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Intensification of 2-phenylethanol production using an aerated system assisted by a membrane-based solvent extraction technique

Intensificación de la producción de 2-feniletanol empleando un sistema aireado asistido por una técnica de extracción de una membrana basada en solvente

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Abstract

The production of 2-phenylethanol (2-PE), a high-valued aroma, has been performed naturally through the bioconversion of L-phenylalanine by the Ehrlich pathway in yeast. Nevertheless, one of the main limitations of this bioconversion is the inhibition of yeast growth by 2-PE. This work evaluated the intensification of 2-PE production in a batch culture using a conventional bioreactor coupled to an *in-situ* product removal (ISPR) process, by means of membrane-based solvent extraction (MBSE). The culture of the native yeast *Kluyveromyces marxianus* ITD0090, using a shaker flask stirred at 150 rpm, produced a maximum concentration of 2-PE of 0.7 g L^{-1} after 24 hours. When the yeast was cultivated in a controlled aerated-stirred bioreactor operated at 0.8 vvm and 350 rpm, the maximum production of 2-PE after 48 h reached 1.49 g L^{-1}. Intensification of the bioprocess was achieved by coupling the controlled bioreactor with a MBSE system, which allowed the continuous recovery of the 2-PE produced, reaching a final titre of 3.02 g L^{-1} after 56 h.

Keywords: aroma, 2-phenylethanol, bioconversion, *Kluyveromyces marxianus*, ISPR.

Resumen

La producción de 2-feniletanol (2-PE), un aroma de alto valor comercial, se puede realizar de forma natural a través de la bioconversión de L-fenilalanina mediante la vía de Ehrlich en las levaduras. Sin embargo, una de las principales limitaciones de esta ruta es la inhibición del crecimiento por la producción del 2-PE. Este estudio evaluó la intensificación de la producción de 2-PE en un cultivo por lotes, utilizando un biorreactor convencional acoplado a un proceso de remoción de producto *in-situ* (ISPR), mediante la extracción de una membrana basada en solvente (MBSE). El cultivo de la levadura nativa *Kluyveromyces marxianus* ITD0090 en matraces agitados a 150 rpm produjo una concentración máxima de 2-PE de 0.7 g L^{-1} después de 24 horas. Cuando la levadura se cultivó en un biorreactor controlando la aireación y agitación se alcanzó una producción máxima de 1.49 g L^{-1} después de 48 h, con una aireación de 0.8 vvm y 350 rpm. La intensificación del bioproceso se logró mediante el acoplamiento del biorreactor controlado con un sistema MBSE, que permitió la recuperación continua del 2-PE producido alcanzando una producción total de 3.02 g L^{-1} después de 56 h.

Palabras clave: aroma, 2-feniletanol, bioconversión, *Kluyveromyces marxianus*, ISPR.

1 Introduction

2-phenylethanol (2-PE; CAS 60-12-8) is a highly-valued aroma and flavor molecule with rose-like notes and a sweet taste (Martínez-Avila *et al*., 2018; Cordero-Soto *et al*., 2020). Notably, it is the second most used alcohol in the cosmetic and perfume industries (Lu *et al*., 2016). Its significance for the pharmaceutical industries has increased in recent decades due to antityrosinase, antimicrobial, and anti-depressive properties (Ueno *et al*., 2019; Zhu *et al*., 2011).

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In the food industry, 2-PE improves the aromatic profile of several fermented beverages, and is used as an additive in candies and non-alcoholic beverages (Domańska *et al*., 2017; Etschmann *et al*., 2002; Królikowski *et al*., 2016). However, while products labeled as "natural" are favored by the consumers, 2-PE has been largely produced by chemical synthesis (Lu *et al*., 2016).

Biological processes using microorganisms or enzymes are considered as natural routes; therefore, they have been proposed as attractive technologies for producing 2-PE (Martínez-Avila *et al*., 2018). In this regard, the bioconversion of L-phenylalanine (L-phe) by following the Ehrlich pathway is a promising method to obtain 2-PE out of a natural route. Several microorganisms have been used to produce 2-PE (Shu *et al*., 2020). However, yeast strains available in culture collections, especially *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* species, have exhibited promising results (Etschmann *et al*., 2005; Gao and Daugulis, 2009; Mei *et al*., 2009; Wang *et al*., 2011; Shu *et al*., 2020; Li *et al*., 2021). Nevertheless, since L-phe is an expensive amino-acid, the economic viability of the bioconversion lies on the use of by-products, obtained out of the food and feed industry, containing L-phe.

K. marxianus species have shown interesting characteristics during a bioconversion such as high growth rate and thermotolerance, favoring the production of the target compound and, thus, minimizing operation costs (Fonseca *et al*., 2008; Lane and Morrissey, 2010; Morrissey *et al*., 2015; Karim *et al*., 2020). Moreover, *K. marxianus*, frequently isolated from food, presents metabolic versatility allowing the assimilation of several substrates such as glucose, lactose, galactose, arabinose, xylose, and inulin (Fonseca *et al*., 2008; Lane and Morrissey, 2010; Morrissey *et al*., 2015; Yamahata *et al*., 2020; Karim *et al*., 2020). It is worth mentioning that *K. marxianus* has achieved GRAS status and Qualified Presumption of Safely (QPS) in the United States and European Union, respectively (Karim *et al*., 2020). For these reasons, its growth has been assessed for the production of ethanol and 2-PE in several environments, especially, wastes from the dairy industry and agro-industrial residues (Conde-Báez *et al*., 2017; Lara-Hidalgo *et al*., 2017; Martínez-Avila *et al*., 2018; Yamahata *et al*., 2020). Other non-conventional yeasts have been evaluated for the bioproduction of 2-PE out of various raw materials (Mierzejewska *et al*., 2019; Rodríguez-Romero *et al*., 2020). Nevertheless, the development of bioprocesses to produce 2-PE is limited by its inhibitory nature during the bioconversion. Additionally, the production of ethanol as a by-product during the bioconversion has influenced the yeast growth, affecting viability during industrial processes, and contributing to the reduction of the production of 2-PE (Shu *et al*., 2020; Haq *et al*., 2020). The critical concentration of 2-PE affecting cell viability has not been defined yet. Growth inhibition of *S. cerevisiae* occurred in batch culture at 2-PE concentrations ranging from 0.6 to 4 g L^{-1} (Lu *et al*., 2016; Stark *et al*., 2003). A concentration of 2.5 g L^{-1} reduced the respiratory capacity of *S. cerevisiae* (Stark *et al*., 2003). The addition of exogenous 2-PE at various concentrations (around 2 g L^{-1}) affected the growth of *S. cerevisiae* W303-1A (Lu *et al*., 2016; Stark *et al*., 2003). By contrast, *Candida glycerinogenes* WL2002-5 showed a higher inhibition tolerance to 2-PE than *S. cerevisiae* W303-1, with no viability reduction in growth at 2 g L^{-1} of 2-PE (Lu *et al*., 2016). On the other side, although the growth inhibition of *K. marxianus* has rarely been assessed, few studies (Adame-Soto *et al*., 2019) have identified that the production of 2-PE is affected by product inhibition. To this end, several studies (Gao and Daugulis, 2009; Etschmann *et al*., 2006) have presented strategies to overcome this inhibiting limitation, obtaining promising results regarding the production of 2-PE.

The intensification of bioconversion processes has been essential to increase product yields and process productivity. *In-situ* product recovery (ISPR) is a promising strategy for the intensification of processes affected by end-product inhibition (Van Hecke *et al*., 2014). Liquid-liquid extraction is frequently used in the downstream recovery of bioconversion products. Depending on the characteristics and needs of the bioproduction process, there are several configurations for placing phases in contact (Van Hecke *et al*., 2014). A promising approach to overcome drawbacks related to the inhibitory nature of 2-PE is its removal and/or recovery from the bioconversion medium during fermentation. ISPR has been demonstrated to recover important inhibitory compounds, such as 2-PE (Adler *et al*., 2011; Etschmann *et al*., 2005; Mihal *et al*., 2012; Šimko *et al*., 2015; Stark *et al*., 2002). Liquid-liquid extraction and adsorption are two of the most commonly reported and effective processes for recovering 2-PE (Etschmann *et al*., 2005; Gao and Daugulis, 2009; Stark *et al*., 2002). However, possible toxicity of the solvent is an important drawback with liquid-liquid extraction.

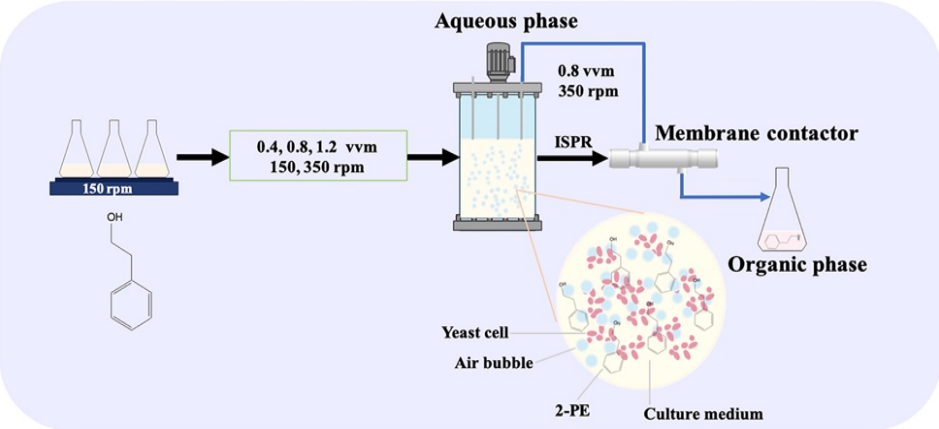

Fig. 1. General scheme of the strategy followed for 2-PE production by *K. marxianus* ITD0090.

Several biocompatible solvents have been studied for 2-PE recovery, including oleic acid, oleyl alcohol and polyprolylene glycole 1200, the most effective solvent for 2-PE production (Etschmann and Schrader, 2006; Stark *et al*., 2002). Product removal can also be achieved using techniques based on solvent immobilization, thus avoiding solvent toxicity. Membrane-based solvent extraction (MBSE) processes have shown promise for the extraction of 2-PE (Adler *et al*., 2011; Mihal *et al*., 2012; Mihal *et al*., 2013). This technique involves placing aqueous bioconversion broth in contact with an organic phase through the pores of a membrane. The porous membrane can be formed of hollow fibers, as a compact membrane contactor with a highly effective exchange area. The stabilized interface between the aqueous and organic phases avoids dispersion of the phases and prevents cells from being in direct contact with the solvents.

This work evaluated the intensification of the bioconversion process for producing 2-PE coupling a conventional batch culture bioreactor to a membrane-based solvent extraction (MBSE) system for the *in-situ* removal of 2-PE. The strategy followed in this work was to produce 2-PE by L-phe bioconversion over a native yeast, *Kluyveromyces marxianus* ITD0090, using a minimal medium culture. To elucidate the intensification of the bioconversion process, cultures were assessed in stirred flasks, aerated-agitated bioreactor and then by coupling a MBSE extraction technique to diminish the yeast inhibition. Figure 1 displays a general scheme showing the strategy followed for the production of 2-PE by *K. marxianus* ITD0090.

2 Materials and methods

2.1 Chemicals

L-phenylalanine (≤ 98%) and 2-phenylethanol (>99%) were provided by Sigma-Aldrich Corporation (St. Louis, MO, USA), $Na_2HPO_4{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$ and citric acid were obtained from J.T. Baker (Phillipsburg, NJ, USA), glucose, yeast extract, and casein peptone were purchased from BD Bioxon (Mexico City, Mexico), and oleyl alcohol was acquired from TCI-Europe (Zwyndrecht, Belgium).

2.2 Yeast, inoculum and culture media preparation

A native yeast strain isolated from natural fermentation of *Agave durangensis* was identified as *K. marxianus* ITD0090 (Adame-Soto *et al*., 2019). Yeast cells were preserved in 30% (v/v) glycerol at -20ºC and activated in yeast extract peptone dextrose medium (YPD; BD Bioxon, Mexico City, Mexico) as a pre-culture medium. After incubation for 24 hours at 30 °C, the yeast cells were transferred to liquid YPD pre-culture medium. The inoculum was prepared using 20% of the previously centrifuged pre-culture medium, and this was then suspended in culture medium containing: 30 g L^{-1} glucose, 8 g L^{-1} L-phenylalanine, 35 g L^{-1} $Na_2HPO_4 \cdot 2H_2O$, 10.5 g L^{-1} citric acid, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 0.17 g L^{-1} yeast extract, adjusted to pH 5 with 5 M HCl. The C/N ratio of the medium was approximatively equal to 17.70 g C $(g\ N)^{-1}$. The inoculum was incubated in 150 mL baffled polycarbonate Erlenmeyer flasks

with a vented top (Corning Incorporated, Corning, NY, USA), at an operating volume of 50 mL, for 12 h at 30 °C and 150 rpm. Inoculum concentration was fixed at 1×10^7 cells per mL.

2.3 *Effect of aeration conditions on 2-PE bioproduction*

In order to assess the synthesis of 2-PE from L-phenylalanine by the native yeast, cultures were conducted in 150 mL shaker flasks containing 50 mL of culture medium. Incubation was carried out in a shaking incubator (Shel Lab, Cornelius, OR, USA) for 24 hours at 30 °C, with agitation of 150 rpm and superficial aeration. Experiments were performed in triplicate.

Controlled cultures were performed with the two yeast strains in a 1-L controlled bioreactor (ADI 1025, Applikon Biotechnology BV, Schiedam, The Netherlands), with a working volume of 0.7 L. The temperature was controlled at 30 °C, and pH was adjusted to 5 with 5 M HCl at the beginning of each experiment. Agitation was performed with a Rushton turbine of six blades, and the bioreactor was aerated by atmospheric air filtered with a 0.45 μm nylon membrane. Different agitation (150 and 350 rpm) and aeration rates (0.4, 0.8 and 1.2 vvm) were tested. All experiments were performed in triplicate.

2.4 *In-situ product removal using MBSE*

Once operation conditions (aeration and agitation) were established in the aerated-stirred bioreactor, bioconversion with the native yeast strain was carried out in a 5-L Biostat® A bioreactor with a working volume of 3-L (Sartorius Stedim Biotech GmbH, Göttingen, Germany). Inoculum and culture medium were prepared as described in section 2.3, the temperature was fixed at 30 °C and the pH at 5.

In-situ extraction was performed using a membrane contactor composed by a hollow-fiber module Liqui-Cel® 2.5×8×50 (3M France, Cergy, France) containing hydrophobic polypropylene fibers (Membrana, Charlotte, NC, USA). Table 1 describes the characteristics of the extraction unit and the experimental setup is depicted in Figure 2.

Table 1. Characteristics of the X50 Liqui-Cel^{TM} commercial hollow fiber module.

Module 2.5x8		**Fibers X50**	
Material	Polypropylene	Material	Polypropylene
Internal diameter	58.4 mm	Internal diameter	220 μm
Internal length	20.3 mm	External diameter	300 μm
Number of fibers	~9800	Effective length	146 mm
Internal surface	~1m^2	Wall thickness	40 μm
		Porosity	40%
		Average pore diameter	0.03 μm

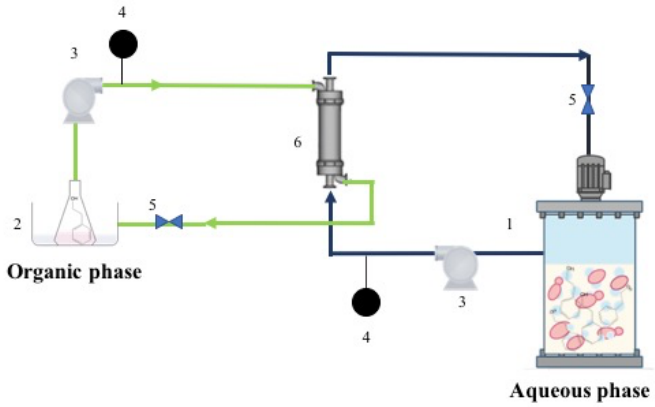

Fig. 2. In-stream product removal system using membrane-based solvent extraction. 1. Bioreactor; 2. Heated bath; 3. Volumetric pumps; 4. Manometers; 5. Pressure control valve; 6. Hollow-fiber membrane contactor.

Oleyl alcohol (CAS 143-28-2) was selected as the organic phase, due to its biocompatibility with yeast and high affinity for 2-PE (Etschmann *et al*., 2003). It circulated counter-currently through the shell side of the module at a flow rate of 12.72 mL min^{-1}. The fermentation medium circulated in and out of the bioreactor through the fiber lumens at a flow rate of 9.67 mL min^{-1}, firstly after 23 h of cultivation and then again after 48 h to avoid product inhibition. This configuration was chosen based on the high viscosity of the organic phase. Given its hydrophobic character, the membrane was preferentially wetted by the organic phase. Hence, a slight overpressure (0.1 bar) was exerted at the fermentation medium side in order to stabilize the interface and prevent the organic phase from flowing across the membrane. 2-PE concentrations were measured as a function of time by collecting samples from the aqueous phase. Sampling changed the volume by less than 5%.

2.5 *Glucose, ethanol, 2-phenylethanol, and cell growth analyses*

Evaluation of 2-PE concentrations in shake flasks and the aerated bioreactor were achieved with a 7820A GC System (Agilent, Waldbronn, Germany), equipped with a CyclosilB (30 m × 0.32 mm × 0.25 μm; J&W Scientific, Palo Alto, CA) column, a flame ionization detector (FID), and an auto-sampler. The carrier gas was helium at a flow rate of 1.5 mL min^{-1}. The detector and injector temperatures were 250 °C and 220 °C, respectively. Quantification was carried out using external standards of ethanol and 2-PE, in the concentration range 0.5-3 g L^{-1}. Glucose consumption was estimated using the technique described by Miller (1959).

During the extractive fermentation experiments, the concentration of 2-PE was measured using a G1530A GC System (Agilent), equipped with an HP-INNOWAX column (Agilent; 30 m × 0.53 mm × 1.00 μm, stationary phase: polyethylene glycol), an FID, and an CTC-PaL Model 1002 LTN CTC SYR automatic injector (Hamilton, Bonaduz, Switzerland). Helium was used as a carrier gas with a flow rate of 10 mL min^{-1}. The oven temperature started at 60 °C, with a ramp of 10 °C min^{-1} up to 220 °C. Injector and FID temperatures were set at 250 °C. Glucose consumption and ethanol production were monitored by an Alliance e2695 high-performance liquid chromatography (HPLC) system (Waters, Molsheim, France), using a HPX-87H Aminex column (300 mm x 7.8 mm x 9 μm; Bio-Rad, Richmond, VA, USA) maintained at 35 °C. L-phenylalanine was quantified by a Dionex Ultimate3000 HPLC-MS system (Thermo Scientific, Germering, Germany) equipped with a Hypersil Gold phenyl column (150 mm × 2.1 mm × 3 μm; Thermo Fisher Scientific, Waltham, MA, USA). All analyses were performed in triplicate.

3 Results and discussion

3.1 *2-phenylethanol production by native yeast K. marxianus ITD0090*

The biosynthesis of 2-PE over *K. marxianus* ITD0090 was first carried out in shake flasks. Figure 3 displays the kinetics of 2-PE produced from L-phenylalanine by *K. marxianus* ITD0090 in batch culture. The aroma compound was detected in the culture after 3 h of bioconversion, reaching a concentration of 0.7 g L^{-1} after 24 h and a productivity rate of 0.029 g L^{-1} h^{-1}. Glucose was totally consumed after 12 h and ethanol production reached a concentration of 12.25 g L^{-1} after 24 h (data not shown). Works performed in shake flasks have reported comparable results regarding the production rate of 2-PE, ethanol, and glucose consumption (Huang *et al*., 2001; Etschmann *et al*., 2003; Adame-Soto *et al*., 2019). Adame-Soto *et al*. (2019) evaluated the production of 2-PE and 2-phenylethylacetate by non-*Saccharomyces* yeasts, including *Saccharomyces cerevisiae, Clavispora lusitaniae, Torulaspora delbrueckii, Kluyveromyces dobzhanskii, Kluyveromyces marxianus*, and *Kluyveromyces* sp. Results showed that *Kluyveromyces marxianus* yeasts produced higher concentrations of 2-PE and 2-phenylethylacetate (2-PEA) than the other species. The production of 2-PEA as a secondary product during the Ehrlich pathway indicated that 2-PE was partly transformed to 2-PEA, based on the oxidative-reductive status of the operating conditions (Morrissey *et al*., 2015). The transformation of 2-PE to 2-PEA has not been clear yet but it has been related to a cell mechanism favored by the decrement of the toxicity of the media (Rodríguez-Romero *et al*., 2020). This mechanistic performance was also detected using *K. marxianus* ITD0090. The production of 2-PEA was here also quantified by CG-MS comparing the mass spectra with those in the NIST database (data not shown).

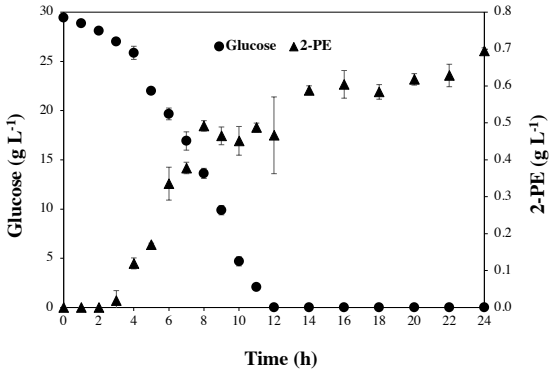

Fig. 3. Kinetics of glucose consumption and 2-phenylethanol production by *K. marxianus* ITD0090 in flasks at 150 rpm, 30°C.

Etschmann *et al*. (2003) assessed the performance of *K. marxianus* yeast (CBS600) obtaining 0.89 g L^{-1} of 2-PE after 41 h of fermentation in a non-optimized medium. Huang *et al*. (2001) observed a concentration of 0.5 g L^{-1} of 2-PE after 16 h of culture with *Pichia fermentans* L-5. Based on these results in shake flasks, the native strain *K. marxianus* ITD0090 has the potential for the biosynthesis of 2-PE. Consequently, culturing in an aerated-agitated bioreactor was performed to improve the production of 2-PE.

3.2 *Effects of aeration and agitation rate on the production of 2-phenylethanol*

To improve the production of 2-PE out of *K. marxianus* ITD0090, culturing was conducted in a controlled bioreactor at 30 °C under the effect of aeration flow rate and agitation rate.

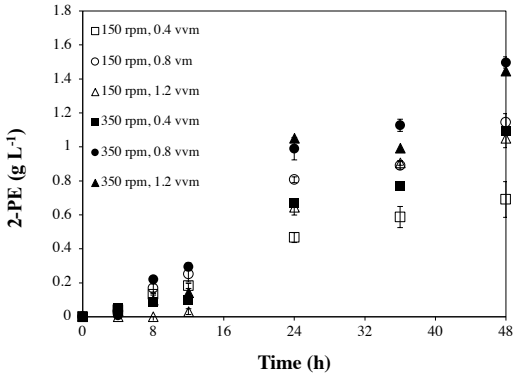

Fig. 4. Effects of air flow rate and agitation rate on the production of 2-PE by *K. marxianus* ITD0090.

Figure 4 displays the kinetics of 2-PE, evaluating six different conditions of agitation and aeration. The production of 2-PE was augmented by increasing the aeration flow rate from 0.4 to 1.2 vvm, at a given agitation rate. A favorable production was obtained at an agitation rate of 350 rpm.

At the lowest level of air supply (0.4 vvm), elevating the agitation rate from 150 to 350 rpm, the production of 2-PE increased from 0.69 to 1.09 g L^{-1} after 48 h of culture. A similar effect occurred at higher aeration flow rates (0.8 and 1.2 vvm), when the agitation rate was increased from 150 to 350 rpm. By varying the aeration rate from 0.4 to 0.8 and 1.2 vvm, at an agitation rate of 150 rpm, the concentration of 2-PE was enhanced from 0.69 g L^{-1} to 1.05 g L^{-1} and 1.14 g L^{-1}, respectively. A similar observation was obtained at an aeration rate of 350 rpm, with an increase in the concentration of 2-PE from 1.09 to 1.5 g L^{-1}. This behavior could be related to oxygen mass transfer from the gas-phase to the aqueous-phase, determined by the volumetric coefficient of oxygen transfer (k_La), which was larger at higher agitation rates (Castillo-Araiza *et al*., 2017). A previous study reported k_La values for the same operating conditions used in this study (Palmerín-Carreño *et al*., 2019). Results showed that maximum k_La values were obtained using 0.8 (24.6 h^{-1}) and 1.2 (15.6 h^{-1}) vvm at 350 rpm; with the larger the k_La values, the greater the production of 2-PE. At 350 rpm, concentrations of 1.49 and 1.44 g L^{-1} 2-PE were obtained at 0.8 and 1.2 vvm, respectively. By increasing the aeration intensity from 0.4 vvm at 150 rpm to 1.2 vvm at 350 rpm, the production of 2-PE was enhanced from 0.69 to 1.5 g L^{-1}, after 48 h of culture. These results allowed an increase in the production of 2-PE by a factor of 2, from 0.014 g L^{-1} h^{-1} to 0.031 g L^{-1} h^{-1} (Table 2). Therefore, the conditions corresponding to 0.8 vvm and 350 rpm were selected as the most favorable operating conditions for the improvement of the bioconversion of 2-PE from L-phenylalanine by *K. marxianus* ITD0090.

3.3 *In-situ 2-phenylethanol removal using membrane-based solvent extraction (MBSE)*

To increase the production of 2-PE in batch fermentation, an ISPR-based strategy was implemented. Bioconversion was conducted in an aerated-agitated bioreactor using the same culture medium and inoculation conditions, an aeration flow

rate of 0.8 vvm, and agitation rate of 350 rpm, a temperature of 30 °C and a controlled pH at 5. The bioreactor was operated independently or after being coupled to a MBSE system for the in-stream removal of 2-phenylethanol. The MBSE was operated after 23 h and 48 h of bioconversion. Figure 5 shows a comparison of the experimental results obtained over time assessing the conventional bioreactor and the ISPR system, in terms of the cell growth, glucose and L-phenylalanine consumption, and ethanol and 2-PE production. After 24 h of fermentation, consumption of glucose was completed, and biomass reached a concentration of 4 g L^{-1}. A maximum concentration of ethanol (9.7 g L^{-1}) was observed after 28 h of culturing (Figure 5a); it, then, decreased during the bioconversion when glucose was totally consumed. To this end, ethanol seems to be used as a carbon and energy source by the yeast, allowing the continual production of 2-PE (Adler *et al*., 2011; Morrissey *et al*., 2015). The uptake rate of L-phenylalanine was low during the bioconversion, having after 48 hours of culturing a concentration of ca. 1.3 g L^{-1}. At this time, the concentration of 2-PE reached a value of 2 g L^{-1}. The molar yield of the bioconversion was equal to 0.999 mol/mol, a value which was close to the stoichiometry of total bioconversion (1 mol/mol). L-phe uptake was similar to the results obtained by Gao and Daugulis (2009) in a single-phase bioconversion. These results were related to the assimilation of nitrogen in organic and its influence in the metabolism of higher alcohols, acetate, and ethyl esters (Rollero *et al*., 2021). The production of these metabolites depends directly on the strain type and also might be related to the C/N ratio (De los Rios-Deras *et al*., 2015; Iñiguez-Muñoz *et al*., 2019; Rodríguez-Romero *et al*., 2020; Rollero *et al*., 2021). Rodríguez-Romero *et al*. (2020) evaluated the production of 2-PE and 2-PEA over non-conventional yeasts using tequila vinasses as substrate considering different C/N ratios (total nitrogen includes organic and inorganic nitrogen). 2-PE was produced and transformed to 2-PEA. A C/N ratio similar to the one used here favored the production of 2-PEA out of 2-PE.

For the coupled system of extractive bioconversion (Figure 5b), glucose was completely consumed after 23 h, and the concentration of 2-PE reached 1.49 g L^{-1}, a value close to the critical concentration for cell viability of other strains (Lu *et al*., 2016; Stark *et al*., 2003). MBSE was started at 23 h of culture, for a duration of 170 min, which reduced the concentration of 2-PE in the bioreactor by 79%. The bioconversion was then continued without MBSE, until a 2-PE concentration of 1.40 g L^{-1} after 48 h. By considering the total amount of 2-PE produced in the aqueous phase during the MBSE coupling, a cumulative concentration of 2-PE of 2.59 g L^{-1} was obtained after 48 h, which was 67% higher than that obtained in a bioreactor without ISPR after the same fermentation time. A second run of MBSE was then carried out for 8 h until 56 h. The concentration of 2-PE in the bioreactor decreased from 1.40 to 0.67 g L^{-1}. After 56 h, the total accumulated amount of 2-PE produced was 3.02 g L^{-1}. The productivity of the bioprocess coupling fermentation and MBSE reached 0.054 g L^{-1} h^{-1}, which was significantly higher than 0.036 g L^{-1} h^{-1}. These results demonstrated that MBSE coupled to fermentation was an efficient technique that could be used to increase the production of 2-PE. Adler *et al*. (2011) evaluated *K. marxianus* CBS600 integrating a MBSE process. The obtained results were similar to the ones obtained here, showing an improvement in the concentration of 2-PE, from 1.4 g L^{-1} in conventional fermentation to 4.0 g L^{-1} in the intensified process.

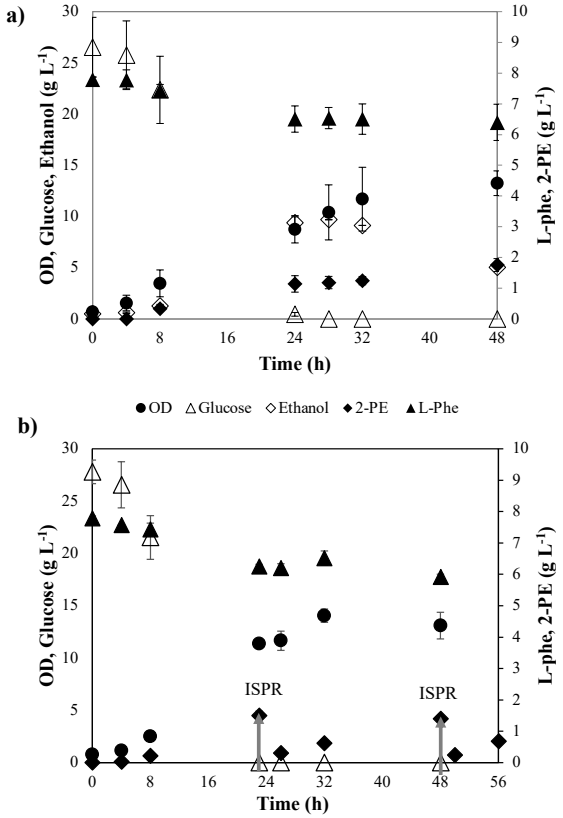

Fig. 5. Kinetics of bioconversion of L-phenylalanine to 2-phenylethanol by *K. marxianus* ITD0090 in aerated batch culture, without (a) and with (b) ISPR using membrane-based extraction with oleyl alcohol.

Shu *et al*. (2020) also obtained a similar result to the obtained in this work, evaluating a semi-continuous fermentation using adsorption ISPR using Hytrel® G3548, and yielding 4.5 g L^{-1} with *S. cerevisiae* BCRC 21812. Mihal *et al*. (2013) reached a concentration of 4.0 g L^{-1} 2-PE in a fed-batch bioreactor using the lyophilized baker's yeast *S. cerevisiae*. Their strategy to improve the process was to combine the membrane extraction with continual extractant regeneration reaching a volumetric 2-PE production of up to 18.6 g L^{-1}. It is worth commenting that even comparing our results with previous reports, the obtained concentrations in this study remained lower (Table 3). Etschmann *et al*. (2006) has obtained the highest production of 2-PE (26.5 g L^{-1}) using a two-phase extraction ISPR with polypropylene glycol 1200 as a compatible organic phase with *K. marxianus* CBS600. Nevertheless, the initial sugar concentration and L-phe concentration were considerably higher than in our bioconversion (60 g L^{-1} and 50 g L^{-1}, respectively). Gao and Daugulis (2009) obtained 20.4 g L^{-1} of 2-PE using the same yeast in a semi-continuous solid-liquid system using Hytrel® 18206 and adding glucose and L-phe after 29 h. Chreptowicz and Mierzejewska (2018) proposed the rapeseed oil as a natural and economical organic phase in a two-phase system, producing 18.50 g L^{-1} with *S. cerevisiae* (JM2014), using a lower concentration of both glucose and L-phe than in previous reports. Results obtained in this work allows the proposal of an economically viable bioprocess with the advantage that after the phase separation the rapeseed oil containing 2-PE can be directly commercialized as a natural product (Chreptowicz and Mierzejewska, 2018). In this sense, as future development, the design of an economically viable process can be proposed, using a more adequate organic phase and using L-phe-based by-products coming from the food and feed industry. The improvement of the production of 2-PE rests on the operation of a continuous operation of the intensified system.

Table 2. Bioconversion performances from L-phenylalanine to 2-phenylethanol by *K. marxianus* ITD0090 at 30°C under different batch culture conditions.

Culture conditions	**Aeration conditions**	**pH**	**Duration (h)**	**2-PE final titre (g L^{-1})**	**2-PE productivity (g L^{-1} h^{-1})**
Flask	NC	NC	24	0.69±0.08	0.029
Bioreactor (1 L)	0.4 vvm, 150 rpm	NC	48	0.69±0.10	0.014
	0.8 vvm, 150 rpm	NC	48	1.14±0.05	0.023
	1.2 vvm, 150 rpm	NC	48	1.05±0.05	0.021
	0.4 vvm, 350 rpm	NC	48	1.09±0.02	0.022
	0.8 vvm, 350 rpm	NC	48	1.49±0.03	0.031
	1.2 vvm, 350 rpm	NC	48	1.44±0.08	0.030
Bioreactor (5 L)	0.8 vvm, 350 rpm	pH 5	48	1.75±0.20	0.036
Bioreactor (5 L) coupled to ISPR	0.8 vvm, 350 rpm	pH 5	48	2.59±0.13	0.050
with two extractions with the MBSE			56	3.02±0.23	0.054

NC: not controlled.

Table 3. Bioconversion of 2-PE from L-phenylalanine by *K. marxianus* with different ISPR and culture conditions.

Yeast	**ISPR**	**2-PE (g L^{-1})**	**Reference**
CBS 600	Two-phase extraction	3.0	Etschmann *et al*., 2003
CBS 600	Two-phase extraction	26.5	Etschmann and Schrader, 2006
CBS 600	Pervaporation	3.47	Etschmann *et al*., 2005
CBS 600	Adsorption	20.4	Gao and Daugulis, 2009
CBS 600	MBSE 4.0	(2-PE +2-PEA)	Adler *et al*., 2011
ITD0090	MBSE	3.02 (aqueous phase)	This study

Conclusions

The native strain *K. marxianus* ITD0090 was a promising yeast for the production of 2-PE. Through different experimental designs, this study evaluated the production of 2-PE, using shake flasks and aerated-agitated bioreactors to improve hydrodynamics and mass transfer. The aerated-agitated bioreactor led to a production of 2-PE of 1.49 g L^{-1} at an aeration flow rate of 0.8 vvm and agitation rate of 350 rpm, the maximum concentration obtained in this current study. This result was related to a C/N ratio of 17.70 g C (g N)$^{-1}$. Control of pH during fermentation increased the efficiency of 2-PE production. Finally, coupling MBSE to batch fermentation increased 2-PE production, reaching a concentration of 3.02 g L^{-1} without glucose feeding. The performance of all fermentation conditions was compared by calculating their volumetric productivities. When the fermentation conditions were controlled for agitation, aeration, and pH, the production of 2-PE was improved by 25%, from 0.029 g L^{-1} h^{-1} to 0.036 g L^{-1} h^{-1}. When the fermentation was coupled to MBSE, the productivity was increased to 0.050-0.054 g L^{-1} h^{-1}. The process was then refined by 80-85%. However, these concentrations remained lower than the reported in previous studies, which operated in batch mode. As a future development, the effect of culture conditions (medium composition, environmental parameters), as well as the use of continuous operation of this integrated system should be implemented to increase the production of 2-PE. In addition, the valorization of by-products containing L-phenylalanine has to be developed in order to reduce the process cost at large scale.

Nomenclature

k_La	volumetric oxygen mass transfer coefficient, h^{-1}

Abbreviations

GC	gas chromatography
GC-MS	gas chromatography coupled to mass spectrometry
HPLC	high-performance liquid chromatography
MBSE	membrane based solvent extraction
2-PE	2-phenylethanol
2-PEA	2-phenylethyl acetate
L-phe	L-phenylalanine
YPD	yeast extract peptone dextrose media
ISPR	*In-situ* Product Removal

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