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Respiro-fermentative metabolism in yeast cultivated in solid-state culture: The Crabtree effect and ethanol production

Metabolismo respirofermentativo en levaduras cultivadas en estado sólido: El efecto Crabtree y la producción de etanol

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Abstract
A strong inoculum in solid-state culture (SSC) is achieved by using a high-density, high-activity microbial population. To determine the best conditions for yeast propagation, the Crabtree effect was analyzed. Inoculum production was carried out in Erlenmeyer flasks with different designs (baffled, coiled and conventional) and filling volumes (20 and 40 %). A 20% filling volume resulted in better air diffusion, leading to improved yeast growth independently of the flask configuration. Yeasts have adapted to produce ethanol under aerobic conditions to compete with other microorganisms. With commercial baker's yeast, the highest ethanol production was achieved at the laboratory scale (195.70±17.25 g ethanol/kg dry matter [DM]). There was no difference in ethanol production between the tubular reactor (171.10±10.87 g ethanol/kg DM) and in the pilot batch bioreactor (165.03±9.90 g ethanol/kg DM) using *S. cerevisiae* yeast ITD00196 as inoculum. Crabtree-positive yeasts have potential for rapid ethanol production in organic waste fermentation without thermochemical or enzymatic pre-treatment by SSC in the laboratory and at pilot-scale.
Keywords: Respiro-fermentative metabolism; Crabtree effect; solid-state culture; ethanol production.

Resumen
Un inóculo fuerte en el cultivo en estado sólido (SSC) se consigue utilizando una población microbiana de alta densidad y gran actividad. Para determinar las mejores condiciones de propagación de las levaduras se analizó el efecto Crabtree. La producción de inóculo se llevó a cabo en matraces Erlenmeyer con diferentes diseños (bafleados, resorte y convencionales) y volúmenes de llenado (20 y 40 %). Un volumen de llenado del 20% condujo a una mejor difusión del aire, al crecimiento de las levaduras y permitió una mejor producción de inóculo independientemente de la configuración del matraz. Las levaduras se han adaptado para producir etanol en condiciones aerobias para competir con otros microorganismos. Con la levadura de panadería comercial se alcanzó la mayor producción de etanol (195.70±17.25 g/kg de materia seca (DM)) a escala de laboratorio. No hay diferencia entre la producción de etanol en el reactor tubular (171.10±10.87 g/kg de DM) y en el biorreactor batch piloto (165.03±9.90 g/kg de DM) utilizando la levadura *S. cerevisiae* ITD00196 como inóculo. Las levaduras con efecto Crabtree-positivo tuvieron un potencial de producción rápida de etanol en la fermentación de residuos orgánicos sin pretratamiento termoquímico o enzimático por SSC en el laboratorio y a escala piloto.
Palabras clave: Metabolismo respiro-fermentativo; efecto Crabtree; cultivo en estado sólido; producción de etanol.

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1 Introduction

Solid-state culture (SSC) is a type of fermentation that takes place on a solid substrate instead of in a liquid medium. The process involves microorganisms, such as fungi, bacteria, and yeasts, which convert the solid substrate into useful products. The advantages of SSC compared to traditional methods are that it is more economical and efficient, uses less energy, and produces higher yields of desired products. In addition, SSC systems are more easily scaled up and can be used for a variety of applications. Solid-state cultures are used to produce enzymes, biofuels, hormones, antibiotics, and other industrial products (Ruíz-Leza *et al*., 2020).

In a SSC, the aim is to recreate the natural characteristics present in the environment in bioreactors. Over time, plants have accumulated large amounts of monosaccharides and polysaccharides, substrates currently used as a source of carbon and energy for cell growth and production of metabolites. Several yeasts developed the metabolic ability needed to assimilate various sources of carbon present in nature. Additionally, the metabolism of certain yeast is regulated by the Crabtree effect (Crabtree, 1929). Crabtree-positive yeast may produce ethanol under aerobic conditions depending on substrate or nutrient availability, leading to a reduction in biomass production (Pronk *et al*., 1996; Hagman *et al*., 2013; Moutsoglou *et al*., 2020). Under anaerobic conditions, acetaldehyde is the last electron accepter and is converted into ethanol under fermentative metabolism. Respiratory metabolism is possible when oxygen is present to act as the final electron acceptor. Both pathways can coexist simultaneously resulting in a mixed (respiro-fermentative) pattern of metabolism (Gancedo and Serrano, 1989; Hagman *et al*., 2013; Moutsoglou *et al*., 2020). The activities of the different pathways can be evaluated by measuring the rates of metabolic formation or substrate uptake. The determination of Crabtree-postitive or -negative yeast can be based on the monitoring of the biomass and ethanol production yields ($Y_{x/s}$ and $Y_{p/s}$, respectively) (Hagman *et al*., 2013).

Saccharomyces cerevisiae is the principal yeast used for the rapid conversion of carbohydrates present in nature into ethanol and CO_2 under anaerobic or aerobic assay conditions (Rozpędowska *et al*., 2011). However, alternative yeasts are being investigated to improve the potential use of different by-products used for ethanol production by SSC (Saucedo-Castañeda *et al*., 1992; Mazaheri *et al*., 2013; Canabarro *et al*., 2017).

Hagman *et al*. (2013) used the Crabtree-positive yeast *S. cerevisiae* as a reference to obtain ethanol and biomass production yields of 0.39 and 0.16 g per gram of glucose, respectively. Conversely, the Crabtree-negative yeast *Kluyveromyces lactis* produced no ethanol and had a biomass production yield of 0.57 g/g. In this article, the yeasts were grown under aerobic conditions maintain a dissolved oxygen concentration above 30%, the only carbon source to be utilized by the studied species was 2% glucose and this behavior depends on the type of yeast used. In the Crabtree-positive strain, glucose was depleted when the maximum concentration of ethanol was obtained and ethanol began to be used as a carbon source. This type of behavior delimits the fermentative and respiratory metabolism. De Deken (1966) reports that the presence of a respiratory metabolism following fermentative metabolism is necessary to compensate for the energy used in cell growth.

Hagman and Piškur (2015) demonstrated that Crabtree-positive yeasts have a mechanism to rapidly uptake glucose, even under conditions of substrate limitation. This rapid absorption is not strictly synchronized with yeast growth, so that this mechanism can easily overcome anabolic activity (cell growth), divert to the pyruvate fermentation route, and consequently accumulate ethanol. Contrary to Crabtree-positive yeasts, Crabtree-negative yeasts exhibit a trade-off in the rates of glucose uptake and carbon flux via anabolic and catabolic ways. (Hagman and Piškur, 2015). When excess glucose is supplied to Crabtree-negative yeast, uncontrolled glucose uptake and ethanol generation is not observed, but instead glucose accumulates in the culture medium. (Alexander and Jeffries, 1990; Fiechter and Seghezzi, 1992).

Therefore, Crabtree-positive yeast have a high potential for fast ethanol production during fermentation of organic substrates in SSC. A lower growth rate of the yeast used in SSC may increase the potential for other native substrate microorganisms to out-compete the target yeast strain.

To minimize the lag phase of the target yeast and minimize native or contaminant organism growth, the inoculum should contain a high concentration of viable cells. Therefore, the conditions for inoculum propagation are of utmost importance to the quality and efficiency of SSC. Laboratory studies frequently use Erlenmeyer flasks for the propagation of microorganisms and inoculum production, allowing flexibility, low cost, and easy operation (Büchs, 2001). However, to obtain high microbial growth, oxygenation is important for the culture. Therefore, factors such as the design of the agitation flask (Marín-Palacio *et al*., 2014) and filling volume (Reynoso-Cereceda *et al*., 2016) are important to enhance microbial growth yield and rate.

Taking these aspects into account, the main aim of this paper was to evaluate the lab-scale production conditions of the inoculum using different flask designs (conventional, coiled, and baffled) and filling volumes (20 and 40 %), to have an inoculum with a high biomass concentration considering the Crabtree effect. Subsequently, the yeasts were then used as inoculum in organic residues fermentation by SSC to know the ethanol production yields in the laboratory scale and finally, the yeast with the highest ethanol production yield was used at pilot-scale.

2 Materials and methods

2.1 Materials

A solid standard mixture (SSM) of the organic fraction of municipal solid waste (OFMSW) was obtained following the methodology proposed by Estrada-Martínez *et al*. (2019). The structural carbohydrates as % dry matter (DM) of SSM were characterized using the methodology reported by Sluiter *et al*. (2011), and were as follows: glucose, 21.16 ± 1.64; fructose, 12.58 ± 0.99; xylose, 2.70 ± 0.80; arabinose, 2.52 ± 0.08 and mannose, 1.39 ± 0.28. The SSM presented an initial pH of 6.46 ± 0.77, an % initial moisture content of 79.57 ± 2.50 and a 35 carbon to nitrogen (C/N) ratio (López-Alcántara *et al*., 2022).

2.2 Yeast and inoculum production

The yeasts employed in this paper were commercial baker's yeast, *Saccharomyces cerevisiae* ITD00196 (Díaz-Campillo *et al*., 2012), *Scheffersomyces stipitis* ATCC58785, *Scheffersomyces stipitis* Y-17104, *Kluyveromyces marxianus* CBS6556, and *Schwanniomyces occidentalis* ATCC26077. The inoculum production assays were carried out with conventional, coiled, and baffled 250 mL Erlenmeyer flasks (Pyrex, Borosilicate glass, USA), as reported by Marín-Palacio *et al*. (2014).

Yeast cultures were assayed using 50 and 100 mL of culture medium (20 and 40% of relative filling volume, respectively). Culture composition was as follows (g/L): glucose, 20; meat peptone, 3.5; yeast extract, 3.0; KH_2PO_4, 2; $MgSO_4{\cdot}7H_2O$, 1 and $(NH_4)_2SO_4$, 0.5 (Saucedo-Castañeda *et al*., 1992), at pH 5 (adjusted with 1 M HCl).

Cultures were incubated on an orbital shaker at 180 rpm for 48 h at 30 ºC with an inoculum of 10^6 cells/mL of the culture medium. Samples were collected periodically throughout incubation and stored at -20 ºC until further analysis by HPLC. Assays were carried out in duplicate.

2.3 SSC at laboratory and pilot-scale for ethanol production

For laboratory scale SSC, inocula were produced in 250 mL Erlenmeyer flasks with the best design for each yeast (baffled flask for baker's yeast, *S. cerevisiae* ITD00196, *S. stipitis* ATCC58785, *S. stipitis* Y-17104, *K. marxianus* CBS6556; conventional flask for *Schw. occidentalis* ATCC26077) with 50 mL (20 % filling volume) of culture medium, using the same medium composition and culture conditions as indicated in inoculum production (Section 2.2).

Tubular bioreactors (TBRs) were packaged with 100 g of the SSM without pre-treatment and incubated at 30ºC for 30 h without airflow. The axenic culture was added at a ratio of 10 % (v/w) of the SSM. Control assays without inocula were performed. In all cases, samples were taken at the initial and final time of the process to estimate carbohydrate consumption and ethanol production.

For pilot-scale SSC, inocula were grown in the 250 mL Erlenmeyer baffled flasks (Section 2.2) and sequentially propagated in 500, then 1000, and then finally 2000 mL baffled Erlenmeyer flasks filled with 100, 200, and 400 mL, respectively (20% filling volume). Each culture was incubated for 12 h at 30ºC and shaken using an orbital shaker (DLAB SK-0330-Pro) at 180 rpm. A 150 L capacity batch bioreactor (length 150 cm x width 24 cm x depth 30 cm) provided with an endless screw-type agitation system (MX/a/2020/001902) was loaded with 25 kg of the SSM without pre-treatment, incubated at 30 ºC for 60 h without airflow and continually agitated at 1 rpm.

Axenic culture of *S. cerevisiae* ITD00196 was used as inocula at a ratio of 10 % (v/w) of SSM without pre-treatment. The bed height of the moist material accounted for almost half the height of the indoor bioreactor (Ruíz-Leza *et al*., 2020). The fermentation monitoring at laboratory and pilot-scale was following the methodology proposed by Estrada-Martínez *et al*. (2019). Samples were obtained at periodic interval times and stored at -20 ºC until further analysis by HPLC. Assays were carried out in duplicate.

2.4 Sampling, carbohydrates, and ethanol determination

In all cultures carried out for inoculum production, 4 mL samples were obtained at periodic interval times (h). An aliquot was for determination of total cell number using a Neubauer chamber. The rest of the sample was centrifuged at 5,300 rpm at 4º C. The pellet was used to determine biomass dry weight and the supernatant was stored at -20 ºC for subsequent HPLC analysis. Once the SSC at laboratory and pilot-scale were completed, 10 g of the fermented substrate were mixed with 40 mL of distilled water in a 250 mL Erlenmeyer flask. The mixture was agitated at 300 rpm for 15 min at 30ºC to extract carbohydrates and ethanol. The mixture was centrifuged at 3,500 rpm for 15 min at 4ºC and the supernatant stored at 4 ºC until HPLC analysis.

The pH, moisture content (Bradley, 2010), carbohydrates, and ethanol concentration were determined following the methodologies previously reported by Estrada-Martínez *et al*., 2019.

The evolution of bioprocess at the pilot-scale was directly monitored through CO_2 and O_2 concentrations. These were monitored through periodic measurement of the gaseous atmosphere obtained from de bioreactor using a GOWMAC Series 580 gas chromatograph and experimental gases concentration pattern was expressed using a simple moving average of 5 points (Saucedo-Castañeda *et al*., 1994; Estrada-Martínez *et al*., 2019).

2.5 *Mathematical considerations*

The microbial growth was simulated using the Logistic mathematical model (Mazaheri *et al*., 2013). The differential equation is presented as follows (1):

$$\frac{dX}{dt} = \mu X\left[1 - \frac{X}{X_{\max}}\right] \tag{1}$$

The integration of equation 1 gives (2):

$$X = \frac{X_{\max}}{1 + (X_{\max} - X_o)/X_o \exp(-\mu t)} \tag{2}$$

Where X is the biomass concentration; X_o and $X_{\max}$ are the initial and maximum biomass (g/L) of the logistic model, respectively; μ (h^{-1}) is the specific growth rate.

The conventional yield coefficient of biomass formation on substrate consumption ($Y_{x/s}$) was estimated at the time when glucose and ethanol were consumed after 48 h of culture (3):

$$Y_{x/s} = \frac{X_f - X_o}{S_o - s_f} \tag{3}$$

Where X_o and X_f are initial and final biomass concentrations after 48 h of culture, respectively. S_o and S_f is the initial and final substrate concentrations (glucose and ethanol) after 48 h of cultivation, respectively. $Y'_{x/s}$ was calculated considering total biomass formation at the time when glucose was consumed after 8-36 h of culture.

The yield coefficient of biomass formation on ethanol consumption ($Y_{x/p}$) was estimated during the respiratory metabolism (RM) phase as indicated in equation 4:

$$Y_{x/p} = \frac{X_{\max} - X_e}{P_{\max} - P_f} \tag{4}$$

Where X_e is the concentration of biomass obtained at the time the highest ethanol concentration ($P_{\max}$) was reached (8-36 h). $X_{\max}$ represents the maximum growth obtained at the end of culture (48 h). $P_{\max}$ is the maximum concentration of ethanol obtained and P_f is the residual ethanol concentration at the end of culture. $X_{\max}$ and $Y_{p/s}$ were calculated as normally performed from the experimental results.

At the pilot-scale, modelling fermentable carbohydrates (FC) and ethanol production were used a typical first-order decay equation (Levenspiel, 1999) and the Gompertz equation (Martínez-Valdez *et al*., 2015), respectively, following the methodology proposed by Estrada-Martínez *et al*. (2019).

2.6 *Statistical analysis*

Data were analyzed using the statistical software Statgraphics Centurion XV.I (Statpoint, Inc.). Significant results identified by performing ANOVA were analyzed with Tukey's HSD test to identify which specific means of the compared groups are different at a 95% significance level.

3 Results and discussion

3.1 *Effect of flask design and filling volume on biomass production*

Three flask designs (conventional, coiled, and baffled) and two filling volumes (20 and 40 %) were evaluated for the inoculum production of *Saccharomyces cerevisiae* ITD00196, baker's yeast, *Scheffersomyces stipitis* ATCC58785, *Scheffersomyces stipitis* Y-17104, *Schwanniomyces occidentalis* ATCC26077, and *Kluyveromyces marxianus* CBS6556, using glucose as a carbon source (Figure 1). The experimental growth data were adjusted to the logistic model (Mazaheri *et al*., 2013). In all cases, a goodness of fit with a coefficient of determination in the range of 0.88 - 0.99 was obtained. The kinetic model is an important consideration for Erlenmeyer flask design. The effect of flask design and filling volume upon biomass production depended on the yeast strain. In general, biomass production was considerably higher with a filling volume of 20 % compared to a filling volume of 40 %.

With a filling volume of 20%, maximum biomass production was obtained with *S. cerevisiae* ITD00196 and baker's yeast, generally independent of the flask design. With the yeasts *K. marxianus* CBS6556 and *Schw. occidentalis* ATCC26077, the lowest and highest biomass production, respectively, were obtained using conventional flask designs (Figure 1). Marín-Palacio *et al*. (2014) reported that flask configuration may affect growth, morphology, and metabolite production; in the cultivation of the bacterium *S. lividans* better results were obtained when using a baffled and coiled flask with a filling volume of 20%. With a fill volume of 40%, lower biomass production was observed with a conventional flask design. Higher growth with lower filling volume is obtained because of higher oxygen transfer in baffled and coiled flasks (Marín-Palacio *et al*., 2014). The variation in flask design (coiled or baffled) increases oxygen transfer, where hydrodynamic improves contact between the gas-liquid phases. In contrast to the conventional Erlenmeyer flask, the air contact surface and the film formed by the liquid medium on the flask are smaller (Reynoso-Cereceda *et al*., 2016).

Growth kinetic parameters were calculated from culture information (Figure 2). It is observed that regardless of the flask design and yeast type, the value of X_{max} was higher in a filling volume of 20% compared to the filling volume of 40 % (Figure 2A). In general, the best conditions for the growth of *S. cerevisiae* ITD00196, baker's yeast, *S. stipitis* ATCC58785, and *S. stipitis* Y-17104 were obtained by using baffled or coiled flasks with a filling volume of 20%. These same conditions could be applied for *K. marxianus* CBS6556, however, low biomass formation yields were obtained for this strain under all conditions assayed.

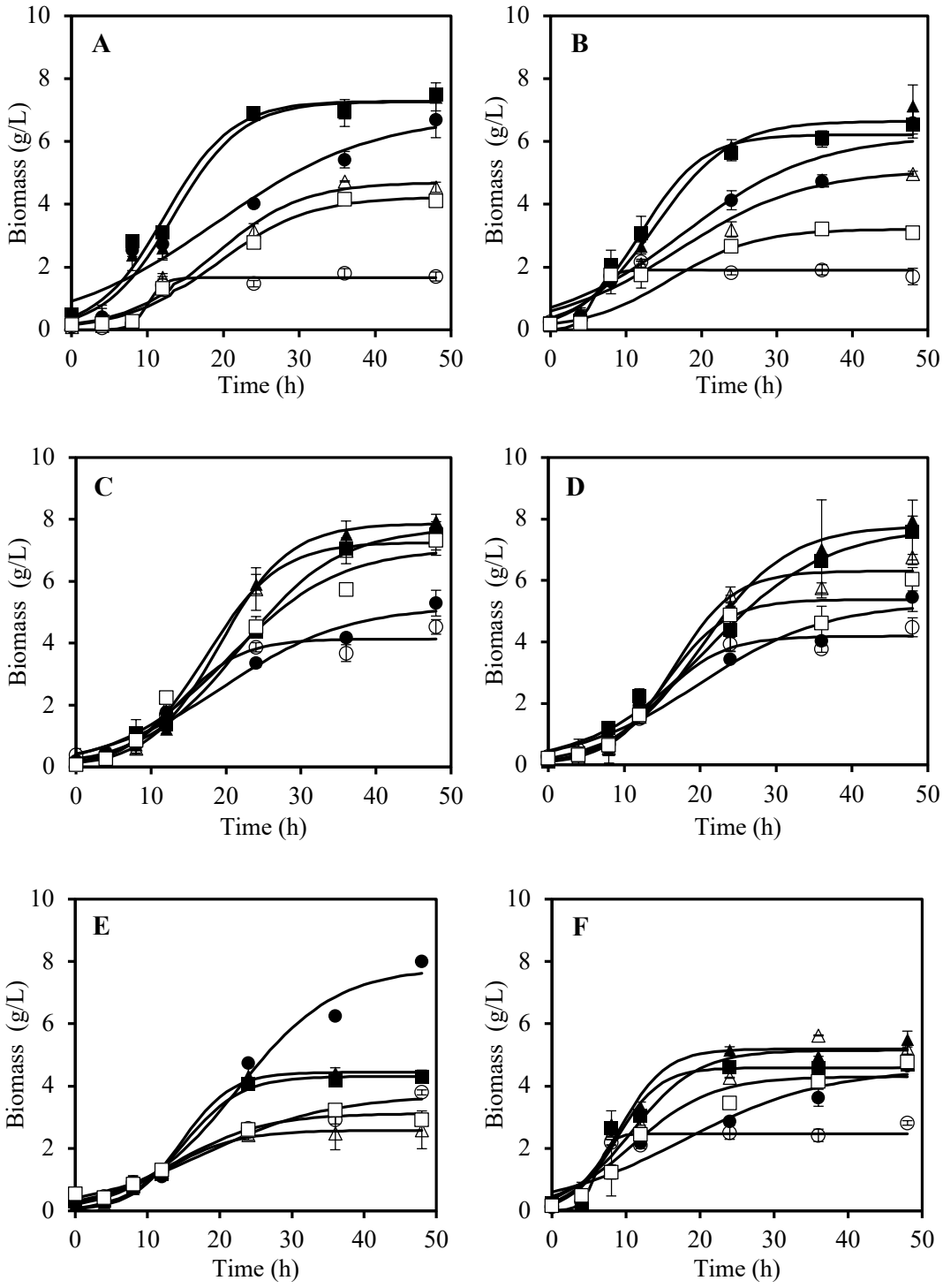

Figure 1. Growth curves in the three flask designs: conventional (●, o), coiled (▲, Δ) and baffled (■, □). Filled and empty symbols correspond to a filling volume of 20 and 40 %, respectively. The continuous line corresponds to the fit of the integral logistic model to the growth curves of the yeasts: A) *S. cerevisiae* ITD00196; B) baker's yeast; C) *S. stipitis* ATCC58785; D) *S. stipitis* Y-17104; E) *Schw. occidentalis* ATCC26077; F) *K. marxianus* CBS6556.

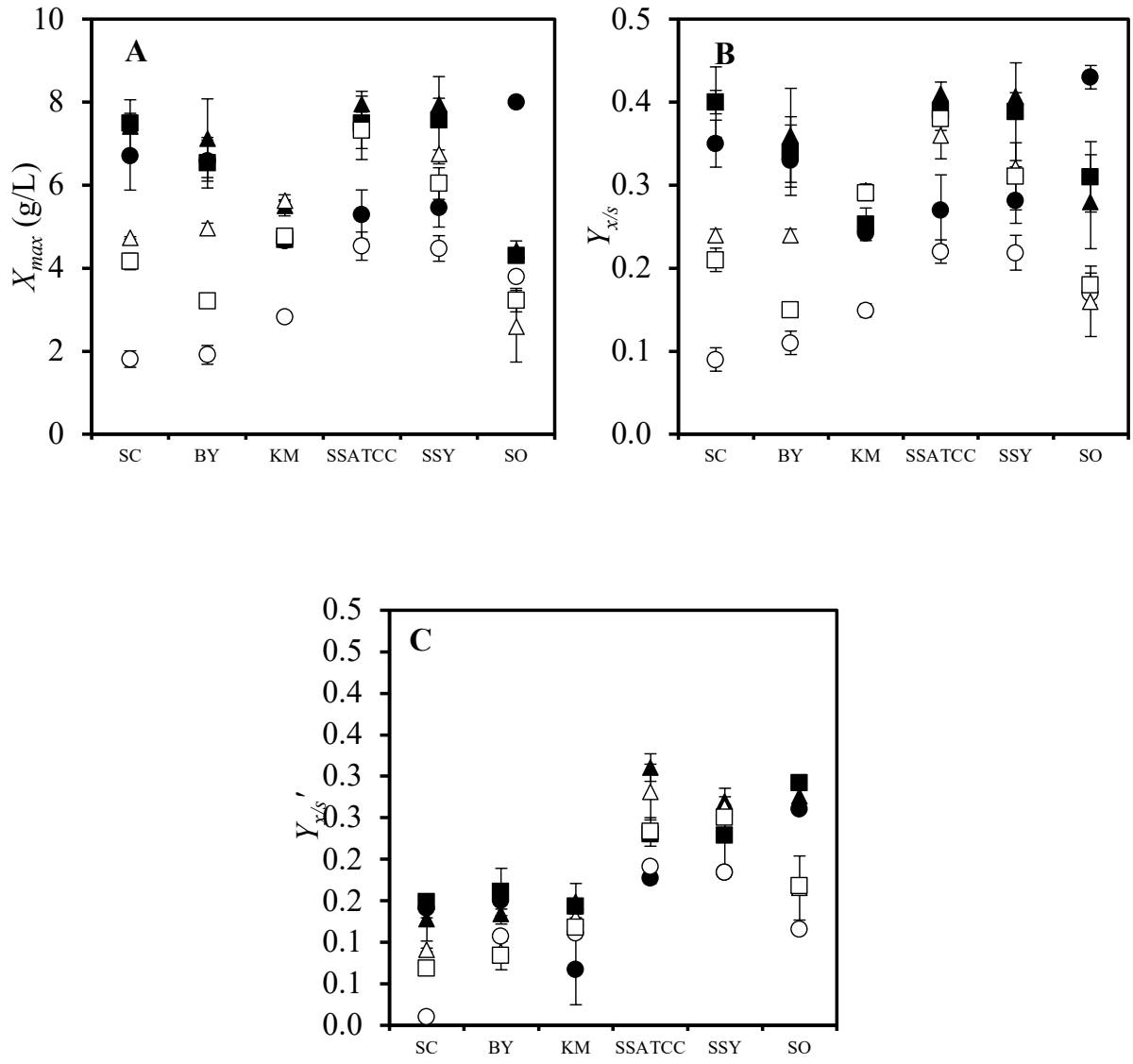

Figure 2. A) $X_{\max}$, B) $Y_{x/s}$ and C) $Y'_{x/s}$ obtained during the yeast assays in the three flask designs: conventional (•, o), coiled (▲, Δ) and baffled (■, □). Filled and empty symbols correspond to a filling volume of 20 and 40 %, respectively. *S. cerevisiae* ITD00196 (SC); baker's yeast (BY); *K. marxianus* CBS6556 (KM); *S. stipitis* ATCC58785 (SSATCC); *S. stipitis* Y-17104 (SSY); *Schw. occidentalis* ATCC26077 (SO).

On the other hand, for *Schw. occidentalis* ATCC26077 the use of conventional flasks was appropriated to obtain the highest biomass concentration, with a filling volume of 20%.

Biomass production efficiency $Y_{x/s}$ determined at the end of culture (48 hr) was higher when using a fill volume of 20%, regardless of the flask type (Figure 2B), similar behavior to that obtained with the $Y'_{x/s}$ yield. The highest yields are obtained with the three designs of the flask for *S. cerevisiae* ITD00196 and baker's yeast, no statistically significant differences were observed between the types of flasks used. The highest performance of *Schw. occidentalis* ATCC26077 (0.43±0.01) with a conventional flask is highlighted. With *S. stipitis* ATCC58785, *S. stipitis* Y-17104, and *K. marxianus* CBS6556 similar values are obtained using coiled and baffled flask designs with a filling volume of 20 and 40 %, the lowest yield corresponds to *K. marxianus* CBS6556. High yields were obtained due to the consumption of glucose and ethanol in most of the experiments. In the case of $Y'_{x/s}$ yield, no statistically significant differences were observed between the type of flask for each of the mentioned yeasts (Figure 2C) when filled at 20%. The $Y'_{x/s}$ yield makes it possible to determine

the type of metabolism of yeasts and thus quantitatively evaluate the Crabtree effect.

3.2 Analysis of the Crabtree effect on culture quality

Simultaneously to the growth kinetics of the different yeast studied (Section 3.1), the kinetics of glucose consumption and ethanol production were obtained for each filling volume (20 and 40 %) and flask design (conventional, coiled, and baffled). In general, a rapid rate of consumption of the carbon source was observed, associated with the formation of ethanol in an early stage of cultivation (8 and 36 h). This phase of fermentative metabolism (FM) was followed by a phase of respiratory metabolism (RM) with the assimilation of ethanol to convert it into biomass. This behavior has been associated with the Crabtree effect and the ability of yeasts to "make-accumulate-consume (ethanol)" (Hagman and Piškur, 2015).

The analysis of the Crabtree effect of *S. cerevisiae* ITD00196, which mainly presents an FM, and *Schw. occidentalis* ATCC26077, which mainly presents an RM under aerobic conditions, is presented below as an example (Figure 3). In the case of *S. cerevisiae* ITD00196, for both filling volumes (Figure 3A and 3B), total glucose consumption and maximum ethanol production is observed at an early stage of cultivation, 8 h for 20% filling volume and 12 h for 40% filling volume. Also, it is observed that ethanol was assimilated as a source of carbon in later stages of cultivation. In most of the yeasts that exhibited the Crabtree effect-positive, glucose was consumed at the point where the highest ethanol production was observed. This represents a border between fermentative and respiratory metabolism (Postma *et al*., 1989; Hagman and Piškur, 2015). This phenomenon is observed in most of the conditions assayed (supplementary material). Due to the rapid ethanol production in Crabtree-positive yeasts, it can be assumed that these yeasts possess many enzymes implicated in the anaerobic glycolysis pathway, even under substrate limitation and aerobic conditions (Hagman and Piškur, 2015).

Conversely, for *Schw. occidentalis* ATCC26077 with a filling volume of 20%, the maximum glucose consumption (14 g/L) was obtained at 24 h and its concentration remains essentially constant until the end of the culture (48 h) (Figure 3C). The maximum ethanol production is reached after 24 h of culture, a concentration that is lower (2.68±0.15 g/L) than that obtained with *S. cerevisiae* ITD00196 (9.51±0.07 g/L) at 8 h of cultivation. However, about 60 % of ethanol was used for cell growth and maintenance. It should be noted that all cultures were performed at a relatively low glucose concentration (20 g/L). Glucose presence in the medium can induce genes related to glycolysis and fermentative metabolism. However, the presence of another substrate (ethanol) can stimulate genes related to secondary metabolism and repress the use of glucose to obtain hydrolytic enzymes (Carmona *et al*., 2002). De Deken (1966) indicated that respiratory metabolism after fermentative metabolism is due to energy compensation necessary for cellular growth. With a filling volume of 40% (Figure 3D), the maximum glucose consumption up to 48 h (15 g/L) is observed. However, the maximum concentration of biomass is achieved at 36 h and that of ethanol at 24 h, which were maintained with little change the rest of the fermentation. In the FM/RM stage, a typical metabolism could be observed, where the yeast grows and fermented at the same rate.

The adequate growth and low ethanol production by *Schw. occidentalis* ATCC26077 could be explained by a strong Pasteur effect (or Crabtree-negative); this characteristic allows maintaining respiratory metabolism under aerobic conditions (Ingledew, 1987) regardless of glucose concentration. The low biomass concentration can be explained by a low activity of the enzyme acetyl-CoA synthetase, the enzyme responsible for the complete oxidation of pyruvate in the Krebs cycle (Pronk *et al*., 1996), so pyruvate accumulation diverted the pathway to ethanol production.

Figure 4 shows the kinetic parameters obtained from ethanol production ($Y_{p/s}$ and $Y_{x/p}$) in the three Erlenmeyer flask designs (conventional, coiled, and baffled) and two filling volumes (20 and 40 %) in the culture kinetics of all yeast studied, using glucose as a carbon source. Although the culture conditions were oriented to biomass production, $Y_{p/s}$ yields very near to the theoretical (0.51) can be observed in Figure 4A, especially for the Crabtree-positive yeasts (*S. cerevisiae* ITD00196, baker's yeast, and *K. marxianus* CBS6556). With a filling volume of 20%, the following strains obtain the best $Y_{p/s}$ results: *S. cerevisiae* ITD00196 (0.50±0.02) using baffled flasks; *S. stipitis* ATCC58785 (0.48±0.03), *S. stipitis* Y-17104 (0.47±0.01), and *Schw. occidentalis* ATCC26077 (0.28±0.01) using conventional flasks. High ethanol production yields can be explained by the accumulation of intracellular pyruvate due to the rapid assimilation of glucose, which contributes to the pyruvate decarboxylase enzyme transforming accumulated pyruvate into acetaldehyde, as the conversion from acetaldehyde to acetyl-CoA is limited and ethanol accumulates (Pronk *et al*., 1996).

Another observed phenomenon is the growth of yeasts from ethanol as a carbon source, a phenomenon that was characterized through $Y_{x/p}$ yield. The yeasts *S. stipitis* Y-17104 (1.40±0.25), *Schw. occidentalis* ATCC26077 (1.02±0.05) and *S. stipitis* ATCC58785 (0.89±0.01) with the highest values of $Y_{x/p}$ and using conventional flasks with a filling volume of 40% (Figure 4B) are highlighted. This characteristic of Crabtree-negative yeasts indicates that the ethanol assimilated for biomass growth was very low. With a filling volume of 40%, the lowest yields were obtained, this could be because a higher concentration of ethanol is used for the growth of the different yeasts studied.

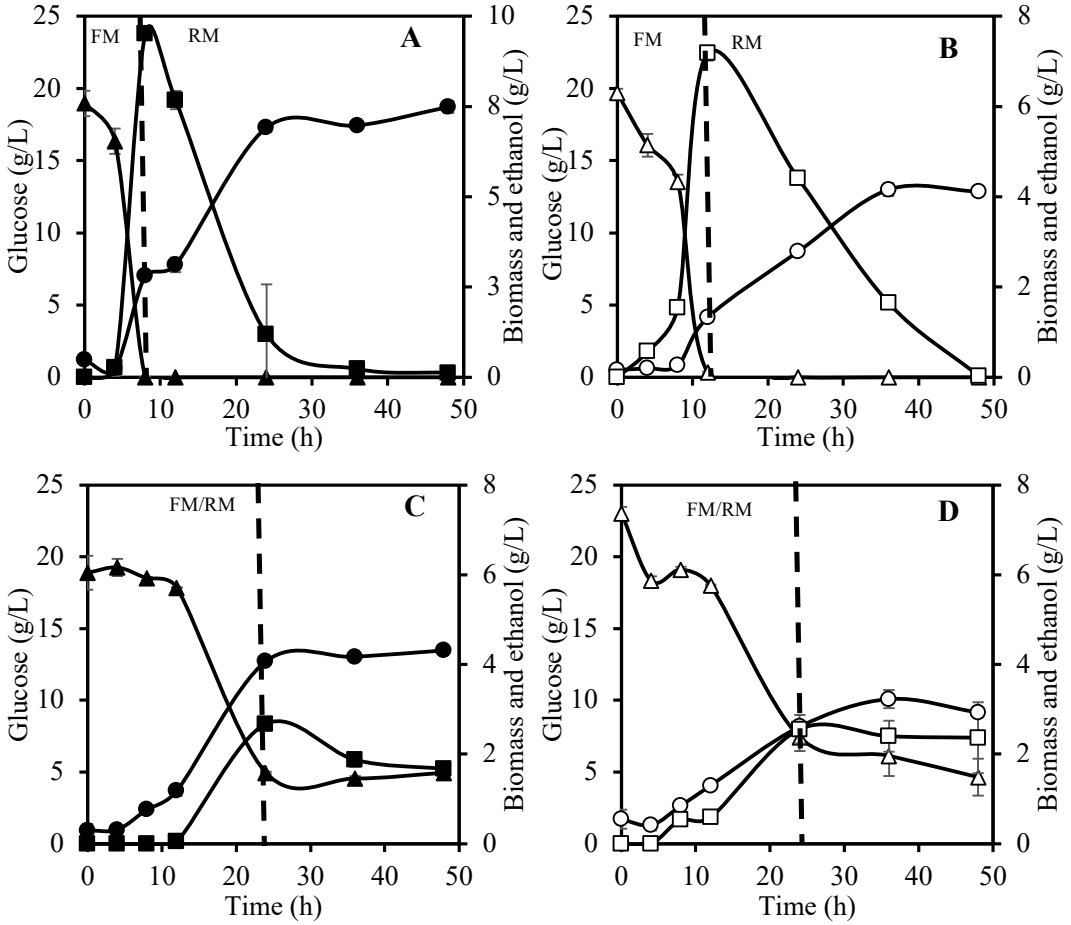

Figure 3. Evolution of the concentration of biomass (•, o), glucose (▲, Δ) and ethanol (■, □) of the *S. cerevisiae* ITD00196 (A and B) and *Schw. occidentalis* ATCC26077 (C and D) yeasts during culture in liquid medium in a baffled flask. The full and empty symbols correspond to a filling volume of 20 and 40 % respectively. FM: Fermentative metabolism and RM: Respiratory metabolism.

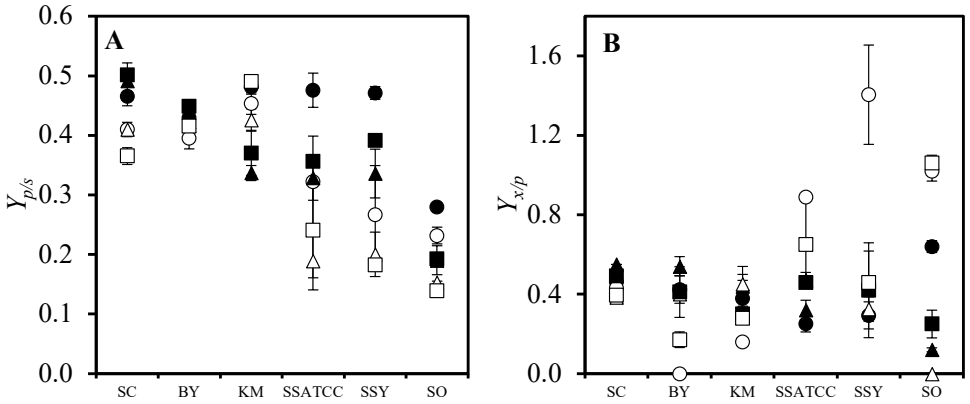

Figure 4. A) $Y_{p/s}$ and B) $Y_{x/p}$ obtained during the yeast assays in the three flask designs: conventional (•, o), coiled (▲, Δ), and baffled (■, □). Filled and empty symbols correspond to a filling volume of 20 and 40 %, respectively. *S. cerevisiae* ITD00196 (SC); baker's yeast (BY); *K. marxianus* CBS6556 (KM); *S. stipitis* ATCC58785 (SSATCC); *S. stipitis* Y-17104 (SSY); *Schw. occidentalis* ATCC26077 (SO).

Table 1. $Y'_{x/s}$ and $Y_{p/s}$ were obtained during the culture of the different yeasts in a liquid medium in a filling volume of 20 %.

Yeasts	$Y'_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)
S. cerevisiae ITD00196	0.13±0.03 - 0.15±0.01	0.47±0.02 - 0.50±0.02
Baker's yeast	0.13±0.01 - 0.16±0.03	0.42±0.01 - 0.45±0.00
K. marxianus CBS6556	0.07±0.04 - 0.15±0.02	0.34±0.01 - 0.48±0.01
S. stipitis ATCC58785	0.18±0.01 - 0.31±0.02	0.33±0.04 - 0.48±0.03
S. stipitis Y-17104	0.18±0.00* - 0.27±0.02	0.34±0.04 - 0.47±0.01
Schw. occidentalis ATCC26077	0.26±0.00 - 0.29±0.00	0.19±0.01 - 0.28±0.01

*Less than 0.01.

With the yields $Y'_{x/s}$ and $Y_{p/s}$ it is possible to quantitatively estimate the degree of Crabtree effect of the yeasts studied. Table 1 shows yields obtained during the culture of the different yeasts in a liquid medium in a baffled flask with a filling volume of 20%. Typical fermentative yields $Y'_{x/s}$ are close to 0.1 (Zhang *et al*., 2019) while respiratory $Y_{x/s}$ yields are close to 0.4, the observed yields of *S. cerevisiae* ITD00196, baker's yeast, and *K. marxianus* CBS6556 yeasts correspond to typical FM yields, while that of *Schw. occidentalis* ATCC26077 is closer to typical RM yields; the first strains behave like yeasts with a Crabtree effect-positive while the latter is identified as Crabtree-negative.

In the inoculum production stage, the yeasts must have the metabolism of the actual target culture conditions. With Crabtree-positive yeasts conditions, is obtained a high cell concentration and a high potential for ethanol production. This avoids contamination by other microorganisms and the rapid utilization of fermentable carbohydrates from organic waste in the bioreactor. However, in the SSC for ethanol production from the SSM of OFMSW, it is important to monitor ethanol production to avoid losses by assimilation.

3.3 Ethanol production in the SSC of the SSM

The ethanol production at laboratory from SSM without pre-treatments and inoculated with axenic cultures were evaluated (Figure 5). Two uninoculated controls were analyzed at the beginning of the culture (C1) and after 30 h of cultivation (C2). Ethanol production of 29.12±2.47 g/kg DM was obtained in C2, which could be due fermentation by the presence of native strains. Comparing the controls with the rest of the yeast, an increase in the yield of ethanol was observed when inoculating with yeast.

Crabtree-positive yeast showed the highest ethanol production; however, no statistically significant differences were observed. Using the baker's yeast as inoculum the highest ethanol production was obtained, attaining 195.70±17.25 g/kg DM (248.04±30.92 L/Ton DM) without pre-treatment. The need to increase the release of fermentable carbohydrates to obtain high yields in ethanol production has been reported, however, increased production costs are associated with this (Tomás-Pejó *et al*., 2008).

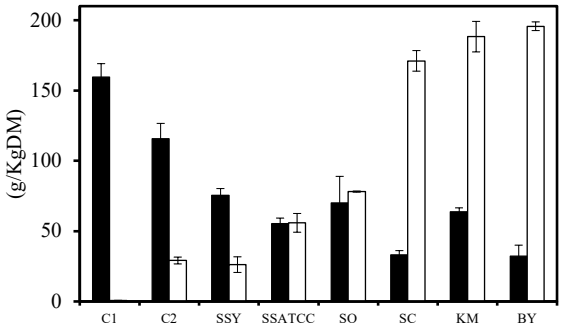

Figure 5. Ethanol (open bars) and fermentable carbohydrates (filled bars) using different yeasts in the fermentation of the SSM without pre-treatment. Control 1 (C1) at the initial time, Control 2 (C2) at the final time (30 h). *S. cerevisiae* ITD00196 (SC); baker's yeast (BY); *K. marxianus* CBS6556 (KM); *S. stipitis* ATCC58785 (SSATCC); *S. stipitis* Y-17104 (SSY); *Schw. occidentalis* ATCC26077 (SO).

Enzymatic hydrolysis, a typical pre-treatment of organic waste, is quite expensive and contributes significantly to the final cost of the biofuel (Klein-Marcuschamer *et al*., 2012; Gómora-Hernández *et al*., 2022). Although Crabtree-negative yeast (*Schw. occidentalis* ATCC26077) does not produce high amounts of ethanol, it can produce amylases (Saucedo-Castañeda *et al*., 1992), particularly pullulanases, capable of hydrolyzing the glucosidic bonds α-1-6 of the pullulan of the starch structure.

Pilot-scale kinetic assays of CO_2 and ethanol production, O_2 and fermentable carbohydrate consumption were completed. (Figure 6). The highest CO_2 concentration (44.17%) was attained at 26 h of culture (Figure 6A). This could be due to the contamination of the substrate by the presence of native strains that consume FC to produce other compounds that are not of commercial interest. The high concentration of CO_2 would not indicate a higher concentration of ethanol because non-ethanol producing microorganisms are present in the SSM. Using first-order decay kinetics, CF consumption was simulated with which a first-order constant (k) of 0.55 h^{-1} and a coefficient of determination of 0.90 (Figure 6B) was achieved. On the other hand, the Gompertz model was used to simulate ethanol production, with a correlation coefficient of 0.99 for the pilot-scale bioreactor was obtained.

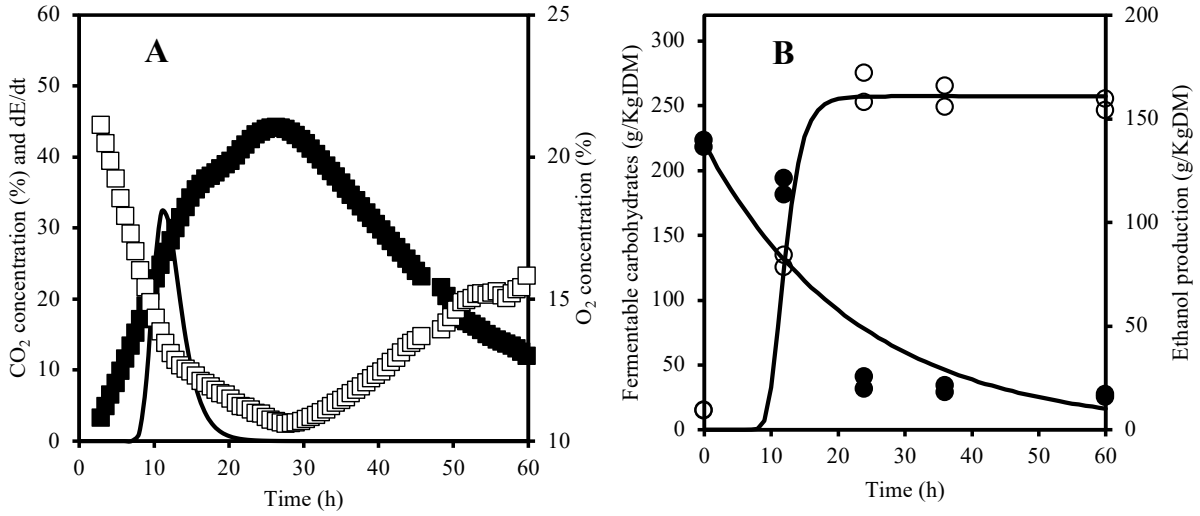

Figure 6. CO_2 (■) and O_2 (□) concentrations in the bioreactor headspace at pilot-scale (A), solid lines represent the ethanol production rate (dE/dt) simulated by differential Gompertz model. Fermentable carbohydrates consumption (•) and ethanol production (o) during the fermentation of SSM without pre-treatment using an *S. cerevisiae* ITD00196 (B) solid lines represent the first-order model for fermentable carbohydrates consumption and integral Gompertz model for ethanol production.

At 24 h, the highest ethanol concentration (165.03±9.90 g/kg DM) was obtained, due to the rapid FC consumption present in the SSM. In contrast to submerged culture (Section 3.2), in SSC no ethanol re-assimilation was observed (Figure 6A), this may be because after 12 h of culture an anaerobic stage was obtained (Figure 6B).

It is important to note that there is no statistically significant difference between ethanol production in TBRs (171.10±10.87 g/kg DM) and the pilot batch bioreactor (165.03±9.90 g/kg DM). The difference in ethanol production is only 3.5% so the process is comparable when using *S. cerevisiae* ITD00196 yeast as inoculum in the use of FC present in the SSM in both scales. The agitation in the bioreactor could help the homogenization of the SSM and improve the speed of the process. The yeast *S. cerevisiae* ITD00196 was selected because it has been characterized for ethanol production (Díaz-Campillo *et al*., 2012). The possibility of producing ethanol with a pilot batch bioreactor using 25 kg WM of the SSM without any thermochemical or enzymatic pre-treatment was demonstrated, however, differences were found that could be diminished with better control of the process, using, for example, a thermal pre-treatment (Estrada-Martínez *et al*., 2019).

Ethanol yields reported in the literature using organic wastes as substrate are similar to those obtained at laboratory and pilot-scale in this paper. Moon *et al*. (2009), evaluated a potential application of food waste for ethanol production by laboratory assays. These authors evaluated the enzymatic hydrolysis of food waste and its use as a substrate for batch ethanol production with the yeast *Saccharomyces cerevisiae*. The maximum ethanol yield of 230 g ethanol/Kg DM was obtained. Alamanou *et al*. (2015), produced 107 g ethanol/Kg DM by fed-batch non-isothermal simultaneous saccharification and fermentation of household food residues as substrate. Household food waste was subjected to acid digestion (dilute sulfuric acid) by microwave. Additionally, enzymatic hydrolysis was performed. Loizidou *et al*. (2017), used enzymatically pre-treated dried household food waste as substrate and fermented with *Saccharomyces cerevisiae* yeast at pilot scale. With this system, they obtained a maximum yield of 188 L of ethanol from one ton of waste (148.33 g ethanol/kg DM). Mahmoodi *et al*. (2018), investigated the use of submerged culture of OFMSW to ethanol production using *Mucor indicus*. The OFMSW was thermal pre-treatment and then enzymatically hydrolyzed; a maximum yield of 191.10 g ethanol/Kg of dry OFMSW was obtained. Karimi and Karimi (2018), studied the ethanol production by anaerobic ethanolic fermentation using kitchen and garden wastes with and without pre-treatment and *Mucor indicus*. These authors obtained 120 L of ethanol per ton of residues without pre-treatment and after dilute acid pre-treatment and enzymatic hydrolysis a maximum yield of 128.84 g of ethanol/kg of DM.

Various uses of raw materials for ethanol production by SSC have been reported in the literature. Saucedo-Castañeda *et al*. (1992), reported a maximum yield of 89.30 g ethanol/kg of dry starch by SSC using a *Schwanniomyces castellii* CBS 2863. Yu *et al*. (2008), achieved a maximum yield of 316 g ethanol/kg in a SSC by mixing dried sweet sorghum dry stalks and H_2SO_4 to obtain anaerobic conditions and fermenting with a baker's yeast mutant AF37X. Mohanty *et al*. (2009), studied the use of Mahula (*Madhuca latifolia* L.) flowers cooked under conventional sterilization conditions and fermented by *Saccharomyces cerevisiae* yeast in a SSC. A maximum yield of 225 g ethanol/kg flower was obtained. Xin *et*

al. (2013), fermented dry horticultural waste by SSC using the yeast *Saccharomyces cerevisiae*. The authors achieved a maximum yield of 62 g ethanol/kg of substrate. The substrate was organosolv-pretreated and enzymatically hydrolyzed using crude fungal enzyme mixtures. Canabarro *et al*. (2017), used saccharified rice bran in solid-state, enzymatically hydrolyzed and fermented in a packed-bed bioreactor. A maximum yield of 139 g ethanol/kg DM was obtained.

The selection of microorganisms and organic matter are important. However, inoculum production with a high concentration of microorganisms is essential for the efficient development of a SSC. The initial incubation conditions in the SSC strongly affect the whole process. SSC is an efficient, cost-effective, promising, and low-cost technology, that makes it an appropriate process for substrate hydrolysis and subsequent ethanol production. In this work, a maximum yield of 195.70±17.25 g ethanol/kg DM or 248.04±30.92 L ethanol/Ton DM was achieved by fermenting an organic substrate without pre-treatment and with commercial yeasts at laboratory and pilot scale in SSC.

Conclusions

A view has been presented to understand the effect of the filling volume using three flask designs on inoculum production. A filling volume of 20% provided the best conditions for the development of inoculums in yeasts used for SSC. Baffled or coiled flasks contribute to high biomass production for yeast exhibiting a Crabtree effect-positive evaluated in terms of $Y'_{x/s}$ and $Y_{p/s}$ during glucose consumption of inoculum cultures. Yeasts with a Crabtree effect-positive have a potential for rapid ethanol production during direct fermentation of SSM.

On the other hand, the use of different yeasts for ethanol production was evaluated by fermenting a SSM without any pre-treatment in SSC at laboratory and pilot-scale. Similar results were obtained in the maximum yield of ethanol production in TBRs and in the batch bioreactor provided with an endless screw-type agitation system. This study shows that the process is more practical for ethanol production, is possible to obtain maximum ethanol yields by SSC comparable to those obtained in conventional submerged cultures and to scale-up more easily than others previously reported.

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