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Isoamyl acetate production during continuous culture of *Pichia fermentans*

Producción de acetato de isoamilo durante el cultivo continuo de *Pichia fermentans*

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Abstract

The yeast *Pichia fermentans* ITD-00165 has demonstrated high potential to produce isoamyl acetate in batch culture. This work aimed to attempt isoamyl acetate production in continuous culture. A bioreactor containing a chemically defined medium was used to develop continuous cultures at three dilution rates (0.25, 0.30 y 0.35 h^{-1}). Isoamyl acetate concentration was measured using an entrapping system based on a bubbling column with n-decane. Results demonstrate that the entrapping system showed a linear correlation between the isoamyl acetate concentration into the culture medium and the absorption rate into the column. Airstream for oxygen supply was successfully used to continuously feed isoamyl alcohol to the culture to diminish the inhibitory effect of the precursor. Finally, it was demonstrated that the continuous culture growing cells assimilated the precursor to reach a steady-state isoamyl acetate production of 0.144 mg L^{-1}.

Keywords: Aroma compound, Isoamyl alcohol, Non-*Saccharomyces* yeasts, banana aroma, Bubbling column.

Resumen

La levadura *Pichia fermentans* ITD-00165 ha demostrado alto potencial para producir acetato de isoamilo en cultivo por lote. Este trabajo tuvo como objetivo intentar la producción de acetato de isoamilo en cultivo continuo. Se utilizó un biorreactor conteniendo un medio químicamente definido para desarrollar cultivos continuos a tres tasas de dilución (0.25, 0.30 y 0.35 h^{-1}) y se determinó la producción de acetato de isoamilo. Para determinar la concentración de acetato de isoamilo en estado estacionario, se implementó un sistema de atrapamiento en una columna de burbujeo con n-decano. Los resultados demostraron la factibilidad de cuantificar la producción de acetato de isoamilo de forma indirecta y que la columna de burbujeo presentó una correlación lineal entre la concentración de estado estacionario en el biorreactor y la tasa de absorción en la columna de burbujeo. Se suministró el precursor (alcohol isoamílico) de forma lenta y continua, aprovechando la corriente de aireación del biorreactor, con lo que se disminuye el efecto inhibitorio del precursor sobre la biomasa. Finalmente, se demostró que las células creciendo en cultivo continuo asimilaron el precursor para obtener una producción de acetato de isoamilo, en el estado estacionario, de 0.144 mg L^{-1}.

Palabras clave: Compuestos de aroma, Alcohol isoamílico, Levaduras non-*Saccharomyces*, aroma a plátano, Columna de burbujeo.

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1 Introduction

Isoamyl acetate (banana-like aroma) is an ester produced by chemical synthesis or by extraction from natural sources (Schrader *et al*., 2004; Carlquist *et al*., 2015). Microbial synthesis of this aroma compound involves the esterification of isoamyl alcohol and acetyl-CoA, which is mediated by alcohol acetyltransferase. (Fujii *et al*., 1994; Yoshimoto *et al*., 1998). Biotechnological synthesis of aroma compounds has increasing importance (Longo and Sanromán, 2006; Mikš-Krajnik *et al*., 2017; Paulino *et al*., 2021) since this is considered a natural method of production (FAO/WHO, 2017). Then, the demand for natural products is growing (Chreptowicz *et al*., 2016), which has increased the interest to study enzymes and microorganisms involved in developing biotechnological processes (Veríssimo *et al*., 2018; Paulino *et al*., 2021).

Aroma compounds such as higher alcohols, esters, and fatty acid derivatives, are obtained from yeasts (Dzialo *et al*., 2017). Thus far, yeast is a generic term referring to a wide variety of single-celled fungi (Nawaz *et al*., 2019). For example, *Pichia* is a yeast genus that produces acetate esters like 2-phenylethyl acetate and isoamyl acetate (Celińska *et al*., 2018). Particularly, *Williopsis saturnus* (Yilmaztekin *et al*., 2009) and *Pichia fermentans* (Sanchez-Castañeda *et al*., 2018; Rentería-Martínez *et al*., 2021) are two non-*Saccharomyces* yeasts with high potential to serve as a basis to develop a biotechnological process. *Williopsis saturnus* was selected as a good producer of isoamyl acetate using a culture medium based on sugar beet molasses (Yilmaztekin *et al*., 2008). Later, this selected strain was grown to produce isoamyl acetate (Yilmaztekin *et al*., 2009), reporting a basal production of 118 mg L^{-1}. Then, they used fuel oil as a source of isoamyl alcohol to be biotransformed into isoamyl acetate, reaching a final production of 354 mg L^{-1}.

In contrast, our workgroup isolated and identified a strain of *Pichia fermentans* (Páez-Lerma *et al*., 2013) which was selected as a prominent candidate to develop a process for isoamyl acetate production (Hernández-Carbajal *et al*., 2013). Subsequent work with this strain demonstrated that isoamyl acetate production increased from 70 to 860 mg L^{-1} using isoamyl alcohol precursor (Rentería-Martínez *et al*., 2016), while biotransformation of leucine permitted to produce 624 mg L^{-1} (Sanchez-Castañeda *et al*., 2018). In addition, it recently produced 2138 mg of isoamyl acetate per liter using a well-aerated batch bioreactor and isoamyl alcohol as a precursor (Rentería-Martínez *et al*., 2021).

On the other hand, continuous culture permits the simultaneous adjustment of thousands of metabolic reactions allowing cell viability at a well-defined growth rate which the experimenter imposes (Thierie, 2016). Then, continuous culture is a powerful experimental tool used to study the production of small molecules, recombinant proteins, as well as to assess the growth rate or metabolism of microorganisms and cultured cells, and for studying evolution (Ong *et al*., 2021). Moreover, continuous culturing systems have been considered a tool for in vivo evolution experiments searching to obtain strains with novel capacities to produce chemicals (Tan *et al*., 2019). Continuous culture has been widely used to study yeasts, mainly *Saccharomyces cerevisiae*. A chaotic multioscillatory metabolic attractor was observed in continuous cultures of *S. cerevisiae* (Roussel and Lloyd, 2007). Chemostat and retentostat cultures of *S. cerevisiae* were used to study the physiological responses to slow growth, low pH, and high CO_2 levels (Hakkaart *et al*., 2020). *Saccharomyces cerevisiae* was grown in continuous culture, finding that ethyl esters production may be correlated with differential expression of the genes involved in aroma pathways (Iñiguez-Muñoz *et al*., 2019).

Co-cultures of *S. cerevisiae* and non-*Saccharomyces* yeasts have also been studied through continuous culture. For example, *Issatchenkia orientalis* was used to enrich continuous cultures of *S. cerevisiae* at different temperatures, allowing the fermentation process beyond the *S. cerevisiae* tolerance temperature limits (Gallardo *et al*., 2011). Accelerostat technique (A-stat) allowed studying the ethanol production from whey by *Kluyveromyces marxianus* (Gabardo *et al*., 2015). Maintenance coefficient and specific product yield parameters were estimated using continuous culture of *Pichia pastoris*, which were then used to design a feeding strategy for culturing the yeast in a fed-batch process (Gautam *et al*., 2021).

Aroma compounds production has also been studied using continuous cultures. *Lactococcus lactis* and *Leuconostoc mesenteroides* were grown in mono and co-cultures using a retentostat to study their role in cheese aroma compounds production (van Mastrigt *et al*., 2019). The yeast *Metschnikowia pulcherrima* produced 2-phenylethanol (650 mg L^{-1}) in continuous culture (Chantasuban *et al*., 2018).

Continuous cultivation allowed to demonstrate that *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Wickerhamomyces anomalus* synthesize ethyl acetate in the mitochondria (Kruis *et al*., 2021).

Considering that there are no previous reports, this work's objective was to attempt isoamyl acetate production by the native yeast *Pichia fermentans* ITD-00165 in continuous culture.

2 Materials and methods

2.1 Yeast strain

The strain *Pichia fermentans* ITD-00165 was used. This yeast was one of the strains isolated from the spontaneous alcoholic fermentation of *Agave duranguensis* (Páez-Lerma *et al*., 2013). It was provided by the Microbial Biotechnology Lab's Culture Collection at the Instituto Tecnológico de Durango.

2.2 Culture medium

The yeast was cultured in a minimal medium reported previously (Flores-Cosío *et al*., 2019; Rentería-Martínez *et al*., 2021). Succinctly, one liter of medium contained: salt solution (960 mL), glucose solution (28.5 mL), vitamin solution (2.5 mL), and trace element solution (1 mL).

Salt solution ($Na_2HPO_4{\cdot}2H_2O$, 1.49 g L^{-1}; K_2HPO_4, 3 g L^{-1}; $(NH_4)_2SO_4$, 3 g L^{-1}; glutamic acid, 1 g L^{-1}) and Glucose solution (700 g L^{-1}) were sterilized separately in an autoclave at 121°C for 15 minutes. The pH of both solutions was adjusted to 5 and, after sterilization, solutions were stored at room temperature until use.

Vitamin solution (aminobenzoic acid, 1 mg L^{-1}; myoinositol, 125 mg L^{-1}; nicotinic acid, 5 mg L^{-1}; pantothenic acid, 5 mg L^{-1}; pyridoxine, 5 mg L^{-1}; thiamine HCl, 5 mg L^{-1}; and biotin, 0.012 mg L^{-1}) and trace element solution ($MgCl_2{\cdot}6H_2O$, 410 mg L^{-1}; $ZnCl_2$, 19.2 mg L^{-1}; $CuCl_2{\cdot}2H_2O$, 0.61 mg L^{-1}; $MnCl_2{\cdot}4H_2O$, 4.45 mg L^{-1}; $CoCl_2{\cdot}6H_2O$, 0.5 mg L^{-1}; $CaCl_2$, 17.37 mg L^{-1}; $FeCl_2{\cdot}4H_2O$, 11.66 mg L^{-1}; $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 0.36 mg L^{-1}; H_3BO_3, 3 mg L^{-1}) were sterilized by filtration through a sterile Nylon membrane (0.20 μm). The pH was adjusted to 5 in both cases. After sterilization, vitamin solution was stored at 4°C, while trace element solution was stored at room temperature until use.

2.3 Recovery and quantification of the isoamyl acetate produced

Isoamyl acetate concentration into the culture medium placed into the bioreactor was measured using an indirect method based on the absorption rate of isoamyl acetate into n-decane. The bioreactor vessel contained 400 mL of culture medium with a specific isoamyl acetate concentration, while the same medium was fed at a rate of 40 mL min^{-1}. The concentrations of isoamyl acetate in the culture medium (C_{IAM}) were 50, 100, 150, 200, and 250 mg L^{-1} to simulate different steady-state concentrations of isoamyl acetate. These experiments were performed in triplicate.

An air stream of 400 mL min^{-1} (1 vvm) was also fed to the bioreactor. Isoamyl acetate has low solubility in water, so it is easily carried away by the airstream. Once it left the bioreactor, the airstream was bubbled into a glass column (2.5 cm diameter × 35 cm high) containing 60 mL of n-decane. Airstream passed through a sintered glass piece to obtain small-size bubbles. Isoamyl acetate is 65 times more soluble in decane than in water (Sánchez-Castañeda *et al*., 2018), so the decane column works like a trap. Fig. 1 shows a diagram of the flow system. Samples of one milliliter were taken from the bubble column every 10 minutes up to 60 min. Samples were analyzed by duplicate by gas chromatography. Isoamyl acetate concentrations in n-decane (C_{IAD}) were plotted against the time, and the least-squares fits were performed (Montgomery *et al*., 2012) to obtain the

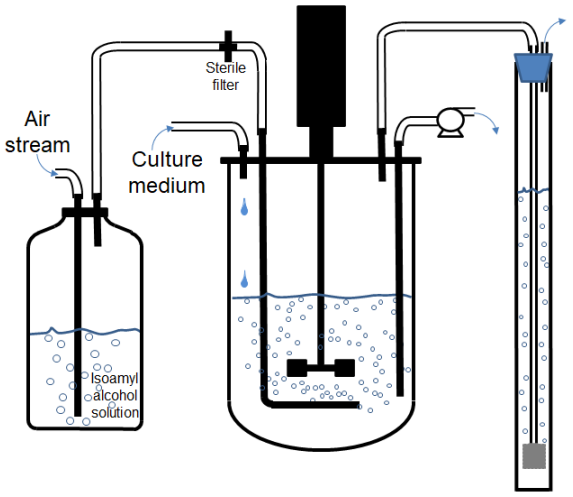

Fig. 1. Bioreaction system for *Pichia fermentans* continuous culture showing the bubbling column containing n-decane (60 mL) to absorb isoamyl acetate from the airstream. The continuous isoamyl alcohol

feeding through the air stream system is also shown.

parameters of the equation:

$$C_{IAD} = \beta_0 + \beta_1 \cdot t \quad (1)$$

Where

C_{IAD} is the isoamyl acetate concentration in n-decane (mg L^{-1}).

t is the time (min).

β_0 is the C_{IAD} at $t = 0$ (mg L^{-1}).

β_1 is the absorption rate of isoamyl acetate into the n-decane (mg L^{-1} min^{-1}).

Least-squares fits included the hypothesis for testing the significance of the slope and intercept. The absorption rate was calculated for each C_{IAM} by triplicate. Finally, absorption rates were plotted against the isoamyl acetate concentrations in the culture medium (C_{IAM}), and a least-squares fit was performed to obtain a standard curve.

The data collected also allowed calculating the overall volumetric mass transfer coefficient (k_Da) of isoamyl acetate from the airstream to decane. It was done using Equation 2.

$$\ln\left(\frac{C^* - C_{IAD}}{C^*}\right) = -k_Da \cdot t \quad (2)$$

Where

C^* is the interfacial concentration of isoamyl acetate at equilibrium, i.e. the solubility of the isoamyl acetate in n-decane (mg L^{-1}).

C_{IAD} is isoamyl acetate concentration in n-decane at time t (mg L^{-1}).

k_Da is the overall volumetric mass transfer coefficient (min^{-1}).

t is the time (min).

The k_Da value was obtained from the slope of a plot of $\ln((C^* - C_{IAD})/C^*)$ against t.

The C_{IAD} was always assumed homogeneous through the decane liquid phase at any given time. It is due to the continuous stirring promoted by airstream flow and the low viscosity of decane (Álvarez *et al.*, 2000).

2.4 Continuous culture

Fermentations were performed with 400 mL of culture medium into the 1 L glass vessel of an Applikon bioreactor (model EZ Control). This bioreactor is equipped with one motor connected to a Rushton impeller, three vertical baffles plates, one air sparger, and one dissolved oxygen sensor, as described previously (Rentería-Martínez *et al.*, 2021).

The bioreactor and the fresh medium bottle (8 L), containing the appropriate volumes of salt solution, were autoclaved. The other nutrient solutions were sterilized as above mentioned and added to the bioreactor and fresh medium bottle, under aseptic conditions, at room temperature. The inoculum was incubated overnight and used to reach a biomass concentration of 14×10^7 cells mL^{-1} at the beginning of the fermentation. It was operated at 120 rpm, 28 °C, and pH of 5. Oxygen was supplied using an airstream of 400 mL min^{-1} (1 vvm) filtered through a sterile Nylon membrane (0.45 μm) previously to be bubbled into the culture medium. Cultures started in batch mode to switch on the flow of fresh medium at six hours.

Continuous cultures were carried out at dilution rates (D) of 0.25, 0.30, and 0.35 h^{-1}. Samples were harvested from the bioreactor outlet flow using collection tubes every three hours to perform analytical determinations. After reaching a first steady state, the isoamyl alcohol feeding was started through the airstream until attaining a second steady state. For this, before entering the bioreactor, airstream was bubbled into a 450 mL glass flask containing 200 mL of an isoamyl alcohol solution (5 g L^{-1}). Isoamyl alcohol (98%) was added to this flask (500 μL every 3 hours) to compensate for the loss of isoamyl alcohol due to the airstream flow. Cell-free experiments were performed by triplicate to determine the accumulation rates of isoamyl alcohol in the culture medium at the three dilution rates used.

Specific aroma production rates were calculated using Equation 3:

$$q_P = \left(\frac{C_X}{C_{IAM}}\right) * D \quad (3)$$

Where:

q_p: specific isoamyl acetate production rate (mgIA gX^{-1} h^{-1}).

C_X: Steady-state biomass concentration (gX L^{-1}).

C_{IAM}: Steady-state isoamyl acetate concentration (mgIA L^{-1}).

D: Dilution rate (h^{-1}).

2.5 Analytical techniques

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

Glucose, acetic acid, 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 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^{-1} 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

Triplicate independent assays were made for developing the method for measuring the isoamyl acetate concentration in the culture medium. Then, linear regressions were performed by least-squares fits, including the hypothesis testing the significance of the slope and intercept. Next, accumulation rates of isoamyl alcohol in the culture medium were determined using a similar strategy. On the other hand, duplicate independent fermentations were carried out for all experimental conditions. Finally, steady-state concentrations, without and with isoamyl alcohol addition, were compared through the one-way analysis of variance (ANOVA) and the Tukey's test. Minitab® 20.4 program was used to carry out all least-squares fit and statistical analysis.

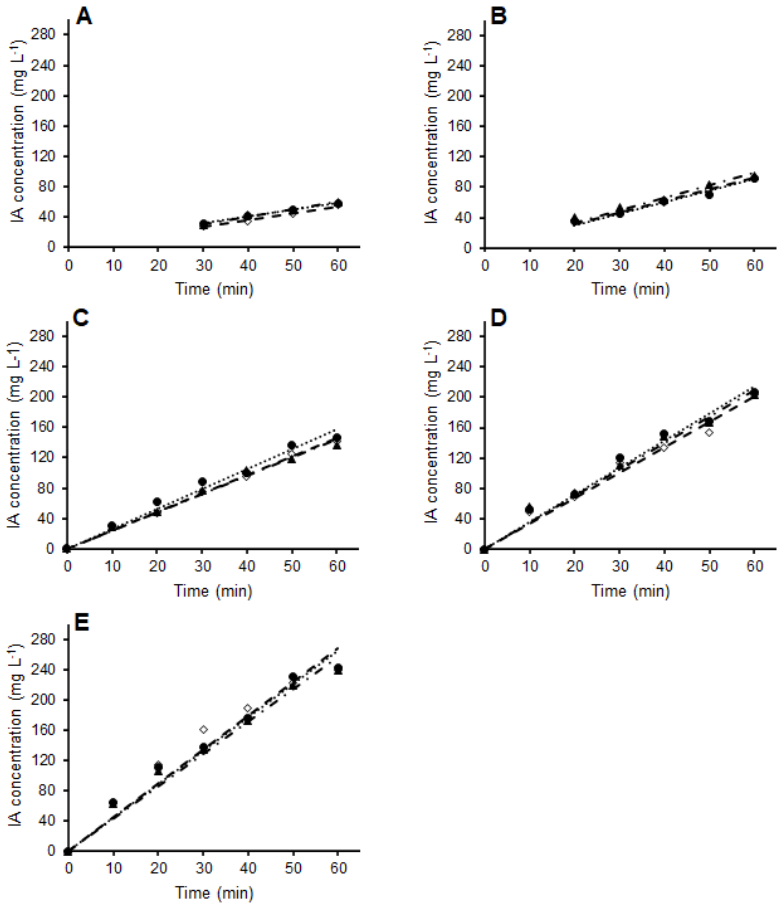

Fig. 2. Isoamyl acetate concentration absorbed into n-decane against the bubble time in the n-decane bubbling column. Culture medium contained isoamyl acetate at constant concentrations of 50 (A), 100 (B), 150 (C), 200 (D), and 250 mg L^{-1} (E). All the graphs show the results of the absorption kinetics performed by triplicate.

3 Results and discussion

3.1 Measurement of isoamyl acetate production

Previous research of our workgroup used a simultaneous fermentation-extraction, placing a layer of decane in direct contact with the culture medium to trap the aroma (Rentería-Martínez *et al*., 2016; Sánchez-Castañeda *et al*., 2018; Rentería-Martínez *et al*., 2021). However, some results indicate that decane could be harming cells (Rentería-Martínez *et al*., 2021). So then, it was decided to avoid direct contact between the decane and the culture medium and try to trap the aroma with the decane in a bubble column. Gas-liquid absorption allows separating some compounds from a gas mixture using an appropriate solvent to reach physical or chemical absorption (Nakao *et al*., 2019; Pakzad *et al*., 2020). The most frequent application of gas-liquid absorption is to remove contaminants, mainly carbon dioxide. However, other examples are separating hydrocarbons by hydrocarbon oil and recovering alcohol vapors (Nakao *et al*., 2019).

Graphs in Fig. 2 show the isoamyl acetate concentration against the bubble time in the n-decane column. Fig. 2A does not show 0-, 10-, and 20-min values since these samples' isoamyl acetate chromatographic peak was not detected. A similar situation is observed in Fig. 2B but only for times 0 and 10 min. It means that the accumulated isoamyl acetate concentration into n-decane was below the instrument's detection limit. Still, after 30 (Fig. 2A) and 20 min (Fig. 2B), the absorption rate of isoamyl acetate into n-decane showed a linear response in

both cases. Similar to that observed in the present work, a linear behavior was previously observed for the CO_2 absorption kinetics in aqueous solutions of 1-dimethylamino-2-propanol 1-dimethylamino-2-propanol, and monoethanolamine (Liu *et al*., 2017).

Results of the least-squares fits included the hypothesis testing for the linear model parameters. It demonstrated statistical evidence ($p<0.05$) that the accumulation of isoamyl acetate is linearly related to the bubble time in the n-decane column. Moreover, the hypothesis tests showed that only the slope value is statistically significant ($p<0.05$). Then, all the least-squares lines have an intercept equal to zero for all isoamyl acetate concentrations assayed. Figures 2A to 2E show three linear absorption kinetics each, all with high repeatability at every isoamyl acetate concentration, with determination coefficient (R^2) values in the range of 0.9858 to 0.9990.

The slopes (isoamyl acetate absorption rates) ranged from 0.908 to 4.484 mg L^{-1} min^{-1}. Then, they were plotted against the isoamyl acetate concentration in the culture medium, finding a linear response when the aroma concentration in the culture medium varies from 50 to 250 mg L^{-1}. Finally, the least-squares fit has p-values that demonstrated strong evidence of linear relation between isoamyl acetate absorption rate and the aroma concentration in the culture medium, but also that intercept was not significant ($p<0.05$). Thus, the final linear regression was:

$$Ab = 0.017 * C_{IAM} \tag{4}$$

Where:

Ab: Absorption rate of isoamyl acetate into n-decane (mg L^{-1} min^{-1}).

C_{IAM}: Isoamyl acetate concentration into culture medium (mg L^{-1}).

This equation has good linearity (R^2=0.9979) into the range of isoamyl acetate concentrations assayed, demonstrating that the indirect method proposed here works to adequately measure isoamyl acetate concentration into the culture medium (Montgomery *et al*., 2012).

On the other hand, the linear fits of the graphs of $\ln((C^* - C_I AD)/C^*)$ against time (data not shown) had non-significant intercepts ($p > 0.05$) and slopes ($k_D a$) in the range of 0.67×10^{-5} to 3.32×10^{-5} min^{-1}. The coefficients of determination of these adjustments ranged from 0.9912 to 0.9991. The $k_D a$ values thus determined were plotted against the isoamyl acetate concentration in the culture medium. It generated a straight line with an R^2 of 0.9978. Then, the $k_D a$ calculations yielded results confirming the linearity of the phenomenon of isoamyl acetate's absorption in decane.

Thus, Equation 2 was used as a standard curve which is graphically shown in Fig. 3. This figure illustrates the confidence interval (95%) and the linear regression's prediction interval (95%). For example, using these data, it is possible to calculate that a measured absorption rate of 2.5 mg L^{-1} min^{-1} corresponds to an isoamyl acetate concentration in the culture medium of 144.8 mg L^{-1}. Therefore, the confidence interval implies a 95% confidence that this value will be between 141 and 148.6 mg L^{-1}. Additionally, the prediction interval indicates that the value will be in the range of 127.7 to 161.9 mg L^{-1} with 95% confidence.

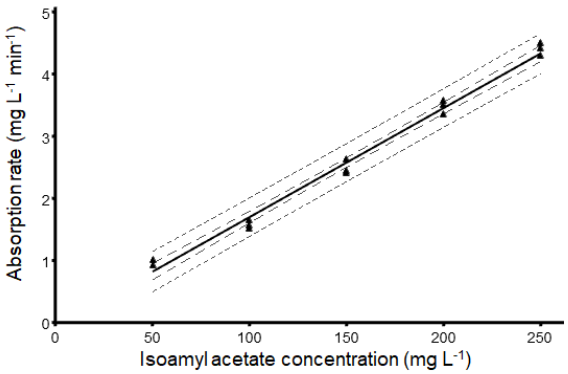

Fig. 3. Graph of the standard curve between isoamyl acetate concentration in the culture medium against the absorption rate into the bubbling column. The graph shows the confidence interval (95%) and the linear regression's prediction interval (95%).

It must be pointed out that results presented here should be considered valid only for the particular conditions employed in the present work (reactor vessel dimensions, medium composition, airflow, absorption column dimensions, and solvent volume). Nevertheless, a similar strategy can be applied for similar cases of volatile molecules easily carried away by air or another gas stream. The bubbling column entrapped the aroma only for measurement purposes in the present work. However, a similar device could be used for aroma recovery, probably employing a multiphase bubbling column as proposed previously to enhance oxygen transfer from the gas phase to the liquid phase (Quijano *et al*., 2020).

3.2 Continuous culture and isoamyl acetate production

Experiments performed to measure the isoamyl alcohol accumulation in the culture medium showed linear responses. The accumulation rates were calculated as the slopes of the linear regressions, with R^2 values ranging from 0.9906 to 0.9927. It yielded non-significant intercepts ($p<0.05$) and accumulation rates of 0.078±0.006, 0.076±0.004, and 0.075±0.011 gIsoamyl alcohol L^{-1} h^{-1} for dilution rates of 0.25, 0.3, and 0.35 h^{-1}, respectively. Differences among the accumulation rates were not significant ($p<0.05$). The maximum isoamyl alcohol concentration reached was around 0.22 g L^{-1} in all the cases. It was obtained between 4.5 and 5 h of operation of the cell-free experiments and remained constant until the end of the assays (6 h). Moreover, the addition of 500 mL of isoamyl alcohol (98%) at three hours did not produce any change in the accumulation of the precursor. Then, the accumulation of isoamyl alcohol depends on the mass transfer from the gas phase to the liquid phase but not on the continuous culture dilution rate.

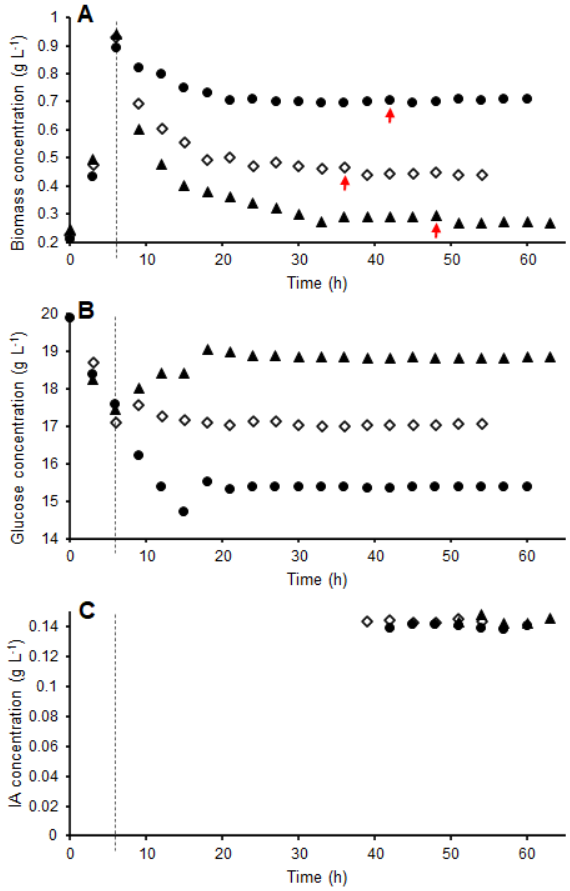

Fig. 4. Biomass (A), glucose (B), and isoamyl acetate (C) concentrations kinetics during the continuous cultures of *Pichia fermentans* ITD-00165 at dilution rates of 0.25 (•), 0.30 (◊), and 0.35 h^{-1} (▲). The red arrows in the biomass concentration kinetics indicate when isoamyl alcohol addition started.

Results obtained during continuous culture are displayed in Fig. 4, which shows the kinetics of growth (Fig. 4A), glucose consumption (Fig. 4B), and isoamyl acetate production (Fig. 4C). As recommended in the literature (Calcott, 1981; Thierie, 2016), all the fermentations operated in batch culture during the first six hours to reach an adequate biomass concentration. Then, the appropriate medium flow rate started at six hours, indicated in the graphs by a dashed vertical line. Thus, the first stage of continuous culture proceeded until it reached a steady state without adding any precursor for isoamyl acetate production. Subsequently, a second continuous culture stage was performed, adding isoamyl alcohol, the adequate precursor for isoamyl acetate production, as demonstrated previously (Rentería-Martínez *et al.*, 2021). Again, steady-states were obtained for all the experimental conditions assayed. Therefore, all fermentations were performed twice with essentially the same results described below.

Fig. 4A exhibits the yeast growth, with an average concentration of 0.92±0.03 g L^{-1} during batch culture, considering the three fermentations performed. Biomass concentrations in Fig. 4A show constant values from 24 to 42 h, from 24 to 36 h, and from 36 to 48 h, which defined the first steady-state for the dilution rates of 0.25, 0.3, and 0.35 h^{-1}, respectively. Thus, isoamyl alcohol feeding started at 36 (D=0.3 h^{-1}), 42 (D=0.25 h^{-1}), and 48 h (D=0.35 h^{-1}), which is indicated by red arrows in Fig. 4A. It was expected a negative effect of isoamyl alcohol on biomass concentration since a toxic effect has been reported against *Saccharomyces cerevisiae* (Martínez-Anaya *et al.*, 2003), *Williopsis saturnus* (Yilmaztekin *et al.*, 2009), and *Pichia fermentans* (Rentería-Martínez *et al.*, 2021). Still, biomass concentration obtained during the second stage at D=0.25 h^{-1} did not change concerning the previous stage, concluding the fermentation at 60 h. A slight but precise diminishing of biomass concentration can be seen in Fig 4A for the dilution rates of 0.30 and 0.35 h^{-1}. It could be attributed to the abovementioned inhibitory effect of the isoamyl alcohol on the biomass.

Rentería-Martínez *et al.* (2021) reported a 65% decrease in biomass concentration, due to an isoamyl alcohol concentration of 0.5 g L^{-1}. As already mentioned, a maximum concentration of isoamyl

alcohol in the culture medium was close to 0.22 g L^{-1} after about five hours of supply to a cell-free system. During continuous cultivation, the biomass assimilates the available isoamyl alcohol, and the effective concentration faced by the biomass must be substantially less than 0.22 g L^{-1}. It must depend on the biomass concentration present to assimilate the precursor. There is no decrease in biomass concentration at D=0.25 h^{-1}. However, decreases of 6.5 (D=0.3 h^{-1}) and 7.2% (D=0.35 h^{-1}) were observed. Thus, it is reasonable to argue that the variation in the impact of isoamyl alcohol is due to the biomass concentration present at each dilution rate rather than a difference in cell resistance.

Data in Fig. 4B show the results for glucose consumption kinetics. Average glucose consumption of 2.6±0.3 g L^{-1} during batch culture was observed, which means a yield of biomass respect of substrate ($Y_{X/S}$) of 0.35 gX gS^{-1}. This yield is low concerning that reported previously for our strain (0.85 gX gS^{-1}) growing on a molasses base medium (Sánchez-Castañeda *et al*., 2018). *Saccharomyces boulardii* showed a yield of 0.458 gX gS^{-1} growing in glucose (González-Figueredo *et al*., 2020). *Kluyveromyces marxianus* yielded 0.13 gX gS^{-1} growing in glucose (Cordero-Soto *et al*., 2021). Nonetheless, similar yields (0.30-0.35 gX gS^{-1}), as obtained here, were reported for four strains of *Pichia pastoris* growth in a minimal medium with glucose as a carbon source (Heyland *et al*., 2011). A yield of 0.65 gX gS^{-1} was reported for *Pichia fermentans* grown in media with glucose (20 g L^{-1}) as a carbon source, supplemented with yeast extract and peptone (Taccari *et al*., 2012). The steady-state glucose concentration was not affected by isoamyl alcohol, as was the case with the biomass concentration. Nonetheless, glucose consumption diminished when the dilution rate increased.

Fig. 4C reveals that isoamyl acetate was not detected during batch cultivation or the first stage of the continuous cultures. *Pichia fermentans* ITD-00165 produced banana aroma in batch culture only after 12 h of incubation without precursor (Sánchez-Castañeda *et al*., 2018). Rentería-Martínez *et al*. (2021) reported an isoamyl acetate basal production (without precursor addition) of 0.07 g L^{-1} in batch culture, using the same strain and culture medium employed here. Those results are according to the result obtained here during batch culture. The low basal production in batch culture must be considered to explain the lack of aroma detection during the continuous culture without precursor. Moreover, the high volatility of isoamyl acetate must also be considered, which means a rapid mass transfer from the liquid phase to the gas phase. Once isoamyl alcohol feeding started, the isoamyl acetate production was measurable at the three dilution rates assayed. It demonstrates that it is possible to use the oxygen supply to supplement isoamyl alcohol in the airstream. This strategy made it possible to provide a small supply of the precursor making the precursor available to produce isoamyl acetate. Isoamyl alcohol availability has been reported to enhance isoamyl acetate production (Quilter *et al*., 2003; Yilmaztekin *et al*., 2009; Rentería-Martínez *et al*., 2021). To our knowledge, this is the first work where isoamyl acetate is trying to produce in continuous culture. Moreover, it is also the first where the precursor continuously feeds into the gas phase avoiding or reducing the abovementioned toxic effect of the isoamyl alcohol. Production of some other aromas has been investigated using continuous culture. For example, phenylalanine has been used as a precursor to produce 2-phenyl ethanol, reporting productions in continuous culture of 2 (Wang *et al*., 2011), 0.6 (Chantasuban *et al*., 2018), and 1.15 g L^{-1} (Drężek *et al*., 2021) On the other hand, Kruis *et al*. (2019) report an ethyl acetate production of 11 g L^{-1}.

Complete continuous culture results are shown in Table 1. It shows that a decrease in glucose consumption was observed as the dilution rate increased. The highest glucose consumption occurred at D=0.25 h^{-1}, which diminished an average of 34 and 73% at dilutions rates of 0.30 and 0.35 h^{-1}, respectively. Glucose consumption decrements were significant ($p<0.05$) from one dilution rate to the other in both culture medium flow changes. Nevertheless, the presence of the precursor did not affect ($p<0.05$) the glucose consumption at each dilution rate.

About yeast growth, like that observed for glucose consumption, the highest growth was at D=0.25 h^{-1}, diminishing as the dilution rate increased. The decreases were significant from one dilution rate to the other for the two culture medium flow changes. Nonetheless, $Y_{X/S}$ presented the inverse pattern since the highest yield was at D=0.35 h^{-1} without precursor. Conversely, yields obtained at D=0.25 h^{-1} were near 35% lower than the highest yield. It should be noted that the presence of precursor did not significantly affect ($p<0.05$) the biomass concentration at D=0.25 h^{-1}.

Ethanol, acetic acid, and glycerol are commonly produced by yeasts still when oxygen is present in the culture medium (Oura, 1977). Yeast ferment sugar to ethanol, glycerol, and compounds derived from

pyruvate such as acetaldehyde, acetic acid, acetoin, succinic acid, among others (Ciani *et al*., 2008; Dzialo *et al*., 2017). Table 1 shows the steady-state concentrations of these compounds obtained at the different dilution rates assayed. Ethanol was the most abundant by-product showing the highest production at D=0.25 h^{-1}, while the lowest production occurred at the dilution rate of 0.30 h^{-1}. Acetic acid and glycerol showed very similar production patterns, which were affected only by the dilution rate, but not by the precursor's presence.

Table 1. Steady-state fermentation parameters during continuous culture of *Pichia fermentans* ITD-00165 at different dilution rates (D).

Consumption or production	**D = 0.25 h^{-1}**		**D = 0.30 h^{-1}**		**D = 0.35 h^{-1}**	
	Without precursor	**With precursor**	**Without precursor**	**With precursor**	**Without precursor**	**With precursor**
Glucose (g L^{-1})	4.524±0.020^{a}	4.519±0.008^{a}	2.966±0.069^{b}	2.985±0.017^{b}	1.217±0.011^{c}	1.219±0.006^{c}
Biomass (g L^{-1})	0.700±0.004^{a}	0.705±0.005^{a}	0.506±0.007^{b}	0.473±0.003^{c}	0.291±0.002^{d}	0.270±0.003^{e}
Ethanol (g L^{-1})	0.733±0.026^{a}	0.751±0.012^{a}	0.240±0.013^{d}	0.227±0.008^{d}	0.665±0.028^{b}	0.624±0.021^{c}
Acetic acid (g L^{-1})	0.263±0.001^{a}	0.265±0.001^{a}	0.245±0.001^{b}	0.252±0.003^{b}	0.258±0.001^{c}	0.258±0.001^{c}
Glycerol (g L^{-1})	0.205±0.007^{a}	0.210±0.003^{a}	0.137±0.026^{b}	0.138±0.036^{b}	0.165±0.007^{b}	0.163±0.011^{b}
Isoamyl acetate (g L^{-1})	ND	0.140b±0.001	ND	0.143±0.001^{a}	ND	0.144±0.003^{a}
Biomass yield (gX gGlucose^{-1} h^{-1})	0.155±0.0002^{d}	0.156±0.001^{d}	0.169±0.009^{c}	0.158±0.001^{d}	0.239±0.002^{a}	0.222±0.003^{b}
Specific Isoamyl acetate (IA) production rate (mgIA gX^{-1} h^{-1})	ND	0.050^{c}±0.001	ND	0.091^{b}±0.001	ND	0.187^{a}±0.005

^{a,b,c,d,e}Different superscripts indicate significant difference (p<0.05) between values in the same row.
ND = Not detected.

Acetic acid production is considered stimulated by the increase of NAD^{+} obtained through glycerol production (Eglinton *et al*., 2002). Glycerol production was highest also at the dilution rate of 0.25 h^{-1}, diminishing with the other dilution rates. Production at D=0.30 h^{-1} was numerically the lowest but without significant difference (p<0.05) concerning that obtained at D=0.35 h^{-1}. Glycerol production is generally associated with NAD^{+} regeneration. Acetic acid production is attributed to low pyruvate carboxylase activity or high aldehyde dehydrogenase activity (Ciani *et al*., 2008).

Finally, aroma production is also shown in Table 1. Isoamyl acetate production was detected only when the precursor was added to the culture medium. Nevertheless, aroma production was expected even without precursor addition since it has been observed in the batch culture of the strain used (Sánchez-Castañeda *et al*., 2018; Rentería-Martínez *et al*., 2021). Steady-state aroma concentration showed a tiny increase (2.5%) when the dilution rate grew from 0.25 to 0.30 and 0.35 h^{-1}. Nevertheless, there were significant (p<0.05) differences between dilution rates when specific aroma production rates (q_P) were calculated.

Table 1 shows significant (p<0.05) q_P increases of 82 (D=0.30 h^{-1}) and 274% (D=0.35 h^{-1}) respect the productivity obtained at 0.25 h^{-1} which occurred despite the decreasing concentration of biomass formed at these dilution rates. It means that the yeast production capacity increases as the specific growth rate also rises. Aroma production could have been limited by the precursor's availability, as reported previously (Calderbank and Hammond, 1994). Isoamyl alcohol was not detected in any sample analyzed by liquid or gas chromatography, implying that the biomass assimilated the total amount of the precursor that reached the culture medium. The isoamyl alcohol source was the solution from which it was carried away by the air stream to feed the bioreactor. Thus, it can be assumed that the amount of precursor that the airstream could carry away was minimal. Then, it is likely that a higher concentration of isoamyl alcohol is required to improve delivery to the bioreactor. Indeed, various concentrations of isoamyl alcohol must be used to characterize the mass transfer from the liquid phase to the air stream and the mass transfer from the air stream to the culture medium in the bioreactor.

Conclusions

It is possible to develop an indirect method for measuring a volatile compound using a liquid trap. In the case of isoamyl acetate, a bubbling column containing decane allowed to absorb this volatile compound efficiently, showing a linear correlation with the aroma concentration in the bioreactor. The standard curve thus defined is particular for the operating conditions here, but a similar strategy can be applied for similar cases.

On the other hand, it was demonstrated the feasibility of taking advantage of the volatility of isoamyl alcohol to continue supplying it as a precursor to producing isoamyl acetate. Continuous providing of isoamyl alcohol diminished their toxic effect on *Pichia fermentans* cells and allowed the biosynthesis of isoamyl acetate.

Collectively, the results of this work demonstrated that the basal production of isoamyl acetate by *Pichia fermentans* is undetectable in continuous culture. However, the yeast can assimilate a continuous supply of the precursor to obtain an appreciable aroma production. Moreover, the yeast production capacity appears to be associated with the specific growth rate.

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