affecting the 2-PEA characteristics (Kim and Park, 2017).

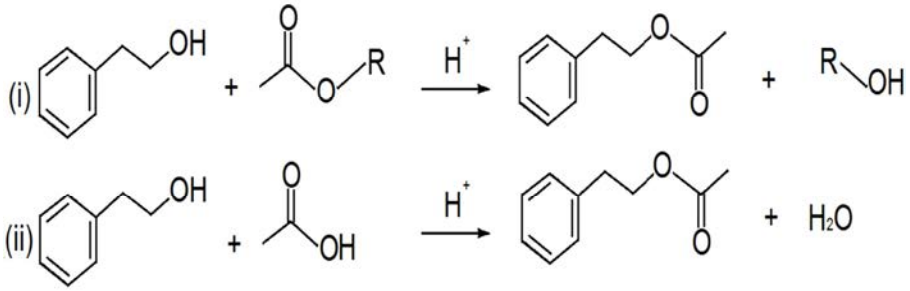


Figure S2.2. Chemical synthesis routes for producing 2-PEA. (i) 2-PE transesterification, (ii) 2-PE esterification.

Although there is no certainty of how much is the world's 2-PE and 2-PEA production, it can be stated that the 2-PE production trend has shown an increase from 7000 ton in 1990 (Etschmann et al., 2002) to 10000 in 2010 (Hua and Xu, 2011). Also, the worldwide volume of use of this additive in fragrance applications was estimated at more than 1000 ton per year in 2008 (Scognamiglio et al., 2012). Hence, it is expected that most of the current 2-PE production is based on the chemical synthesis from benzene and styrene and in a lower extent via plant extraction from natural sources (Carlquist et al., 2015). Similarly, 2-PEA production levels are unknown, but it is estimated its consumption as additive in fragrance applications have ranged from 100-1000 ton per year in 2008 (McGinty et al., 2012).

On the other hand, biotechnological production of 2-PE and 2-PEA has been studied by means of two approaches. In the case of 2-PE, the predominant approach has been the biosynthesis. In general, 2-PE bioproduction has been performed by fungi like *Aspergillus oryzae* (Masuo et al., 2015), bacteria as *Enterobacter sp* (Zhang et al., 2014), but mainly by yeasts such as *S. cerevisiae* (Eshkol et al., 2009; Kim et al., 2014; Stark et al., 2002), *K. marxianus* (Conde-Báez et al., 2017; Fabre et al., 1998; Gao and Daugulis, 2009), *Yarrowia lipolytica* (Vong et al., 2016) or other non-conventional yeast like *Metschnikowia pulcherrima* (Chantasuban et al., 2018), *Aureobasidium pullulans* or *Pichia kudriavzevii* (Chreptowicz et al., 2018). So far, *S. cerevisiae* and *K.*

marxianus have proved to be one of the most effective 2-PE producers (Carlquist et al., 2015; Morrisey et al., 2015).

Similarly, 2-PEA bioproduction has been focused on the use enzymes as catalysts in the esterification and transesterification of 2-PE. In this sense, lipases have been the most used in the combined esterification-transesterification process using different media (Kim and Park, 2017; Kuo et al., 2014a, 2014b; Tan et al., 2011). In a lesser extent, the 2-PEA bioproduction via *de novo* synthesis (Viana et al., 2011) and using precursors (Etschmann et al., 2005; Guo et al., 2017) has also been studied.

In this sense, the most efficient biotechnological route leading to 2-PE and 2-PEA is the bioconversion of L-phenylalanine (L-Phe) via the Ehrlich pathway (Etschmann et al., 2002). Nevertheless, the biosynthesis of 2-PE and 2-PEA can also be achieved by *de novo* synthesis starting from simple sugars through the Shikimate pathway. Nonetheless, the efficiency of this path is not high enough to be used as a productive alternative by itself (Carlquist et al., 2015).

The technology used for the L-Phe biotransformation into 2-PE and 2-PEA has been typically developed for SmF processes. However, the main drawback of this technique is the 2-PE inhibition of the yeast cells, limiting the final product concentrations and space-time yields (Wang et al., 2011a).

To overcome this restraint, strategies such as strain development (*e.g.*, metabolic engineering, system biology, genome shuffling), culture optimization and cells immobilization have been used as traditional ways to increase 2-PE production in SmF systems (Etschmann et al., 2015, 2004; Kin et al., 2014). Notwithstanding, the most studied alternatives focus on the in situ product removal (ISPR) techniques (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 mediums as substrates, and, in general, reagent grade solvents or complex reaction systems leading to energy consuming and costly productive processes.

Regarding the use of SSF for producing these compounds, to the best of the recent knowledge, no attempts have been reported yet.

References

Białecka-Florjańczyk, E., Krzyczkowska, J., Stolarzewicz, I., Kapturowska, A., 2012. Synthesis of 2-phenylethyl acetate in the presence of *Yarrowia lipolytica* KKP 379 biomass. J. Mol. Catal. B Enzym. 74, 241–245.

Carlquist, M., Gibson, B., Yuceer, Y.K., Paraskevopoulou, A., Sandell, M., Angelov, A.I., Gotcheva, V., Angelov, A.D., Etschmann, M.M.W., 2015. Process engineering for bioflavour production with metabolically active yeast - a mini-review. Yeast 32, 123–143.

Červeňanský, I., Mihal, M., Markoš, J., 2017. Potential application of perfusion and pertraction for in situ product removal in biocatalytic 2-phenylethanol production. Sep. Purif. Technol. 183, 11–20.

Chantasuban, T., Santomauro, F., Gore-Lloyd, D., Parsons, S., Henk, D., Scott, R., Chuck, C., 2018. Elevated production of the aromatic fragrance molecule, 2-phenylethanol, using *Metschnikowia pulcherrima* through both *de novo* and *ex novo* conversion in batch and continuous modes. Chem. Technol. Biotechnol. https://doi.org/10.1002/jctb.5597

Chreptowicz, K., Sternicka, M.K., Kowalska, P.D., Mierzejewska, J., 2018. Screening of yeasts for the production of 2-phenylethanol (rose aroma) in organic waste-based media. Lett. Appl. Microbiol. 66, 153–160.

Conde-Báez, L., Castro-Rosas, J., Villagómez-Ibarra, J.R., Páez-Lerma, J.B., Gómez-Aldapa, C., 2017. Evaluation of Waste of the Cheese Industry for the Production of Aroma of Roses (Phenylethyl Alcohol). Waste and Biomass Valorization 8, 1343–1350.

Eshkol, N., Sendovski, M., Bahalul, M., Katz-Ezov, T., Kashi, Y., Fishman, A., 2009. Production of 2-phenylethanol from L-phenylalanine by a stress tolerant *Saccharomyces cerevisiae* strain. J. Appl. Microbiol. 106, 534–542.

Etschmann, M.M.W., Bluemke, W., Sell, D., Schrader, J., 2002. Biotechnological production of 2-phenylethanol. Appl. Microbiol. Biotechnol. 59, 1–8.

Etschmann, M.M.W., Huth, I., Walisko, R., Schuster, J., Krull, R., Holtmann, D., Wittmann, C., 2015. Improving 2-Pheylethanol and 6-Pentyl-a-Pyrone production with fungi microparticle-enhanced cultivation (MPEC). Yeast 32, 145–157.

Etschmann, M.M.W., Sell, D., Schrader, J., 2005. Production of 2-Phenylethanol and 2-Phenylethylacetate from L-Phenylalanine by coupling whole-cell biocatalysis with organophilic pervaporation. Biotechnol. Bioeng. 92, 624–634.

Etschmann, M.M.W., Sell, D., Schrader, J., 2004. Medium optimization for the production of the aroma compound 2-Phenylethanol using genetic algorithm. J. Mol. Catal. B Enzym. 29, 187–193.

Fabre, C.E., Blanc, P.J., Goma, G., 1998. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. Biotechnol. Prog. 14, 270–274.

Gao, F., Daugulis, A.J., 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by *Kluyveromyces marxianus*. Biotechnol. Bioeng. 104, 332–339.

Guo, D., Zhang, L., Pan, H., Li, X., 2017. Metabolic engineering of *Escherichia coli* for production of 2-Phenylethylacetate from L-phenylalanine. Microbiol. Open e486, 1–6.

Hua, D., Lin, S., Li, Y., Chen, H., Zhang, Z., Du, Y., Zhang, X., Xu, P., 2010. Enhanced 2-phenylethanol production from L-phenylalanine via in situ product adsorption. Biocatal. Biotransformation 28, 259–266.

Hua, D., Xu, P., 2011. Recent advances in biotechnological production of 2-phenylethanol. Biotechnol. Adv. 29, 654–660.

Kim, B., Cho, B.-R., Hahn, J.-S., 2014. Metabolic engineering of *Saccharomyces cerevisiae* for the production of 2-phenylethanol via Ehrlich pathway. Biotechnol. Bioeng. 111, 115–124.

Kim, H., Park, C., 2017. Enzymatic synthesis of phenethyl ester from phenethyl alcohol with acyl donors. Enzyme Microb. Technol. 100, 37–44.

Kin, T.-Y., Lee, S.-W., Oh, M.-K., 2014. Biosynthesis of 2-phenylethanol from glucose with genetically engineered *Kluyveromyces marxianus*. Enzyme Microb. Technol. 61–62, 44–47.

Kuo, C.H., Chen, G.J., Chen, C.I., Liu, Y.C., Shieh, C.J., 2014a. Kinetics and optimization of lipase-catalyzed synthesis of rose fragrance 2-phenylethyl acetate through transesterification. Process Biochem. 49, 437–444.

Kuo, C.H., Liu, T.A., Chen, J.H., Chang, C.M.J., Shieh, C.J., 2014b. Response surface methodology and artificial neural network optimized synthesis of enzymatic 2-phenylethyl acetate in a solvent-free system. Biocatal. Agric. Biotechnol. 3, 1–6.

Masuo, S., Osada, L., Zhou, S., Fujita, T., Takaya, N., 2015. *Aspergillus oryzae* pathways that convert phenylalanine into the flavor volatile 2-phenylethanol. Fungal Genet. Biol. 77, 22–30.

McGinty, D., Vitale, D., Letizia, C.S., Api, A.M., 2012. Fragrance material review on phenethyl acetate. Food Chem. Toxicol. 50, S491–S497.

Morrisey, J.P., Etschmann, M.M.W., Schrader, J., Billerbeck, G.M., 2015. Cell factory applications of the yeast *Kluyveromyces marxianus* for the biotechnological production of natural flavour and fragrance molecules. Yeast 32, 3–16.

Okuniewska, P., Domańska, U., Więckowski, M., Mierzejewska, J., 2017. Recovery od 2-phenylethanol from aqueous solutions of biosynthesis using ionic liquids. Sep. Purif. Technol. 188, 530–538.

Scognamiglio, J., Jones, L., Letizia, C.S., Api, A.M., 2012. Fragrance material review on phenylethyl alcohol. Food Chem. Toxicol. 50, S224–S239.

Sendovski, M., Nir, N., Fishman, A., 2010. Bioproduction of 2-Phenylethanol in a Biphasic Ionic liquid Aqueous system. J. Agric. Food Chem. 58, 2260–2265.

Stark, D., Kornmann, H., Münch, T., Sonnleitner, B., Marison, I.W., Von Stockar, U., 2003. Novel type of in situ extraction: Use of solvent containing microcapsules for the bioconversion of 2-phenylethanol from L-phenylalanine by *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 83, 376–385.

Stark, D., Münch, T., Sonnleitner, B., Marison, I.W., Stockar, U. von, 2002. Extractive Bioconversion of 2-Phenylethanol from L-Phenylalanine by *Saccharomyces cerevisiae*. Biotechnol. Prog. 18, 514–523.

Tan, H.S.G., Yu, B., Curran, P., LIU, S.Q., 2011. Lipase-catalyzed synthesis of natural aroma-active 2-phenylethyl esters in coconut cream. Food Chem. 124, 80–84.

Viana, F., Belloch, C., Vallés, S., Manzanares, P., 2011. Monitoring a mixed starter of Hanseniaspora vineae-Saccharomyces cerevisiae in natural must: Impact on 2-phenylethyl acetate production. Int. J. Food Microbiol. 151, 235–240.

Vong, W.C., Au Yang, K.L.C., Liu, S.Q., 2016. Okara (soybean residue) biotransformation by yeast *Yarrowia lipolytica*. Int. J. Food Microbiol. 235, 1–9.

Wang, H., Dong, Q., Guan, A., Meng, C., Shi, X., Guo, Y., 2011a. Synergistic inhibition effect of 2-phenylethanol and ethanol on bioproduction of natural 2-phenylethanol by *Saccharomyces cerevisiae* and process enhancement. J. Biosci. Bioeng. 112, 26–31.

Wang, H., Dong, Q., Meng, C., Shi, X. ai, Guo, Y., 2011b. A continuous and adsorptive bioprocess for efficient production of the natural aroma chemical 2-phenylethanol with yeast. Enzyme Microb. Technol. 48, 404–407.

Zhang, H., Cao, M., Jiang, X., Zou, H., Wang, C., Xu, X., Xian, M., 2014. De-novo synthesis of 2-phenylethanol by *Enterobacter sp*. CGMCC 5087. BMC Biotechnol. 14, 30.

Chapter 7

Bioproduction of 2-phenethyl alcohol and 2-phenethyl acetate through the solid-state fermentation of sugarcane bagasse

Part of this chapter has been published in: Martínez, O., Sánchez, A., Font, X., Barrena, R. 2018. Bioproduction of 2-phenylethanol and 2-phenethyl acetate by *Kluyveromyces marxianus* through the solid-state fermentation of sugarcane bagasse. Applied microbiology and biotechnology. doi.org/10.1007/s00253-018-8964-y.

7.1 Overview

Since 2-PE and 2-PEA production via SSF has not been reported yet, in this chapter, the aim was to assess the feasibility of the SSF of sugarcane bagasse supplemented with L-Phe for obtaining 2-PE and 2-PEA in a residue-based system using *K. marxianus* as inoculum. With this purpose, the 0.5 L continuously air supplied reaction system was used, and all experiments were undertaken under sterile conditions.

The first objective was to perform a screening of some operational variables to identify those more relevant to the productive process in a batch operation mode. A second objective included the evaluation of several operational strategies to improve the 2-PE and 2-PEA production, testing complementary strategies such as glucose additions or modifying the feeding strategy.

7.2 Results

As previously stated in chapter 1, SSF processes are influenced by biological and physicochemical factors affecting the strain growth and the metabolization of the products (Rodriguez-Leon et al., 2008). Some of 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}^{Tot}) [$mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS}] (calculated as accumulated 2-PE and 2-PEA production per initial dry mass 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 per initial moles of L-Phe), L-Phe conversion ($X_{L\text{-}Phe}$) [% $mol_{L\text{-}Pher}$ $mol^{-1}_{L\text{-}Phe0}$] (the number of L-Phe moles reacted per 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 total productivity (2-PE and 2-PEA) per reactor volume).

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.

7.2.1 Influence of operational variables on the production

Initial experiments consisted of the evaluation of the effects of six operational variables on the performance index P_{sub}^{Tot}. 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 7.1). pH, MC and inoculum load were kept at 5.3, 68% and 10^8 CFU g^{-1}_{TS} respectively, based on the results found in chapter 5. T and P_d were assessed between 30-37°C and 1.55 and 3.1% (dry basis) respectively, considering the results of prior studies in SmF (Etschmann et al., 2003; Gao and Daugulis, 2009; Garavaglia et al., 2007). On the other hand, 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 the results found in chapter 5.

From Figure 7.1(a) it is evident that three out of the four assessed variables significantly influence the combined 2-PE and 2-PEA production. In Figure 7.1(b) it can be seen that operating at 30°C coincides with the highest production, while a temperature increase only induces a reduction in P_{sub}^{Tot}. Similarly, when molasses were added, an apparent adverse effect is produced in the P_{sub}^{Tot} trend (Figure 7.1(c)). Furthermore, from Figure 7.1(d) the effect of the air supply in the production is presented. For the evaluated interval, an increase in S_{AFR} is directly related to a higher production.

As detailed in Figure 7.1(a) and (b), one of the most significant variables affecting the process is the temperature. However, the trend found here is the opposite to the described by some authors (Etschmann et al., 2005; Garavaglia et al., 2007) for SmF systems. In this sense, although high temperatures contribute 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 temperature for the strain as found by authors like Eshkol et al. (2009) and as it has occurred in this case.

Table 7.1. Performance indices of the experiments in the factorial design N°1.

Run	Evaluated variables				Performance indices					
					P_{sub}^{i} (mg_i g^{-1}_{TS})			Y_{mol} ($mol_{2\text{-PE+2-PEA}}$ $mol^{-1}_{L\text{-Phe0}}$)	% $X_{L\text{-Phe}}$ ($mol_{L\text{-Phe Reacted}}$ $mol^{-1}_{L\text{-Phe0}}$)	$Y_{s\text{-}t}$ ($mg_{2\text{-PE+2-PEA}}$ L^{-1} h^{-1})
	T (°C)	P_d (%)	S_{AFR} (L h^{-1} g^{-1}_{TS})	M_d (%)	2-PE	2-PEA	Total			
1	30	1.55	0.05	0	8.9	2.0	10.9	0.80	51.8	9.4
2	37	1.55	0.05	0	5.5	1.1	6.6	0.49	31.2	9.9
3	30	3.10	0.05	0	9.5	0.7	10.2	0.44	35.2	10.9
4	37	3.10	0.05	0	3.6	1.3	4.9	0.20	20.7	9.3
5	30	1.55	0.10	0	9.4	1.0	10.4	0.82	57.9	10.3
6	37	1.55	0.10	0	8.4	1.4	9.8	0.76	53.2	6.2
7	30	3.10	0.10	0	10.6	1.2	11.8	0.51	36.3	4.5
8	37	3.10	0.10	0	6.3	1.7	8.0	0.33	30.0	7.3
9	30	1.55	0.05	20	6.1	0.6	6.7	0.51	66.7	6.4
10	37	1.55	0.05	20	3.9	0.5	4.4	0.34	48.7	4.2
11	30	3.10	0.05	20	6.5	0.6	7.1	0.31	38.4	6.6
12	37	3.10	0.05	20	3.7	0.4	4.1	0.17	30.7	3.8
13	30	1.55	0.10	20	7.2	0.8	8.0	0.64	70.5	7.5
14	37	1.55	0.10	20	6.0	1.2	7.2	0.56	47.4	6.8
15	30	3.10	0.10	20	8.6	0.9	9.5	0.44	33.9	8.4
16	37	3.10	0.10	20	7.8	0.9	8.7	0.40	38.1	7.7

Data computed at the end of the fermentation at 72 h. Results are the mean value of duplicates with a standard deviation less than 10%. 2-PE: 2-phenylethanol; 2-PEA: 2-phenethyl acetate; P_d: L-phenylalanine dose; M_d: sugar beet molasses dose; S_{AFR}: specific air flow rate; Y_{mol}: molar yield; P_{sub}^{i}: cumulative production; $X_{L\text{-Phe}}$: L-phenylalanine conversion; $Y_{s\text{-}t}$: space-time yield.

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 maintain a specific dissolved oxygen concentration (Chreptowicz et al., 2016), in SSF it plays a much more substantial role 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). 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) as well as for the fruit-like compounds in chapter 5.

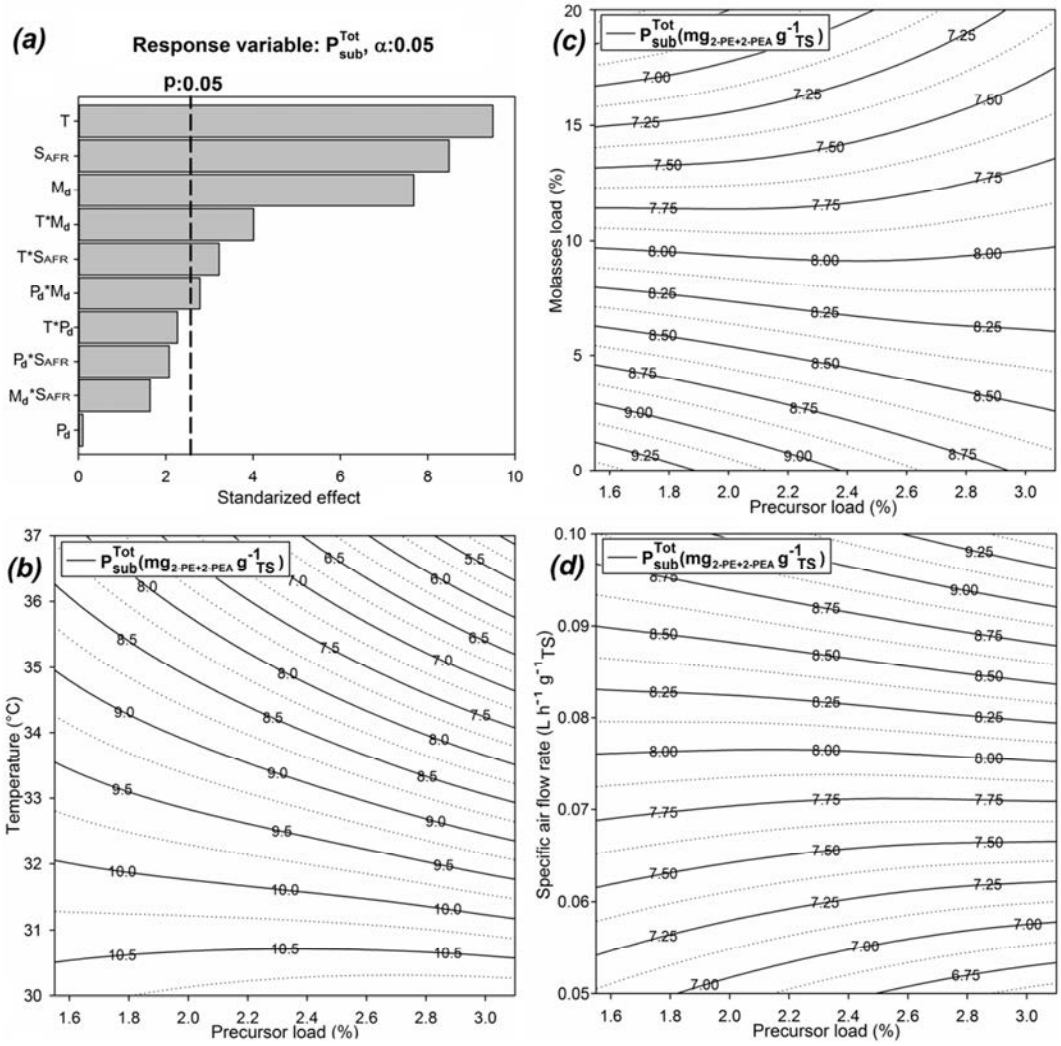


Figure 7.1. (a) Pareto chart and variables effects on the cumulative production of 2-PE and 2-PEA for the solid-state fermentation of sugarcane bagasse with *K. marxianus* for design N°1. (b) temperature effect, (c) molasses load effect, (d) specific air flow rate effect.

On the other hand, using sugar beet molasses in SSF as a useful sugars complement supplier have been previously confirmed (Jiménez-Peñalver et al., 2016). Unfortunately, molasses 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) (Table 5.1) and it is estimated around 68% of these are not amino acid-N based (Olbrich H, 2006), leading to contents of other sources around

10% of the total available nitrogen. Finally, although the L-Phe dose plays a critical role in the process since it is the primary source for the biotransformation, at the evaluated interval, the found trend suggests its effect on P_{sub}^{Tot} is very limited. Under this scenario, L-Phe dose needs further evaluation to find the most appropriate levels for improving the 2-PE and 2-PEA production.

In this sense, 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 7.2). Here, T, S_{AFR}, M_d and Ino_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 results of Design N°1. 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 in chapter 5), and P_d was set between 3 and 6 % (dry basis) as a manner of supplying an excess of precursor.

Table 7.2. Performance parameters of the experiments in the factorial design N°2.

Run	Evaluated variables			Performance indices					
				P_{sub}^{i} (mg_{Prod} g^{-1}_{TS})			Y_{mol} (% $mol_{2\text{-}PE+2\text{-}PEA}$ $mol^{-1}_{L\text{-}Phe0}$)	$X_{L\text{-}Phe}$ ($mol_{L\text{-}Phe\ Reacted}$ $mol^{-1}_{L\text{-}Phe0}$)	$Y_{s\text{-}t}$ ($mg_{2\text{-}PE+2\text{-}PEA}$ L^{-1} h^{-1})
	MC (%)	pH	P_d (%)	2-PE	2-PEA	Total			
1	60	4.8	3.0	4.7	2.5	7.2	0.29	29.0	8.6
2	76	4.8	3.0	8.7	3.0	11.7	0.26	20.3	8.6
3	60	6.0	3.0	5.1	1.8	6.9	0.27	20.5	7.7
4	76	6.0	3.0	8.5	3.5	12.0	0.28	30.5	9.0
5	60	4.8	6.0	4.5	2.6	7.1	0.26	24.6	8.2
6	76	4.8	6.0	8.2	4.5	12.7	0.23	21.3	8.3
7	60	6.0	6.0	3.5	2.0	5.5	0.20	40.0	6.4
8	76	6.0	6.0	7.9	5.2	13.1	0.24	19.2	9.0

Data computed at the end of the fermentation at 72 h. Results are the mean value of duplicates with a standard deviation less than 10%. 2-PE: 2-phenylethanol; 2-PEA: 2-phenethyl acetate; P_d: L-phenylalanine dose; MC: moisture content; Y_{mol}: molar yield; P_{sub}^{i}: cumulative production; $X_{L\text{-}Phe}$: L-phenylalanine conversion; $Y_{s\text{-}t}$: space-time yield.

As shown in Figure 7.2(a), MC is the only variable of the group with a significant influence on P_{sub}^{Tot}. It can also be seen in Figure 7.2(b) and (c) how pH and

P_d contour plots remain almost vertical lines, with a limited impact on P_{sub}^{Tot}, suggesting a limited effect on the bioproduction of 2-PE and 2-PEA.

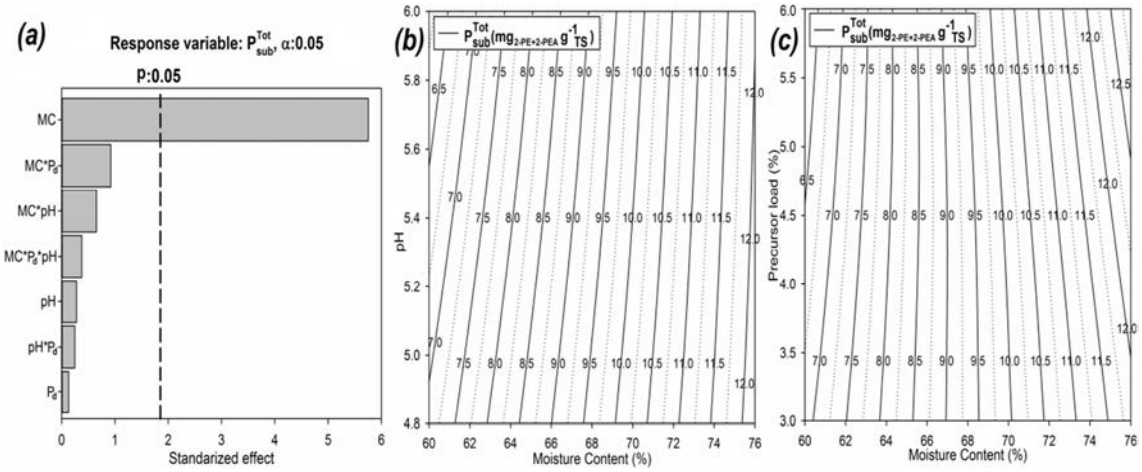


Figure 7.2. (a) Pareto chart and variables effects on the cumulative production of 2-PE and 2-PEA for the solid-state fermentation of sugarcane bagasse with *K. marxianus* for design N°2. (b) pH and MC effects, (c) L-Phe dose and MC effects.

From design N°2 the only significant variable that can be extracted is the MC. In this case, data indicates that the higher the MC, the higher the production (Figure 7.2(b) and (c)), reaching the maximum values when working near the water holding capacity of the substrate at 76%. As it has been previously stated (Chapter 5), in general, MC plays a significant role in SSF influencing other parameters like the substrate porosity and the water availability, and these, in turn, affect the aerobic/anoxic condition of the fermentation. While a high MC would contribute to the formation of anaerobic zones (favoring the alcohol species), a low MC would lead to a slower development of the process; but at the same time, with higher porosity, it would promote an aerobic environment ideal for the *K. marxianus* growth. Moreover, during the fermentation, some water is produced, which added to the mass loss (due to the organic matter degradation) could induce to exceed the WHC of the media if the MC level is to close to this limit. Hence, selection of MC becomes a tradeoff to maximize the production, avoiding problems like exceeding the WHC with the consequent leachate generation, or the excessive ethanol production and its synergistic inhibition of the strain.

Having in mind the above aspects, and considering the MC control is very limited along the SSF process, using an intermediate initial MC seems to be 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.

Differently, although initial pH is set up based on the strain needs, some microorganisms such as *K. marxianus*, are more flexible to pH changes (Lane and Morrissey, 2010). That is how P_{sub}^{Tot} is expected to be less affected by pH fluctuations as found in other studies (Etschmann and Schrader, 2006; Fabre et al., 1998; Garavaglia et al., 2007). Coming back to L-Phe dose, it is clear that there is no a direct relationship between the L-Phe availability and the 2-PE and 2-PEA production. That was evident from the design N°2, where the further increase in P_d was assessed to identify if the production could be enhanced due to a higher precursor availability.

As observed in Figure 7.2(c), P_{sub}^{Tot} reaches the maximum production levels in the highest levels of the evaluated interval, reaching 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% (Table 7.1 and Table 7.2). This data suggests that *K. marxianus* is not using the L-Phe surplus efficiently for producing 2-PE and 2-PEA. Consequently, the subtle increase in production when using higher L-Phe loads may not compensate the waste of unreacted precursor. Under this scenario, different alternatives to increase the efficiency of *K. marxianus* for metabolizing the precursor were decided to be evaluated. In practice, contents below 3% seem to be adequate for running the process without wasting significant amounts of L-Phe.

Based on the 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 Figure 7.3, under these conditions, the production rapidly grows during the first 25-26 h of processing, coinciding with the oxygen uptake rate (OUR) and CO_2 production (CO_2^P) profiles. In this phase, the respiration quotient (RQ) rapidly falls from 2.2 to 1.05 to stay almost constant in the remaining fermentation time. Also, after the peak of maximum activity, the highest cells count is found (Figure 7.3(b)). At that point, the total productivity suddenly decreases due to the depletion of the sugar content with the corresponding slowdown in the L-Phe conversion (Figure 7.3(a)), reaching a maximum P_{sub}^{Tot} of 16 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} after 72 h. As seen, at that point, the main product corresponds to 2-PE, reaching around 75% of the total production. Given the marked change in the

production after 24-26 h, the maximum Y_{s-t} also agrees with this inflection point. Since L-Phe transformation coincides with the growth phase, it is expected that the end of this stage could serve as a reference point for alternative changes in the process strategy.

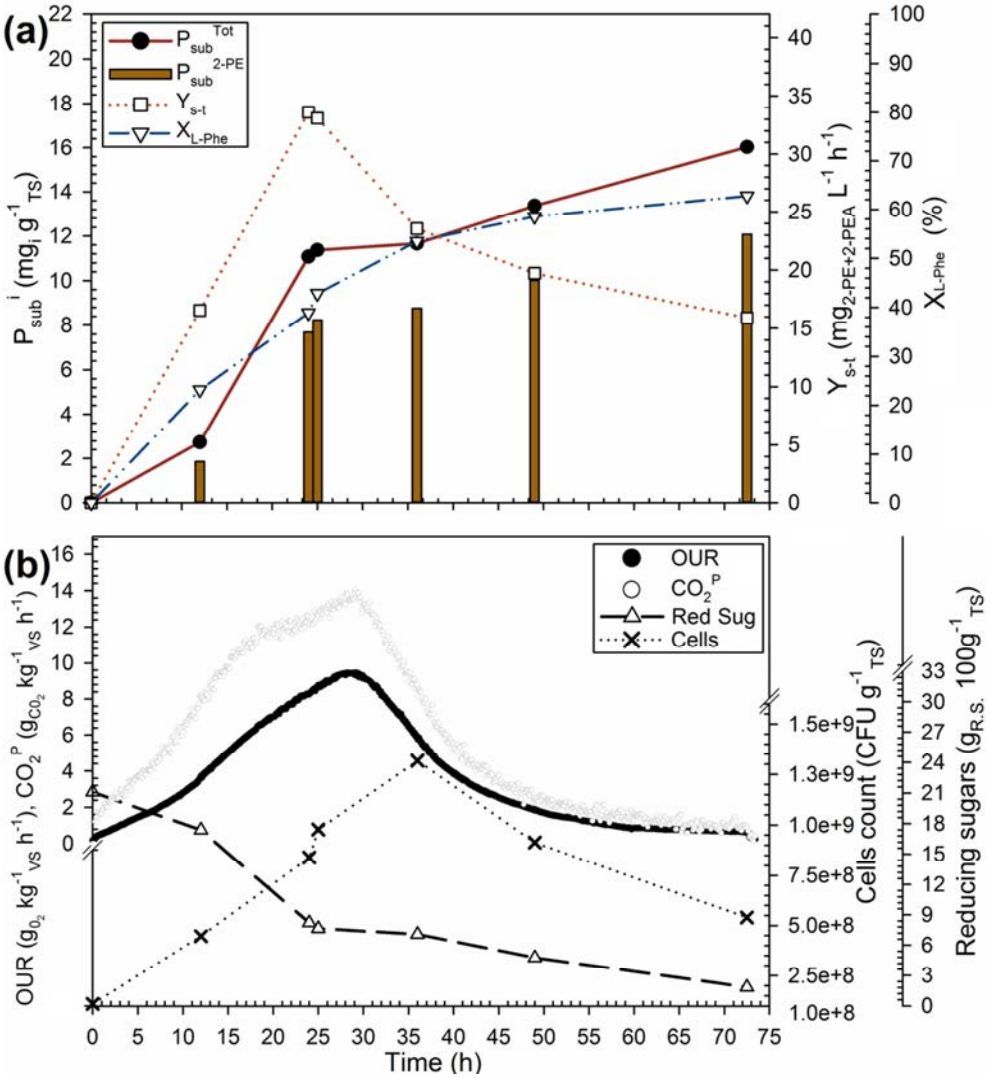


Figure 7.3. Time course of the solid-state fermentation of sugarcane bagasse for producing 2-PE and 2-PEA in the reference scenario E_1. (a) P_{sub}^{Tot}, P_{sub}^{2-PE}, Y_{s-t} and X_{L-Phe}; (b), OUR, CO_2^P, cells count and reducing sugars.

7.2.2 Production enhancement based on operating conditions and glucose addition

The strategies implemented in this section are focused on the effect of S_{AFR}, temperature and the addition of glucose as complementary carbon source on the 2-PE and 2-PEA production. As seen in section 7.2.1, S_{AFR} and temperature are two of the most significant variables affecting the process, so they could be further analyzed to

improve the production of the rose-like species. Also, considering that the production is growth-dependent, the addition of glucose for supplying fresh sugars to the media could be an alternative to promote the *K. marxianus* activity for more extended periods of time. Having in mind that for the reference scenario (E_1) the available sugars are consumed close to the point of maximum activity (also near the maximum *K. marxianus* growth), and that at that point the 2-PE and 2-PEA productivity is suddenly reduced, by using this inflection point as reference for implementing changes in the process seems to be adequate.

Therefore, the evaluated strategies comprised changes devoted to analyze the fermentation before and after this point. Thus, the S_{AFR} was increased up to 0.14 L h^{-1} g^{-1}_{TS} as strategy for enhancing the production during the growth phase (strategies E_2 and E_2^*), but it has also been used to promote a less aerobic environment in the post-maximum growth phase (strategies E_4 and E_4^*) 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 (Boer et al., 2007; Romagnoli, 2014).

Similarly, the temperature has been increased from 30°C to 40°C at the point mentioned above as a second alternative for promoting a less aerobic environment in the post-maximum growth phase (strategies E_3 and E_3^*), considering the results found in chapter 5 for producing fruit-like aromas when working at high temperatures. In the same way, glucose additions have been performed at the selected inflection point, and they have been done for each of the previously described scenarios (strategies E^*).

Table 7.3 shows a summary of characteristics of the experiments comprising the strategies based on operating conditions and glucose addition. The remaining operational variables were set at the same initial conditions than the reference scenario E_1.

Figure 7.4 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}^{Tot} 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}^{Tot} and Y_{s-t} neither when rising temperature (E_3) nor by reducing S_{AFR} in 1/3 (E_4).

Table 7.3. Characteristics of the strategies based on operating conditions and glucose addition.

Strategy	Performed changes				Objective	Maximum P_{sub}^{i} ($mg_i\ g^{-1}_{TS}$)		
	Growth phase (G)	After 25 h (post-maximum growth phase)						
	S_{AFR} ($L\ h^{-1}\ g^{-1}_{TS}$)		T (°C)	Glucose added		2-PE	2-PEA	Total
E_1	0.10	NC	NC	-	Reference scenario	12.08	3.94	16.03
E_2	0.14	NC	NC	-	G enhancement	15.49	3.16	18.65
E_3	0.10	NC	40	-	Less aerobic	8.50	6.98	15.48
E_4	0.10	0.03	NC	-	Less aerobic	11.34	4.39	15.73
E_1^*	0.10	NC	NC	85% R.S. at t_o	Extending *K. marxianus* activity by Glu	13.05	4.78	17.83
E_2^*	0.14	NC	NC	85% R.S. at t_o	G enhancement + Extending *K. marxianus* activity by Glu	13.57	6.29	19.86
E_3^*	0.10	NC	40	85% R.S. at t_o	Less aerobic + Extending *K. marxianus* activity by Glu	13.75	5.42	19.17
E_4^*	0.10	0.03	NC	85% R.S. at t_o	Less aerobic + Extending *K. marxianus* activity by Glu	12.85	7.35	20.20
E_5^*	0.10	NC	NC	100% R.S. at t_o[a]	Extending *K. marxianus* activity by Glu (Long-term)	16.22	4.86	21.08

T_0:30°C, pH_0:4.8, MC_0:66%, Inoculum: $10^8 CFU\ g^{-1}_{TS}$, L-Phe_0: 2% (dry basis), 2.5% for E_5^*. t_0: time zero; G: growth phase; NC: oo 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}^{Tot}: cumulative production. [a]A second glucose addition with the same characteristics was made at 48 h.

These latter strategies stimulate the modification of the O_2 transfer, increasing the respiration quotient far from a pure aerobic process toward a less aerobic environment (RQ > 1.9). However, at the moment changes are made, the available sugars are low enough (<8%) to be further exploited by the strain. Thus, the process might be considered finished 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.

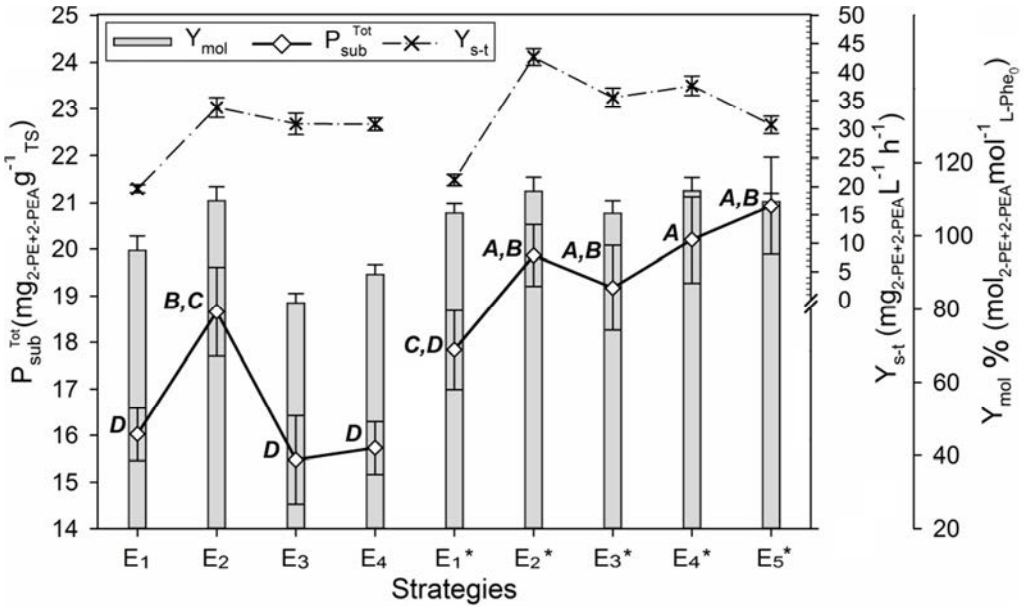


Figure 7.4. Performance indices of the strategies based on operating conditions and glucose addition for improving the 2-PE and 2-PEA production. P_{sub}^{Tot} and Y_{mol} are the maximum values in time while Y_{s-t} is the maximum value after the growth phase. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$).

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 by using glucose additions at the end of the growth phase (as fresh sugar source) was also estimated to identify alternatives to increase the combined 2-PE and 2-PEA production. Hence, in E_1^* and E_2^* glucose was added such that it represented 85% of initial sugar content, and operational conditions were kept unchanged along the fermentation. Under those conditions, the maximum P_{sub}^{Tot} increased by 11% and 24% respectively until a maximum of 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}^{Tot} relative to E_1 was obtained. In general, in these strategies, the RQ trend in the interval 24 to 48 h shows that the environment is less aerobic (RQ > 1.85), suggesting that the objective of these strategies is fulfilled.

For instance, in E_4^* (Figure 7.5) the first 24 h are similar to those found in E_1 (This phase is equal for both strategies). However, after the glucose addition and reduction of S_{AFR}, P_{sub}^{Tot} has grown to a maximum of 20.2 $mg_{2\text{-PE+2-PEA}}$ g^{-1}_{TS} after 49 h of processing, reaching a 2-PE content close to 64% (Figure 7.5(a)). It can also be observed that in this strategy, glucose addition has allowed to *K. marxianus* remaining active for a more extended period than in E_1, reaching a number of cells close to 1.3 10^9 CFU g^{-1}_{TS} (Figure 7.5(b)). Besides, this activity has remained almost unchanged until the available sugars were almost depleted, just after the 2-PE and 2-PEA productivity started to fall together with the L-Phe consumption rate.

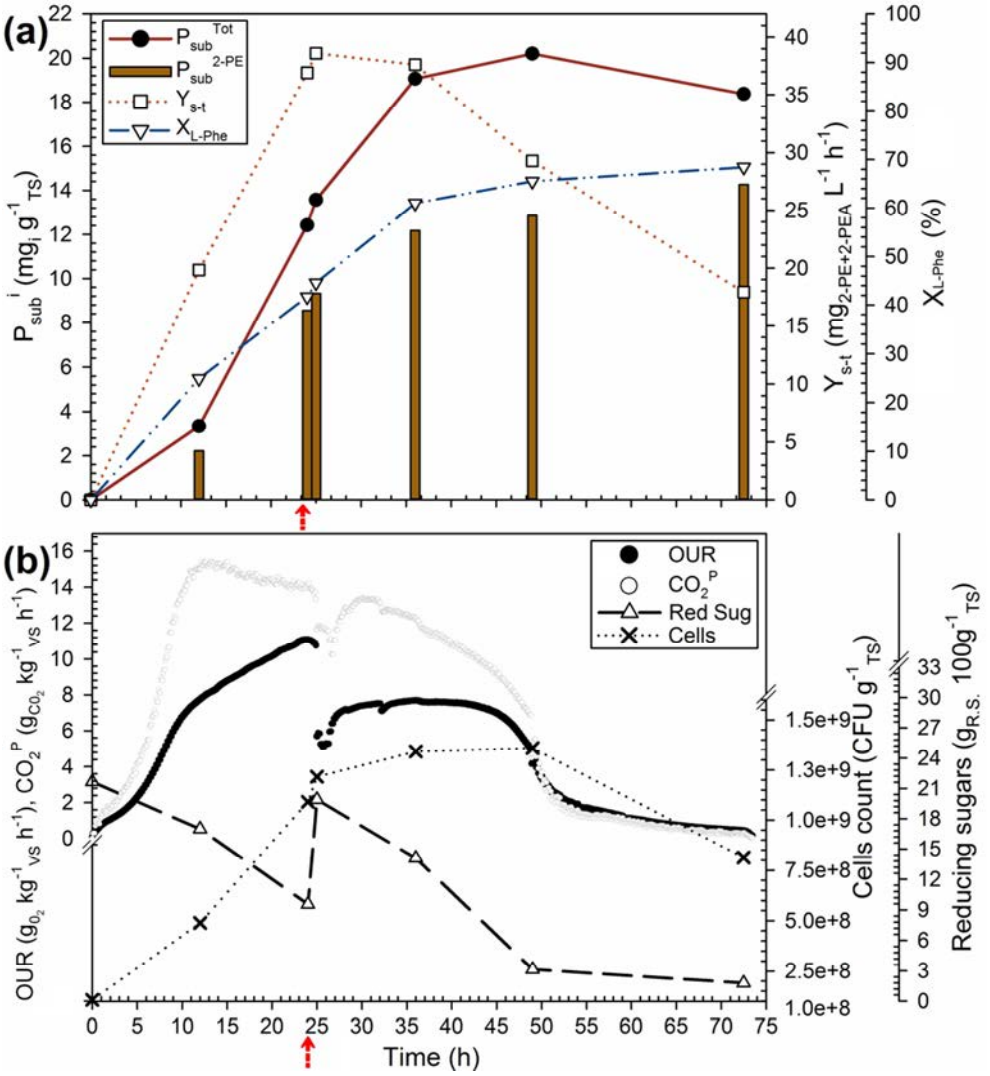


Figure 7.5. Time course of the solid-state fermentation of sugarcane bagasse for producing 2-PE and 2-PEA in scenario E_4^*. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-PE}}$, $Y_{s\text{-}t}$ and $X_{L\text{-Phe}}$; (b), OUR, CO_2^P, cells count and reducing sugars. Dash arrows indicate the addition of glucose.

Considering that these results suggest that P_{sub}^{Tot} could be improved by promoting the *K. marxianus* activity, 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 Figure 7.4, in E_5^* there are no significant changes in the maximum 2-PE and 2-PEA production compared to the previous scenarios where glucose was added. Moreover, as it was found in these strategies, Y_{mol} is around the average of these strategies (109 %) with similar $X_{L\text{-}Phe}$ values.

Figure 7.6(a) and (b) show the time course of the SSF for the strategy E_5^*. As expected, the point in which maximum *K. marxianus* activity was achieved is similar to E_1. At this point, the microbial activity coincided with the highest cell counts for the strain ($1.3\ 10^9$ CFU g^{-1}_{TS}) (Figure 7.6(b)). Nevertheless, differently than E_1, when glucose was supplied, an additional increase in activity was found, but it did not coincide with *K. marxianus* growth (as it has occurred in E_4^*); instead, cells count have remained at the same level during the time there were enough sugars to be consumed.

Nevertheless, the effect on P_{sub}^{Tot}, $X_{L\text{-}Phe}$ and $Y_{s\text{-}t}$ was an extra increase lasting up to 36 h of fermentation (Figure 7.6(a)). At this point, the activity started falling, and the performance parameters had had only subtle changes, even when at 48 h a new glucose addition was performed. In that case, there were no significant improvements regarding the 2-PE and 2-PEA production, and the maximum P_{sub}^{Tot} was obtained after 72 h of processing when available sugars were entirely exhausted. In general, in strategy E_5^* the 2-PE content has reached up to 76% (Figure 7.6(a)), and as it has happened in E_4^*, it has continuously grown even after the maximum P_{sub}^{Tot} is achieved, suggesting that the 2-PEA is being transformed into other species or it is being consumed in this last stage of the fermentation.

Once the process was split in the point of maximum activity for *K marxianus*, two different aspects could 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 P_{sub}^{Tot}. Similarly, the use of glucose to provide additional sugars 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 was available in the media.

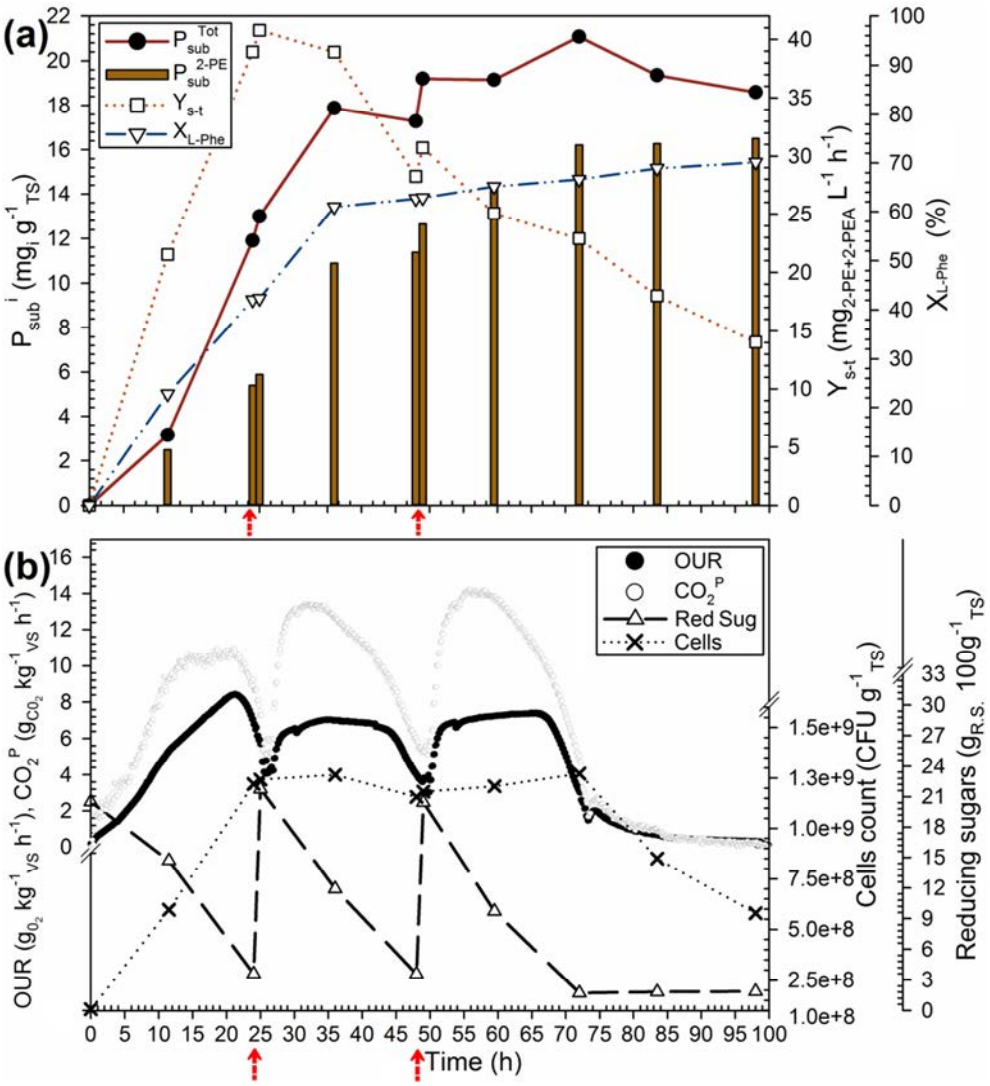


Figure 7.6. Time course of the solid-state fermentation of sugarcane bagasse for producing 2-PE and 2-PEA in scenario E_5^*. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-}PE}$, $Y_{s\text{-}t}$ and $X_{L\text{-}Phe}$; (b), OUR, CO_2^P, cells count and reducing sugars. Dash arrows indicate the addition of glucose.

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 (Pan et al., 2014; Wang et al., 2013) 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.

At the same time, as shown in Chapter 6, the specific *K. marxianus* strain used in this study was able to produce up to 3.8 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} in a system based on SCB as only carbon source, proving both biotransformation mechanisms are working in parallel. 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, in aerobic and anoxic environments, since Y_{mol} is beyond 100% for these scenarios (Figure 7.4)

7.2.3 Production enhancement based on the feeding strategy

Table 7.4 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 sugar content provided by glucose with sugars coming from partial additions of the same substrate (SCB). 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 (E_1), 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.

The performance of the strategies E_6 to E_8 compared to the reference strategy (E_1) is shown in Figure 7.7. As it is observed, the use of a split feeding seems to increase the maximum production levels, in particular when the load is split into more than two fractions (E_7 and E_8). The trend shows how the fed-batch strategy helps to increase P_{sub}^{Tot} until a maximum of 18.4 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} in E_8. This suggests that the fed-batch strategy succeeds replacing the glucose additions presented in the previous

section to increase the 2-PE and 2-PEA combined production. In general, by doing this, Y_{s-t} is also improved reaching mean values around 29 $mg_{2\text{-PE}+2\text{-PEA}}$ L^{-1} h^{-1}.

Table 7.4. Characteristics of the strategies based on the feeding of sugarcane bagasse.

Strategy	Main characteristics	Residue & L-Phe loads[a]	Maximum P_{sub}^{i} (mg_i 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)
t_0: time zero; 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}^{Tot}: cumulative production. [a]The feeding percentage is referred to the total load of a batch fermentation, equivalent to 96g of prepared substrate per replicate.

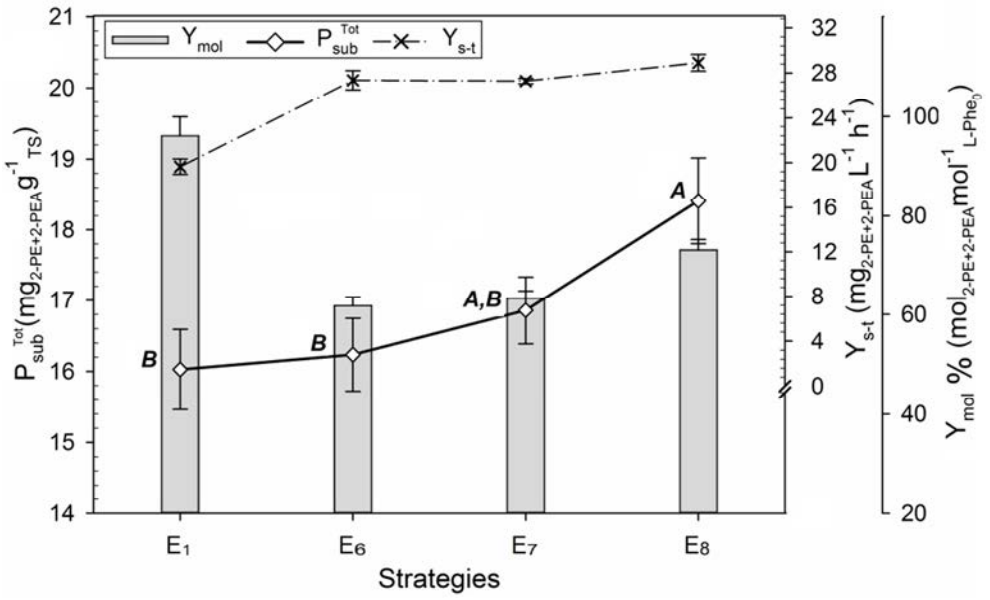


Figure 7.7. Performance indices of the strategies based on the feeding strategy for improving the 2-PE and 2-PEA production. P_{sub}^{Tot} and Y_{mol} are the maximum values in time while Y_{s-t} is the maximum value after the growth stage. Different capital letters indicate significant differences between the evaluated groups ($p<0.05$).

As detailed in Figure 7.8, when using a fed-batch of two splits, the first 24 h of processing are analogous to the batch scenario E_1, reaching the first peak of maximum activity at 24 h with similar P_{sub}^{Tot} and OUR (13.3 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} and 9.7 g_{O2} kg^{-1}_{VS} h^{-1} respectively). At that point the addition of the remaining 50% of the substrate load induces a dilution effect as shown in chapter 6 (*K. marxianus* cell and P_{sub}^{Tot} fall), and the yeast uses the available sugars supplied to grow up to 1.7 10^9 CFU g^{-1}_{TS} promoting a further 2-PE and 2-PEA production that reaches a maximum of 16.2 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} after 42 h of fermentation.

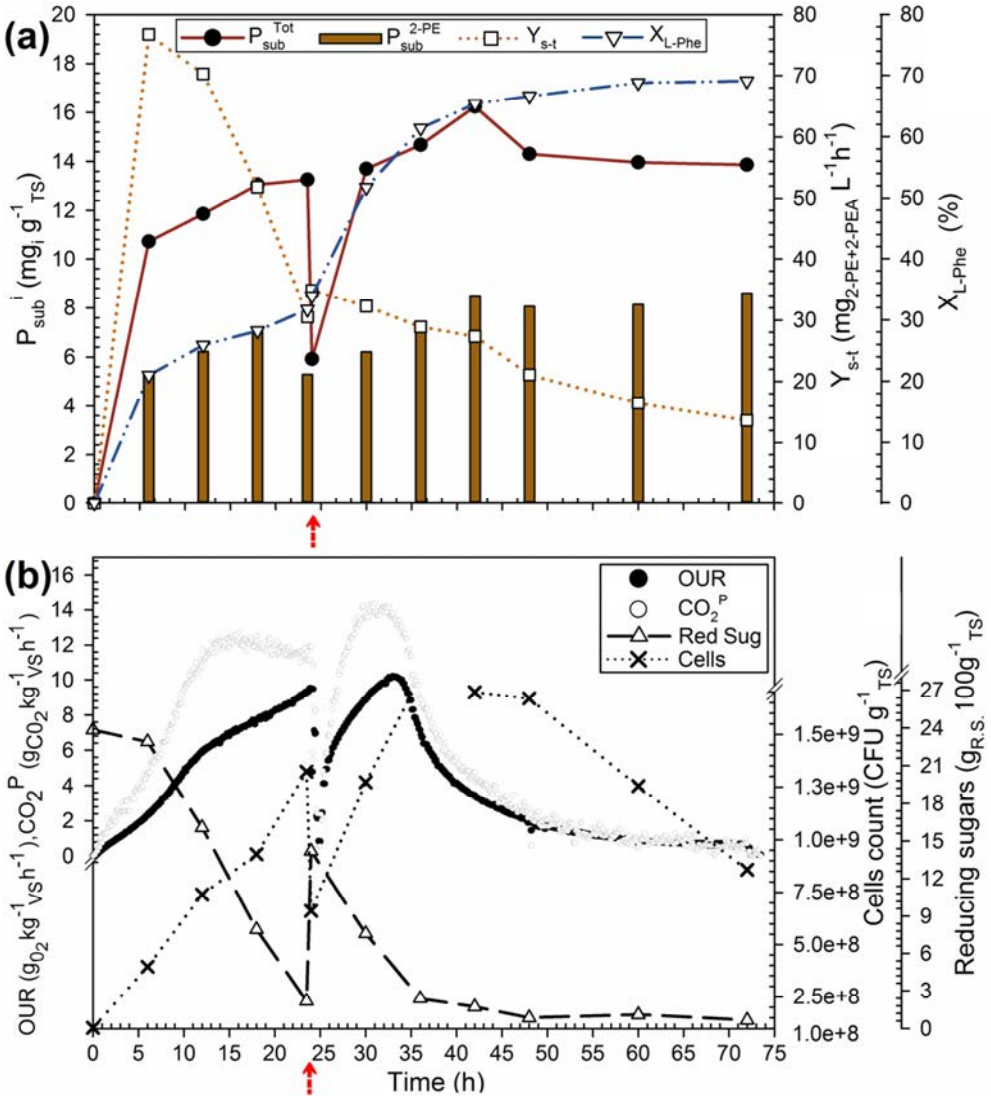


Figure 7.8. Time course of the solid-state fermentation of sugarcane bagasse for producing 2-PE and 2-PEA in fed-batch mode, strategy E_6. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-}PE}$, $Y_{s\text{-}t}$ and $X_{L\text{-}Phe}$; (b) OUR, CO_2^P, cells count and reducing sugars. Dash arrows indicate the addition of fresh substrate.

Although the maximum P_{sub}^{Tot} obtained in E_6 is not significantly different from E_1, there were differences in the L-Phe consumption ($X_{L\text{-}Phe}$ reached 70%), and in the distribution of the products. While in E_1 the primary product is 2-PE, in E_6 both products are almost equally distributed (2-PE 52%, 2-PEA 48%) in the point of maximum production.

The time course of the strategy E_7 is shown in Figure 7.9. 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}^{Tot} is presented (Figure 7.9(a)), 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 P_{sub}^{Tot} at 48 h. After this point, a subtle reduction in P_{sub}^{Tot} is presented until the end of the fermentation as occurred in the previous strategies E_4^*, E_5^* and E_6. It can also be observed that $X_{L\text{-}Phe}$ continuously grows, but the considerable changes occur when fresh SCB is added to the media, coinciding with the *K. marxianus* growth (Figure 7.9(b)).

From Figure 7.9(b) it can also be observed that there is a direct relationship between cells count and the strain activity. Differently than in E_4^* and E_5^*, in E_7 *K. marxianus* population agrees with the OUR and CO_2^P profiles, growing with the increase of activity. Moreover, 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. Again, as occurred in E_6, the maximum P_{sub}^{Tot} is not significantly different from the obtained in E_1, but the ratio of products has changed, with similar contents for each of them at the end of the fermentation (53% 2-PE, 47% 2-PEA) and a subtle increase in $X_{L\text{-}Phe}$ up to 72% after 72 h.

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}^{Tot} and a higher $X_{L\text{-}Phe}$ (Figure 7.10(a)) as occurred in E_6 and E_7. The main difference lies in the time *K. marxianus* remain active, which seems to be a direct function of the sugars availability (Figure 7.10(b)). As seen, in E_8 the maximum combined 2-PE and 2-PEA production is reached close to 60 h of processing. At that point, the L-Phe consumption almost ceases due to the depletion of the carbon source and P_{sub}^{Tot} and $Y_{s\text{-}t}$ start falling

as occurred in the other fed-batch scenarios. This time, the $X_{L\text{-}Phe}$ reaches 79% after 72 h, and the maximum P_{sub}^{Tot} has increased a 9.2% compared to the obtained in E_7. Besides, as occurred in E_6 and E_7 products distribution after 72 h is around 50% each (55% 2-PE, 45% 2-PEA).

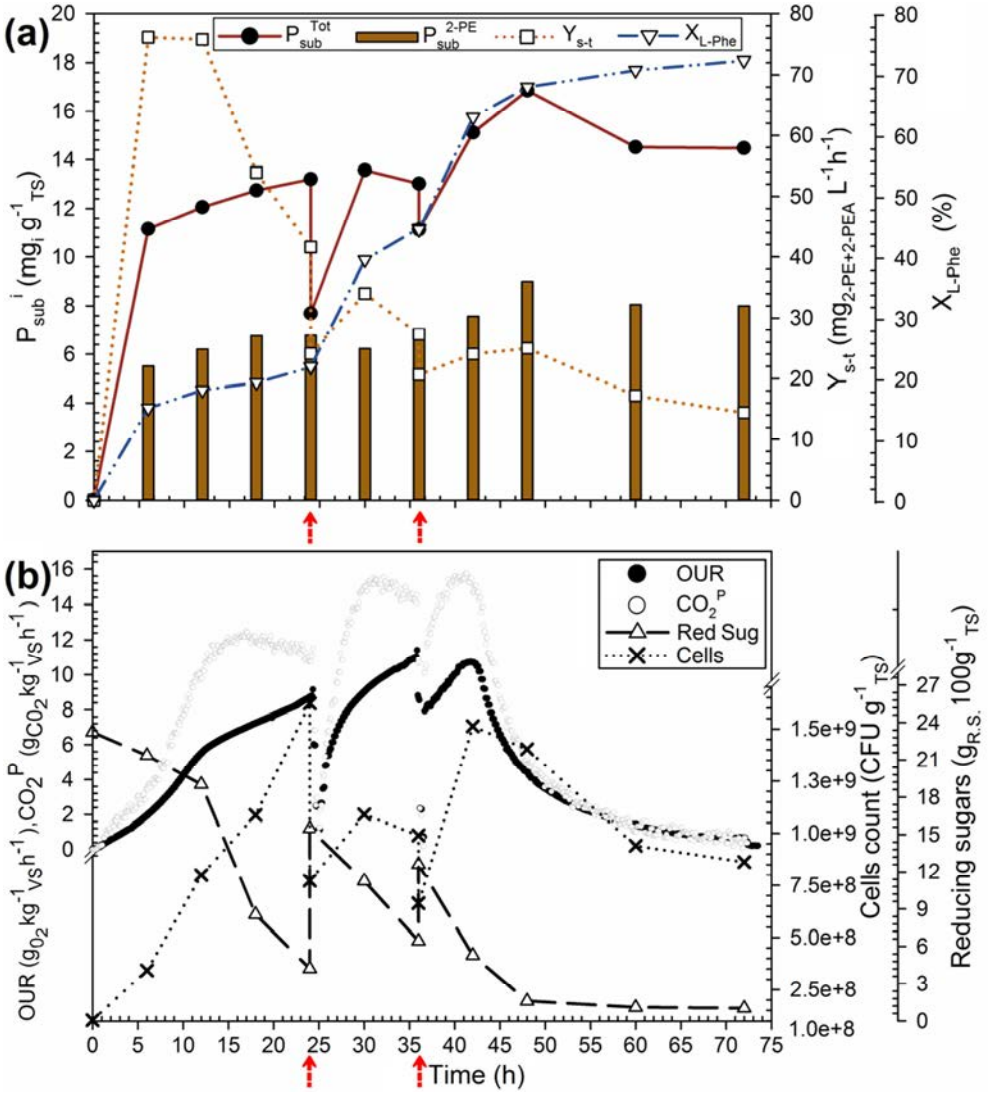


Figure 7.9. Time course of the solid-state fermentation of sugarcane bagasse for producing 2-PE and 2-PEA in fed-batch mode, strategy E_7. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-}PE}$, $Y_{s\text{-}t}$ and $X_{L\text{-}Phe}$; (b) OUR, CO_2^p, cells count and reducing sugars. Dash arrows indicate the addition of fresh substrate.

In these fed-batch strategies, despite 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 (Figure 7.7). 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).

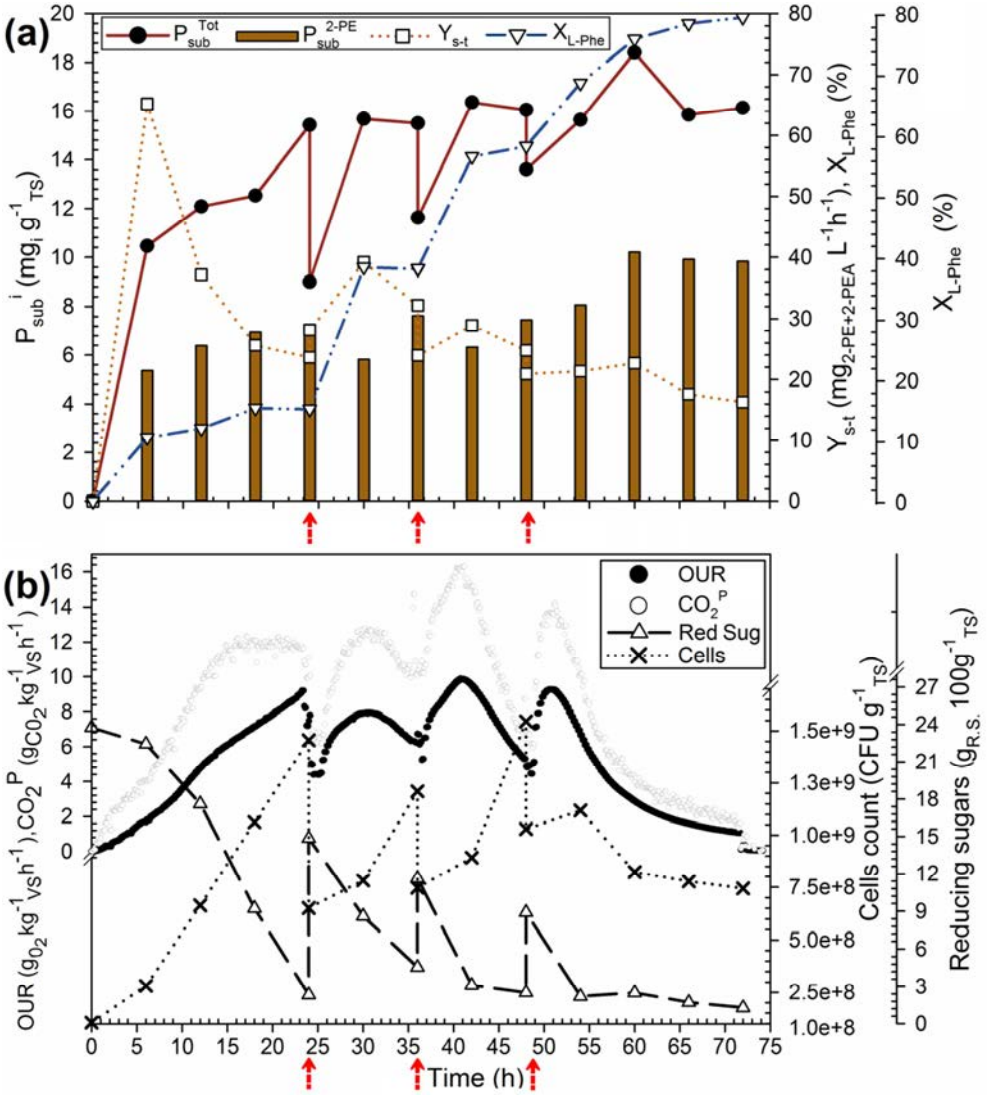


Figure 7.10. Time course of the solid-state fermentation of sugarcane bagasse for producing 2-PE and 2-PEA in fed-batch mode, strategy E_8. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-}PE}$, $Y_{s\text{-}t}$ and $X_{L\text{-}Phe}$; (b) OUR, CO_2^P, cells count and reducing sugars. Dash arrows indicate the addition of fresh substrate.

Another remarkable 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 that *K. marxianus* is able to keep active and consuming L-Phe at higher 2-PE concentrations than in SmF.

As seen in Figure 7.11, the batch process (E_1) reaches equivalent 2-PE liquid concentrations higher than the most common inhibitory limit found in SmF of almost 4 g/L (beyond 60% higher) (Etschmann and Schrader, 2006; Stark et al., 2003b; Wang et al., 2011a). The same occurs when the strategy is changed to the addition of glucose (E_5^*) remaining at higher equivalent 2-PE liquid concentrations during more than 40 h of processing without any adverse effect in the *K. marxianus* activity. At the same time, for the SSF fed-batch operations, 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. Intrinsically, this operating mode avoids high products concentrations during most of the process limiting the effect of the 2-PE on *K. marxianus* cells.

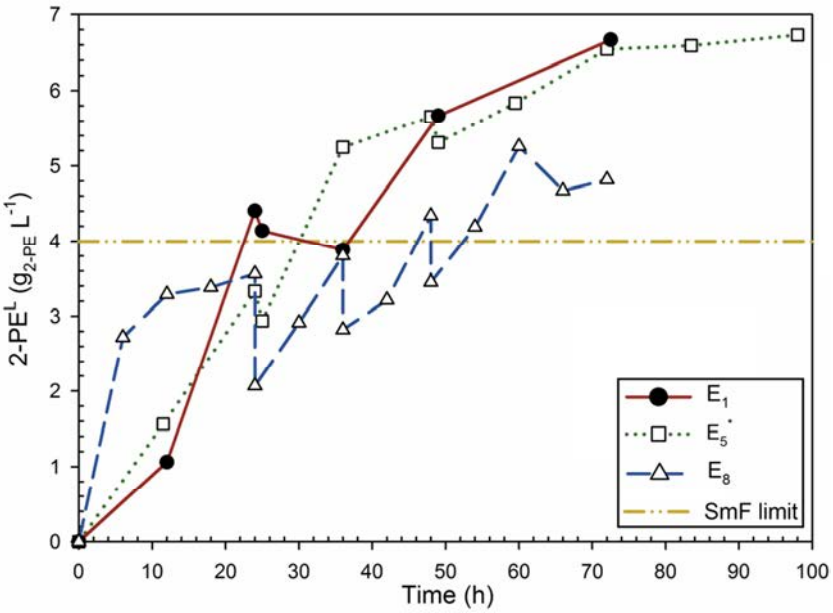


Figure 7.11. Equivalent 2-phenylethanol concentration in the liquid interface (2-PE^L) of the solid-state fermentation of sugarcane bagasse with *K. marxianus*. SmF: submerged fermentation. 2-PE^L computed based on the moisture content of the solid media. E_i: evaluated strategies.

This data suggests 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 or moisture content along the residue and due to the fermentation). Also, under these conditions, 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.

Final remarks

It can be stated that the selected *K. marxianus* has used the agro-industrial residue sugarcane bagasse as sole carbon source for the biotransformation of L-phenylalanine into 2-PE and 2-PEA through SSF. In general, using SSF fed-batch processes have resulted in a simple and reliable way to increase the 2-PE and 2-PEA production.

Similarly than the fruit-like case, the results here presented for the lab-scale SSF would serve as base for the developments of the bioprocess at larger scales.

References

Białecka-Florjańczyk, E., Krzyczkowska, J., Stolarzewicz, I., Kapturowska, A., 2012. Synthesis of 2-phenylethyl acetate in the presence of *Yarrowia lipolytica* KKP 379 biomass. J. Mol. Catal. B Enzym. 74, 241–245.

Boer, V.M., Tai, S.L., Vuralhan, Z., Arifin, Y., Walsh, M.C., Piper, M.D.W., De Winde, J.H., Pronk, J.T., Daran, J.M., 2007. Transcriptional responses of *Saccharomyces cerevisiae* to preferred and nonpreferred nitrogen sources in glucose-limited chemostat cultures. FEMS Yeast Res. 7, 604–620.

Chen, H., 2013. Modern Solid State Fermentation, First Edition. Ed. Springer, New York.

Chreptowicz, K., Wielechowska, M., Główczyk-Zubek, J., Rybak, E., Mierzejewska, J., 2016. Production of natural 2-phenylethanol: From biotransformation to purified product. Food Bioprod. Process. 100, 275–281.

Deive, F.J., Costas, M., Longo, M.A., 2003. Production of a thermostable extracellular lipase by *Kluyveromyces marxianus*. Biotechnol. Lett. 25, 1403–1406.

Eshkol, N., Sendovski, M., Bahalul, M., Katz-Ezov, T., Kashi, Y., Fishman, A., 2009. Production of 2-phenylethanol from L-phenylalanine by a stress tolerant *Saccharomyces cerevisiae* strain. J. Appl. Microbiol. 106, 534–542.

Etschmann, M.M.W., Bluemke, W., Sell, D., Schrader, J., 2002. Biotechnological production of 2-phenylethanol. Appl. Microbiol. Biotechnol. 59, 1–8.

Etschmann, M.M.W., Schrader, J., 2006. An aqueous-organic two-phase bioprocess for efficient production of the natural aroma chemicals 2-phenylethanol and 2-phenylethylacetate with yeast. Appl. Microbiol. Biotechnol. 71, 440–443.

Etschmann, M.M.W., Sell, D., Schrader, J., 2005. Production of 2-Phenylethanol and 2-Phenylethylacetate from L-Phenylalanine by coupling whole-cell biocatalysis with organophilic pervaporation. Biotechnol. Bioeng. 92, 624–634.

Etschmann, M.M.W., Sell, D., Schrader, J., 2003. Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. Biotechnol. Lett. 25, 531–536.

Fabre, C.E., Blanc, P.J., Goma, G., 1998. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. Biotechnol. Prog. 14, 270–274.

Gao, F., Daugulis, A.J., 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by *Kluyveromyces marxianus*. Biotechnol. Bioeng. 104, 332–339.

Garavaglia, J., Flôres, S.H., Pizzolato, T.M., Peralba, M.D.C., Ayub, M.A.Z., 2007. Bioconversion of L-phenylalanine into 2-phenylethanol by *Kluyveromyces marxianus* in grape must cultures. World J. Microbiol. Biotechnol. 23, 1273–1279.

Jiménez-Peñalver, P., Gea, T., Sánchez, A., Font, X., 2016. Production of sophorolipids from winterization oil cake by Solid-state fermentation: Optimization, monitoring and effect of mixing. Biochem. Eng. J. 115, 93–100.

Lane, M.M., Morrissey, J.P., 2010. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. Fungal Biol. Rev. 24, 17–26.

Masuo, S., Osada, L., Zhou, S., Fujita, T., Takaya, N., 2015. *Aspergillus oryzae* pathways that convert phenylalanine into the flavor volatile 2-phenylethanol. Fungal Genet. Biol. 77, 22–30.

Medeiros, A.B.P., Pandey, A., Vandenberghe, L.P.S., Pastorel, G.M., Soccol, C.R., 2006. Production and recovery of aroma compounds produced by solid-state fermentation using different adsorbents. Food Technol. Biotechnol. 44, 47–52.

Olbrich H, 2006. The molasses, Biotechnologie Kempe GmbH. Berlin.

Pan, X., Qi, H., Mu, L., Wen, J., Jia, X., 2014. Comparative metabolomic-based metabolic mechanism hypothesis for microbial mixed cultures utilizing cane molasses wastewater for higher 2-Phenylethanol production. J. Agric. Food Chem. 62, 9927–9935.

Rodriguez-Leon, J., Soccol, C.R., Pandey, A., Rodriguez, D., 2008. Factors affecting solid-state fermentation, in: Pandey, A., Soccol, C.R., Larroche, C. (Eds.), Current Developments in Solid-State Fermentation. Springer, New Delhi, p. 521.

Romagnoli, G., 2014. The Ehrlich pathway for amino acid catabolism in yeasts. PhD thesis. Technische Universiteit Delft.

Stark, D., Zala, D., Münch, T., Sonnleitner, B., Marison, I.W., Von Stockar, U., 2003. Inhibition aspects of the bioconversion of L-phenylalanine to 2-phenylethanol by *Saccharomyces cerevisiae*. Enzyme Microb. Technol. 32, 212–223.

Wang, H., Dong, Q., Guan, A., Meng, C., Shi, X., Guo, Y., 2011. Synergistic inhibition effect of 2-phenylethanol and ethanol on bioproduction of natural 2-phenylethanol by *Saccharomyces cerevisiae* and process enhancement. J. Biosci. Bioeng. 112, 26–31.

Wang, Q., Song, Y., Jin, Y., Liu, H., Zhang, H., Sun, Y., Liu, G., 2013. Biosynthesis of 2-phenylethanol using tobacco waste as feedstock. Biocatal. Biotransformation 31, 292–298.

Wittmann, C., Hans, M., Bluemke, W., 2002. Metabolic physiology of aroma-producing *Kluyveromyces marxianus*. Yeast 19, 1351–1363.

Chapter 8

Operational strategies for improving the 2-phenethyl alcohol and 2-phenethyl acetate production via solid-state fermentation of sugarcane bagasse

Part of this chapter will be submitted for publication: Martínez, O., Sánchez, A., Font, X., Barrena, R. 2018. Operational strategies for improving the 2-phenylethanol and 2-phenethyl acetate bioproduction in a residue-based solid-state fermentation system.

8.1 Overview

As it has been shown in chapter 7, SSF of SCB supplemented with L-Phe presented promising characteristics to become a feasible alternative for bioproducing 2-PE and 2-PEA. However, results exposed in chapter 7 are limited regarding the scale and the sterilization of the substrate. Thus, as stated in chapter 6, these constraints limit the development of the SSF technology reducing its efficiency and sustainability.

Therefore, following the same precepts of chapter 6, here, it was decided to assess the 2-PE and 2-PEA bioproduction from the operational strategies standpoint as a tool to overcome the constraints above. As it has been found in chapters 6 and 7, fed-batch (FB) operation has succeeded to enhance the tested SSF processes, so its implementation in this stage has been considered again.

At the same time, sequential-batch mode (SB) has been implemented as a potential alternative operational strategy. This operating mode has served as a reliable way to operate different SSF processes in semi-continuous regime (Cerda et al., 2017a; Xu et al., 2017) showing motivating results regarding the productivity and global efficiency. As detailed before (chapter 6), implementation of alternatives approaches to static-batch SSF is also a way to reduce the typical adverse effects found due to the scale-up of SSF processes. Therefore, the evaluation of these strategies at different process scales become of significant importance.

Thus, this chapter aimed to assess some operational strategies such as static-batch, fed-batch and sequential-batch to improve the combined 2-PE and 2-PEA production in the SSF of sugarcane bagasse supplemented with L-phenylalanine by means of *K. marxianus*. Also, the performance of these strategies on two different scales (1.6 and 22 L) has been examined through the use of various indices. With this purpose, the selected strategies were tested using the non-sterilized substrate. In both cases, reactors were neither temperature-controlled nor isolated, expecting a dynamic temperature profile induced by the metabolic heat generated in the solid media. Table 8.1 contains the main description of the evaluated operational strategies.

Table 8.1. Characteristics of the evaluated operational strategies for producing 2-PE and 2-PEA.

Experiment	Corresponding Strategy	Characteristic	Substrate & L-Phe load*
Batch	Static substrate (Reference condition)	Substrate remains static along the fermentation	100% at t_0
FB-33	Fed-batch operation with load split in three parts	Limiting nutrient availability + temperature control by means of a partial feeding	33% at t_0 33% at t_1 33% at t_2
SB	Sequential-batch replacing 78% of the load	Operating the process in a semi-continuous mode by replacing the substrate at the given points[a]	100% at t_0 78% at t_R

*t_0: At the beginning of the process; t_1: maximum activity point after t_0; t_2: maximum activity point after t_1; t_R: maximum activity point after the substrate replacement of each cycle in the sequential-batch strategy. The reference for 100% load is the maximum amount of substrate and L-Phenylalanine used in the static-batch, *i.e.*, 300g for 1.6 L and 2.5kg for 22 L.

[a]Substrate replacement in the sequential-batch strategy is performed once the process reaches the maximum activity by withdrawing 78% of the fermented substrate (234g for 1.6 L, 1950g for 22 L), and replacing it with fresh prepared substrate that is mixed with the remaining 22% as a manner of inoculum.

8.2 Results

The process performance has been analyzed through the same indices used in chapter 6 (C_{air} and I_{Req}) and 7 (P_{Sub}^{i}, $Y_{s\text{-}t}$ and Y_{mol}), and the average maximum productivity (P_t) [$mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{TS}\ h^{-1}$] (computed as the maximum P_{sub}^{Tot} per spent hour to reach this maximum point). As occurred in chapter 7, results express the 2-PE and 2-PEA content found in both, the solid-liquid interface and in the gas phase. The presence of the products in the gas phase was always in the range 4-5% of the total production.

8.2.1 Strategies at 1.6 L scale

These experiments consisted of evaluating the effect of the operational strategies shown in Table 8.1 on $P_{Sub}^{2\text{-}PE}$ and P_{Sub}^{Tot} working with variable temperature in a non-isolated 1.6 L reactor. Initial pH, MC, S_{AFR}, L-Phe load, and inoculum load were set at 4.8, 68%, 0.14 L h^{-1} g^{-1}_{TS}, 3.5% (dry basis) and 10^8 CFU g^{-1}_{TS} respectively according to the results found in chapter 7. Experiments have been developed in duplicate.

Figure 8.1 shows the time course of the static-batch strategy at 1.6 L scale. As seen, the process starts with a 9 h lag-phase, followed by a rapid increase in activity

lasting around 15 h. At that point, activity falls due to the depletion of available sugars, and after 40 h of processing, activity and temperature have fallen such that the process could be considered finished (Figure 8.1(b)).

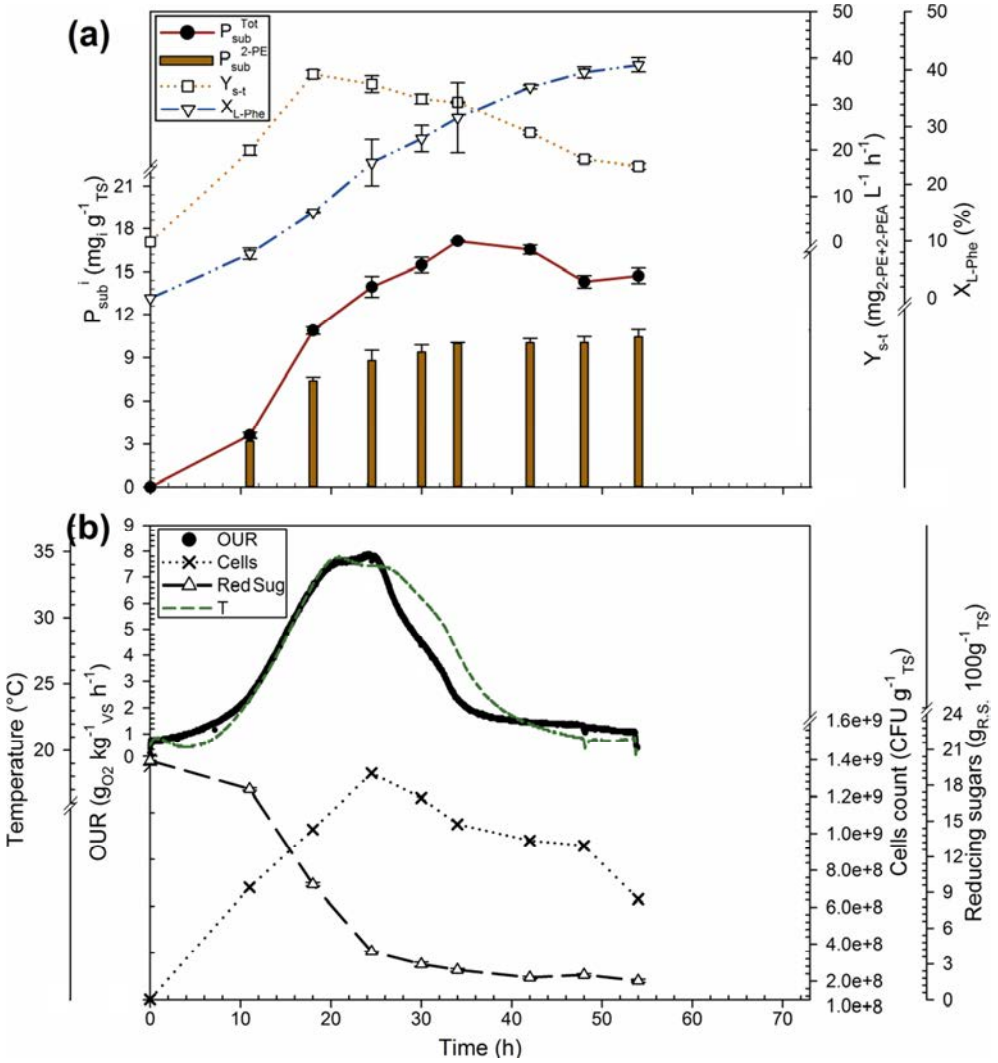


Figure 8.1. Time course of the solid-state fermentation of sugarcane bagasse supplemented with L-phenylalanine in batch mode at 1.6 L scale. (a) P_{sub}^{Tot}, P_{sub}^{2-PE}, Y_{s-t} and X_{L-Phe}; (b) OUR, temperature, cells count and reducing sugars.

At the same time, 2-PE and 2-PEA combined productivity shows a continuous increasing trend (Figure 8.1(a)), particularly high during the *K. marxianus* exponential growth phase, reaching the maximum P_{Sub}^{Tot} after 34 h (17.2 $mg_{2-PE+2-PEA}$ g^{-1}_{TS}) and coinciding with the decrease of the microbial activity and the *K. marxianus* population. Although P_{Sub}^{2-PE} continuously grows to reach a maximum of 10.4 mg_{2-PE} g^{-1}_{TS} after 54 h, a subtle reduction in P_{Sub}^{Tot} is observed in the last 20 h of processing, suggesting that

2-PEA is being consumed or transformed into other unidentified compounds once there were no more available sugars.

When operating the SSF using fed-batch mode (Figure 8.2), the reactor was initially filled with a third of the substrate load for a batch (equivalent to 100 g), and therefore, the process started more slowly since the increase in temperature has taken a longer time (Figure 8.2(d)). As a result, the OUR_{Max} obtained after 18 h of fermentation only reached 1.6 g_{O2} kg^{-1}_{VS} h^{-1} and a maximum temperature of 23°C, significantly different to the achieved in the batch mode. At that point, the second substrate fraction was added (second 100 g), and a rapid increase in both OUR and P_{Sub}^{Tot} was observed.

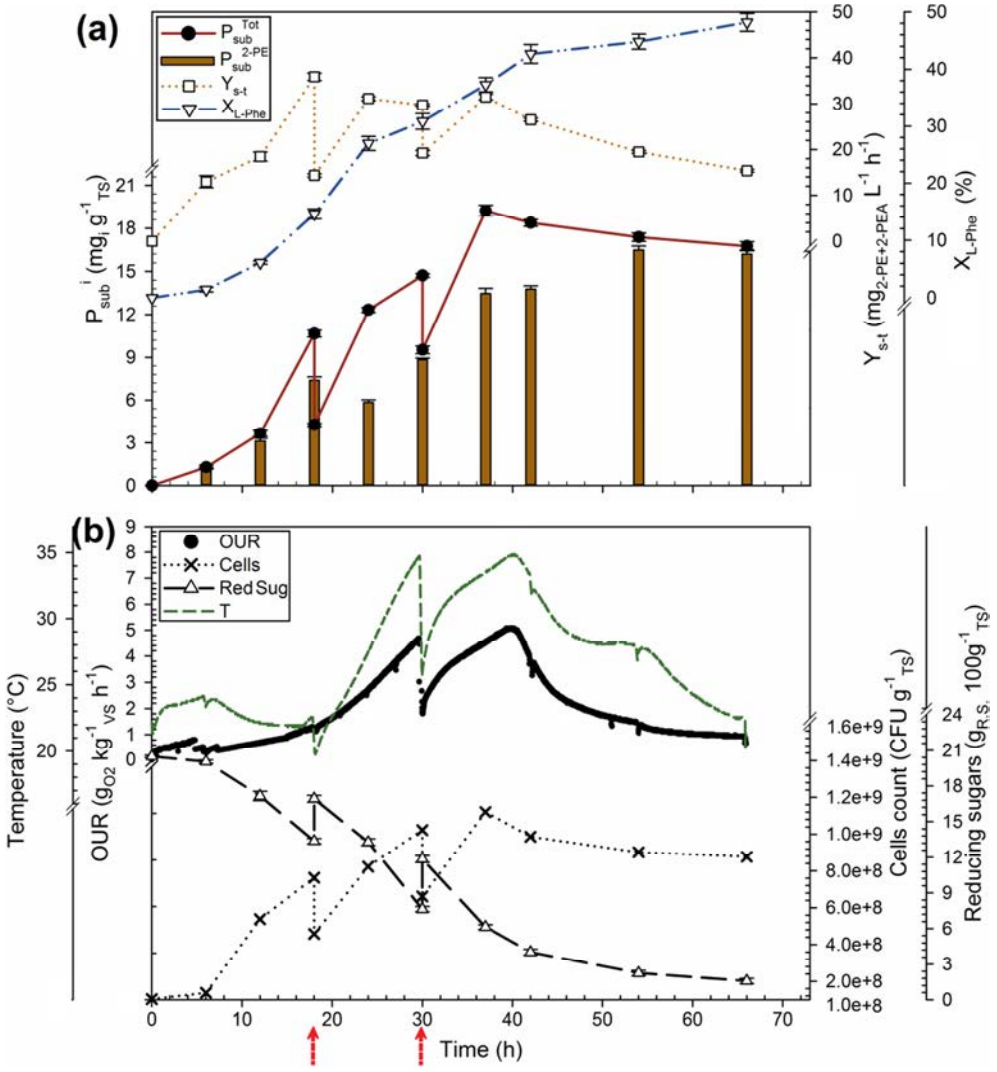


Figure 8.2. Time course of the solid-state fermentation of sugarcane bagasse supplemented with L-phenylalanine in fed-batch mode at 1.6 L scale. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-}PE}$, $Y_{s\text{-}t}$ and $X_{L\text{-}Phe}$; (b) OUR, temperature, cells count and reducing sugars. Dash arrows indicate the addition of fresh substrate.

In this phase, the maximum 2-PE and 2-PEA production has reached 14.7 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} after 30 h of fermentation. Again, after the addition of fresh substrate (last 100 g), the fast increase in activity was accompanied by a high 2-PE and 2-PEA productivity (Figure 8.2(a)). This time, the maximum P_{Sub}^{Tot} (19.2 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS}) was achieved around 37 h of processing just after the maximum OUR and temperature were achieved (5 g_{O2} kg^{-1}_{VS} h^{-1} and 34.9°C respectively) matching with the depletion of available sugars. As occurred in batch mode, after the maximum P_{Sub}^{Tot} was reached, a subtle drop was observed although the 2-PE content was still growing until the end of the fermentation after 66 h. This effect has also been observed in the SSF at constant temperature as shown in chapter 7.

Figure 8.3 shows the time course of the sequential-batch operation with 78% of replacement. In this strategy, the SSF started as a static-batch with a 100% substrate and L-Phe load at time zero (equivalent to 300 g), and it was allowed to proceed until the system reached the maximum activity (6.4 g_{O2} kg^{-1}_{VS} h^{-1}), coinciding with the maximum temperature (30°C) as done before for the previous strategies (growth cycle 1).

At that moment, the first substrate replacement was performed, and due to the dilution effect, the OUR, temperature, *K. marxianus* population and P_{Sub}^{Tot} have fallen significantly. However, the system has succeeded recovering itself due to the SCB and L-Phe supply, and after 17 h of the replacement, the maximum activity, temperature and P_{Sub}^{Tot} levels were achieved again. This time, positioning around 7.5 g_{O2} kg^{-1}_{VS} h^{-1}, 39°C and 20.7 $mg_{2\text{-}PE+2\text{-}PE}$ g^{-1}_{TS} respectively (growth cycle 2). As seen in Figure 8.3(a), two more successive growth cycles have been undertaken (for a total of 4 cycles), and no significant differences among these latter have been found regarding their global behavior.

As seen in Figure 8.4, T 1.6 L there are no significant differences among the evaluated strategies when comparing the maximum P_{sub}^{Tot} reached in each scenario. In this sense, although FB and SB modes reach higher P_{sub}^{Tot} than the batch mode, the differences are minimized mainly due to the reduced production in the first growth cycle of the SB strategy, which is significantly different from the successive three cycles (p 0.025).

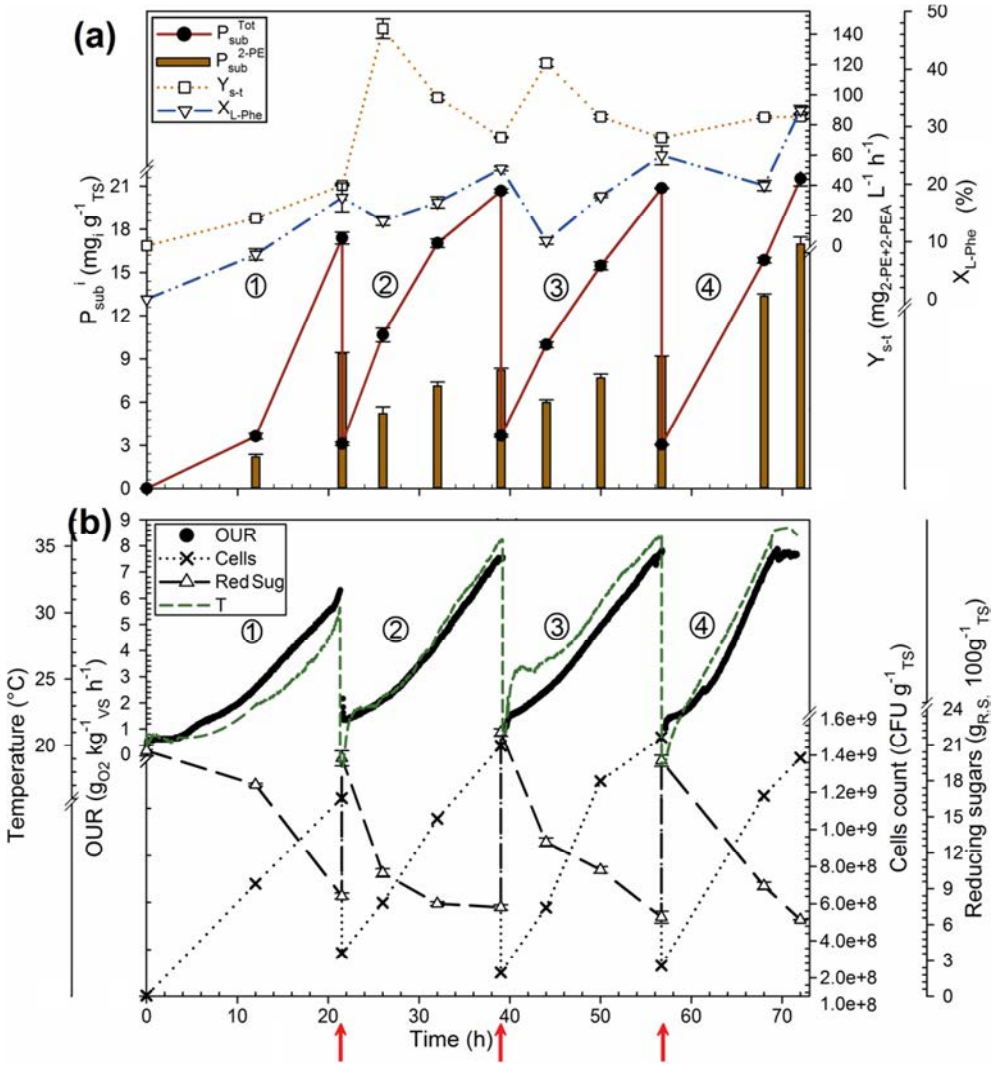


Figure 8.3. Time course of the solid-state fermentation of sugarcane bagasse supplemented with L-phenylalanine in sequential-batch mode at 1.6 L scale. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-}PE}$, $Y_{s\text{-}t}$ and $X_{L\text{-}Phe}$; (b) OUR, temperature, cells count and reducing sugars. Continuous arrows indicate the replacement points.

Nonetheless, without considering the results of this cycle (only cycles 2 to 4), the differences become significant, and the trend indicates that the highest production was achieved in the SB, followed by the FB and the batch mode. Thus, by using alternative strategies to the static-batch, improvements in the maximum P_{sub}^{Tot} reached an increase of 12.3% and 17.5% for FB and SB respectively. However, the increase in the total production (2-PE+2-PEA) was not linked with the products ratio. As it is observed, the accumulated 2-PE content in the point of maximum production was close to 60% for the batch and SB strategies (58 and 57% respectively). Differently, in FB the 2-PE content has reached 70%.

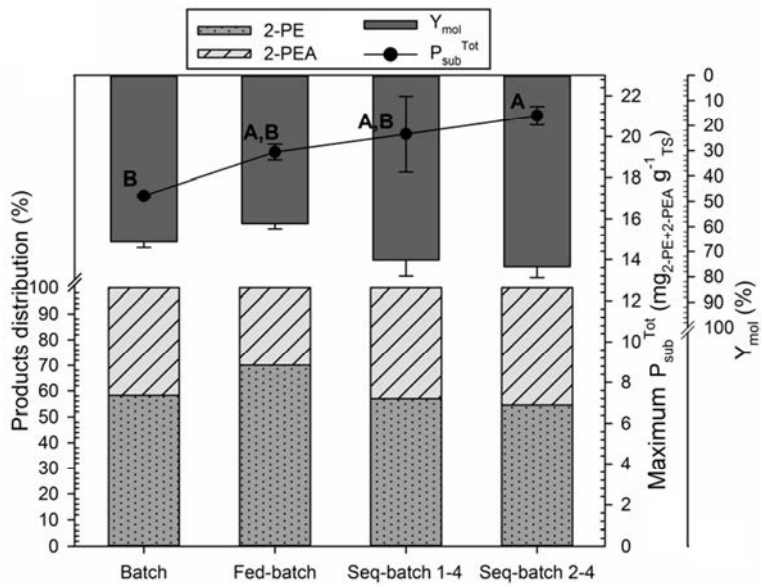


Figure 8.4. Production indices for the evaluated operational strategies at 1.6 L scale in the point of maximum production. P_{sub}^{Tot}: total cumulative production; Y_{mol}: molar yield; 2-PE: 2-phenethyl alcohol; 2-PEA: 2-phenethyl acetate.

As occurred at constant temperature (Chapter 7), this variation might be related to the metabolic routes that might be promoted by each of the strategies, and the consumption of the L-Phe through those routes. In this sense, the Y_{mol} indicates that in batch and SB modes, 70% of the supplied L-Phe could be transformed into 2-PE and 2-PEA. On the contrary, in FB this yield only reaches 59% suggesting that the *de novo* transformation is not promoted in the same way when using FB.

As summarized in Figure 8.5, the performance of the strategies can also be seen from the point of view of the efficiency of use of the resources involved in the fermentation. One of these resources is the time. Although the maximum P_{sub}^{Tot} is a direct index of the 2-PE and 2-PEA production in each strategy, it only provides information about the maximum concentration levels achieved. However, taking in mind the time taken to reach the maximum P_{sub}^{Tot}, the average maximum productivity can be analyzed. Thus, through the index P_t it could be stated that the higher average productivity is achieved in batch and FB modes (0.496-0.493 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} h^{-1} respectively) being almost twice of the average productivity for the SB (0.280 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} h^{-1}).

In this sense, the productivity suddenly changes in SB since the time spent in the process is increased with every new cycle. Nonetheless, by comparing the strategies in a

similar base of time (*e.g.*, considering the availability of 80 h of processing), SB mode would provide the highest amount of products ($mg_{2\text{-}PE+2\text{-}PEA}$); a 96% more than the batch, and 75% more than the FB mode. This result is a consequence mainly of the combined effect produced by the reduction of the lag-phase in SB (from 9 h to 4 h) and the ability to process a larger mass of substrate in the same period as described by Xu et al., (2017).

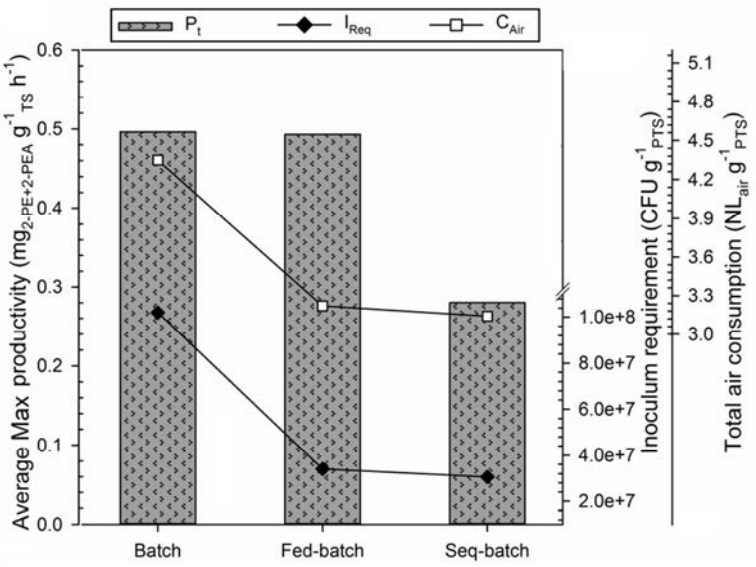


Figure 8.5. Effectiveness indices of the evaluated operational strategies at 1.6 L scale in the point of maximum production. P_t: average maximum productivity; I_{Req}: inoculum requirement; C_{Air}: total air consumption.

Furthermore, the use of resources like the inoculum is also affected by the selected strategy. In this regard, I_{Req} points out the economy obtained for FB and SB strategies compared to the batch case. As observed in Figure 8.5, the decrease in the inoculum needs ranges between 67-70% for these strategies when they are referred to the total mass of the processed substrate. As stated by Cheirsilp and Kitcha, (2015) the inoculum savings could play a key role in the implementation at industrial scale, enhancing the economy and sustainability of the SSF process.

Similarly, regarding the air consumption, operating in FB or SB modes results in a substantial saving of this resource. As seen, at this scale, the tested FB could save until 26% of air and SB a 28% compared to the batch scenario. These results are in accordance with the findings of chapter 6, corroborating that the implementation of

these strategies also contributes to the development of the technology as a large-scale application.

8.2.2 Strategies at 22 L scale

Experiments in this section involved the evaluation of the selected operational strategies on $P_{Sub}^{2\text{-}PE}$ and P_{Sub}^{Tot} considering the effect of working with variable temperature in a non-isolated steel 22 L reactor. Here, the increase in scale implied working with eightfold the mass used in the section 8.2.1 while keeping unchanged the geometry and the height to diameter ratio than the 1.6 L system (h:ϕ of 2). Initial pH, MC, S_{AFR}, L-Phe load, and inoculum load were set at 4.8, 68%, 0.14 L h^{-1} g^{-1}_{TS}, 3.5% (dry basis) and 10^8 CFU g^{-1}_{TS} respectively as done for the 1.6 L experiments. In this case, experiments were performed in duplicate for batch, and fed-batch, while SB results represent a single run.

Figure 8.6 summarizes the time course of the batch strategy at 22 L scale. It can be seen that the batch process shows a similar trend to the one found at 1.6 L with a rapid growth phase from 12 to 30 h. It is at the end of this phase when the maximum P_{sub}^{Tot} is reached, coinciding with the OUR_{Max}, maximum temperature (8.3 g_{O2} kg^{-1}_{VS} h^{-1} and 37.8°C respectively) and the depletion of the available sugars. In this case, the maximum P_{sub}^{Tot} was 16.8 $mg_{2\text{-}PE+2\text{-}PEA}$ $g^{-1}_{TS,}$ and as occurred at 1.6 L, this value has dropped at the end of the fermentation, even that the $P_{sub}^{2\text{-}PE}$ has continued to grow up to 11.6 $mg_{2\text{-}PE}$ g^{-1}_{TS}.

In the fed-batch mode (Figure 8.7), the global behavior found at 1.6 L was also maintained. At this scale, the process has taken some additional time in the lag phase (16 h), but after this, the microbial activity was much higher than at 1.6 L, allowing the temperature to get values up to 40.2°C after the successive substrate additions (Figure 8.7(b)). As occurred at 1.6 L, the strain has grown more due to the split of the substrate (Figure 8.7(b)), contributing to an increase in the productivity, similarly than in the FB SSF of chapters 6 and 7. In this scenario, the maximum P_{sub}^{Tot} has been reached after the maximum OUR peak (11 g_{O2} kg^{-1}_{VS} h^{-1}) obtained after the successive substrate feeding, as occurred at 1.6 L and in FB mode at constant temperature as described in chapter 7.

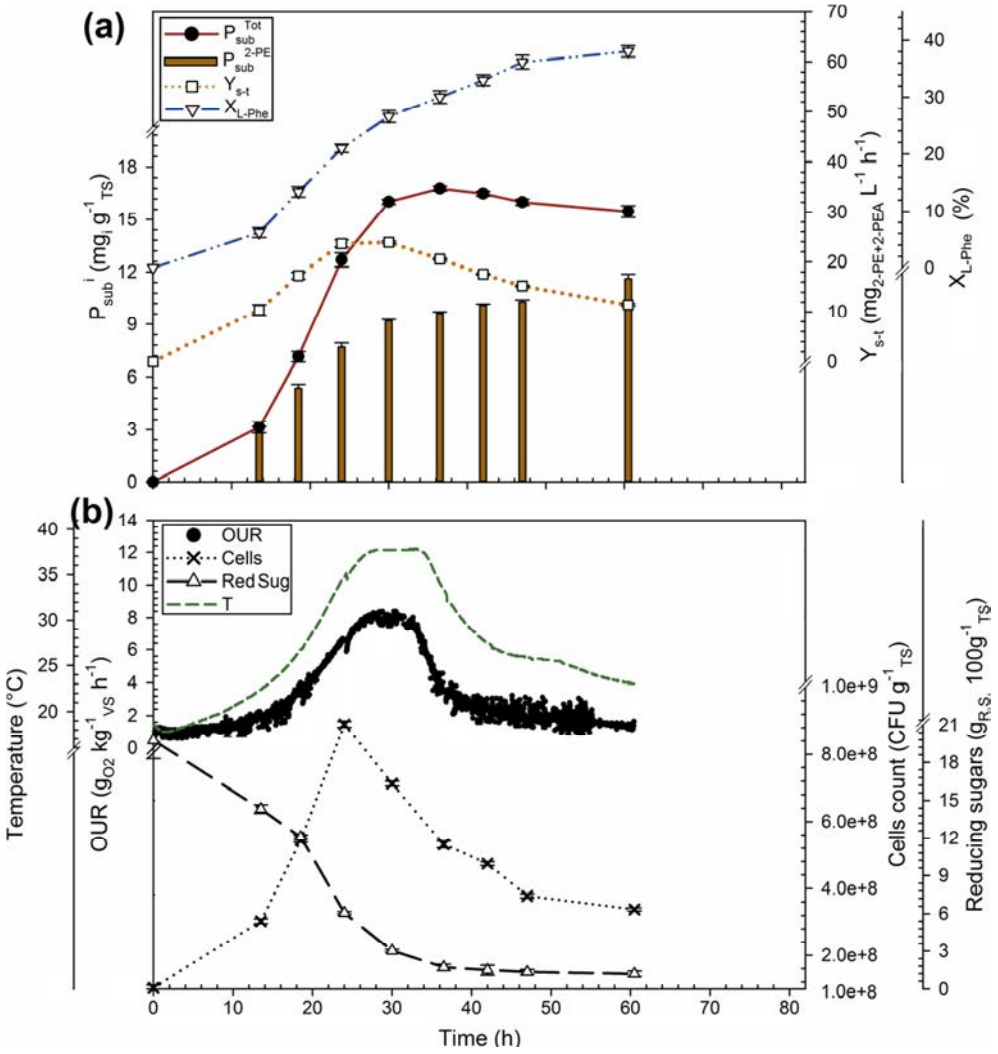


Figure 8.6. Time course of the solid-state fermentation of sugarcane bagasse supplemented with L-phenylalanine in batch mode at 22 L scale. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-}PE}$, $Y_{s\text{-}t}$ and $X_{L\text{-}Phe}$; (b) OUR, temperature, cells count and reducing sugars.

At 22L, the SB strategy was evaluated through the development of five consecutive growth cycles (Figure 8.8). As occurred at 1.6 L, the first cycle corresponded to a static-batch with 100% of the substrate and L-Phe load (2.5 kg), that proceeded until the maximum OUR and temperature were achieved after 21.5 h of processing. At that point, the substrate replacement was undertaken (withdrawing 1.95 kg of the reactor content), so the second growth cycle was started. As it can be seen in Figure 8.8, for cycles 2 to 5, the maximum P_{sub}^{Tot} coincides with the maximum microbial activity as well as the maximum temperature, reaching a mean of 17.9 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS}. In the same interval, the mean maximum OUR and temperature were 12.6 g_{O2} kg^{-1}_{VS} h^{-1} and 33.1°C respectively.

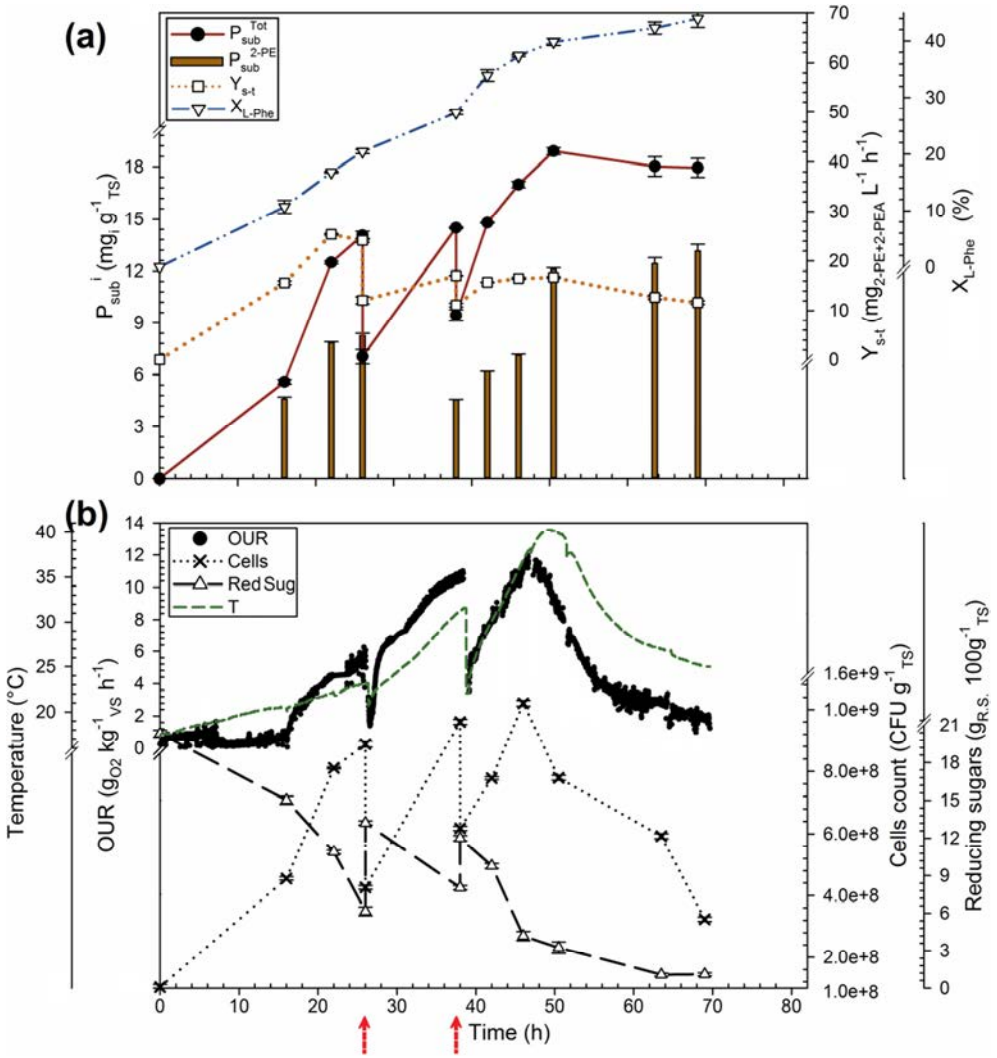


Figure 8.7. Time course of the solid-state fermentation of sugarcane bagasse supplemented with L-phenylalanine in fed-batch mode at 22 L scale. (a) P_{sub}^{Tot}, P_{sub}^{2-PE}, Y_{s-t} and X_{L-Phe}; (b) OUR, temperature, cells count and reducing sugars. Dash arrows indicate the addition of fresh substrate

Compared to the SB at 1.6 L, there are no significant differences in the general behavior of the SB strategy. However, the main difference lies in the maximum P_{sub}^{Tot} obtained at this scale. As observed, even that the OUR reached higher levels at 22 L, it was not directly related neither to a higher temperature nor a higher *K. marxianus* growth as occurred at 1.6 L scale (Figure 8.3(b) and Figure 8.8(b)).

While at 1.6 L *K. marxianus* was able to reach up to 1.5 10^9 CFU g^{-1}_{TS}, at 22 L the maximum growth was almost half with 8.0 10^8 CFU $g^{-1}_{TS.}$ Similarly, although at 22 L it was processed a higher mass of substrate, temperate was not able to achieve the maximum levels found at 1.6 L (around 36°C) just reaching 33°C. These differences

may suggest that the change in scale could promote a higher activity of other microorganisms presented in the solid media. These microorganisms might be consuming valuable nutrients in parallel with *K. marxianus*, limiting the growth of the strain, and therefore, limiting the 2-PE and 2-PEA production.

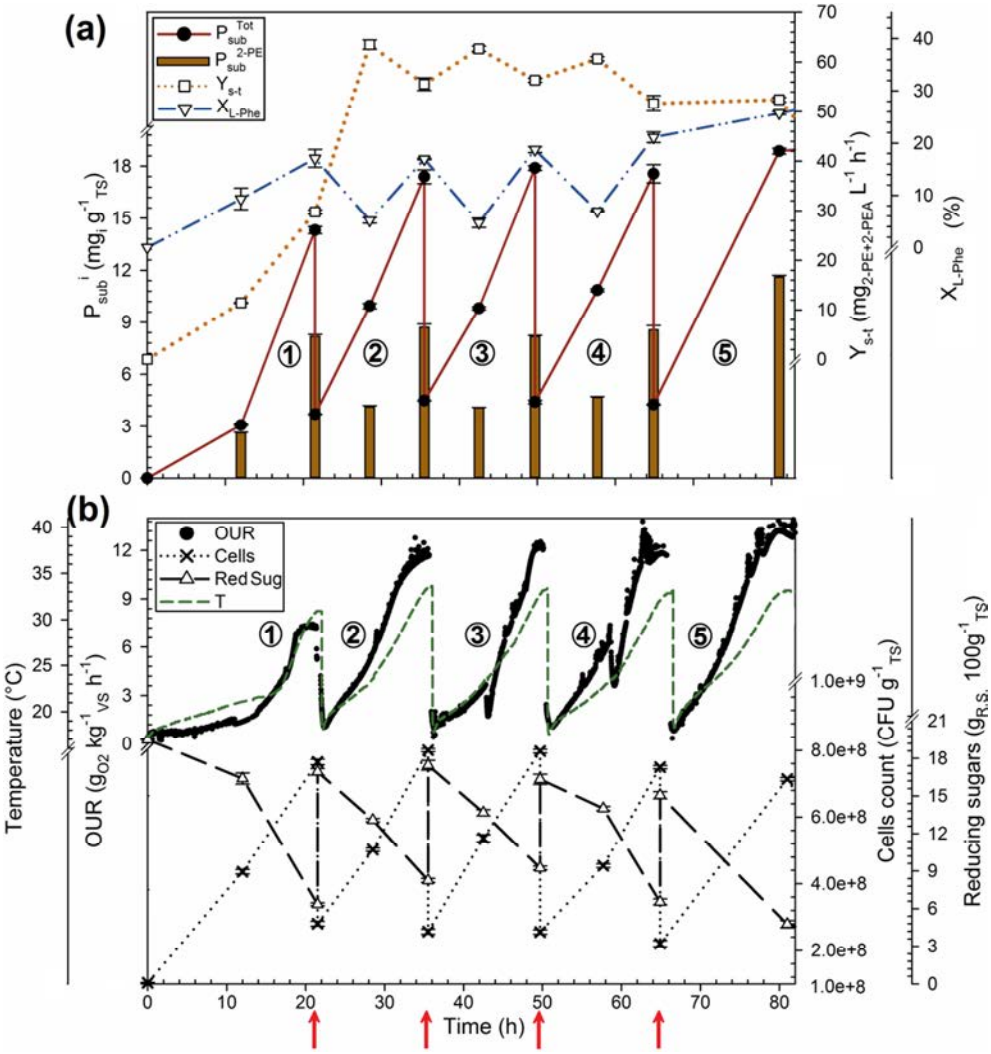


Figure 8.8. Time course of the solid-state fermentation of sugarcane bagasse supplemented with L-phenylalanine in sequential-batch mode at 22 L scale. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-}PE}$, $Y_{s\text{-}t}$ and $X_{L\text{-}Phe}$; (b) OUR, temperature, cells count and reducing sugars. Continuous arrows indicate the replacement points.

As seen in Figure 8.9, in terms of the Tukey test, at 22 L the assessed strategies reach identical maximum P_{sub}^{Tot} levels. Differently to 1.6 L scale, the SB strategy was not able to reach higher accumulated productions, and consequently, no significant differences are found in this regard. At the same time, the products distribution has

conserved the same trend found at 1.6 L. In this case, batch and SB produced around 55% of 2-PE at the point of maximum production, while using FB this percentage has reached 64%. Once more, this change was accompanied with a lower Y_{mol} for the FB mode (64%) that compared to the other two strategies is still 8% lower, as found at 1.6 L.

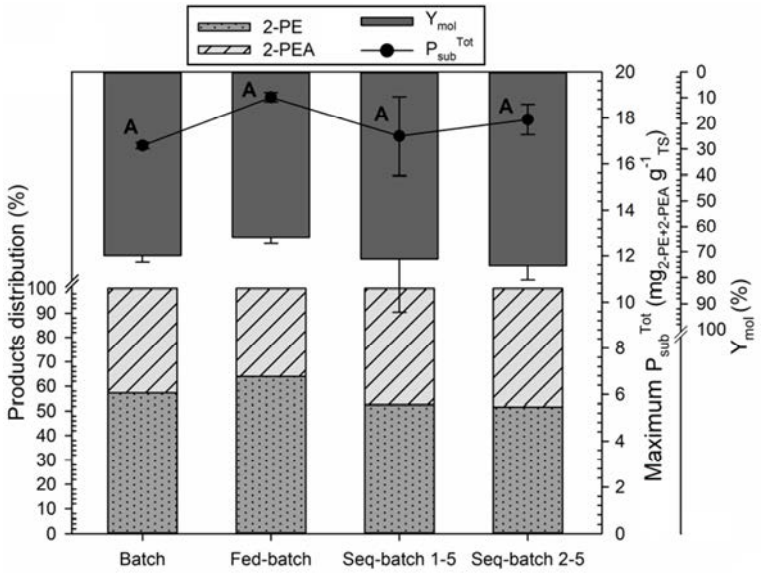


Figure 8.9. Production indices for the evaluated operational strategies at 22 L scale in the point of maximum production. P_{sub}^{Tot}: total cumulative production; Y_{mol}: molar yield; 2-PE: 2-phenethyl alcohol; 2-PEA: 2-phenethyl acetate.

It was also found that the effectiveness performance indices have behaved similarly at both scales (Figure 8.10). Thus, at 22 L the SB mode reached an average productivity of 0.213 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}{}_{TS}\ h^{-1}$, less than half of the productivity achieved in the batch (0.456 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}{}_{TS}\ h^{-1}$). In the midpoint, the FB strategy has reached 0.378 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}{}_{TS}\ h^{-1}$.

Although the average productivity of FB and SB resulted worse than in batch mode, they were compensated with higher space-time yields ($Y_{s\text{-}t}$) as occurred in other SSF processes using sequential-batch approaches (Xu et al., 2017). In this case, the $Y_{s\text{-}t}$ in the point of maximum production was 20.6 $mg_{2\text{-}PE+2\text{-}PEA}\ L^{-1}\ h^{-1}$ in batch mode, while it reached 49.2 $mg_{2\text{-}PE+2\text{-}PEA}\ L^{-1}\ h^{-1}$ in SB, 2.3 times higher. For FB, the change in the maximum $Y_{s\text{-}t}$ fluctuates based on the number of splits made, but in general, increases are lower than in SB mode, reaching values ranging from 2 to 80% if the data obtained in chapter 7 is also analyzed.

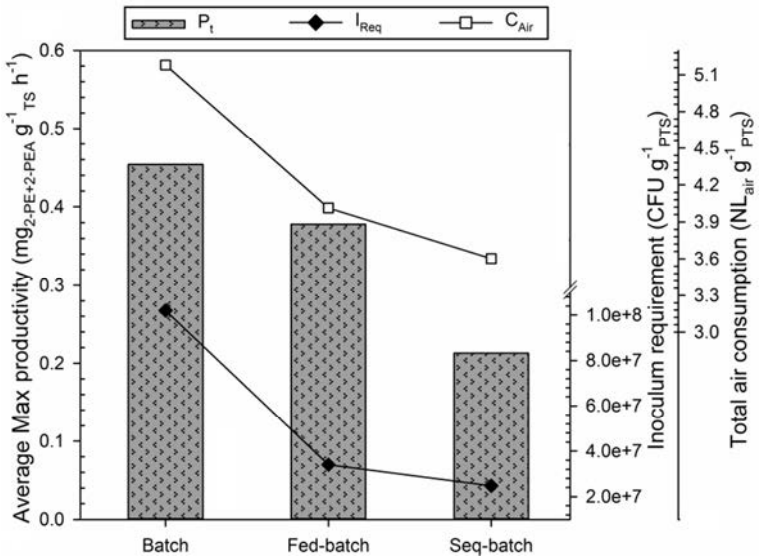


Figure 8.10. Effectiveness indices of the evaluated operational strategies at 22 L scale in the point of maximum production. P_t: average maximum productivity; I_{Req}: inoculum requirement; C_{Air}: total air consumption.

Furthermore, as occurred at 1.6 L, the implementation of alternatives strategies to a batch SSF has confirmed their advantages for saving some of the resources used in the fermentation. At this scale, since five growth cycles were tested for the SB strategy, the inoculum required for processing these cycles was 2.5 10^7 CFU per gram of processed substrate. Under these conditions, the inoculum required for processing the same amount of substrate using the batch mode would be 76% higher than the one needed in a sequential-batch approach.

Likewise, the total air consumption per gram of processed substrate has kept the same saving levels found at 1.6 L scale. Here, FB could save up to 22% of air, and SB up to 30% compared to the air needed for processing the same amount of substrate in through a batch process.

8.2.3 Scale-up effects

Based on the results found at the two selected scales, it can be stated that, although the global behavior of the strategies has been preserved, the process scale-up has induced some significant changes in the global performance of the SSF. As detailed in Figure 8.11, the maximum P_{sub}^{Tot} achieved in the different strategies has suffered a

reduction of 1.9% (batch), 1.7% (fed-batch) and 14.5% (sequential-batch) being significantly different only for SB mode.

It is also seen that the average productivity has changed due to the increase in scale. The fall in P_t has reached 8.5% in batch and 23-24% for FB and SB. Although these results point out a loss in the effectiveness of the SSF due to the scaling of the process, they also suggest that the use of alternative operating modes reduce the adverse effects when compared to the reference static-batch SSF.

For instance, by implementing a FB mode, it is plausible to reach higher P_{sub}^{Tot} levels at 22 L than in batch mode at 1.6 L scale and at constant temperature at 0.5 L. Also, by using a SB operation mode, the SSF would work with higher Y_{s-t} at 22 L than both, batch and FB. In this sense, despite the lower effectiveness of the process at this scale, SB has a Y_{s-t} between 56-61% higher than the one found for batch and FB at 1.6 L.

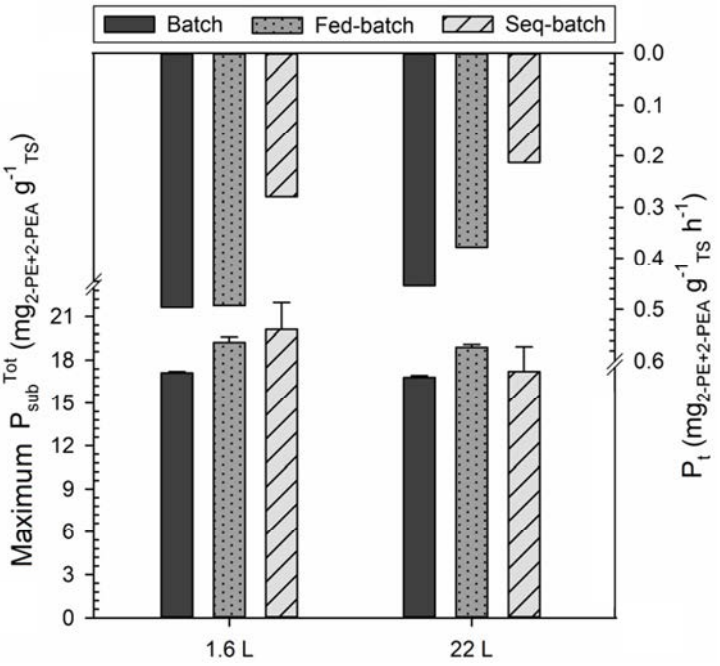


Figure 8.11. Scaling-up effects on the 2-PE and 2-PEA bioproduction for the evaluated strategies. P_{sub}^{Tot}: total cumulative production; P_t: average maximum productivity.

Having these results in mind, it could be stated that by using a fed-batch approach is a simple way to enhance the SSF for 2-PE and 2-PEA production in short-term operations. In fact, FB is the evaluated strategy that better maintain the performance indices after an increase in scale. However, the use of this strategy for

large-scale applications could promote some difficulties similar than those presented for a batch process. For instance, the time required to prepare a new batch (or fed-batch) could significantly reduce the global productivity of the process. Besides, from the three strategies, FB is the one requiring more time to achieve the maximum P_{sub}^{Tot} levels, which would reduce its performance when processing a significant amount of material.

Differently, SB presents some characteristics that help to overcome these drawbacks. Hence, although the evaluated SB works with lower average productivity than the batch and fed-batch modes, it is compensated by a global reduction of the processing time induced by the decrease of the lag-phase in each growth cycle, and the reduction in the preparing time required for running each cycle (it is expected to be lower than the time required to prepare a batch or fed-batch). Consequently, results show that the bioproductive approaches here proposed could serve as a starting point for the further scaling to pilot plant considering the inherent advantages and handicaps of each strategy.

8.2.4 Extended sequential-batch

Based on the results obtained in sections 8.2.1 and 8.2.2, it would be expected that the sequential-batch is an appropriate strategy for bioproducing 2-PE and 2-PEA via SSF in a long-term approach. Nonetheless, the reproducibility of the strategy is essential for the development above. Thus, the process needs to be assessed in a more extended period. With this purpose, it was decided to evaluate this strategy at 1.6 L scale increasing the growth number cycles. A total of seven cycles were carried out (in duplicate). As it was explained in section 8.2.1, Initial pH, MC, S_{AFR}, L-Phe load, and inoculum load were set at 4.8, 68%, 0.14 L h^{-1} g^{-1}_{TS}, 3.5% (dry basis) and 10^8 CFU g^{-1}_{TS} respectively.

Figure 8.12 shows the time profile of the strategy. As detailed, the process starts as a static-batch, and after a short lag-phase (7 h) a rapid increase in the microbial activity allows the system to reach the maximum OUR and temperature levels after 20 h of fermentation (7.9 g_{O2} kg^{-1}_{VS} h^{-1} and 35°C respectively) (Figure 8.12(b)). As it was done before, this point was selected to perform the substrate replacement substituting 78% of the reactors load. As expected, the substrate replacement induced a dilution effect, but the levels found in the first growth cycle were successfully recovered in the

subsequent cycles. As seen in Figure 8.12(a), the P_{sub}^{Tot} reached during the first three growth cycles was very stable with a mean of 20.1 $mg_{2\text{-}PE+2\text{-}PEA}$ $g^{-1}{}_{TS}$. Also, both, production levels and microbial activity has been consistent with the trends found in the experiments of section 8.2.1.

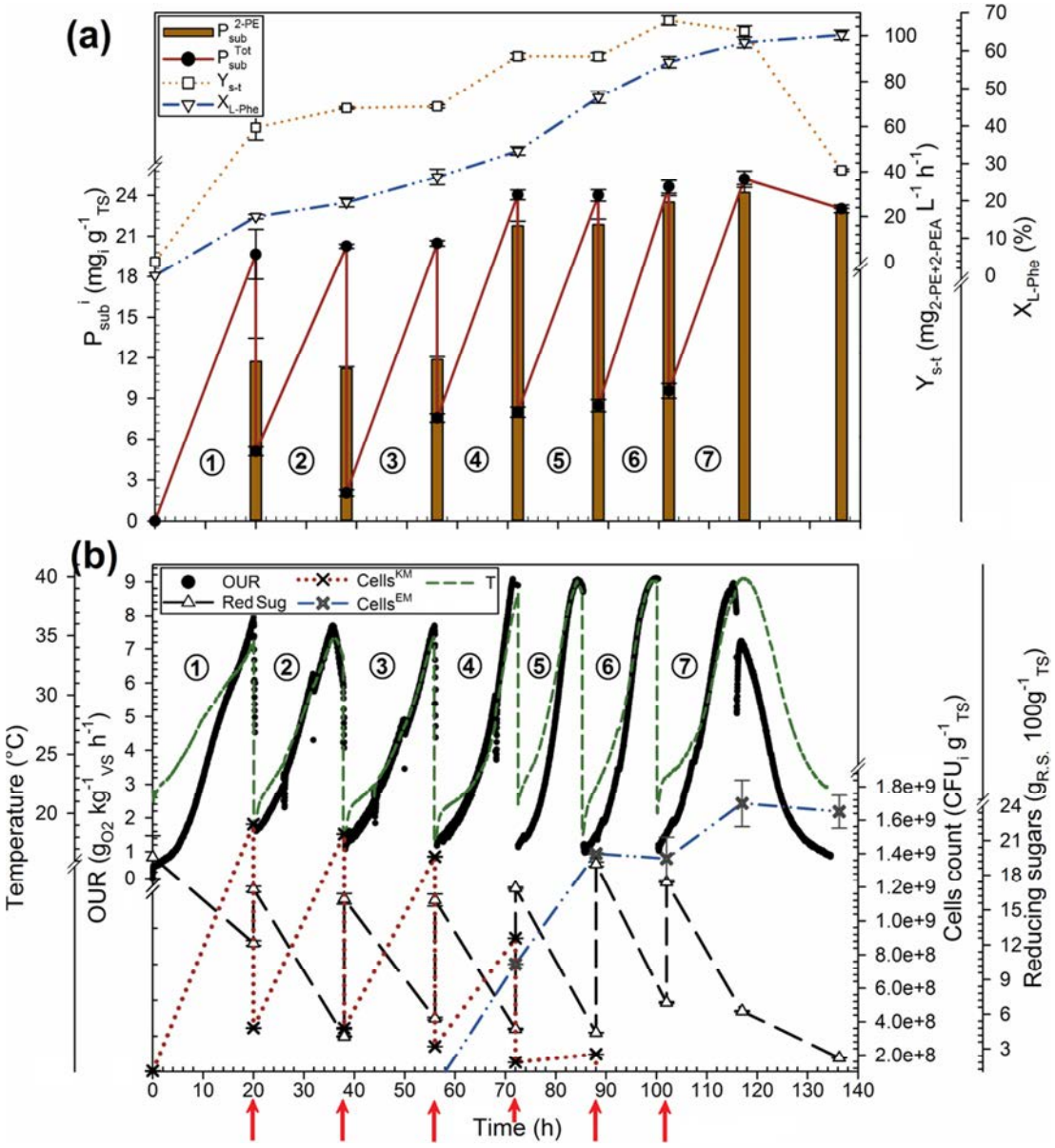


Figure 8.12. Time course of the extended sequential-batch mode at 1.6 L scale. (a) P_{sub}^{Tot}, $P_{sub}^{2\text{-}PE}$, $Y_{s\text{-}t}$ and $X_{L\text{-}Phe}$; (b) OUR, temperature, cells count and reducing sugars. Continuous arrows indicate the replacement points.

However, the system has suffered a significant change from the fourth growth cycle; visible from the different monitoring parameters. As observed in Figure 8.12(a),

at the point of maximum activity, P_{sub}^{Tot} has suddenly improved to 24.0 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS}, a 19.4% higher than the preceding cycles. Nonetheless, this intensification was also accompanied by the increase of OUR_{Max} (9.0 g_{O2} kg^{-1}_{VS} h^{-1}), maximum temperature (39.1°C), and L-Phe consumption (34%). On the contrary, at the same point, the unexpected decrease in the *K. marxianus* population was significantly higher than in the previous cycles, reaching 8.9 10^8 CFU g^{-1}_{TS} which is almost 36% less than in cycle 3 (Figure 8.12(b)).

In this scenario, it was evident the *K. marxianus* population should not be related to the observed increase in biological activity, suggesting that some other(s) microorganism(s) were highly active in the solid media at that point of the fermentation. In this sense, it was also clear that working with the not-sterilized residue would imply the presence of other microorganisms in the media, and it was expected their activity would contribute in the loss of effectiveness compared to a sterile process. Nevertheless, the inoculum load used through these experiments was high enough to consider the *K. marxianus* the predominant species during the fermentation process.

As it can be observed from the results of chapter 6 and those in sections 8.2.1 and 8.2.2, this assumption could be valid given the length of the evaluated fermentation processes. Nevertheless, by extending the fermentation in a not-sterile environment seems to affect the *K. marxianus* ability to maintain a consistent population level. Although this effect was expected to some extent, it resulted more remarkable that the process is boosted instead of being hindered by this change.

Analyzing the subsequent growth cycles, it is found that the system follows a consistent trend in which P_{sub}^{Tot} reach a maximum of 24.0, 24.6 and 25.2 $mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{TS} for cycles 5 to 7 (Figure 8.12(a)). Again, in these cycles, OUR_{Max} levels range 9 g_{O2} kg^{-1}_{VS} h^{-1} with a maximum temperature between 38.7 and 40.1°C. Additionally, another significant change occurred regarding the time required by the system to reach these maximum levels. While in the first three growth cycles (and those of section 8.2.1) this time was over 18 h, for cycles 4 to 7 it has taken a mean of 15.1 h, a 16% less. Because of these changes, a significant increase in $Y_{s\text{-}t}$ has also occurred passing from a mean of 65.5 to 97.6 $mg_{2\text{-}PE+2\text{-}PEA}$ L^{-1} h^{-1}, a 49% more.

The changes in the system had another two crucial implications to be pointed out. First, as seen from Figure 8.12(a), the 2-PE contribution on P_{sub}^{Tot} has radically turned from a mean of 57.7% to 93.3% in cycles 4 to 7, suggesting that the

bioconversion of this product has been favored. Secondly, it was observed the fading of *K. marxianus* on the solid media simultaneously with the appearance of a second emergent microorganism (EM) distinguishable through the monitoring of the Petri dishes.

As seen in Figure 8.12(b), *K. marxianus* only reached a maximum growth of 2.0 10^8 CFU g^{-1}_{TS} in cycle 5, and it was impossible to identify it in the subsequent cycles. At the same time, the emergent microorganism has grown continuously from cycle 4 (in cycle 3 the EM was detected in very low concentrations, two orders of magnitude below the *K. marxianus* one) surpassing the *K. marxianus* in the fifth cycle and maintaining the cells number around 1.5 10^9 CFU g^{-1}_{TS}. As a result, it could be stated that the process run with *K. marxianus* was stable just during three cycles, (four considering the results of section 8.2.1) but the reasons behind the presented changes cannot be deduced by the information provided so far.

Due to the outstanding results obtained here, the EM was isolated and identified from two independent samples following the procedure described in section 3.4.5 in chapter 3. As a result, with a confidence of 99%, the EM was identified as the yeast *Pichia kudriavzevii*. To verify the ability of this yeast for producing 2-PE and 2-PEA, and to corroborate the role played for this strain in the extended sequential-batch runs, it was decided to evaluate the SSF process by using *P. kudriavzevii* in batch mode using the 0.5 L system under sterile conditions.

In this sense, the SSF was evaluated (in triplicate) under the conditions exposed in Table 8.2, keeping the remaining operational condition identical to those exposed at the end of section 7.2.1in chapter 7. Thus, S_{AFR} was set in 0.10 L h^{-1} g^{-1}_{TS}, MC in 66%, initial pH at 4.8, 2% L-Phe (dry basis). The SSF process was undertaken at two temperature levels considering the optimum temperature for *K. marxianus* and the highest temperature achieved in the extended sequential-batch experiments. Similarly, two different inoculums were used. One with only *P. kudriavzevii*, and the second, corresponding a 1:1 mixture of both species (approximately 5 10^7 CFU g^{-1}_{TS} of each one).

Figure 8.13 shows the time course of the evaluated scenarios with *P. kudriavzevii*. As seen in Figure 8.13(a), compared to the SSF using *K. marxianus*, the processes in which *P. kudriavzevii* was involved have obtained significantly higher production levels, reaching a maximum P_{sub}^{Tot} ranging between 19.3 and 21.8 $mg_{2\text{-PE+2-}}$

$_{PEA}$ g$^{-1}_{TS}$ which is in average, a 30.5% higher than the obtained for *K. marxianus* at the same conditions. Also, it was found that, as occurred in the last cycles of the extended sequential-batch, the 2-PE production with *P. kudriavzevii* was favored over 2-PEA, constituting, in average, the 95.6% of the total production (Figure 8.13(b)).

Table 8.2. Variables used to evaluate the *P. kudriavzevii* performance for producing 2-PE and 2-PEA.

Variable	Low level	High level
Temperature (°C)	30	38
Inoculum load (CFU g$^{-1}_{TS}$)	10^8 -50% *P. kudriavzevii* -50% *K. marxianus*	10^8 -100% *P. kudriavzevii*

On the other hand, regarding the microbial activity, it could be seen that *P. kudriavzevii* has grown better at 30°C reaching levels above 1.2 10^9 CFU g$^{-1}_{TS}$, and at 38°C reaching up to 8.0 10^8 CFU g$^{-1}_{TS}$ (Figure 8.13(c)). As observed, the OUR profile at both temperatures was not particularly different, reaching OUR_{Max} close to 8 g_{O2} kg$^{-1}_{VS}$ h^{-1}. Finally, it is evident that the improvement in the performance parameters of the SSF process is not limited to the production, but, considering that *P. kudriavzevii* takes less time to reach the levels above, the global productivity is enhanced as well.

Having in mind that the sequential-batch experiments has been performed in duplicate, at different time periods and using a different material for each reactor, the most promising hypothesis about the origin of the *P. kudriavzevii* is that it was an indigenous microorganism in the SCB. In fact, the analysis of inoculated Petri dishes, using the liquid extract obtained from the stored SCB (following the procedure described in section 3.4.4), has shown that *P. kudriavzevii* was present. Thus, it can be hypothesized that the particular conditions of the process have allowed it to grow.

In this sense, it could be stated that *P. kudriavzevii* strains have been found in wide variety of media like soils (Koutinas et al., 2016), food and some derivatives (Osorio-Cadavid et al., 2008; Talukder et al., 2016; Zheng et al., 2018), food wastes and compost (Dandi et al., 2013; Yuan et al., 2017), wine (del Mónaco et al., 2016), fermentation starters (Choi et al., 2017; Greppi et al., 2015; Toivari et al., 2013), but also in the juice extracted from sugarcane (Dhaliwal et al., 2011). Thus, as mentioned before, the origin of this strain could be linked to its survival in the SCB leftovers. For

instance, Chelliah et al., (2016) were able to obtain *P. kudriavzevii* from a frozen *idli* batter sowing the ability of this strain to survive in extreme conditions.

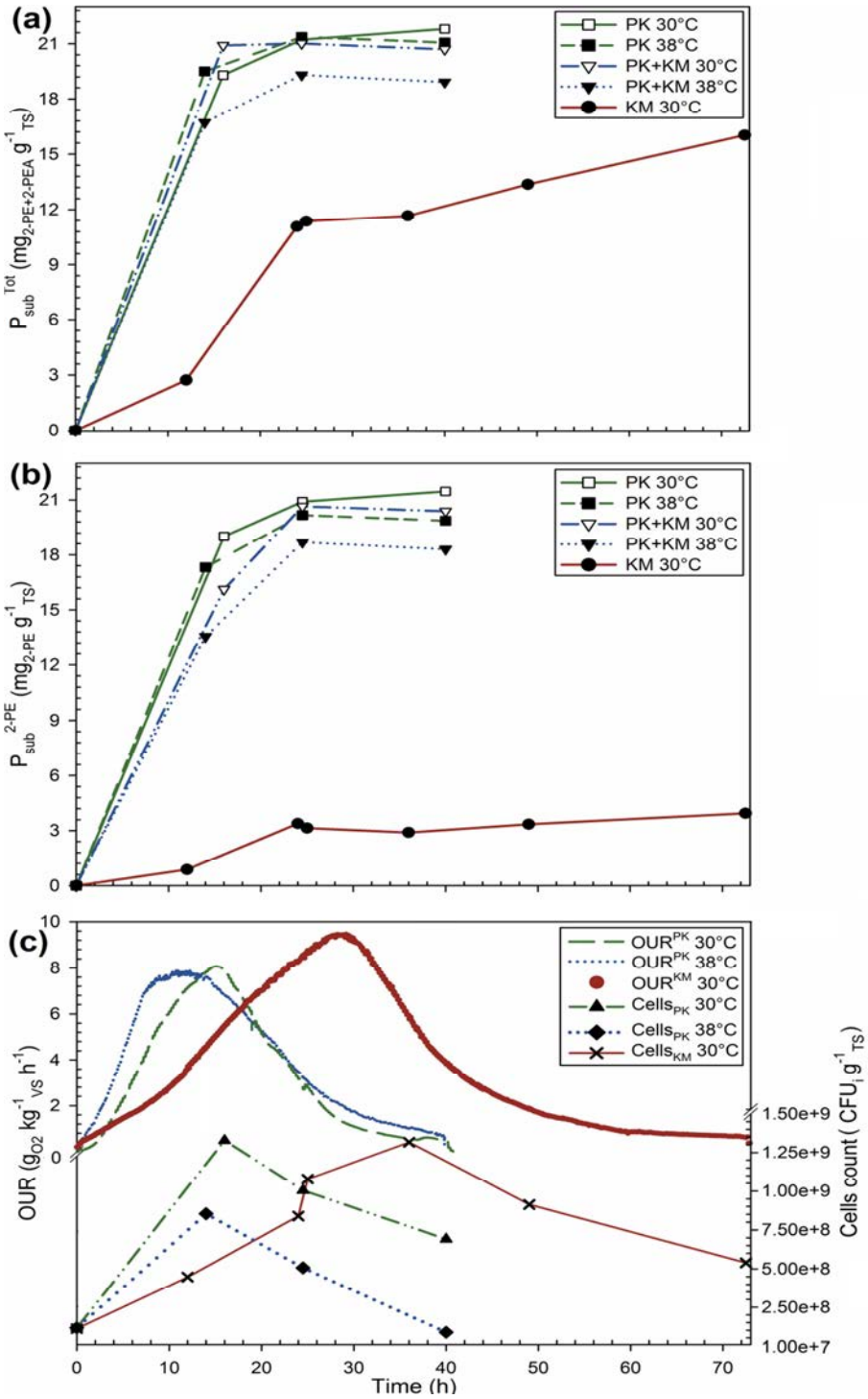


Figure 8.13. Time course of the SSF of *Pichia kudriavzevii* at 0.5 L and constant temperature for producing 2-PE and 2-PEA. (a) P_{sub}^{Tot}, (b) $P_{sub}^{2\text{-PE}}$, (c) OUR and cells count. KM: *K. marxianus*; PK: *P. kudriavzevii*.

However, the specific reasons inducing the *P. kudriavzevii* growth are still unknown. In this regard, the assumed hypothesis is that the SB mode has provided to *P. kudriavzevii* the time to adapt itself to the solid-media such that it could thrive and compete with *K. marxianus* for the available nutrients. As seen, *P. kudriavzevii* has shown to be a high growth rate yeast (at least it grows faster than *K. marxianus*). Moreover, it has grown well at 30 and 38°C suggesting that it is tolerant in this temperature range, and its ability to survive in adverse conditions become evident from the results of the extended sequential-batch. Besides, these characteristics are in accordance with previous reports indicating the multi-stress tolerance capability of *P. kudriavzevii* strains (Koutinas et al., 2016; Radecka et al., 2015; Talukder et al., 2016).

In general, *P. kudriavzevii* strains have been tested for different purposes, including the heavy metal removal (C. Li et al., 2016; Chunsheng Li et al., 2016), the enhancement of composting (Nakasaki et al., 2013), as an antimicrobial agent (Chelliah et al., 2016), or in the biodiesel production (Sankh et al., 2013). However, the main application of *P. kudriavzevii* has been the bioethanol production (Mukherjee et al., 2017; Radecka et al., 2015; van Rijswijck et al., 2017; Yuan et al., 2017). Regarding the feasibility for producing 2-PE, some recent studies (Chreptowicz et al., 2018; Mu et al., 2014; van Rijswijck et al., 2017) have shown that some *P. kudriavzevii* strains are able to use different sugars as nutrients source for obtaining, among others, 2-PE through SmF processes. Thus, based on this hypothesis, the indigenous *P. kudriavzevii* here identified could be a promising alternative in the biotransformation of L-Phe into 2-PE and 2-PEA not only because of its performance but also considering the potential to withstand and succeed in complex matrices like the tested solid media.

8.2.5 Products recovery

Considering that 2-PE and 2-PEA are mainly presented in the solid-liquid interface (up to 95%), it would be needed a recovery step after the SSF. One of the options would be a solid-liquid extraction using a solvent able to recover both products. According to different literature, when 2-PE is produced via SmF, several solvents have been used. For instance, Stark et al., (2002) have proposed using oleic acid due to its partition coefficient with 2-PE and its favorable handling properties. Also, Etschmann and Schrader, (2006) have employed PPG 1200 showing high recovery rates. Sendovski

et al., (2010) proposed the use of ionic liquids as a more biocompatible alternative than organic solvents while Chreptowicz et al., (2016) used ethyl acetate as an environmentally friendly and low-cost solvent.

However, considering the nature of the media containing the products, it seems adequate to perform a particular assessment of the used solid media. With this purpose, it was decided to evaluate three different solvent alternatives by using two designs of experiments as detailed in Table 8.3. As seen, selected solvents were water, ethyl acetate and methanol. Also, the evaluation was carried out considering the effect of two of the main factors affecting the extraction process, temperature and the solid:solvent ratio (Sol:Liq).

Table 8.3. Main characteristics of the designs of experiments for the evaluation of the 2-PE and 2-PEA recovery step.

DOE	Type	Solvents[a]	Factors	Levels			N° runs	Response variable[b]
				1	2	3		
N°1	Full Factorial 3 levels	Water-Ethyl Acetate	Temperature/ Sol:Liq ratio	25°C/ 1:4	35°C/ 1:7	45°C/ 1:10	9_{each}	2-PE+2-PEA recovery
N°2	Box-Behnken	Water-Methanol	Temperature/ Sol:Liq ratio/ %Methanol	25°C/ 1:4/ 0	35°C/ 1:7/ 50	45°C/ 1:10/ 100	15	

[a]In DOE N°1 water and ethyl acetate were evaluated independently each other. In DOE N°2 the evaluation was performed on the mixture water:methanol using the established concentrations. [b]For the analysis, the response variable was normalized referring the data to the highest value found in both sets of DOE.

At the same time, the extraction assessment was performed by using a sample collected at the point of maximum production in the sequential-batch test at 22 L scale (section 8.2.2). Specifically, it was collected in the growth cycle 5, corresponding at 81 h of processing. Each extraction run was undertaken in duplicate using 200 mL sealed plastic containers where 10g of fermented material were placed. The selected solvent was added to the weighted sample according to the given Sol:Liq ratio and the containers were put in an orbital shaker at 220 rpm for 30 min at the given temperature, performing two successive extractions. After each extraction, the supernatant was collected and processed as explained in section 3.4.3 of Chapter 3. Results were

expressed as the mean concentration of each duplicate ($mg_{2\text{-PE+2-PEA}}$ g^{-1}_{TS}), normalized respect to the highest value found in both sets of data (24.6 $mg_{2\text{-PE+2-PEA}}$ g^{-1}_{TS}).

Figure 8.14 contains the response surfaces of the evaluated scenarios in DOE N°1. As it can be observed, when using water as solvent (Figure 8.14(a)), the recovery of both products is better at low Sol:Liq ratio, reaching almost 91% of the maximum recovery levels. It is also evident that at the evaluated interval, the temperature of extraction does not significantly affect the extraction process. Analogous behavior is found with ethyl acetate (Figure 8.14(b)). As seen, the best performance of this solvent is found at 1:10 Sol:Liq ratio, reaching an 88% of the maximum recovery levels. As it occurs with water, the horizontal lines in the contour plot suggest the temperature effect is negligible in the evaluated interval.

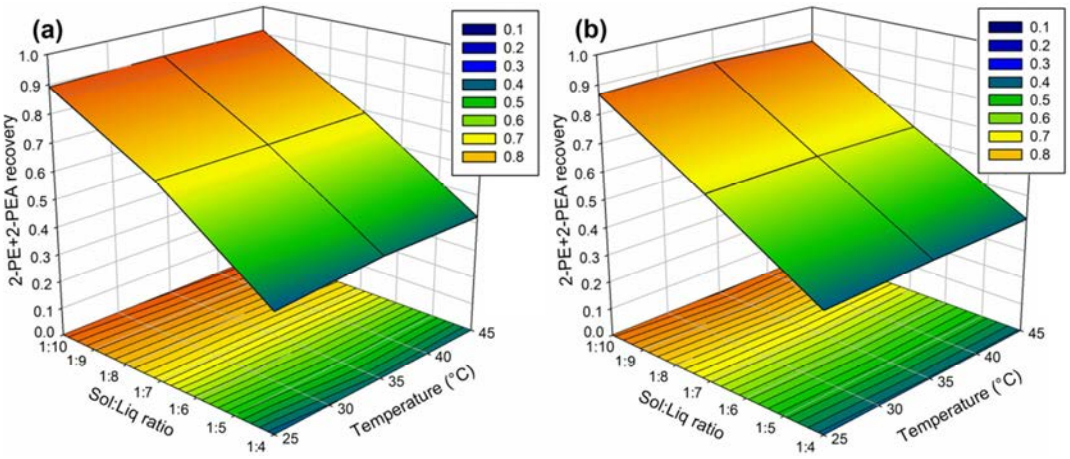


Figure 8.14. Response surfaces for products recovery in DOE N°1. (a) Water; (b) ethyl acetate.

A similar trend is found in the mixture water-methanol. As seen in Figure 8.15(a) the highest recovery levels are obtained with a Sol:Liq ratio of 1:10, with negligible changes due to the temperature variation. Differently, the effect of the methanol concentration has a substantial effect on the total recovery. As observed in Figure 8.15(b), the trend suggests that the higher the methanol content, the higher the 2-PE and 2-PEA recovery almost independently of the temperature, and with slight changes regarding the Sol:Liq ratio along the evaluated interval.

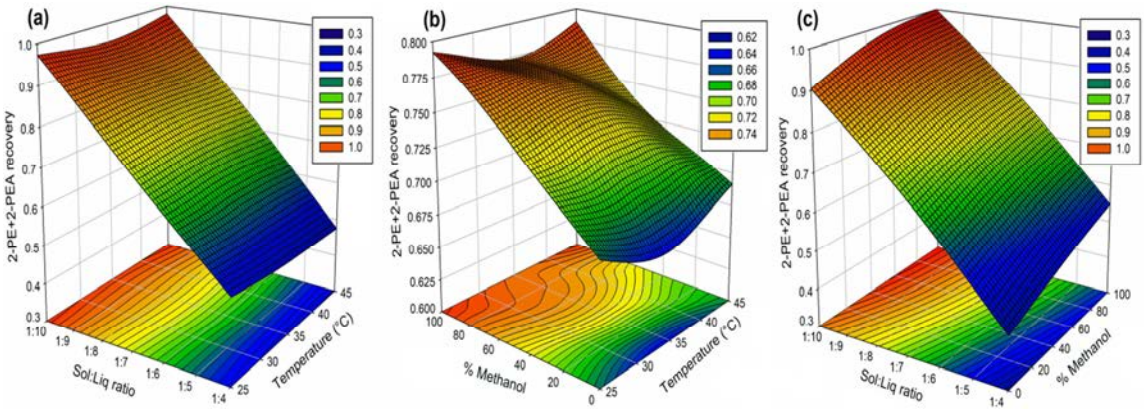


Figure 8.15. Response surfaces for products recovery in DOE N°2. (a) Sol:Liq Vs temperature; (b) % methanol Vs temperature; (c) Sol:Liq Vs % methanol.

As it is detailed in Figure 8.15(c), the maximum recovery levels have been achieved using pure methanol in a 1:10 Sol:Liq ratio at 35°C (24.6 $mg_{2\text{-PE+2-PEA}}$ g^{-1}_{TS}). These results suggest that, among the evaluated solvents, methanol is the best recovering the products from the solid matrix. As seen in Figure 8.16, the differences between solvents are significant, and by using methanol, the increase in the recovery would reach up to 12.3% and 18.0% compared to the use of water and ethyl acetate respectively. Having this in mind, the selection of the solvent would be focused on the balance between the recovery efficiency and the possible adverse effects of using one or another solvent. For instance its cost or the effects on the environment where the final residue is disposed.

In this sense, the use of more environmentally friendly solvents like water would be an option. Nevertheless, the relatively high recovery efficiency of water is a consequence of the 2-PE and 2-PEA concentrations in the liquid extracts, which are not substantially high (compared to the solubility of these species in water at the extraction conditions). Thus, for 2-PE the maximum concentrations range the 0.36 $g_{2\text{-PE}}$ L^{-1}, while for 2-PEA they are around 0.46 $g_{2\text{-PEA}}$ L^{-1}.

Another remarkable aspect of the recovery process is that, in general, the use of two successive extractions is effective enough to recover up to 97% of the quantified content of the products. Hence, in the first extraction a mean of 60-65% of products are recovered, and in the second another 30-32% is collected.

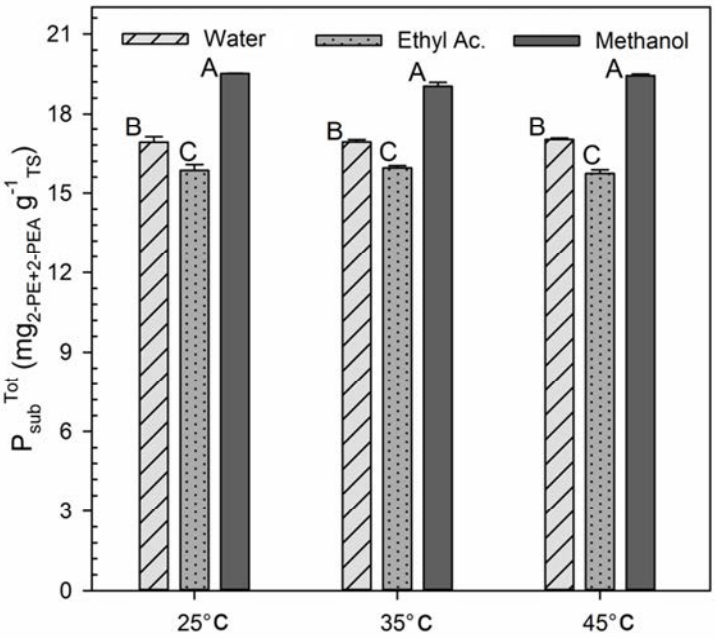


Figure 8.16. Comparison of the solvents performance in the recovery of 2-PE and 2-PEA at different temperatures. Data evaluated at 1:7 Sol:Liq ratio. P_{sub}^{Tot}: total cumulative production.

8.2.6 Final biological stability of the fermented substrates

The aerobic respiration indices for the residues obtained after the fermentation process using the different strategies at 22 L scale are presented in Table 8.4. It can be observed that the initial substrate has a high potential to be aerobically degraded, and it is no significantly different than the one analyzed in chapter 6. Thus, this residue could be classified as a residue with high biodegradability considering its dynamic respiration index (DRI) is higher than 5 g_{O2} kg^{-1}_{VS} h^{-1} (Barrena et al., 2011).

As it was found in chapter 6, the fermented residues have been degraded to a significant extent due to the SSF with *K. marxianus*. It can be observed that the lower DRI corresponds to the batch strategy, very close to 1 g_{O2} kg^{-1}_{VS} h^{-1}, point where an organic residue can be considered stable (Barrena et al., 2011). However, as occurred with the residue used for the fruit-like production, the fed-batch strategy produces a residue less degraded than in the batch case, but this time, not so far of the aforementioned limits.

Table 8.4. Aerobic respiration indices of the residues after the solid-state fermentation for producing 2-PE and 2-PEA.

Strategy	DRI_{24h} (g_{O2} kg^{-1}_{VS} h^{-1})	DRI_{24h} (g_{O2} kg^{-1}_{TS} h^{-1})	OUR_{Max-1h} (g_{O2} kg^{-1}_{VS} h^{-1})	At_4 (g_{O2} kg^{-1}_{TS})
Initial residue	5.2 ± 0.2	4.9 ± 0.2	5.7 ± 0.2	280.2 ± 10.3
Static-batch	1.0 ± 0.1	1.0 ± 0.1	1.9 ± 0.2	78.4 ± 9.6
Fed-batch 33%	1.2 ± 0.1	1.2 ± 0.1	2.5 ± 0.2	83.5 ± 4.6
Sequential-batch[a]	2.9 ± 0.1	2.6 ± 0.1	3.0 ± 0.2	194.1 ± 6.8
After products recovery[b]	1.1 ± 0.1	1.0 ± 0.1	1.8 ± 0.2	90.7 ± 3.7

DRI: Dynamic respiration index; At_4: cumulative index after 4 days; OUR_{Max-1h}: 1 h average maximum oxygen uptake rate. Indices have been computed based on the dynamic respirometric analysis at 37°C. [a]Sample obtained in the 4th growth cycle at the point of maximum microbial activity. [b]Sample obtained in the sequential-batch and processed after the products extraction under the best conditions exposed in section 8.2.5.

On the contrary, the residue collected at the point of replacement in the sequential-batch mode is apparently the less non-stabilized organic fraction, and additional processing would be needed before its final use. Nevertheless, since the 2-PE and 2-PEA production implies a further step for the products recovery, the effect of the extraction with methanol (as exposed in section 8.2.5) have induced a decrease in the activity of the residue. As observed, after the extraction process the DRI_{24h} has fallen almost 60%, reaching levels analogous to those obtained through the batch approach.

8.2.7 SSF compared to SmF approaches

Comparing the performance of SSF and SmF systems is not straightforward due to the intrinsic characteristics of each approach. While SmF indices are commonly referred to the volume of the liquid phase, in SSF the typical base of calculation is the dry mass of substrates (*i.e.* the dry matter content). Thus, comparing among these dissimilar systems is not an easy task, and the need for suitable indices is needed.

Here, this assessment has been done by means of three performance indices which provide information on different aspects of the process, and that can be computed in both systems. First, the space-time yield which indicates the volumetric productivity of each system. Secondly, the mass precursor yield ($Y_{L\text{-}Phe}$) [$mg_{2\text{-}PE+2\text{-}PEA}$ $g^{-1}_{L\text{-}Phe0}$] which is an index of the precursor consumption efficiency. Finally, the glucose yield (Y_{Glu}) [$mg_{2\text{-}PE+2\text{-}PEA}$ g^{-1}_{Sugar}] which indicates the sugars consumption efficiency.

Table 8.5 summarizes the above performance indices of several SmF systems for bioproducing 2-PE and 2-PEA. Similarly, Table 8.6 shows the same indices for the most representative evaluated strategies exposed in chapters 7 and 8.

As detailed in Table 8.5, SmF processes have a wide range of $Y_{L\text{-}Phe}$ between 0.09 and 0.73 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{L\text{-}Phe0}$. However, most of these systems reach $Y_{L\text{-}Phe}$ below 0.51 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{L\text{-}Phe0}$. Differently, in the SSF scenarios (Table 8.6), the higher $Y_{L\text{-}Phe}$ values suggest that these approaches require less L-Phe to produce the same amount of products than in a SmF process, or in other words, these approaches work with higher precursor consumption efficiency. Thus, it is expected SSF to be better than SmF regarding the use of the precursor.

Moreover, if the efficiency of the use of the available sugar is analyzed, Y_{Glu} indicates that there is not a clear trend among the SmF systems. While complex systems with ISPR reach values as high as 190 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{Sugar}$, some others are not able to reach 30 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{Sugar}$ (mean value 69.5 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{Sugar}$) suggesting that the efficiency of sugars consumption depends on several factors linked to the particular ISPR technique. On the contrary, evaluated SSF using only SCB as substrate show a consistent trend producing between 52 up to 107 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{Sugar}$, with a mean of 84.8 $mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{Sugar}$. As expected, this index shows the advantage of using only organic residues as carbon source in a SSF process, considering that, in average, it requires fewer sugars for producing the same products through a SmF system.

Finally, comparing the systems regarding the $Y_{s\text{-}t,}$ some aspects can be pointed out. In general, SmF present better performance than SSF with a mean of 73.8 $mg_{2\text{-}PE+2\text{-}PEA}\ L^{-1}\ h^{-1}$ compared to the 32.8 $mg_{2\text{-}PE+2\text{-}PEA}\ L^{-1}\ h^{-1}$ of the evaluated SSF approaches. This difference of near 44% is a direct consequence of the integration of sophisticated ISPR techniques that promote the further 2-PE and 2-PEA production. Differently, the evaluated SSF techniques are limited regarding the in situ product recovery. In this sense, these approaches aimed to keep the system as simple as possible to promote the sustainability of the processes. As seen in Table 8.6, the most promising alternatives seem to be those using operational strategies such as fed-batch and sequential-batch, which are still prone to be optimized in many aspects.

Table 8.5. Performance indices of some SmF systems for 2-PE and 2-PEA production.

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
Synthetic media	B	0.25	0.35	28.6	28.6	(Fabre et al., 1998)
	B	5.0	0.09	27.7	57.4	(Gao and Daugulis, 2009)
	B	0.5	0.15	13.1	17.5	(Yin et al., 2015)
	B	6.2	0.72	156.5	50.0	(Chreptowicz et al., 2016)
	B + ads	0.25	0.51	51.4	171.4	(Mei et al., 2009)
	B + BILS	0.25	0.75	150.0	41.7	(Sendovski et al., 2010)
	FB	3.6	0.32	13.8	86.4	(Stark et al., 2002)
	FB	2.0	0.25	122.5	43.8	(Serp et al., 2003)
	Imm. FB	2.0	0.19	190.0	65.5	(Serp et al., 2003)
	Liq-liq ILAL	15.0	0.44	24.3	156.3	(Mihal et al., 2012)
Grape must	B	2.0	0.23	3.8	9.6	(Garavaglia et al., 2007)
Tobacco waste	B	0.5	NA	31.6	17.6	(Wang et al., 2013)
Whey cheese	B	0.1	NA	NA	13.3	(Conde-Báez et al., 2017)
Cassava wastewater	B	0.25	0.24	66.5	18.5	(Oliveira et al., 2015)
Molasses	B + ads	0.5	0.60	66.0	169.2	(Hua et al., 2010)
Molasses + Glu	B + OP	2.4	0.65	97.5	234.0	(Etschmann et al., 2005)

V_R: Reactor volume; $Y_{L\text{-}Phe}$: mass precursor yield; Y_{Glu}: glucose yield; $Y_{s\text{-}t}$: space-time yield; B: batch; FB: fed-batch; SB: sequential-batch; ads: adsorbents; BILS: biphasic ionic liquid system; Imm: immobilized; ILAL: internal loop air-lift; OP: organic pervaporation; Glu: glucose; L-Phe: L-phenylalanine. Prod: quantified products (2-PE: 2-phenethyl alcohol; 2-PEA: 2-phenethyl acetate.). NA: not available.

[a]Computed based on the total L-Phe added. [b]Computed based on the total glucose or sugars used during the fermentation. [c]Computed based on the reaction volume. [*]computed at maximum production point.

Table 8.6. Performance indices of the evaluated SSF systems for 2-PE and 2-PEA bioproduction.

Substrate	Configuration	V_R (L)	$Y_{L\text{-}Phe}^{a*}$ ($g_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{L\text{-}Phe0}$)	Y_{Glu}^{b*} ($mg_{2\text{-}PE+2\text{-}PEA}\ g^{-1}_{Sug0}$)	$Y_{s\text{-}t}^{c*}$ ($mg_{2\text{-}PE+2\text{-}PEA}\ L^{-1}\ h^{-1}$)	Reference
Sugarcane bagasse	B	0.5	0.76	51.6	15.9	This study
	B + Glu	0.5	0.78	38.6	22.9	This study
	FB-33	0.5	0.53	66.1	25.0	This study
	B	1.6	0.59	87.4	30.5	This study
	B	22	0.59	86.7	20.6	This study
	FB-33	1.6	0.69	99.3	31.5	This study
	FB-33	22	0.63	95.5	16.8	This study
	SB	1.6	0.65	107.0	71.6	This study
	SB	22	0.60	84.9	60.7	This study

V_R: Reactor volume; $Y_{L\text{-}Phe}$: mass precursor yield; Y_{Glu}: glucose yield; Max $Y_{s\text{-}t}$: space-time yield at maximum production point; B: Batch; FB-33: fed-batch 33%; SB: sequential-batch; Glu: Glucose; L-Phe: L-phenylalanine; 2-PE: 2-phenethyl alcohol; 2-PEA: 2-phenethyl acetate.
[a]Computed based on the total L-Phe added. [b]Computed based on the total sugars used during the fermentation. [c]Computed based on the reactor volume. [*]Computed at maximum production point.

Final remarks

The use of alternative operating modes in SSF has been proven as a suitable strategy for the bioproduction of 2-phenethyl alcohol and 2-phenethyl acetate in a residue-based system. Simultaneously, sequential-batch and fed-batch strategies have shown a significantly better performance regarding the use of some of the resources involved in the fermentation, such as the inoculum requirement and the air consumed per gram of processed substrate. Consequently, due to the potential of the proposed strategies to enhance the SSF performance, they could serve as a starting point for the further scaling to pilot plant scale considering the inherent advantages and handicaps of each strategy.

The growth of the yeast *P. kudriavzevii* in subsequent stages of the sequential-batch operation possess in evidence an opportunity to enhance the 2-PE production via SSF. The outstanding ability of this strain to survive and succeed is a potential asset to be exploited in the development of future processes.

References

Barrena, R., Gea, T., Ponsá, S., Ruggieri, L., Artola, A., Font, X., Sánchez, A., 2011. Categorizing Raw Organic Material Biodegradability Via Respiration Activity Measurement: A Review. Compost Sci. Util. 19, 105–113.

Cerda, A., Gea, T., Vargas-García, M.C., Sánchez, A., 2017. Towards a competitive solid state fermentation: Cellulases production from coffee husk by sequential batch operation and role of microbial diversity. Sci. Total Environ. 589, 56–65.

Cheirsilp, B., Kitcha, S., 2015. Solid state fermentation by cellulolytic oleaginous fungi for direct conversion of lignocellulosic biomass into lipids: Fed-batch and repeated-batch fermentations. Ind. Crops Prod. 66, 73–80.

Chelliah, R., Ramakrishnan, S.R., Prabhu, P.R., Antony, U., 2016. Evaluation of antimicrobial activity and probiotic properties of wild-strain *Pichia kudriavzevii* from frozen idli batter. Yeast 33, 385–401.

Choi, D.H., Park, E.H., Kim, M.D., 2017. Isolation of thermotolerant yeast *Pichia kudriavzevii* from nuruk. Food Sci. Biotechnol. 26, 1357–1362.

Chreptowicz, K., Sternicka, M.K., Kowalska, P.D., Mierzejewska, J., 2018. Screening of yeasts for the production of 2-phenylethanol (rose aroma) in organic waste-based media. Lett. Appl. Microbiol. 66, 153–160.

Chreptowicz, K., Wielechowska, M., Główczyk-Zubek, J., Rybak, E., Mierzejewska, J., 2016. Production of natural 2-phenylethanol: From biotransformation to purified product. Food Bioprod. Process. 100, 275–281.

Conde-Báez, L., Castro-Rosas, J., Villagómez-Ibarra, J.R., Páez-Lerma, J.B., Gómez-Aldapa, C., 2017. Evaluation of Waste of the Cheese Industry for the Production of Aroma of Roses (Phenylethyl Alcohol). Waste and Biomass Valorization 8, 1343–1350.

Dandi, N.D., Dandi, B.N., Chaudhari, A.B., 2013. Bioprospecting of thermo- and osmo-tolerant fungi from mango pulp-peel compost for bioethanol production. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 103, 723–736.

del Mónaco, S.M., Rodríguez, M.E., Lopes, C.A., 2016. *Pichia kudriavzevii* as a representative yeast of North Patagonian winemaking terroir. Int. J. Food Microbiol. 230, 31–39.

Dhaliwal, S.S., Oberoi, H.S., Sandhu, S.K., Nanda, D., Kumar, D., Uppal, S.K., 2011. Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzevii*. Bioresour. Technol. 102, 5968–5975.

Etschmann, M.M.W., Schrader, J., 2006. An aqueous-organic two-phase bioprocess for efficient production of the natural aroma chemicals 2-phenylethanol and 2-phenylethylacetate with yeast. Appl. Microbiol. Biotechnol. 71, 440–443.

Etschmann, M.M.W., Sell, D., Schrader, J., 2005. Production of 2-Phenylethanol and 2-Phenylethylacetate from L-Phenylalanine by coupling whole-cell biocatalysis with organophilic pervaporation. Biotechnol. Bioeng. 92, 624–634.

Fabre, C.E., Blanc, P.J., Goma, G., 1998. Production of 2-phenylethyl alcohol by *Kluyveromyces marxianus*. Biotechnol. Prog. 14, 270–274.

Gao, F., Daugulis, A.J., 2009. Bioproduction of the aroma compound 2-phenylethanol in a solid-liquid two-phase partitioning bioreactor system by *Kluyveromyces marxianus*. Biotechnol. Bioeng. 104, 332–339.

Garavaglia, J., Flôres, S.H., Pizzolato, T.M., Peralba, M.D.C., Ayub, M.A.Z., 2007. Bioconversion of L-phenylalanine into 2-phenylethanol by *Kluyveromyces marxianus* in grape must cultures. World J. Microbiol. Biotechnol. 23, 1273–1279.

Greppi, A., Krych, Ł., Costantini, A., Rantsiou, K., Hounhouigan, D.J., Arneborg, N., Cocolin, L., Jespersen, L., 2015. Phytase-producing capacity of yeasts isolated from traditional African fermented food products and PHYPk gene expression of *Pichia kudriavzevii* strains. Int. J. Food Microbiol. 205, 81–89.

Hua, D., Lin, S., Li, Y., Chen, H., Zhang, Z., Du, Y., Zhang, X., Xu, P., 2010. Enhanced 2-phenylethanol production from L-phenylalanine via in situ product adsorption. Biocatal. Biotransformation 28, 259–266.

Koutinas, M., Patsalou, M., Stavrinou, S., Vyrides, I., 2016. High temperature alcoholic fermentation of orange peel by the newly isolated thermotolerant *Pichia kudriavzevii* KVMP10. Lett. Appl. Microbiol. 62, 75–83.

Li, C., Yu, J., Wang, D., Li, L., Yang, X., Ma, H., Xu, Y., 2016. Efficient removal of zinc by multi-stress-tolerant yeast *Pichia kudriavzevii* A16. Bioresour. Technol. 206, 43–49.

Li, C., Zhang, D., Ma, N., Wang, D., Li, L., Yang, X., Xu, Y., 2016. Comparison study on Copper bioaccumulation by growing *Pichia kudriavzevii* and *Saccharomyces cerevisiae*. Environ. Prog. Sustain. Energy 35, 1353–1360.

Mei, J., Min, H., Lü, Z., 2009. Enhanced biotransformation of L-Phenylalanine to 2-Phenylethanol using an in situ product adsorption technique. Process Biochem. 44, 886–890.

Mihal, M., Veres, R., Markoš, J., Stefuca, V., 2012. Intensification of 2-phenylethanol production in fed-batch hybrid bioreactor: Biotransformation and simulations. Chem. Eng. Process. Process Intensif. 57–58, 75–85.

Mu, L., Hu, X., Liu, X., Zhao, Y., Xu, Y., 2014. Production of 2-phenylethanol by microbial mixed cultures allows resource recovery of cane molasses wastewater. Fresenius Environ. Bull. 23, 1356–1365.

Mukherjee, V., Radecka, D., Aerts, G., Verstrepen, K.J., Lievens, B., Thevelein, J.M., 2017. Phenotypic landscape of non-conventional yeast species for different stress tolerance traits desirable in bioethanol fermentation. Biotechnol. Biofuels 10, 1–19.

Nakasaki, K., Araya, S., Mimoto, H., 2013. Inoculation of *Pichia kudriavzevii* RB1 degrades the organic acids present in raw compost material and accelerates composting. Bioresour. Technol. 144, 521–528.

Oliveira, S.M., Gomes, S.D., Sene, L., Christ, D., Piechontcoski, J., 2015. Production of Natural Aroma By Yeast in Wastewater of Cassava Starch Industry. J. Brazilian Assoc. Agric. Eng. 35, 721–732.

Osorio-Cadavid, E., Chaves-López, C., Tofalo, R., Paparella, A., Suzzi, G., 2008. Detection and identification of wild yeasts in Champús, a fermented Colombian maize beverage. Food Microbiol. 25, 771–777.

Radecka, D., Mukherjee, V., Mateo, R.Q., Stojiljkovic, M., Foulquié-Moreno, M.R., Thevelein, J.M., 2015. Looking beyond *Saccharomyces*: The potential of non-conventional yeast species for desirable traits in bioethanol fermentation. FEMS Yeast Res. 15, 1–13.

Sankh, S., Thiru, M., Saran, S., Rangaswamy, V., 2013. Biodiesel production from a newly isolated *Pichia kudriavzevii* strain. Fuel 106, 690–696.

Sendovski, M., Nir, N., Fishman, A., 2010. Bioproduction of 2-Phenylethanol in a Biphasic Ionic liquid Aqueous system. J. Agric. Food Chem. 58, 2260–2265.

Serp, D., Von Stockar, U., Marison, I.W., 2003. Enhancement of 2-phenylethanol productivity by *Saccharomyces cerevisiae* in Two-Phase Fed-Batch fermentations using solvent immobilization. Biotechnol. Bioeng. 82, 103–110.

Stark, D., Münch, T., Sonnleitner, B., Marison, I.W., Stockar, U. von, 2002. Extractive Bioconversion of 2-Phenylethanol from L-Phenylalanine by *Saccharomyces cerevisiae*. Biotechnol. Prog. 18, 514–523.

Talukder, A.A., Easmin, F., Mahmud, S.A., Yamada, M., 2016. Thermotolerant yeasts capable of producing bioethanol: isolation from natural fermented sources, identification and characterization. Biotechnol. Biotechnol. Equip. 30, 1106–1114.

Toivari, M., Vehkomäki, M.L., Nygård, Y., Penttilä, M., Ruohonen, L., Wiebe, M.G., 2013. Low pH D-xylonate production with *Pichia kudriavzevii*. Bioresour. Technol. 133, 555–562.

Van Rijswijck, I.M.H., Wolkers - Rooijackers, J.C.M., Abee, T., Smid, E.J., 2017. Performance of non-conventional yeasts in co-culture with brewers' yeast for steering ethanol and aroma production. Microb. Biotechnol. 10, 1591–1602.

Wang, Q., Song, Y., Jin, Y., Liu, H., Zhang, H., Sun, Y., Liu, G., 2013. Biosynthesis of 2-phenylethanol using tobacco waste as feedstock. Biocatal. Biotransformation 31, 292–298.

Xu, D., Yao, H., Xu, Z., Wang, R., Xu, Z., Li, S., Feng, X., Liu, Y., Xu, H., 2017. Production of ε-poly-lysine by *Streptomyces albulus* PD-1 via solid-state fermentation. Bioresour. Technol. 223, 149–156.

Yin, S., Zhou, H., Xiao, X., Lang, T., Liang, J., Wang, C., 2015. Improving 2-Phenylethanol production via Ehrlich pathway using genetic engineered *Saccharomyces cerevisiae* strains. Curr. Microbiol. 70, 762–767.

Yuan, S.-F., Guo, G.-L., Hwang, W.-S., 2017. Ethanol production from dilute-acid steam exploded lignocellulosic feedstocks using an isolated multistress-tolerant *Pichia kudriavzevii* strain. Microb. Biotechnol. 10, 1581–1590.

Zheng, X., Li, K., Shi, X., Ni, Y., Li, B., Zhuge, B., 2018. Potential characterization of yeasts isolated from Kazak artisanal cheese to produce flavoring compounds. Microbiologyopen 7, 1–12.

Chapter 9

Conclusions & Future work

General conclusions

- It was identified that sugarcane bagasse presents suitable characteristics as raw material for running a solid-state fermentation using the yeast *K. marxianus* to produce aroma compounds.
- *K. marxianus* has proven its efficiency for the biosynthesis of volatile compounds of fruit-like characteristics using only the selected agro-industrial residues as sole substrate for the solid-state fermentation.
- *K. marxianus* was able to use the sugarcane bagasse as sole carbon source for the biotransformation of L-phenylalanine into 2-PE and 2-PEA through SSF system under non-sterile conditions. Promising results were obtained at 22 L scale.
- These results are a step forward in the development of the bioprocess towards its implementation as a large-scale application, and they also show the importance of the alternative operational strategies as tools for the improvement of the SSF's efficiency, and their potential in the industrial development of similar bioprocesses.
- These findings suggest that the principle of "from waste to product" could be applied for obtaining these valuable aroma compounds in a more sustainable and environmentally friendly way.

Section 1. Fruit-like compounds

- *K. marxianus* has also demonstrated its ability to grow in this solid media under very different conditions, using the available nutrients for the production of a variety of aroma compounds according to the operational parameters. Thus, in an optimized batch process at sterile conditions, the maximum total cumulative volatile production was achieved at 40°C, 0.14 L h^{-1} g^{-1}_{TS}, 35% molasses (dry basis) was 161 mg_{Vol} per gram of dry substrate (g^{-1}_{TS}) producing mostly alcohol species. On the contrary, in the same system at 30°C 0.11 L h^{-1} g^{-1}_{TS}, 25% molasses, the production of ester compounds was maximized (47.6 mg_{ester} g^{-1}_{TS}), producing a pleasant fruity odor derived from the higher ester species content.
- The production of fruit-like compounds via SSF has been studied under different process strategies using as substrate a non-sterilized mixture of sugarcane bagasse/sugar beet molasses and the GRAS strain *K. marxianus*.

- In these systems, the use of SSF fed-batch strategies resulted in a more productive way to obtain the value-added fruit-like compounds, enhancing the amount of ester species in the accumulated volatiles. The synergy among substrate availability, the ability to keep an aerobic environment and temperature control are the main responsible for the success of the fed-batch strategies for improving the ester species selectivity.
- While the average total volatile production was 120 mg_{Vol} g^{-1}_{TS}, fed-batch operation promoted the highest increases in the ester content up to 57 mg_{Est} g^{-1}_{TS}, an 88 and 59% more than in the static-batch and intermittent mixing modes respectively.
- The influence of the scaling-up was strategy-dependent. These effects are more evident when a static-batch mode is used, and it is slightly reduced by using intermittent mixing. On the contrary, by means of a fed-batch strategy, the scaling effects are minimized at the evaluated conditions.

Section 2. Rose-like compounds

- At lab-scale, it was shown that by using SSF fed-batch processes result in a simple and reliable way to increase the 2-PE and 2-PEA production.
- The use of alternative operating modes in SSF has been proven as a suitable strategy for the bioproduction of 2-phenethyl alcohol and 2-phenethyl acetate in a residue-based system at bench-scale.
- In those systems, the fed-batch strategy has shown increases of 12.2 and 12.5% in the maximum 2-PE and 2-PEA production respect to the maximum levels achieved for the reference batch operation at 1.6 and 22 L respectively. On the other hand, sequential-batch provided sustained fermentations lasting 72 and 81 h, with increases of 17.5 and 2.4% at 1.6 and 22L respectively.
- Simultaneously, sequential-batch and fed-batch strategies have shown a significantly better performance regarding the use of some of the resources involved in the fermentation, such as the inoculum requirement and the air consumed per gram of processed substrate. These savings have reached values ranging 66-75% for the inoculum needs and 20-30% regarding the air consumption compared to the batch mode.

- The loss of production due to the increase in scale was limited to less than 2% for batch and fed-batch modes, while it was 14% for sequential-batch operation.
- During the evaluation of an extended sequential-batch strategy, the appearance of the indigenous yeast *Pichia kudriavzevii* has shown substantial enhancements in the bioproduction of 2-phenethyl alcohol.
- The preliminary results shows that, at the same conditions, *P. kudriavzevii* is able to transform the L-phenylalanine into 2-PE significantly better than *K. marxianus*, reaching up to 30% more in a batch fermentation at sterile conditions.

Future work

As the first attempt to produce aroma compounds via Solid-state fermentation in our group, and with a scarce literature on this topic, much future work is still to be performed.

- The growth of the yeast *P. kudriavzevii* in subsequent stages of the sequential-batch operation possess in evidence an opportunity to enhance the 2-PE production via SSF. The outstanding ability of this strain to survive and succeed is a potential asset to be exploited in the development of future processes.
- The proposed SSF approaches here presented are still prone to be improved in several aspects. For instance, some parameters of the operational strategies such as fed-batch and sequential-batch can be modified to optimize the production of the desired products (*e.g.*, number of substrate splits, replacement percentage, the point of replacement)
- A significant effort is required to find a suitable method to recover the different aroma compounds, such that the suggested approaches could be complemented.
- This work has been focused on the use of only one substrate as main raw material, but the integration of other organic fractions could promote synergies that end up with improvements of the SSF process (*e.g.*, using material of complementary characteristics, providing to the solid media better conditions than the ones using only one substrate).