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Enhancing isoamyl acetate biosynthesis by *Pichia fermentans*

Mejorando la biosíntesis de acetato de isoamilo por *Pichia fermentans*

O. Rentería-Martínez[1], J.B. Páez-Lerma[1], J.A. Rojas-Contreras[1], J. López-Miranda[1], M.A. Martell-Nevárez[2], N.O. Soto-Cruz[1]*

[1]*Tecnológico Nacional de México/Instituto Tecnológico de Durango. Felipe Pescador 1830 Ote., Z.C. 34080, Durango, México.*
[2]*Facultad de Ciencias Forestales, Universidad Juárez del Estado de Durango. Río Papaloapan y Blvd. Durango S/N, Z.C. 34120, Durango, México.*

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Abstract

Microbial production of aroma compounds is a promising alternative to extracting plants or chemical synthesis. The yeast *Pichia fermentans* ITD-00165 has demonstrated a high potential to produce isoamyl acetate. The yeast was cultured in a bioreactor on a chemically defined medium to evaluate the influence of airflow (0.5, 0.75, and 1.0 vvm) and agitation speed (150, 175, and 200 rpm) on the isoamyl acetate production. Isoamyl acetate was retained using an *in situ* entrapping system, which was obtained, adding a cap of n-decane in a ratio of 80:20 (medium:decane). This work demonstrated the relevance of well-controlled aeration to enhance isoamyl acetate production using isoamyl alcohol as a precursor. Culture operating near to 0.71 vvm and 168 rpm (k_La = 10.6 h^{-1}) achieved production of 2.138 g L^{-1}, which means an almost 2.5-fold increase in respect to the higher value previously reported. On the other hand, the minimum production occurred at the best aeration conditions (k_La = 13.2 h^{-1}), meaning that excessive aeration could negatively affect the aroma production. Nevertheless, it appears that the concomitant ethanol production negatively affects aroma production.

Keywords: Non-*Saccharomyces* yeasts, banana aroma, aeration, extractive fermentation.

Resumen

La producción microbiana de compuestos aromáticos es una alternativa prometedora a la extracción de plantas o la síntesis química. La levadura *Pichia fermentans* ITD-00165 ha demostrado un alto potencial para producir acetato de isoamilo. La levadura se cultivó en un biorreactor en un medio químicamente definido para evaluar la influencia del flujo de aire (0.5, 0.75 y 1.0 vvm) y la velocidad de agitación (150, 175 y 200 rpm) sobre la producción de acetato de isoamilo. El acetato de isoamilo se retuvo utilizando un sistema de atrapamiento *in situ*, el cual se obtuvo agregando una capa de n-decano en una proporción de 80:20 (medio:decano). Este trabajo demostró la relevancia de una aireación bien controlada para mejorar la producción de acetato de isoamilo utilizando alcohol isoamílico como precursor. El cultivo que opera cerca de 0.71 vvm y 168 rpm (k_La = 10.6 h^{-1}) logró una producción de 2.138 g L^{-1}, lo que significa un aumento de casi 2.5 veces con respecto al valor más alto previamente publicado. Por otro lado, la producción mínima se dio en las mejores condiciones de aireación (k_La = 13.2 h^{-1}), lo que significa que una aireación excesiva podría afectar negativamente la producción de aroma. Sin embargo, parece que la producción concomitante de etanol afecta negativamente la producción de aroma.

Palabras clave: Levaduras non-*Saccharomyces*, aroma a plátano, aireación, fermentación extractiva.

1 Introduction

Food, cosmetic, tobacco, and pharmaceutical industries utilize aroma compounds to manufacture many of their products (Achmon *et al*., 2014; Kim *et al*., 2014; Chreptowicz *et al*., 2016). Natural aroma compounds are extracted from plants, but this way has some inconveniences such as uncertain access to raw materials, low yields, high waste generation, and high production costs. Nevertheless, regulations on food additives establish that aromas coming from microbial or enzymatic processes are cataloged as natural products, which has increased the importance of the production of these compounds through biotechnology (FAO/WHO, 2012).

* *Corresponding author. E-mail*: nsoto@itdurango.edu.mx
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Microbial production of aroma and other flavors is a promising alternative to extracting plants or chemical synthesis, which are the actual primary sources (Schrader *et al*., 2004; Carlquist *et al*., 2014; Paulino *et al*., 2021). The growing demand for natural products comes from increasing the consumers' apprehensive attitude towards synthetic compounds (Sánchez-Castañeda *et al*., 2018). It has led to the development of research related to the study of enzyme technology (Veríssimo *et al*., 2018) and microorganisms capable of producing aromas by biotransformation of added precursors (Palmerín-Carreño *et al*., 2019).

Yeasts are recognized for their capacity to benefit food products (Nawaz *et al*., 2020) and produce a wide range of aroma compounds such as higher alcohols, esters, fatty acid derivatives, among others (Dzialo *et al*., 2017). Some works used *Saccharomyces* yeasts (Kondo *et al*., 2012; Tian *et al*., 2015; Shen *et al*., 2016), but non-*Saccharomyces* yeasts have gain recognition as potential producers of aroma compounds. Then, there have been efforts for developing biotechnological processes to produce aroma compounds. For example, 2-phenylethanol and 2-phenyl ethyl acetate were produced by *Kluyveromyces marxianus* (Etschmann *et al*., 2005; Adler *et al*., 2011; Adame-Soto *et al*., 2019). A co-culture *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* (Fan *et al*., 2019) was used to produce ethyl acetate, while *Williopsis saturnus* (Yilmaztekin *et al*., 2009) and *Pichia fermentans* (Rentería-Martínez *et al*., 2016; Sanchez-Castañeda *et al*., 2018) have been used to produced isoamyl acetate.

Esters can be produced by fermentation and are characterized mainly by their sweet, fruity, and floral aroma, significantly influencing beverages and foods' sensory properties. Particularly, isoamyl acetate has a characteristic banana aroma and is a particularly important ester utilized in developing products from the food, cosmetic, and pharmaceutical industries (Abbas, 2006). Production of isoamyl acetate occurs mainly by chemical synthesis, but there is an increasing importance of biotechnological processes (Longo and Sanromán, 2006): enzymatic synthesis (Torres *et al*., 2009; Mhetras *et al*., 2010; Osorio-Viana *et al*., 2014; Vakili *et al*., 2020; Paulino *et al*., 2021) and fermentation (Yilmaztekin *et al*., 2009; Rentería-Martínez *et al*., 2016; Sánchez-Castañeda *et al*., 2018). Microbial synthesis of isoamyl acetate is performed intracellularly by the esterification of the isoamyl alcohol and acetyl-CoA in a reaction mediated by the enzymes alcohol acetyltransferases I and II (Fuji *et al*., 1994; Yoshimoto *et al*., 1998; Procopio *et al*., 2011).

Studies carried out in our workgroup showed that the yeast *Pichia fermentans* ITD-00165, isolated from Agave duranguensis juice during spontaneous alcoholic fermentation (Páez-Lerma *et al*., 2013), has high potential to produce isoamyl acetate (Hernández Carbajal *et al*., 2013; Rentería-Martínez *et al*. 2016; Sanchez-Castañeda *et al*., 2018). Nevertheless, the synthesis of aromas depends on the genus and the species of microorganisms. It is also necessary to provide an adequate environment for its growth and the production of metabolites of interest. Thus, it is essential to consider the parameters of fermentation, such as dissolved oxygen's role. Rojas *et al*. (2001) demonstrated that the yeasts *Hanseniaspora guilliermondii*, *Pichia anomala*, and *Hanseniaspora uvarum* produce isoamyl acetate in the presence of oxygen. The previous relevant studies on isoamyl acetate production by yeasts were performed using i) a bioreactor stirred at 100 rpm, but without airflow or precursor (Yilmaztekin *et al*., 2008), ii) shake flasks (100 rpm) with precursor addition but without airflow (Yilmaztekin *et al*.,2009), and iii) aerated flasks (1 vvm) with precursor but without agitation (Rentería-Martínez *et al*. 2016; Sanchez-Castañeda *et al*., 2018). Rentería-Martínez *et al*. (2016) also found that isoamyl alcohol is a better precursor than leucine since isoamyl alcohol supplement (1 g L^{-1}) yielded an increment of 9.7 times, while leucine addition (4 g L^{-1}) allowed an increase of 6.2 times. Previous studies have attempted to increase isoamyl acetate production solely by adding a precursor but neglecting an adequate control of the fermenter aeration. Then, the results reported here are the first obtained by adding a precursor (isoamyl alcohol) and performing the fermentation in a bioreactor operating under systematically controlled conditions of aeration (airflow and agitation speed).

This work aimed to enhance isoamyl acetate biosynthesis by the native yeast *Pichia fermentans* ITD-00165 in a well-aerated bioreactor.

2 Materials and methods

2.1 Yeast strain

The strain *Pichia fermentans* ITD-00165 was used. It was one of the strains isolated from the spontaneous alcoholic fermentation of *Agave duranguensis* (Páez-

Lerma *et al*., 2013) and was provided by the Microbial Biotechnology Lab's Culture Collection at the Instituto Tecnológico de Durango.

2.2 Culture medium

The chemically defined medium used was reported previously by Flores-Cosío *et al*. (2019). It contained, per liter: glucose solution, 28.5 mL (as the only carbon source); salt solution, 960 mL; vitamin solution, 2.5 mL; and trace element solutions, 1 mL each. The pH of all solutions was adjusted to 5 before mixing.

Glucose solution was made in a 700 g L^{-1} stock. It was prepared by dissolving 700 g of glucose with stirring and heating. The solution cooled down to room temperature, the pH was set, and then made up to 1 L. The salt solution contained (g L^{-1}): $Na_2HPO_4{\cdot}2H_2O$, 1.49 g; K_2HPO_4, 3 g; $(NH_4)_2SO_4$, 3 g; glutamic acid, 1 g. Glucose and salt solutions were sterilized separately in an autoclave at 121 °C for 15 minutes and stored at room temperature until use.

Vitamin solution included (mg L^{-1}): aminobenzoic acid, 1; myoinositol, 125; nicotinic acid, 5; pantothenic acid, 5; pyridoxine, 5; thiamine HCl, 5; and biotin, 0.012. Trace element solutions were prepared separately and each contained (mg L^{-1}): $MgCl_2{\cdot}6H_2O$, 410; $ZnCl_2$, 19.2; $CuCl2{\cdot}2H_2O$, 0.61; $MnCl_2{\cdot}4H_2O$, 4.45; $CoCl_2{\cdot}6H_2O$, 0.5; $CaCl_2$, 17.37; $FeCl_2{\cdot}4H_2O$, 11.66; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.36; H_3BO_3, 3. Vitamins and trace element solutions were filtered through a sterile Nylon membrane (0.20 μm) and stored at 4 °C until use.

The precursor (isoamyl alcohol) was gradually added to maintain a concentration near 0.5 g L^{-1} during the fermentation. Isoamyl acetate was retained using a simultaneous fermentation/liquid-liquid extraction system as an *in situ* entrapping system. It was obtained by adding a cap of decane in a ratio of 80:20 (medium:decane), according to that described previously (Sánchez-Castañeda *et al*., 2018). These authors discussed all relevant aspects of this entrapping system.

2.3 Airflow and agitation speed

A 3^2 complete factorial design allowed the assessment of the influence of airflow (0.5, 0.75, and 1.0 vvm) and agitation speed (150, 175, and 200 rpm) on the isoamyl acetate production by *P. fermentans*. Fermentations were performed into an Applikon bioreactor model EZ Control equipped with a 1 L glass vessel, which contained 650 mL of culture medium. The bioreactor has a 140 W motor (0-2000 rpm), one Rushton impeller of 6 flat blades (Dim = 4.5 cm), three vertical baffle plates, an L-type air sparger, and a polarographic dissolved oxygen sensor. Culture conditions were 28 °C, pH of 5.0, and inoculum size of $14{\times}10^7$ cells mL^{-1}. The airstream was filtered through a sterile 0.45 μm Nylon membrane. Fermentations were performed by duplicate during 24 h, taking samples from the aqueous phase (5 mL) and organic phase (1 mL) every 3 h.

2.4 Measurement of the volumetric oxygen transfer coefficient (k_La)

The dissolved oxygen (DO) electrode (AppliSens O_2 Sensor Z010023525) was calibrated at 28 °C using distilled water at two oxygen saturation points, 0 and 100% DO. Water at 0% DO was obtained bubbling nitrogen (N_2) for 30 min, while the air was bubbled for 30 min to prepare water at 100% DO. Subsequently, oxygen saturation concentrations (C_L^*) in the culture medium and the culture medium+decane were estimated. An airflow of 325 mL min^{-1} (0.5 vvm) was bubbled into the culture medium at 28 °C and 150 rpm for one hour. Then, the calibrated DO sensor was introduced into the medium, and the %DO was registered. This procedure was repeated four times and was also applied to the culture medium+decane (80:20). The C_L^* was estimated using Equation 1.

$$C^* = \frac{\%DO}{100} C_w^* \tag{1}$$

where

C^*: Oxygen saturation concentration into the culture medium or the culture medium+decane (mg L^{-1}).

%DO: Value obtained by the DO sensor (% saturation).

C_w^*: Oxygen saturation concentration in pure water at 28 °C (8.077 mg O_2 L^{-1}) was calculated from the data reported by Battino *et al*. (1983).

The determination of k_La was made, without and with decane presence, for all aeration conditions of the factorial design. The k_La was measured in cell-free conditions using the dynamic gassing-out method described previously (Tribe *et al*., 1995). Concisely, oxygen was eliminated from the culture medium bubbling N_2 (200 mL min^{-1}) until the DO reached only minimum levels (1-2%). Then, the air was fed at different flow rates according to the experimental design. Dissolved oxygen measurements were registered each minute until reaching a constant

value. The data collected allowed calculating the k_La using Equation 2.

$$\ln\left(1-\frac{C_L}{C^*}\right)=-k_La\cdot t \quad (2)$$

where
C^*: Oxygen saturation concentration into the culture medium or the culture medium+decane (mg L^{-1}).
C_L: Oxygen concentration at time t (mg L^{-1}).
k_La: Volumetric oxygen transfer coefficient (min^{-1}).
t: Time (min).

The k_La value was obtained from the slope of a graph of t vs. $\ln\left(1-\frac{C_L}{C^*}\right)$.

All determinations assumed that the liquid phase was well-mixed. The DO sensor has a response time (20-30 s) that always was significantly lower than the inverse of k_La values obtained. Then, the potential influence of DO electrode dynamics on the k_La was neglected.

2.5 *Analytical techniques*

Aqueous phase samples from the bioreactor were split into two portions. One milliliter was filtered through a 0.45 μm Nylon membrane and frozen until analysis of glucose, glycerol, ethanol, isoamyl alcohol, and isoamyl acetate. The other portion (4 mL) was used for biomass measurement by dry weight. Organic phase samples were frozen until analysis of isoamyl alcohol and isoamyl acetate.

Glucose, glycerol, and ethanol concentrations were determined by liquid chromatography in an instrument Agilent series 1200 equipped with a refractive index detector and a BioRad HPX-87H HPLC ion exclusion column (300 × 7.8 mm I.D.). Sulfuric acid (5 mM) was the mobile phase at a flow rate of 0.6 mL min^{-1}. The column oven was maintained at 60 °C, while 40 °C was the refractive index detector's temperature. One microliter was the injection volume.

Isoamyl acetate and isoamyl alcohol concentrations were measured by gas chromatography, using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass selective detector. An HP-Innowax column (length, 60 m; inside diameter, 0.25 mm; film thickness, 0.5 μm; stationary polyethylene glycol phase) allowed the separation of the compounds. High purity helium was used as the mobile phase at a constant flow of 1 mL min^{-1}. Injector and detector temperatures were 180 and 280 °C, respectively. The oven temperature was programmed as follows: initial temperature of 40 °C, followed by a ramp of 5 °C/min to reach 180 °C. The injection (1 μL) was made in split mode (1:50), while the mass detector was used in scan mode with an ionization voltage of -70 eV.

2.6 *Statistical analysis*

Duplicate independent fermentations were carried out for all experimental conditions, and all samples were analyzed in duplicate. Results were reported as means±standar deviation. The one-way analysis of variance (ANOVA) and the Tukey's test were ($p<0.05$) performed with the program Statistica 7.0.

3 Results and discussion

3.1 *Effect of airflow and agitation speed*

Preliminary experiments showed a diminishing of 65, 80, and 96% on the final concentration of biomass of *Pichia fermentans* after 24 h of incubation, caused by the presence of 0.5, 1.5, and 2.5 g isoamyl alcohol per liter, respectively. It was then decided to maintain the isoamyl alcohol concentration below 0.5 g L^{-1} by gradually addition along the 24 hours of fermentation. Some investigations have highlighted the importance of isoamyl alcohol as a precursor of isoamyl acetate (Yilmaztekin *et al*., 2009; Quilter *et al*., 2003). Nonetheless, the toxicity of this alcohol has also been reported. Strains of *Saccharomyces cerevisiae* tolerated up to 5 g L^{-1} in the medium without loss of viability (Martínez-Anaya *et al*., 2003). Still, the viability of *Williopsis saturnus* was slightly diminished with the presence of 1 g isoamyl alcohol per liter, according to results reported by Yilmaztekin *et al*. (2009). It aids in avoiding the toxic effect but could also promote a better utilization since it has been demonstrated that alcohol acetyltransferase in yeasts became saturated with the addition of isoamyl alcohol in more than 1 g L^{-1} of fusel oil (Quilter *et al*., 2003) or 0.4 g L^{-1} (Calderbank and Hammond, 1994). Nevertheless, it should be noted that the activity of the acetyltransferases can significantly differ between yeast species and strains (Dzialo *et al*., 2017).

Isoamyl acetate production was detected from the three hours of fermentation, showing the highest experimental production (2.138±0.046 g L^{-1}) at aeration conditions of 0.75 vvm and 175 rpm, using isoamyl alcohol as a precursor (Table 1).

Table 1. Isoamyl acetate production by *Pichia fermentans* ITD-00165 at 24 h of fermentation under different aeration conditions.

Airflow (vvm)	**Agitation speed (rpm)**	k_La (h^{-1})	**Isoamyl acetate (g L^{-1})**
0.5	150	5.7	1.401 ± 0.001^{cd}
	175	4.2	1.608 ± 0.058^{bc}
	200	8.2	1.061 ± 0.133^{e}
0.75	150	9.4	1.709 ± 0.012^{b}
	175	11.3	2.138 ± 0.046^{a}
	200	9.8	1.304 ± 0.107^{d}
1.0	150	4.9	0.901 ± 0.100^{ef}
	175	12.0	0.740 ± 0.057^{f}
	200	13.2	0.923 ± 0.024^{e}

[a,b,c]Different superscripts indicate significant difference ($p<0.05$).

This table shows that the highest production is 20 to 65%, significantly higher ($p<0.05$) than any other experimental value. The highest production is also 30 times greater than the obtained in this work without precursor addition (0.070 ± 0.004 g L^{-1}), as seen in Table 2. The amount achieved without precursor addition is considered the basal production from this yeast strain (synthesis de novo), which comes from esterification of the acetyl group from acetyl coenzyme A and isoamyl alcohol, both produced during glucose catabolism (Sánchez-Castañeda *et al*., 2018). Table 1 also reveals that 0.75 vvm was the aeration that permitted the best results, although a substantial drop (~40%) occurred when agitation speed switched from 175 to 200 rpm. On the other hand, 1.0 vvm was the aeration rate that yielded the lowest productions.

Table 2 shows a comparison of the relevant studies aiming to increase the isoamyl acetate production by yeasts. Yilmaztekin *et al*. (2009) reported an increment of three times in isoamyl acetate production (from 0.118 to 0.354 g L^{-1}) using fusel oil as isoamyl alcohol source in a stirred bioreactor without airflow. Later, Rentería-Martínez *et al*. (2016) used aerated flasks without agitation and adding one dose (4 g L^{-1}) of L-leucine or gradual addition of isoamyl alcohol, which allowed to increase the isoamyl acetate production up to 0.55 and 0.860 g L^{-1} supplementing leucine and isoamyl alcohol, respectively. It should be noted that leucine and isoamyl alcohol are two different precursors since leucine is a nutrient that can be used as a nitrogen source in the cell metabolism, while isoamyl alcohol is a compound reported as toxic to yeasts (Martínez-Anaya *et al*., 2003; Yilmaztekin *et al*., 2009). Leucine enters the Ehrlich pathway, where, like other amino acids, it is converted into its corresponded fusel alcohol, the isoamyl alcohol (Hazelwood *et al*., 2008). It is then esterified with acetyl-CoA by the action of the enzyme alcohol acetyltransferase (Fujii *et al*., 1994; Yoshimoto *et al*., 1998) to yield isoamyl acetate. Sánchez-Castañeda *et al*. (2018) added L-Leucine as a precursor in aerated flasks without agitation producing 0.624 g L^{-1}. Thus, the results presented here demonstrate the relevance of a well-aerated bioreactor, combining airflow and agitation, since our highest production is almost 2.5 times better than the higher value previously reported.

On the other hand, the process is not a simple bioconversion of isoamyl alcohol to isoamyl acetate. The isoamyl alcohol fed to the fermentation esterifies with acetyl coenzyme A produced during glucose catabolism. However, the yeast produced significant concentrations of ethanol and glycerol concomitantly with the isoamyl acetate production. Fig. 1A shows the fermentation kinetics at moderate aeration (0.75 vvm - 175 rpm), where the highest isoamyl acetate production occurred, while Fig. 1B presents the results at high aeration conditions (1 vvm - 175 rpm), where the lowest production was observed. Glucose consumption was remarkably similar since the initial amount of substrate was almost depleted in 24 h, showing practically the same pattern at both aeration conditions.

Nonetheless, there are substantial differences among both fermentation kinetics. Isoamyl acetate was produced continuously in both fermentations. However, the final concentrations reached for isoamyl acetate were 2.9 times higher at moderate aeration than that produced at high aeration conditions.

Table 2. Increment of isoamyl acetate production by yeasts using precursors for bioconversion.

Fermentation system	Airflow (vvm)	Agitation speed (rpm)	Precursor	Increment[(1)]	Reference
Shake flasks	-	100	Isoamyl alcohol[(2)]	3	Yilmaztekin *et al*., 2009
Flasks	1	-	Leucine	6.2	Rentería-Martínez *et al*. 2016
Flasks	1	-	Isoamyl alcohol	9.7	
Flasks	1	-	Leucine	6.9	Sanchez-Castañeda *et al*., 2018
Bioreactor	0.5 - 1.0	150 - 200	Isoamyl alcohol	30.5	Present work

[(1)]Number of times relative to the production obtained without precursor. [(2)]As fusel oil.

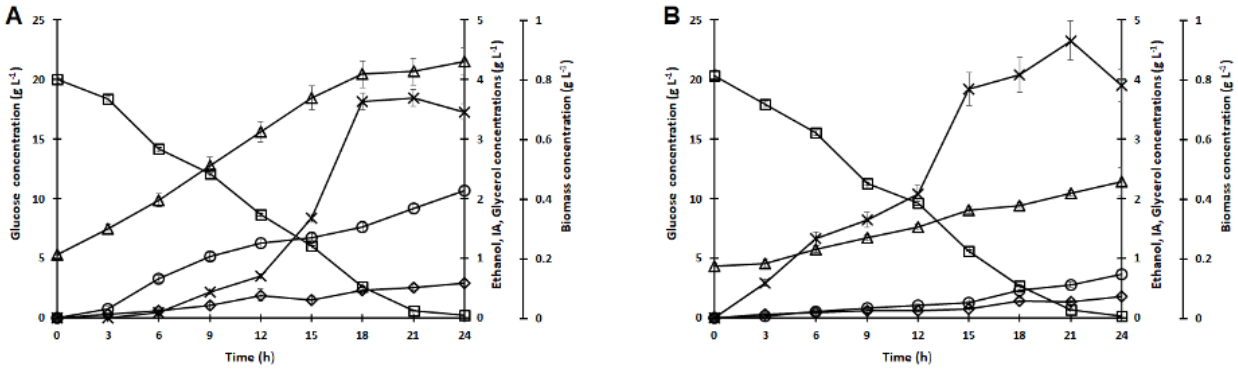

Fig. 1. Fermentation kinetics at (A) 0.75 vvm and 175 rpm, and (B) 1 vvm and 175 rpm. Glucose (□), biomass (△), ethanol (×), glycerol (⋄), and isoamyl acetate (◯).

Iñiguez-Muñoz *et al*. (2019) concluded that aeration (0.5 vvm) significantly reduced ester production by *Saccharomyces cerevisiae* during agave juice fermentation. They also mentioned that aeration could limit the availability of the acetyl group's donor for esterification (acetyl coenzyme A), which may be used for yeast growth. However, it seems that the case of *Pichia fermentans* is different since the specific growth rate was significantly higher ($p<0.05$) at moderate aeration (0.182 ± 0.0001 h^{-1}) than that observed at high aeration (0.116 ± 0.014 h^{-1}). At the same time, the biomass produced at moderate aeration (0.65 ± 0.02 g L^{-1}) was also greater significantly ($p<0.05$), concerning that produced at high aeration conditions (0.28 ± 0.04 g L^{-1}). The availability of acetyl coenzyme A probably limits the production of isoamyl acetate by *Pichia fermentans*, but how this occurs remains investigated.

Another factor to consider is the ethanol production differences between both fermentations. Ethanol production at moderate aeration occurred slowly up to 12 h, but more quickly from 12 to 18 h, reaching its maximum concentration (3.69 ± 0.04 g L^{-1}) at 21 h of incubation. In contrast, ethanol production at high aeration occurred more intensely from the beginning of fermentation and reached a maximum concentration (4.65 ± 0.33 g L^{-1}) at 21 h of incubation. Like that happened with growth, the production of isoamyl acetate and glycerol could also be diminished by ethanol production. The inhibitory effect of ethanol in fermentation for aroma production has been reported previously by Mihal' *et al*. (2012), who studied L-phenylalanine's biotransformation 2-phenyl ethanol (rose-like aroma) by *Saccharomyces cerevisiae*. They concluded that ethanol formation during fermentation contributed as an inhibitor to end the process.

3.2 *Isoamyl acetate production and the k_La*

The C^* values obtained into the culture medium and culture medium+decane were 7.66 ± 0.05 and 9.21 ± 0.12 mg O_2 L^{-1}, respectively.

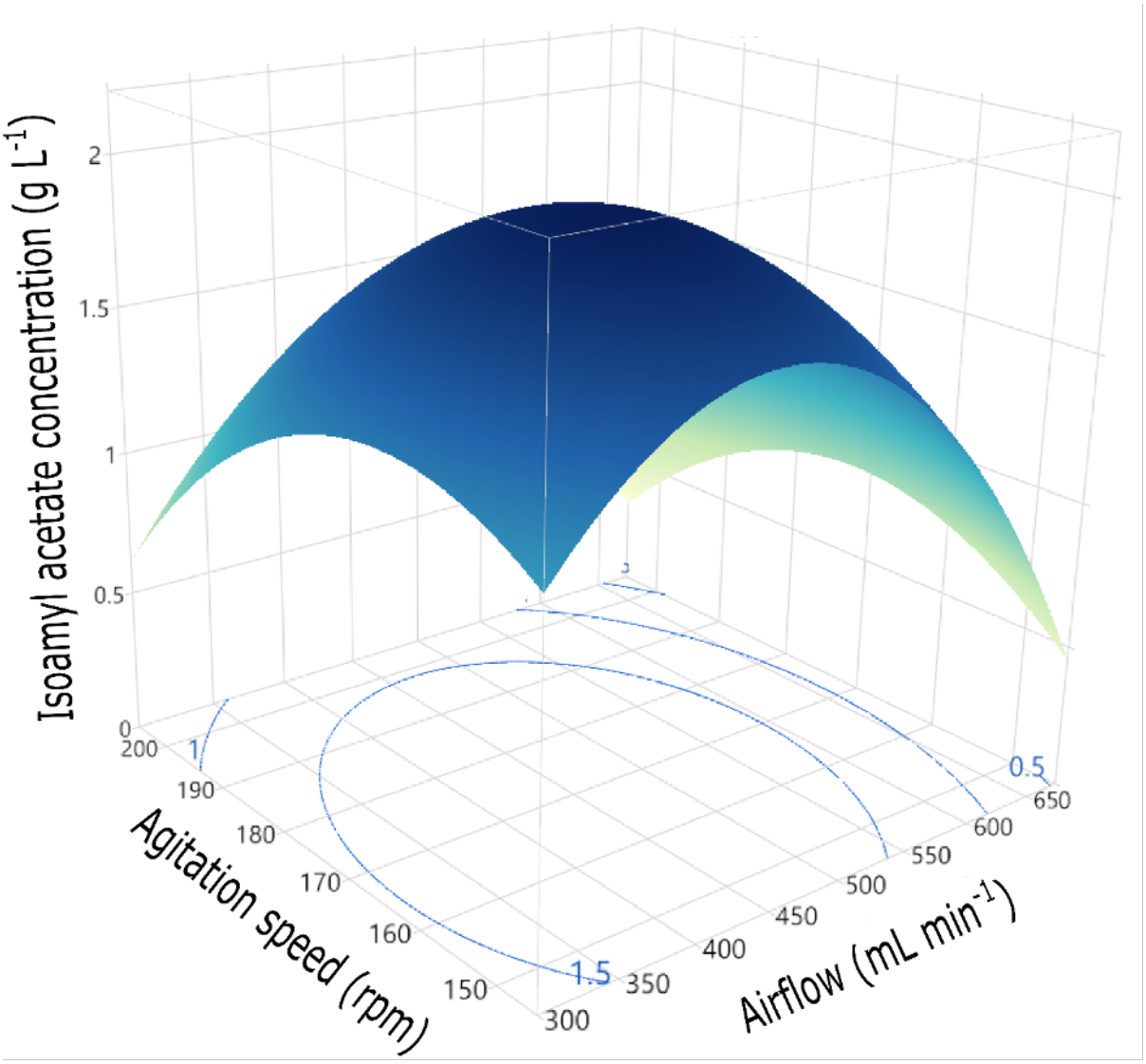

Fig. 2. Contour plot of a quadratic fit to the experimental data of isoamyl acetate production by *Pichia fermentans*.

Something similar was reported by Nielsen *et al*. (2003), who found that an organic solvent (hexadecane) improved the oxygen solubility in mixtures with water. They pointed out that the higher oxygen solubility in the organic solvent increments the mixture's oxygen absorption capacity.

Fig. 2 shows the response surface of a quadratic fit (r^2=0.9467) to the experimental data of isoamyl acetate production, which allows an overview of the results from the factorial design. Fig. 2 reveals that isoamyl acetate production enhanced when the airflow was increased from 325 to 465 mL min^{-1} (0.5 to 0.75 vvm) and the agitation from 150 to 170 rpm. However, subsequent increases in airflow and agitation speed resulted in sharp decreases in isoamyl acetate production. It means that excessive aeration negatively affected isoamyl acetate production.

The volumetric oxygen transfer coefficient (k_La) is the appropriate measure to determine the aeration efficiency, and bioreaction systems have been studied extensively, including the characterization of two (Nielsen *et al*., 2003) and three-phase (Quijano *et al*., 2020) abiotic systems. The k_La was determined under all aeration conditions.

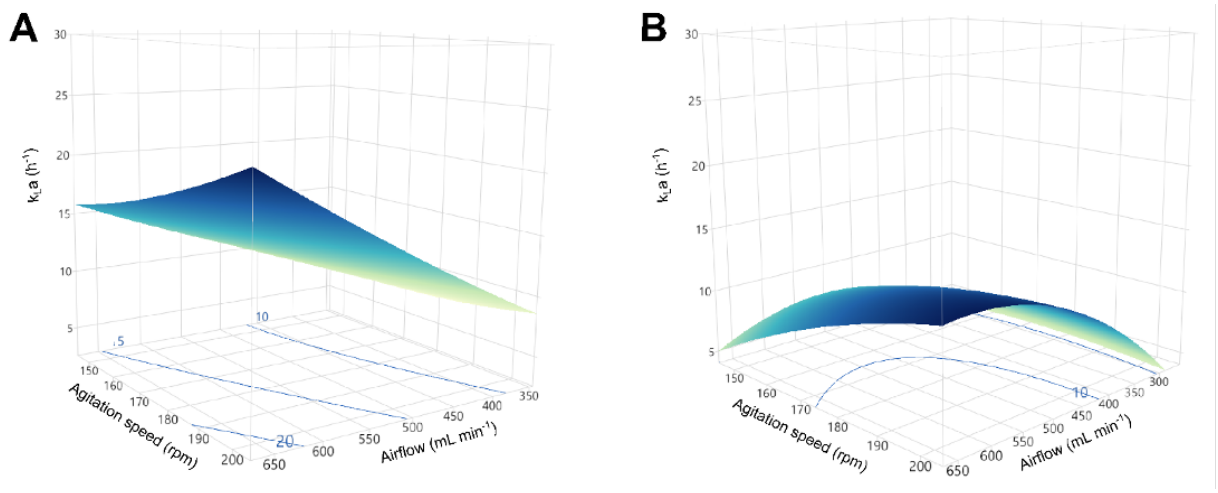

Fig. 3. Contour plots for the volumetric oxygen transfer coefficient (k_La) in the culture medium without (A) and with (B) added decane. Units of k_La are min^{-1}.

Fig. 3A presents the culture medium's k_La (quadratic fit, r^2=0.9181) without decane, ranging from 6.7 to 20.6 h^{-1}, while Fig. 3B shows that values (quadratic fit, r^2=0.8602) for the medium with decane were from 5.3 to 12.5 h^{-1}. This range for k_La values is similar to that reported (2.4 to 13.2 h^{-1}) for similar ranges of airflow (0.4 to 1.2 vvm) and agitation speed (150 to 250 rpm) by Palmerín-Carreño *et al*. (2019) using polypropylene glycol 1200 as the organic phase. As would be expected, Fig. 3A shows that k_La values increase with increasing stirring speed and airflow. Still, it is observed that the bioreactor used is slightly more dependent on airflow than on agitation speed. On the other hand, decane significantly diminished the k_La values since the drop in the k_La value due to decane presence was greater when agitation and aeration conditions were more intense. (Fig. 3B). Some works informed previously about decrements in k_La when organic solvents were added to a mineral medium (Dumont *et al*., 2006; Nielsen *et al*., 2003; Torres-Martínez *et al*., 2009; Jiménez-González *et al*., 2015). Jiménez-González *et al*. (2015) pointed out a controversy regarding immiscible organic solvents' effect on the k_La in triphasic systems (liquid-liquid-gas). They compared their results against other published, concluding that all solvents that diminish the k_La have dynamic and kinematic viscosities at least 3.5 times higher than those of water. In contrast, one solvent that improves the k_La (dodecane) has values close to those of the water. Nonetheless, data reported by Wohlfarth (2017) allows estimating that the decane's dynamic viscosity at 28 °C is 0.82 mPa s, while the kinematic viscosity is obtained by dividing the dynamic viscosity by the density (0.73 g mL^{-1}), resulting in 1.14 mm^2 s^{-1}. These values are comparable to those of the water. Thus, the results presented here contribute to the abovementioned controversy since decane reduced the k_La values from 22.1 to 13.2 h^{-1} (0.5 vvm - 150 rpm) and from 7.3 to 5.7 h^{-1} (1 vvm - 200 rpm).

The maximum production of isoamyl acetate is at the zone around 465 mL air min^{-1} (0.71 vvm), and 168 rpm (Fig. 2), where the k_La is near to 10.6 h^{-1} (Fig 3B), while the minimum production observed was at the conditions of the best aeration (k_La = 13.2 h^{-1}). Aeration has been used trying to improve aroma production by fermentation processes. Lehnert *et al*. (2008) used different oxygen supply conditions, based on the k_La determination, finding that oxygen supply was an influential tool to control the fermentation's degree and the flavor formation by immobilized *Saccharomyces cerevisiae*. *Proteus vulgaris* isolated from a Livarot cheese changed its aroma compound production from a sulphur/rotten egg note to a fruity/fresh and cheese notes by oxygen supply to the fermentation (Deetae *et al*., 2011).

It was previously concluded (Sanchez-Castañeda *et al*., 2018) that n-decane has good biocompatibility with the strain used and that the *in situ* production/extraction system employed exhibits a low loss of isoamyl acetate by stripping. The agitation and airflow used in the present work can be considered lightweight compared to the conditions used by other authors who used more intense conditions such as 200-800 rpm and 0.5-2 vvm (Nielsen *et al*., 2003); 100-500 rpm and 0.25 vvm (Jiménez-González *et al*.,

2015); and 300-900 rpm and 0.22-1.33 vvm (Aldric *et al*., 2009). The aqueous and organic phases were observed well differentiated at a macroscopic level, with a slight mixing zone between them (150 rpm and 0.5 vvm). As agitation and aeration increased, the mixing zone's thickness also increased, and small decane droplets were observed dispersed in the aqueous phase. Likewise, having obtained an increase of ~20% in C^* when decane was present may indicate a more intense mixing between the phases at the microscopic level. It means that yeast cells could also have more intense contact with n-decane, affecting yeast's metabolic behavior. Then, the effect of decane on the yeast and the loss of isoamyl acetate by stripping in a well-agitated system are two areas for further research.

Conclusions

This work demonstrated the relevance of well-controlled aeration to enhance the production of isoamyl acetate by *Pichia fermentans* using isoamyl alcohol as a precursor. Controlled aeration can only be reached by combining airflow and agitation in a bioreactor, which, operating near 0.71 vvm and 168 rpm, achieved production of 2.138 g L^{-1}. It is an almost 2.5-fold increase concerning the higher value previously reported. Isoamyl acetate production showed a maximum amount at moderate aeration conditions (k_La = 10.6 h^{-1}). The minimum production occurred at the best aeration conditions (k_La = 13.2 h^{-1}), meaning that excessive aeration could negatively affect the aroma production.

Nevertheless, the process is more complex than a simple bioconversion of isoamyl alcohol to isoamyl acetate since significant amounts of glycerol and ethanol are concomitantly produced. It appears that ethanol accumulation negatively affects aroma production. Still, decane's potential effect on yeast physiology and the loss of isoamyl acetate by stripping are also areas for further research.

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