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Ethanol production from Mexican fruit wastes using a new *Saccharomyces cerevisiae* strain

Producción de etanol a partir de desechos frutales mexicanos utilizando una nueva cepa de *Saccharomyces cerevisiae*

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Abstract
In the Mexican croplands are generated large amounts of agroindustrial wastes that are usually not exploited. Damaged fruits wasted in the municipality of Tres Valles, Veracruz, are an excellent feedstock to produce ethanol, since do not need a sophisticated pretreatment and have high fermentable sugar concentrations. In this work is described ethanol production from damaged fruits by a new strain of *Saccharomyces cerevisiae* isolated from *Agave* sp. wastes. Fermentations were carried out in batch and repeated batch cultures using biocatalysts formed by *S. cerevisiae* AP1 cells immobilized into alginate-coated polyester fiberfill. Biocatalysts showed a high fermentative capability at reducing sugar concentrations higher than 30 g L^{-1}. In batch cultures, with 32.58 g reducing sugar L^{-1}, was produced up to 15.39 g ethanol L^{-1} at 16 h, with a volumetric productivity of 0.962 g L^{-1} h^{-1} and a fermentation efficiency of 94.77%. Instead in a 5-cycle repeated batch fermentation, with a reducing sugar content among 30 to 43 g L^{-1}, ethanol production in each cycle was fast, higher than 15 g L^{-1}, with fermentation efficiencies higher than 80%, and with volumetric productivities from 2.5 to 2.9 g L^{-1} h^{-1} after second cycle. Afterwards five cycles of repeated batch fermentation, total ethanol production was 95.41 g L^{-1} in just 44 h process.
Keywords: ethanol, *Saccharomyces cerevisiae*, immobilization, damaged fruits, polyester fiberfill.

Resumen
En los campos de cultivos mexicanos se generan grandes cantidades de desechos agroindustriales que generalmente no son aprovechados y actúan como una fuente de contaminación. Los desechos frutales generados en el municipio de Tres Valles, Veracruz, son una excelente materia prima para producir etanol, ya que no requieren pretratamientos sofisticados y contienen altas concentraciones de azúcares fermentables. En este trabajo se describe la producción de etanol a partir de desechos frutales utilizando una nueva cepa de *Saccharomyces cerevisiae* aislada de residuos de *Agave* sp. Las fermentaciones se realizaron en cultivos por lote y por lotes repetidos utilizando biocatalizadores conformados por células de *S. cerevisiae* AP1 inmovilizadas en fibra de poliéster recubierta con alginato. Los biocatalizadores mostraron una alta capacidad fermentativa a concentraciones de azúcares reductores superiores a 30 g L^{-1}. En los cultivos por lote se produjo hasta 15.39 g etanol L^{-1} en 16 horas, con una productividad volumétrica de 0.962 g L^{-1} h^{-1} y una eficiencia de fermentación de 94.77%. En cambio, en una fermentación de cinco lotes repetidos, con un contenido de azucares reductores de 30-43 g L^{-1}, la producción de etanol fue rápida, superior a 15 g L^{-1}, con eficiencias de fermentación superiores al 80%, y con productividades volumétricas de 2.5-2.9 g L^{-1} h^{-1} a partir del segundo ciclo. Después de cinco ciclos de fermentación por lotes repetidos, la producción total de etanol fue de 95.41 g L^{-1} en 44 h de proceso.
Palabras clave: etanol, *Saccharomyces cerevisiae*, inmovilización, desechos frutales, fibra de poliéster.

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1 Introduction

Humanity has historically satisfied its energy needs thanks to the combustion of fossil fuels, bringing as major collateral damage the generation of greenhouse gases and the release of particles into the atmosphere. 73% carbon dioxide produced is worldwide generated by the combustion of fossil fuels (Wildenborg and Lokhorst, 2005). Moreover, fossil fuels are a finite resource, it is estimated that they will finish in the next 40-50 years (Vohra *et al*., 2014). Faced with the imminent energy crisis, the biofuels emerge as an alternative to reduce the use of fossil fuels. Nowadays, ethanol is the most widely used biofuel since it is used as an additive or substitute of gasolines. Ethanol to be a non-fossil fuel presents some economic, environmental and social benefits, such as: i) when it is blended with gasoline, the octane number of the mixture increases; ii) it having a high oxygen content (35%) improves the combustion efficiency; iii) its combustion reduces the emissions of carbon monoxide, volatile organic compounds, sulfur oxides and particles; iv) it forms oxidation by-products less toxic than the by-products formed from other alcohols; v) it is not toxic for the environment; vi) it is generated from renewable agricultural products; vii) its production encourage the regional engineering, investigation and development; viii) its production creates jobs and increase the level of services for the rural population (Aldana-González *et al*., 2022; Balat *et al*., 2008; Demirbas, 2009; Sarris and Papanikolaou, 2016).

The world's largest ethanol producers are the United States of America and Brazil with a market share of 58% and 28%, respectively, whereas Europe and rest of the world only produce 14% (Bayrakci Ozdingis and Kocar, 2018). Ethanol generated in the United States of America and Brazil is produced with corn glucose and sugarcane sucrose, respectively. Meanwhile, ethanol produced in Europe is made with wheat, sugar beet and wastes from the wine industry (Balat *et al*., 2008). Currently, ethanol produced from cereals and plants with a high content of fermentable sugars is economically viable (Mondragón-Cortez *et al*., 2022). However, these feedstocks are essential agro-foods by the human population. When food is used to produce a biofuel, a moral conflict is created. Therefore, alternative feedstocks for ethanol production that do not compromise the food supply, such as the agroindustrial wastes, have been explored. Countries with great agronomic activity are also great agro-waste generators, and this makes them potential candidates to develop a thriving ethanol industry.

Mexico has 24.6 million hectares for agriculture, is the eleventh-largest producer of food, the eleventh-largest producer of agricultural crops, the third-largest producer of mango with 2,156,040 tons, the ninth-largest producer of pineapple with 1,271,521 tons and the twelfth-largest producer of watermelon with 1,194,033 tons in the world (SIAP, 2022). In 2006, 76 million tons of organic wastes were annually generated in Mexico, of which 79% were primary crop residues (corn straw, sorghum straw, top/leaves of sugarcane and wheat straw) and 21% were secondary crop residues (sugarcane bagasse, corncobs, maguey bagasse, and coffee pulp) (Carrillo-Nieves *et al*., 2019; Valdez-Vazquez *et al*., 2010). Mexico has 15 states with a high potential for generating energy (via combustion and fermentation) from lignocellulosic organic wastes, and the state of Veracruz is one the them (Valdez-Vazquez *et al*., 2010). Veracruz has the largest fruit-growing area in Mexico. The municipality of Tres Valles, Veracruz, was distinguished for producing 44.5 tons of pineapple and 3.04 tons of mango per hectare (SIAP, 2020). Local farmers have calculated that during the harvest season 25-50% of the pineapple production, 30% of the watermelon production and 35% of the mango production per hectare were lost by environmental factors such as strong rains and winds, generating high volumes of non-marketable fruits. These damaged fruits are a non-lignocellulosic waste with high concentration of fermentable sugars that have not exploited by the municipality. Damaged fruits are usually left to rot on the fields propitiating the growth of insects that damage crops. Therefore, the aim of this study was to evaluate the potential use of fruits wastes generated on Mexican croplands as a substrate to produce ethanol via fermentation using a new *Saccharomyces cerevisiae* strain isolated from *Agave* sp. wastes. Besides developing a biocatalyst with *S. cerevisiae* cells immobilized into alginate-coated polyester fiberfill in order to improve the fermentative capability and the reusability of *S. cerevisiae* cells in repeated batch cultures.

2 Material and methods

2.1 Fruit wastes and pretreatment

Damaged fruits (mango, watermelon and pineapple) were collected from fruit fields from the municipality of Tres Valles, Veracruz, Mexico, during months April-May 2019. Fruit wastes were washed with distilled water and stored at 4°C to prevent their decomposition.

To extract the fermentable sugars from damaged fruits, 250 g of each fruit were grinded until a semi-solid paste was formed. Semi-solid pastes of each waste were mixed and filtered by mechanical compression using a mesh formed with a four-layer gauze. The liquid extract (300-350 mL, pH 4.0-4.2), which still contained suspended solids, was gauged with distilled water at 500 mL. This juice contained 47.204 ± 1.04 g L^{-1} of reducing sugars and was utilized to prepare the ethanol production medium.

2.2 Immobilization support

Polyester fiberfill (PF), so-called Dacron, with a 1 cm of thick was used to immobilize yeast cells. This compressed fiber was bought in a local textile store. PF was cut in cubes

of approximately 1 cm^3 and the ends were sewn to avoid cubes lose their structure during fermentation. Prior their use, PF cubes were washed with boiling and distilled water, squeezed and oven-dried at 80°C for 48 hours. Dried PF cubes was brought to room temperature before use them.

2.3 *Isolation and molecular identification of yeast*

The yeast strain utilized in this study was isolated from *Agave* sp. wastes derived from the agave spirits industry by a serial dilution technique using yeast extract peptone dextrose (YPD) agar and potato dextrose agar (PDA). It was purified by repetitive streaking on YPD agar. Plates were incubated at 30°C for 3 days.

Yeast genomic DNA (gDNA) was extracted according to Cenis (1992) from 18-hour YPD liquid cultures incubated at 30°C and 200 rpm. Kit Wizard SV Gel and PCR Clean-Up System (Promega, Madison, EUA) was used to clean up and purify the gDNA following the manufacturer's indications. DNA integrity was verified by electrophoresis on 0.7% agarose gel. Ribosomal DNA (rDNA) was amplified by polymerase chain reaction (PCR) using ITS4-B (5'-CAG GAG ACT TGT ACA CGG TCC AG-3') (Gardes and Bruns, 1993) and ITS5 (5'- GGA AGT AAA AGT CGT AAC AAG G-3') (White *et al*., 1990) that are primers for 25s-rDNA and 18s-rDNA conserved sequences respectively. The amplified products were purified using Kit Wizard SV Gel and PCR Clean-Up System (Promega, Madison, EUA) and submitted for identification by sequencing to Macrogen (Seoul, South Korea).

The nucleotide sequence obtained was analyzed in a standard nucleotide-nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) homology search. The phylogenetic analysis was performed with the GenBank database using the MUSCLE iteration method (multiple sequence alignment by log-expectation) limiting to the first 20 hits of the alignment with more than 94% coverage for the estimation of the relationship between species/genus, the phylogenetic tree was built with the MEGA (Molecular Evolutionary Genetics Analysis) (www-megasoftware.net) software based on a "Neighbor-Join Tree" test.

2.4 *Culture media*

Yeast was firstly grown on PDA plates at 30°C for 3 days. Then, yeast cells (3 to 4 loopful from plates) were inoculated into 500 mL Erlenmeyer flacks with 200 mL of grown medium, which contained per liter: 10 g glucose, 5 g peptone and 1 g yeast extract; pH of cultures was maintained to 5.0-5.2 with a 0.1M acetate buffer. Growth cultures was covered with cotton plugs and incubated at 160 rpm and 30°C for 3 days up to reaching a concentration of 1.1×10^7 cell per milliliter. This biomass was utilized as inoculum for the solid-state cultures on PF cubes. A two-fold concentrated growth media (2x), except for peptone, was formulated to increase the cell concentration of yeast and to allow the attachment of biomass into PF cubes.

The ethanol production medium composition was similar to that growth medium, except for glucose, which was replaced by reducing sugars from damaged fruits juice. All culture mediums were sterilized in an autoclave at 121°C and 1.1 atm during 15 min before use.

2.5 *Solid-state fermentation*

Four dried PF cubes were placed in 500 mL Erlenmeyer flasks with cotton plugs and were sterilized at 121°C for 20 min. After sterilization, moisture content (wet basis) of each cube was adjusted to 92 ± 5.46 % by adding 8.4 mL per cube of 2x grown medium inoculated with 2.7×10^6 yeast cells per milliliter. Solid-state cultures were incubated at 30°C for 3 days until reaching 1.5×10^9 cell per cube.

2.6 *Yeast immobilization*

PF cubes with attached yeast biomass were covered with a thin layer of alginate in order to prevent the detachment of adhering biomass using the entrapment method reported by Ellaiah *et al*. (2004) with some modifications. Briefly, PF cubes with attached biomass were placed into a sterile solution of sodium alginate (3%, w/v) at room temperature for approximately 30 seconds, then they were transferred to a 0.2 M $CaCl_2$ solution at 4°C for 5 min in order to favor the alginate gelation. Alginate-coated PF (ACPF) cubes were washed several times with a sterile distilled water and used for the ethanol production.

2.7 *Effect of suspended solid and sterilization on the reducing sugar concentration*

The effect of suspended solid and sterilization on the final amount of reducing sugars in the ethanol production medium was evaluated by a completely randomized 2^2 factorial design with two levels of juice suspended solids (with or without) and two levels of sterilization (with or without). Four replicates of each treatment were tested. Juice suspended solids was removed by centrifugation at 4000 rpm and sterilization was carried out at 121°C and 1.1 atm during 15 min. Table 1 shows the experimental matrix for 2^2 factorial design.

2.8 *Batch fermentation*

Batch fermentation was carried out in 1L Erlenmeyer flasks with cotton plug containing 600 mL of production medium with three different concentrations of reducing sugars from damaged fruits juice, 11.25 ± 0.074, 22.86 ± 0.147 and 32.58 ± 4.913 g L^{-1}. All flasks were inoculated with six alginate-coated PF cubes with attached yeast (ACPF cubes).

Table 1. 2^2 factorial design planning matrix and responses obtained.

Treatment	X1	X2	Reducing sugar (g/L)*
1	WTs	WTste	46.873
	WTs	WTste	45.896
	WTs	WTste	47.866
	WTs	WTste	48.192
2	WTs	Wste	54.544
	WTs	Wste	54.707
	WTs	Wste	56.450
	WTs	Wste	59.924
3	Ws	WTste	45.749
	Ws	WTste	48.192
	Ws	WTste	47.329
	Ws	WTste	47.546
4	Ws	Wste	58.453
	Ws	Wste	58.453
	Ws	Wste	54.278
	Ws	Wste	58.404

WTs = without solids; Ws = with solids; WTste = without sterilization; Wste = with sterilization. *Reducing sugar quantified in each treatment.

Cultures were incubated at 30°C and 160 rpm during one week. 2-mL samples from broth were withdrawn at 0, 6, 12, 24, 48, 72, and 96 hours. Batch experiments were performed in duplicate.

2.9 Reuse of biocatalyst in batch fermentation

5-cycle repeated batch fermentation was conducted in 1L Erlenmeyer flasks with cotton plug containing 600 mL of production medium with a reducing sugar concentration ranged from 30 to 43 g L^{-1}. Immobilized biomass previously used in batch fermentation with 32.58 g L^{-1} of reducing sugars was harnessed as an inoculum in these experiments. Repeated batch cultures were incubated at 30°C and 160 rpm. Each fermentation cycle lasted 24 hours. At the end of each batch, immobilized biomass was recovered by filtration and transferred to a fresh production medium. During each cycle, 2-mL samples were collected at 0, 6, 8, 16 and 24 hours. Repeated batch fermentation was carried out in duplicate.

2.10 Analytical methods

Yeast biomass was determined by cell counting with a Neubauer chamber. Before counting, cells were stained with methylene blue (Alfenore *et al*., 2002). Yeast biomass was expressed as number of cells per cube.

Reducing sugars from damaged fruit juice and fermentation samples were spectrophotometrically quantified at 540 nm using the 3,5-dinitrosalicylic acid method (Miller, 1959). Calibration was performed using standard solutions of glucose (R^2 = 0.998). Reducing sugar concentration was expressed as grams per liter.

Ethanol was quantified based on the methodology stated by Seo *et al*. (2009). In this methodology, ethanol was extracted with tri-*n*-butyl phosphate (TBP, Sigma Aldrich), and then was oxidized by chromium (VI) to form chromium (III) and acetic acid (Magrí *et al*., 1997). The latter was spectrophotometrically measured at 580 nm.

Fermentation samples were centrifuged at 1400 rpm for 10 min at 20°C. Supernatant (500 μL) was mixed with 500 μL of TBP. Mixture was vigorously homogenized in a vortex mixer for 10 minutes, and then was centrifuged at 1400 rpm for 10 min at 20°C. After centrifugation, the organic phase (the upper layer) was recovered and mixed with 500 μL of dichromate reagent. Reaction mixture was vortexed for 10 minutes at room temperature, and then was centrifuged at 1400 rpm for 10 minutes at 4°C. The resulting aqueous phase (the lower layer) was carefully withdrawn from mixture and its absorbance was measured at 580 nm. Ethanol concentration was calculated using a calibration curve from standard solutions of ethanol (R^2 = 0.989). Ethanol concentration was reported as grams per liter.

A Perkin Elmer Lambda 35 UV/Vis spectrophotometer was utilized to measure the absorbance during the determination of reducing sugars and ethanol.

2.11 Fermentation parameters and ethanol kinetic model

Sugar conversion (X_S), ethanol yield ($Y_{P/S}$), volumetric ethanol productivity (Q_P) and fermentation efficiency (η) were calculated to characterize the fermentation process (Pacheco *et al*., 2010; Singh *et al*., 2013). Table 2 showed equations used for the calculation of these parameters.

Table 2. Characterization parameters of ethanol production during the damage fruit juice fermentation.

Parameter	Equation and symbol	
Sugar conversion (%)	$X_S = \frac{S_0 - S_F}{S_0}(100)$	(1)
Ethanol yield (g g^{-1})	$Y_{P/S} = \frac{P_F}{S_0 - S_F}$	(2)
Volumetric ethanol productivity (g L^{-1} h^{-1})	$Q_P = \frac{P_{\max}}{t_F}$	(3)
Fermentation efficiency (%)	$\eta = \frac{Y_{P/S}}{Y_{th}}(100)$	(4)

Where S_0 and S_F are the reducing sugar concentrations (g L^{-1}) at the beginning and the end of fermentation, respectively. P_F is the ethanol concentration at the end of process (g L^{-1}), P_{max} is the maximum ethanol concentration (g L^{-1}) and t_F is time (h) where the maximum ethanol concentration was reached. Y_{th} is the theoretical ethanol yield, 0.51 g g^{-1}.

The ethanol production was modeled by the modified Gompertz equation (Eq. 5).

$$P = P_{\max} \cdot \exp\left[-\exp\left(\frac{R_{pm} \cdot \exp(1)}{P_{\max}}\right)(\lambda - t) + 1\right] \quad (5)$$

Where R_{pm} is the maximum ethanol production rate (g L^{-1} h^{-1}), λ is the lag-phase time (h) and t is the fermentation time (h) (Shuler *et al.*, 2017). The software OriginPro 2017 was used to estimate the equation parameters applying the Levenberg-Marquardt method for non-linear regression. The goodness of fit between experimental data and model was measured by the determination coefficient (R^2).

2.12 Statistical analysis

Statistical analysis from the completely randomized 2^2 factorial design was done by the IBM SPSS statistic 19.0 software with a confidence level of 95%. Assumption of homogeneity of variance was verified by the Levene's test (p-value = 0.347). Normal distribution of errors was confirmed by Kolomogorov-Smirnov test using the Statgraphics 18 software (p-value = 0.603). Tukey's HSD post hoc test was used to determine differences between treatments ($\alpha = 0.05$).

3 Results and discussion

Roadmap for ethanol production from Mexican fruit waste is depicted in Figure 1. Process involves six stages, which are: isolation and molecular identification of yeast from wastes derived from the agave spirits industry, fruit wastes harvest, waste treatment, chemical characterization of wastes, development of a biocatalyst with immobilized yeast cells, and fermentation.

3.1 Yeast identification

Phylogenetic analysis of the yeast used in this work was performed against with other 20 *Saccharomyces* species retrieved from GenBank showed that the sequence of this strain is closely related to *S. cerevisiae* (Figure 2). An identity greater that 94% was found with a sequence of 553 nucleotides, therefore the strain was identified and designated as *S. cerevisiae* strain AP1.

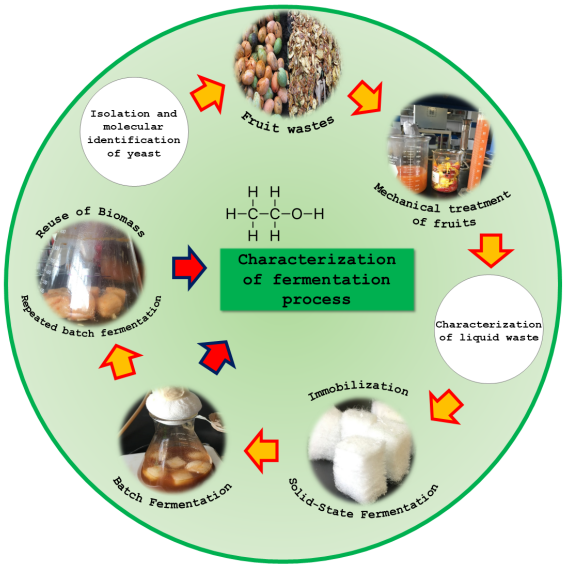

Figure 1. Different stages of ethanol production from damaged fruit juice.

3.2 Effect of suspended solid and sterilization on the reducing sugar concentration

Damaged fruit juice formulated from equal proportions of mango, watermelon and pineapple wastes is a promising feedstock for producing ethanol, since it is a low-cost by-product and contains in average 47.204 ± 1.04 g L^{-1} of reducing sugars. However, this juice contains a significant number of solid particles hard to separate by filtration. In order to assess whether these suspended solids may disturb the reducing sugar concentration after sterilizing the production medium, a completely randomized 2^2 factorial design was performed considering suspended solids and sterilization as factors. According to analysis of variance of factorial design, sterilization impacted significantly on the reducing sugar concentration in medium (p-value < 0.0001), but suspended solids did not (p-value = 0.590). Interaction among factors was also not significant on the reducing sugar content in medium (p-value = 0.588).

Tukey's HSD post hoc test showed that the reducing sugar concentration in treatments without sterilization remained unchanged at 47.204 g L^{-1}. However, in treatments with sterilization the sugar concentration increased 1.2-fold with respect to those without heating (Figure 3). This increase of the reducing sugar concentration after sterilization could have been due to breakdown of some non-reducing disaccharides, such as sucrose, into reducing monosaccharides, such as glucose and fructose. Wann *et al.* (1997) concluded that hydrolysis of sucrose, at autoclave sterilization temperature and pressure, was highly dependent on pH, favoring in acid conditions. Similar conclusions were

drawn during the acid hydrolysis of sucrose from sweet sorghum syrup and Nipa Sap (Klasson *et al*., 2022; Nguyen *et al*., 2016).

Preliminary studies about the chemical characterization of damaged fruit juice have revealed that this feedstock contains mainly glucose (from 27 to 66 g L^{-1}), fructose (from 44 to 56 g L^{-1}) and sucrose (from 16 to 48 g L^{-1}) as fermentable sugars (data not shown); proportion of these sugars in the juice can vary depending on season of the year, ripening stage and stage of decay of fruits.

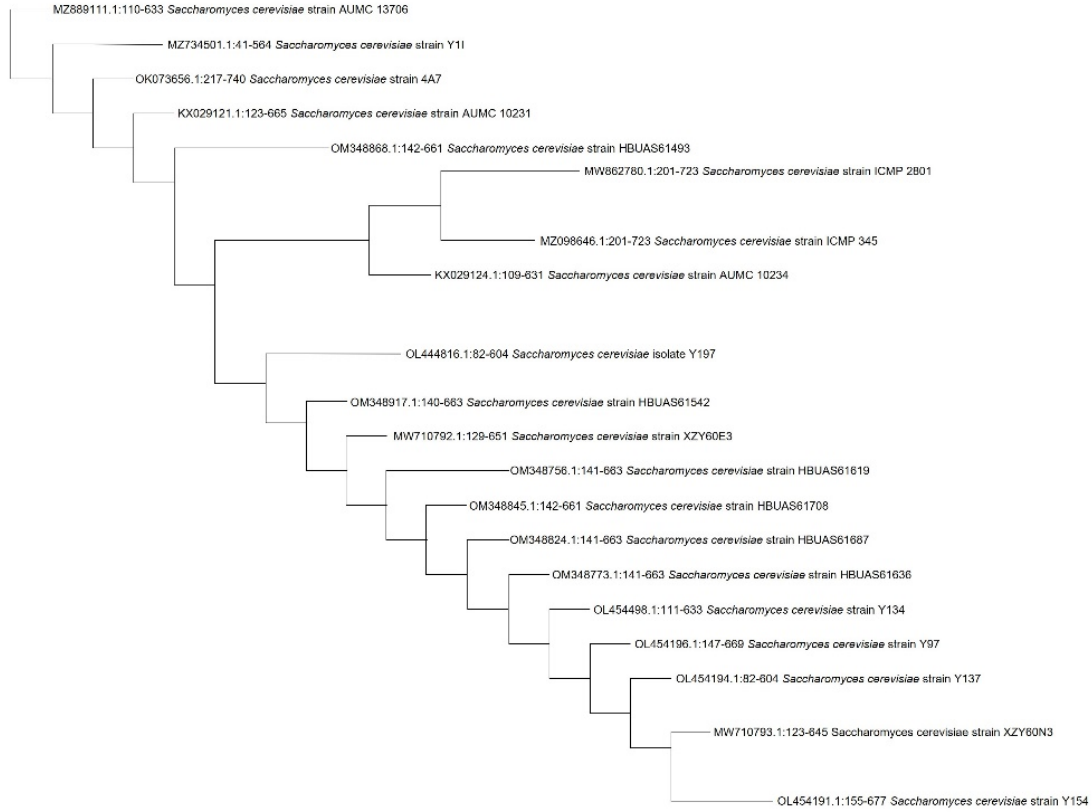

Figure 2. Phylogenetic analysis of the Internal Transcribed Spacer regions of *S. cerevisiae* AP1 with other 20 species of *S. cerevisiae*. The phylogenetic tree was built with the MEGA Software (www-megasoftware.net) based on a "Neighbor-Join Tree" test, the 20 hits used meet at least 94% coverage.

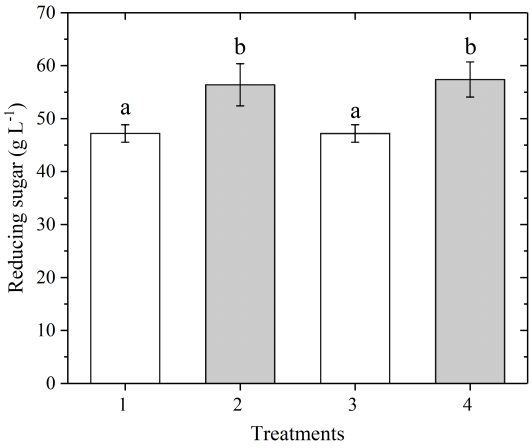

Figure 3. Effect of suspended solid and sterilization on the reducing sugar concentration. Values are expressed as means ± confidence intervals. Different superscript letters indicate significant differences among means (p-value < 0.05).

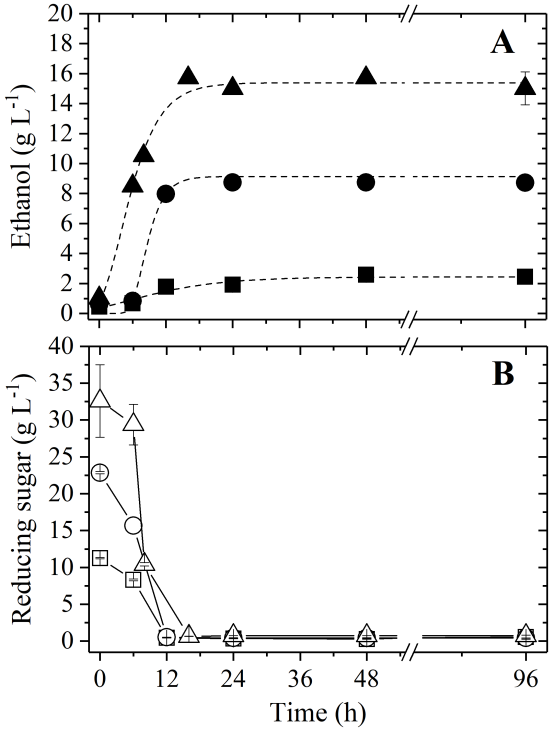

Figure 4. Ethanol production (A) and sugar consumption (B) during batch fermentation with immobilized *S. cerevisiae* biomass into polyester fiberfill cubes. Black-filled triangles (▲), circles (●) and squares (■) depict the ethanol concentration produced with 11.25, 22.86 and 32.58 g L^{-1} of reducing sugars, respectively. On the other hands, white triangles (Δ), circles (o) and squares (□) symbolize the residual concentration of reducing sugars. Dash lines (- - -) represent the fit of experimental data to the modified Gompertz model.

3.3 Batch fermentation of damaged fruit juice

The capability of ACPF cubes for transforming sugars from damaged fruits to ethanol was evaluated in batch experiments, at different initial concentrations of reducing sugars. Figure 4 depicts both ethanol production and reducing sugar consumption profiles at three different initial concentrations of reducing sugars.

It can be seen in Figure 4 that ethanol production by ACPF cubes was highly dependent on the initial concentration of carbon source. Indeed, ethanol production was increased with increasing the initial concentration of reducing sugars, as stated by Pinheiro *et al*. (2008) during the cashew apple juice fermentation by free cells of *S. cerevisiae* Saf-Instant. Moreover, ethanol production began immediately after inoculation of ACPF cubes to the production medium, with the exception of cultures with 22.86 g L^{-1} of reducing sugars where a 5.73 ± 0.843 h lag-phase (λ) was observed (Figure 4A). This lag-phase may be due more to an error in the ethanol quantification than a cellular adaptation period, since there was no a lag-phase in consumption of reducing sugars. In ethanol fermentation, lag periods at the beginning of process are frequently observed when either cells are not adapted to environment or are exposed to high substrate concentrations (mainly sugars), or are in the presence of substances than inhibit their growth (El-Dalatony *et al*., 2016; Jong-Sub *et al*., 2013).

Reducing sugar consumption by ACPF cubes in batch fermentations was according to ethanol production (Figure 4B). The stage of accelerated consumption of reducing sugars, and therefore the phase of accelerated production of ethanol, happened in the first 12 h of fermentation. In this period of time the sugar uptake rates were 0.89 ± 0.235, 1.86 ± 0.383 and 2.12 ± 0.653 g L^{-1} h^{-1} for cultures with 11.25, 22.86 and 32.58 g L^{-1} of reducing sugars, respectively. By contrast, Wu *et al.* (2014) found out that the sugar uptake rate of *S. cerevisiae* Wu-Y2 was independent on the initial concentration of reducing sugars; in glucose/galactose mixtures (20 g L^{-1}) with different proportions of monosaccharides. *S. cerevisiae* Wu-Y2 always consumed glucose and galactose at a rate of 1.25 g L^{-1} h^{-1} and 0.83 g L^{-1} h^{-1}, respectively, during the accelerated production of ethanol, regardless of the initial concentrations of monosaccharides in mixtures.

At the end of each batch fermentation, ACPF cubes had consumed more than 95% of the initial reducing sugars (Table 3).

Regarding ethanol production, evolution of this alcohol during batch experiments was satisfactorily modeled with the modified Gompertz equation; R^2 values for all production curves were greater than 0.94 (Figure 4A). In fermentations with an initial concentration of reducing sugars of 11.25, 22.86 and 32.58 g L^{-1}, the maximum ethanol concentration (P_{max}) was 2.44 ± 0.11, 9.14 ± 0.802 and 15.39 ± 0.267 g L^{-1}, respectively (Table 3). These P_{max} values were reached in fermentation times ranged from 16 to 48 h (Table 3); indeed, by increasing the initial concentration of reducing sugars in cultures, the time of P_{max} decreased. Volumetric ethanol productivity and ethanol yield (or fermentation efficiency) increased 19.2-fold and 2.13-fold when the initial concentration of reducing sugars increased from 11.25 up to 32.58 g L^{-1}, respectively (Table 3). Firoozi *et al*. (2022) fermented sugar beet molasses (about 80 g L^{-1}) with *S. cerevisiae* PTCC cell immobilized on L-lysine coated magnetite nanoparticles, and after 34 h, they yielded 0.43 gram of ethanol per gram of sugar, which was equivalent to a fermentation efficiency of 85.90%. Whereas ACPF cubes, in just 16 h, were able to yield 0.48 gram of ethanol per gram of sugar, i.e. fermentation efficiency of 94.77%, in cultures with 32.58 g L^{-1} of reducing sugars.

Table 3. Kinetic and stoichiometric parameters of batch fermentations of damage fruit juice with immobilized *S. cerevisiae* biomass.

	Initial sugar concentration (g L^{-1})		
Parameter	11.25	22.86	32.58
Maximum ethanol production time (t_F, h)	48	24	16
Sugar conversion (X_S, %)	95.57 ± 0.03	98.061 ± 0.013	97.72 ± 0.353
Maximum ethanol concentration (P_{max}, g L^{-1})*	2.44 ± 0.11	9.136 ± 0.802	15.39 ± 0.267
Yield ($Y_{P/S}$, g g^{-1})	0.227 ± 0.01	0.408 ± 0.034	0.483 ± 0.075
Volumetric productivity (QP, g L^{-1} h^{-1})	0.05 ± 0.002	0.381 ± 0.033	0.962 ± 0.026
Fermentation efficiency (η, %)	44.57 ± 0.02	79.921 ± 6.734	94.77 ± 8.828

*P_{max} was calculated by the modified Gompertz equation using non-linear regression ($R^2 > 0.94$)

Demiray *et al*. (2018) fermented a hydrolysate of pomegranate peel which content of reducing sugars was 12.01-14.14 g L^{-1} using *S. cerevisiae* cells and generated 4.37 g ethanol L^{-1} after 6 h, whilst ACPF cubes produced only 2.44 g ethanol L^{-1} after 48 h in similar culture conditions. Casabar *et al*. (2019) used a fungal hydrolysate of pineapple fruit peel wastes high in reducing sugars, 285.67 ± 17.21 g L^{-1}, as feedstock to generate ethanol by means of *S. cerevisiae* cultures. These authors apprised a maximum ethanol concentration of 5.98 ± 1.01 g L^{-1} at 24 h fermentation. On the other hand, ACPF cubes at 24 h were able to produce 1.53-fold more ethanol to that reported by Casabar *et al*. (2019), using an initial concentration of reducing sugars an order of magnitude lower. Abdullah *et al*. (2015) found a maximum ethanol concentration of 18.67 ± 1.60 g L^{-1} in fermentation of a sugar-rich oil palm frond juice (100.46 g L^{-1} of total sugar) after 24 h. Nevertheless, ACPF cubes achieved an ethanol production of 15.39 g L^{-1} in 16 h fermentation using a damaged fruits juice with only 32.58 g L^{-1} of reducing sugars.

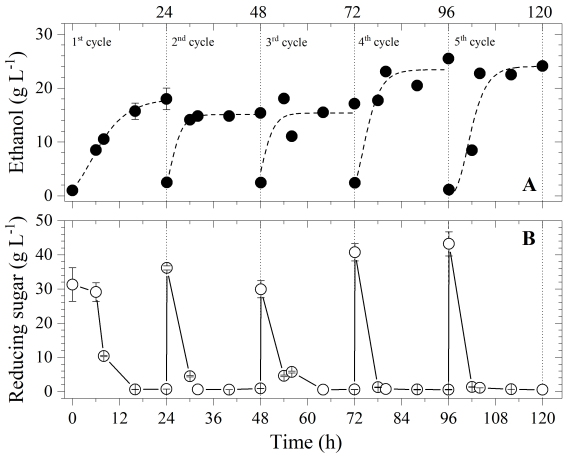

Figure 5. Profiles of ethanol production (A) and sugar consumption (B) throughout the 5-cycle repeated batch fermentation with immobilized *S. cerevisiae* biomass into polyester fiberfill cubes. Black-filled circles (•) and white circles (o) represent the concentrations of ethanol produced and sugar consumed, respectively. Dash lines (- - -) represent the fit of experimental data to the modified Gompertz model.

3.4 Reuse of ACPF cubes in batch fermentation

Due to ACPF cubes demonstrated to be efficient biocatalysts to ferment sugar concentrations greater than 30 g L^{-1} in batch experiments. Ethanol production was carried out in a five-batch repeated process using damaged fruit juice with a reducing sugar concentration among 30 to 43 g L^{-1} in order to evaluate stability and reusability of these biocatalysts. Figure 5 shows the ability of ACPF cubes to produce ethanol from damaged fruit juice in a repeated batch fermentation scheme.

In a repeated batch process, ACPF cubes were successfully able to ferment sugars from damaged fruit juice. The sugar conversion throughout process was higher than 97% (Figure 5B). Fermentation efficiency of each production cycle was higher than 80% and ethanol yield values were brought near to the theoretical ethanol yield, 0.51 g g^{-1} (Table 4). Volumetric ethanol productivity gradually increased in each production cycle from 1.130 to 2.914 g L^{-1} h^{-1}. Volumetric ethanol productivity values for the last four cycles were in average 2.41-fold higher than the first one. No batch exhibited a lag-phase thanks to the adaptation of *S. cerevisiae* AP1 cells to the fermentation environment (Figure 5), as stated by Plessas *et al*. (2007) during ethanol production with *S. cerevisiae* cells immobilized on orange peel.

Furthermore, fermentative capability of ACPF cubes was enhanced in each batch; either by decreasing the time of reaching P_{max} (from 18 to 8 h) or increasing P_{max} (from 15 to 23.3 g L^{-1}) (Table 4). In the first three production cycles, P_{max} values were statistically similar, ranging from 15.12 to 18.08 g L^{-1} (Figure 5A). Instead, in the fourth and fifth cycles, ethanol concentration increased considerately up to P_{max} values of 23.456 ± 2.511 and 23.309 ± 0.858 g L^{-1}, respectively (Figure 5A). At the end of repeated batch fermentation, ACPF cubes yielded 95.41 g ethanol L^{-1} in 44 h.

Table 4. Kinetic and stoichiometric parameters of repeated-batch fermentation of damage fruit juice by immobilized *S. cerevisiae* biomass into polyester fiberfill cubes.

Parameter	1st cycle	2nd cycle	3rd cycle	4th cycle	5th cycle
Maximum ethanol production time (t_F, h)	16	6	6	8	8
Initial sugar concentration (g L^{-1})	31.333 ± 4.913	36.196 ± 0.590	29.943 ± 2.500	40.781 ± 2.530	43.212 ± 5.501
Sugar conversion (X_S, %)	97.773 ± 0.359	98.016 ± 0.032	97.991 ± 0.169	98.367 ± 0.102	98.544 ± 0.189
Maximum ethanol concentration (P_{max}, g L^{-1})*	18.077 ± 1.619	15.118 ± 0.510	15.446 ± 3.100	23.456 ± 2.511	23.309 ± 0.858
Yield ($Y_{P/S}$, g g^{-1})	0.590 ± 0.102	0.426 ± 0.016	0.526 ± 0.127	0.585 ± 0.150	0.547 ± 0.059
Volumetric productivity (Q_P, g L^{-1} h^{-1})	1.130 ± 0.101	2.520 ± 0.085	2.574 ± 0.215	2.877 ± 0.314	2.914 ± 0.107
Fermentation efficiency (η, %)	109.595 ± 14.495	83.553 ± 3.099	103.222 ± 15.276	114.649 ± 18.930	107.328 ± 11.474

*P_{max} was calculated by the modified Gompertz equation using non-linear regression (R^2 >0.90)

It is worth noticing that ACPF cubes were reused without any metabolic regeneration step, i.e. they did not cultivate in a nutrient-enriched culture medium prior to fermentations. Despite not metabolically regenerating ACPF cubes, these never lost their fermentative capability in any batch, on the contrary, ethanol production improved in the last batches (Figure 5). A fermentative capability similar to ACPF cubes was reported by Liu *et al*. (2020) with *S. cerevisiae* 1308 cell immobilized onto sheet cotton fibers packed in porous hallow balls during fermentation of cassava supernatant (about 225 g total sugar L^{-1}). In that study, biocatalysts produced about 100 g ethanol L^{-1} in each cycle of a six repeated batch cultures with efficiencies higher than 92%. It has also been reported that *S. cerevisiae* NCIM 3640 cells immobilized on sugarcane pieces are efficient biocatalyst to produce ethanol from sugar cane juice (161.6 g reducing sugar L^{-1}) and molasses (151.6 g reducing sugar L^{-1}) in a repeated batch process. Babu *et al*. (2012) found out that *S. cerevisiae* NCIM 3640 cells attached to sugarcane pieces yielded in a stable way about 72.65-76.28 g ethanol L^{-1} in at least six repeated batch experiments, showing fermentation efficiencies higher than 91% and volumetric ethanol productivities up to 2.36 g L^{-1} h^{-1} after 30 h. Newly, Erkan Ünsal *et al*. (2023) tested the fermentative capability of *S. cerevisiae* ATCC 36858 cells immobilized on 3D-printed nylon spheres and they uncovered that these biocatalysts were able to produce over 37 g ethanol L^{-1} in fifteen cycles of repeated batch fermentation using a production medium with 100 g glucose L^{-1}. Nevertheless, biocatalysts gradually diminished their capacity for producing ethanol and uptake glucose after the seventh cycle.

El-Dalatony *et al*. (2016) remarked that the ability of *S. cerevisiae* ATCC 204679 cells immobilized into alginate beads to ferment *Chlamydomonas Mexicana* YSL008 biomass drastically decreased through seven cycles of repeated batch fermentation due to lack of essential nutrients that the production medium did not provide to the cells; decreasing ethanol production from 8.73 g L^{-1} at the first cycle to below 1 g L^{-1} at the seventh cycle. Drawback that was solved incubating biocatalysts in YPD medium for 12 h prior to each production cycle. After regenerating biocatalysts, ethanol production only remained stable four cycles, reaching a maximum ethanol concentration of 9.7 g L^{-1}, a volumetric productivity of 0.138 g L^{-1} h^{-1} and a fermentation efficiency of 79.5%. On the other hand, Bautista *et al*. (2022) successfully produced ethanol from sweet corn stalk juice (137.95-180.62 g total sugar L^{-1}) in batch system with *S. cerevisiae* TISTR 5020 cells immobilized in cotton balls, generating 62.12 g ethanol L^{-1} with a fermentation efficiency of 88.19%. However, these biocatalysts were not efficient to produce ethanol in three-cycles of repeated batch fermentation, which causing a decrease of fermentation efficiency up to 60.33%; this possible due to either a catabolite repression or a product inhibition or a high osmotic pressure in production medium.

Conclusions

In this study, ethanol production from Mexican fruit wastes using immobilized *S. cerevisiae* cells was investigated. In addition, a new strain of *S. cerevisiae* isolated from *Agave* sp. wastes was identified and classified as strain AP1. Fruit wastes demonstrated to be a suitable feedstock for the production of ethanol because they have a high content of fermentable sugars, they did not require a sophisticated pretreatment and they are found in large quantities in Mexican's cropland. On the other hand, Polyester fiberfill coated with alginate proved to be an excellent material for yeast immobilization, since it allowed the attachment of a large number of cells, it did not limit nutrient uptake not the ethanol release and it always maintained its structural integrity. Biocatalysts formed by *S. cerevisiae* AP1 cells immobilized into alginate-coated polyester fiberfill (ACPF)

cubes exhibited a high fermentative capacity, which was enhanced at high sugar concentrations. At reducing sugar concentrations greater than 30 g L^{-1}, ACPF cubes were able to yield about 0.48 g ethanol per g of sugar, showing fermentation efficiencies over 80%, regardless of the type of fermentation. Nevertheless, volumetric ethanol productivity enhanced from 0.962 to 2.914 g L^{-1} h^{-1} with increasing of reuse cycles of ACPF cubes, which led shorter maximum production times, from 16 h at the first cycle to 8 h at the fifth cycle. At the end of five cycles of repeated batch fermentation, ACPF cubes yielded in average 95.41 g ethanol L^{-1} in just 44 h process. In summary, it can be concluded that ACPF cubes are efficient biocatalysts to transform fruit wastes to ethanol; the higher the reducing sugar concentration, the higher the ethanol production and the shorter fermentation time. However, more studies must be carried out to determine feasibility of ACPF cubes to ferment agroindustrial wastes at the pilot plant level.

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