J Biochem Tech (2018) 9(1): 1-7
ISSN: 0974-2328

Comparative analysis of the kinetic growth parameters and ethanol production in non-*Saccharomyces* yeasts at the bioreactor level using *Agave cupreata* juice

González-Hernández Juan Carlos*, Hernández Esparza Manjury Jatziry, Luis Alberto Madrigal-Perez, Juan Alfonso Salazar-Torres, Radames Trejo Valencia

Received: 13 October 2016 / Received in revised form: 04 January 2018, Accepted: 04 January 2018, Published online: 09 January 2018

Abstract

The mezcal production in the state of Michoacán, located in central Mexico, is considered an informal activity with little to no regulation. Therefore, the object of this study was to analyze fermentation using a variety of yeast strains at the bioreactor level in order to establish statistical differences in the production of ethanol and biomass. *Kluyveromyces marxianus*, *Zygosaccharomyces bailli*, *Zygosaccharomyces rouxi*, *Pichia kluyveri* and *Isstchenkia terrícola* were added to *Agave cupreata* juice to carry out the fermentation process, it was placed in a bioreactor. Throughout this process, samples were taken every four hours in order to measure biomass, ethanol production, acetic acid, reducing sugars, glucose, fructose, pH, and cell growth. The yeasts studied had a significant ethanol production (50.16, 35.52, 36.37, 41.67 and 47.87 g/L, respectivaly). *P. kluyveri* biomass yield was 0.13 g/g, which indicates that it has great potential for the industrial production of ethanol using *Agave cupreata* juice.

Keywords: Mezcal, bioreactor, ethanol, non-Saccharomyces yeasts

González-Hernández Juan Carlos*, Hernández Esparza Manjury Jatziry, Luis Juan Alfonso Salazar-Torres

Tecnológico Nacional de México, Instituto Tecnológico de Morelia. Lab. Bioquímica. Ave. Tecnológico # 1500. C. P. 58120. Morelia, Michoacán, México

*Tel: (+52) 443 312 1570. Ext. 231
* E-mail: jcgonzal@itmorelia.edu.mx

Alberto Madrigal-Perez

Instituto Tecnológico Superior de Ciudad Hidalgo, Av. Ing. Carlos Rojas Gutiérrez #2120. C. P. 61100. Ciudad Hidalgo, Michoacán, México.

Radames Trejo Valencia

Tecnológico Nacional de México, Instituto Tecnológico de Minatitlán, Blvd. Institutos Tecnológicos S/N Col. Buena Vista Norte C.P. 96848. Minatitlán, Veracruz. México.

Introduction

The production of mezcal in the central Mexican state of Michoacán is elaborated artisanally with *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts present in the environment. Consequently, the yields are relatively low and the product is highly variable. Due to the non-standardization of the production process in this region, mezcal with different physical and chemical characteristics is obtained. Therefore, it is necessary to develop standard methods for mezcal production in Michoacán in order to produce a high quality product. Throughout history, techniques have been implemented using microorganisms in fermentation processes to fabricate a wide variety of goods, including enzymes, beverages, drugs, fuels, among others (Arrizon 2006). Yeasts are the ideal microorganisms for the production of mezcal, since they are responsible for alcoholic fermentation. These can be present in Agave or in the vinasses found in the environment causing spontaneous alcoholic fermentation.

Agave mezcal fermentation includes *S. cerevisiae* and non-*Saccharomyces species* (Damián et al., 2010). The spontaneous fermentations that occurs naturally without any external inoculation causes variations in the final product as well as undesired products such as glycerol, organic acids, and volatile compounds, which in addition to being toxic, directly influence the taste and aroma of the mescal (Caceres 2008). For this reason, it recommended to use yeast inoculums for which an optimum environment can be provided in order to produce the desired compounds, and thus, obtain an efficient yield in a shorter period of time. Therefore, it is vital to determine yeast species and genera that provide effective fermentation speed, sugar consumption, and flexibility for the control of mezcal sensory quality (De León et al., 2008).

Materials and Methods

Substrate source and culture medium preparation

In order to obtain *A. cupreata* juice, leaves were heated in an autoclave at 121 ° C for 20 minutes (Damián et al., 2010). Posteriorly, they were ground so as to obtain juice, which was subsequently filtered. To prepare the culture medium, 1600 mL of the *A. cupreata* juice was used. The sugar concentration

was adjusted to 12 ° degrees Brix implementing refractometry according to the classic sugar industry methodology by implementing an ABBE refractometer and adding distilled water. $(NH_4)2H_2PO_4$ salts were added in 0.1% concentration and the pH was adjusted to 5.5 Lastly, It was placed in the autoclave and sterilized for 20 minutes at 15 lbs / in at 121 ° C (Damián et al., 2010).

Microorganisms and preparation of the inoculum

Five yeast strains were isolated from spontaneous fermentations taken from local Mezcal production plants located in Etúcuaro, Michoacán, Mexico. The strains isolated and characterized by molecular thechniques were *K. marxianus var*. Drosophilarum, *Z. bailii*, *Z. rouxi*, *P. kluyveri* and *I. terrícola* (Damián et al., 2010). The strains were placed on Petri dishes with YPD medium, which consisted of 20% agar 10% casein bacto peptone, 10% yeast extract, 10% glucose, and 10% Agave juice. They were placed in a laminar flow hood (CV-2) under sterile conditions. Subsequently, they were incubated for two days at a temperature of 30 ° C and then cooled. For the formulation of the inoculum, 100 mL of *A. cupreata* juice was hydrolyzed, filtered, sterilized, and posteriorly placed in a 250 mL Erlenmeyer flask. By means of a culture loop, three to four fresh strains of the colonies present in the Petri dishes were used to inoculate the medium. All of these procedures took place under sterile conditions. After inoculation, the flask was placed in a shaking incubator (Shaker Incubator CVP-100B) at a temperature of 28 ° C For 24 h at 180 rpm (Damián et al., 2010).

Fermentation conditions in the bioreactor

The samples were fermented in a stirred tank type bioreactor (Applikon ADI 1010 Bio Controller). During this process samples were taken to evaluate cell growth and ethanol production as response variables. The fermentations were carried out for each yeast in duplicate by taking samples every 4 h for 60 h. The pH of the culture media was adjusted to 5.0; however, this was not a parameter that was monitored during fermentation. The concentration of reducing sugars in the hydrolyzed medium was adjusted to 12 °Brix with distilled water. The medium was enriched with ammonium phosphate $(NH_4)2H_2PO_4$ salts [1 g / L] as nitrogen source for the yeasts. For each test, an initial concentration of 3 X 10^6 cells / mL was inoculated into 1500 mL of fermentation medium in a 3000 mL pre-sterilized bioreactor, shaken at 150 rpm and heated to 28 ° C for 60 hours, an oxygen supply of 290 mL/min (0.1933 vvm). Samples were taken every four hours, measuring cell growth yeast doubling time (td), substrate consumption (glucose and fructose), ethanol production, and, acetic acid were calculated (Martínez-Corona 2016).

Analytical methods

Cell viability was determined by cell counting using a Neubauer chamber and trypan blue (1%), taking into account that the initial cell count was $3X10^6$ cells / mL (Martínez-Corona 2016). The concentration of reducing sugars in the hydrolyzed medium in addition to the dilution process was calculated using the DNS oxide reduction, which was performed using a modified version of methodology (González-Hernández 2012). In the presence of reducing sugars, 3, 5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid, resulting in a color change, which is measured by absorbance at a wavelength of 540 nm. Reducing sugars were measured taking samples every four hours throughout the fermentation process using this phenomenon. The concentration of sugars constituting the hydrolyzed medium and the substrates in the fermentation samples were estimated every eight hours using Megazyme® (glucose and fructose) enzyme assays. The main fermentation products were calculated implementing enzymatic test with kits Megazyme® for ethanol and acetic acid. Changes in sample absorbance were measured on a Jenway 6305® UV-Visible spectrophotometer.

Logistic equation

Cell growth adjustment with the logistic equation was made with the model proposed by Verhulst in 1838, which proposes a model in which growth slows as density increases, called "logistic model or logistic equation" (Martínez et al., 2004). The logistic equation proposed by Verhulst is described below, which is a population differential with respect to time, which, when integrated, gives an approximation of real population behavior.

$$\frac{dN}{dt} = \mu N \left(1 - \frac{N}{N_{máx}}\right)$$

Where:
μ= Specific growth speed
N= Population
$Nmáx$ = Maximum population reached in a determined time

The integrated equation is shown below:

$$N(t) = \frac{N_o \; N_{máx}}{(N_{máx} - N_o)e^{-\mu t} + N_o}$$

Where:
μ= Specific growth rate
No = Original population
$Nmáx$ = Maximum population reached in a determined time
t = time

Application of unstructured model

Due to the fact that the cultivation medium used was Agave juice, which is characterized by high levels of fructose and glucose, these two sugars were taken as limiting substrates. Furthermore, ethanol was taken as a product, being the product with the greatest quantity due to the type of microorganism used, as well as inhibition due to the same, and a product partially linked to growth, due to the fact that it had the greatest adjustment. In summary, behavior is subject to the following characteristics: Monod Model, Inhibition by product (Et-OH), Two limiting substrates (Cs1= Glucose and Cs2 = Fructose), partially linked to growth. Below are the equations used for the characteristics used before: Substrate consumption equation.

$$\frac{dC_s}{dt} = -Y_{\frac{s}{x}} \frac{dC_x}{dt}$$

Note: given that there are two substrates, the equation is the same for both, with only the subscript changing in Cs from 1 to 2, where 1 is glucose and 2 is fructose. Equation for biomass production with inhibition by product and two limiting substrates.

$$\frac{dC_x}{dt} = \mu_{máx} \left(\frac{C_{S1_o}}{k_{S1} + C_{S1_o}}\right)\left(\frac{C_{S2_o}}{k_{S2} + C_{S2_o}}\right)\left(1 - \frac{Cp}{Cpmáx}\right)^n Cx$$

Product formation:

$$\frac{dP}{dt} = \alpha \frac{dC_s}{dt} + \beta Cx$$

Where:
Cs = Concentration of substrate.
Cx = Concentration of biomass.
$CS1o$ = Original glucose concentration
$CS2o$ = Original fructose concentration
Cp = Original product concentration.
$Cpmáx$ = Maximum ethanol concentration produced by the yeast.
$kS1$ = Saturation constant or affinity for glucose.
$kS2$ = Saturation constant or affinity for fructose.
α = Production coefficient associated with growth (product yield over biomass).
β = Production coefficient unassociated with growth (specific rate of product formation).

Statistical analysis

One-way ANOVA tests were carried out for each of the variables corresponding to each of the different strains and then a Tukey-Kramer test was performed using JMP 6.0® software.

Results

In Table 1, the kinetic parameters of microbial growth of the strains used in the present study were determined. Characteristics of the different growth stages obtained from the experiments shown are in Fig. 1. From the results obtained, the yeasts that were used in this study all demonstrated distinct characteristics. *K. marxianus* presented its exponential phase at 16 h, as did the yeasts *Z. bailli* and *I. terrícola*. In contrast, *Z. rouxi* and *P. kluyveri* ended their exponential phase at 12 h (Fig. 1). The results obtained show that *K. marxianus*, *Z. bailli*, and *I. terrícola* demonstrated the shortest doubling times (td) and had their exponential phase at 12 h. While *Z. rouxi* and *P. kluyveri*, which presented their exponential phase at 16 h, had a longer doubling time. *P. kluyveri* demonstrated the longest duplication time. The specific growth rate (μ) was also recorded and the doubling time of each of the yeasts was used to carry out the relevant calculation.

Yeasts *Z. rouxi* and *P. kluyveri*, showed a lower specific speed of growth while the remaining yeasts used, *K. marxianus*, *Z. bailli*, and *I. terrícola*, recorded a higher specific growth rate. On the basis of the results obtained, the division speed (δ) was recorded, in which the yeasts *Z. rouxi* and *P. kluyveri* obtained relatively low cell division times unlike the other yeasts.

K. marxianus reported the highest division rate. The biomass/substrate yield (Yx/s) was determined on the basis of the final hour of the fermentation (hour 60). Yeasts *Z. rouxi* and *P. kluyveri* showed the high yields with 0.11 and 0.13 (g/g) respectively, while *Z. bailli* obtained the lowest yield (0.02 g/g).
Table 1 also shows the product/substrate yield (Yp/s) for each yeast, which was calculated based on the time at which the maximum ethanol production was obtained. For each of the yeasts this value was distinct. *K. marxianus*, *Z. rouxi* and *I. terricola* yeasts recorded the highest ethanol production at 40 h, while at 36 h *Z. bailli* presented the highest ethanol production. On the other hand, *P. kluyveri* recorded the highest ethanol production at 28 h.

Based on the results obtained, *K. marxianus* recorded the highest product/substrate yield followed by *P. kluyveri* (0.80 and 0.61 respectively) while *Z. bailli* having the lowest yield. Statistical analysis was performed for each of the parameters recorded and a variance analysis was obtained. A Tukey-Kramer analysis of the product yield/substrate (Yp/s) of *K. marxianus* differed statistically *Z. bailli*, *Z. rouxi*, *P. kluyveri* and *I. terricola*. Whereas, *Z. rouxi* and *I. terricola* did not differ statistically from each other (Table 2).

Table 1.- Kinetic growth parameters of microbial non-*Saccharomyces yeasts*.

Yeast	td (h)	μ (1/h)	δ (1/h)	Y x/s (g/g)	Y p/s (g/g)
K. marxianus	1.33	0.519	0.749	0.04	0.80
Z. bailli	1.35	0.513	0.741	0.02	0.34
Z. rouxi	1.70	0.407	0.588	0.10	0.41
P. kluyveri	1.82	0.380	0.548	0.13	0.61
I. terricola	1.35	0.511	0.737	0.03	0.40

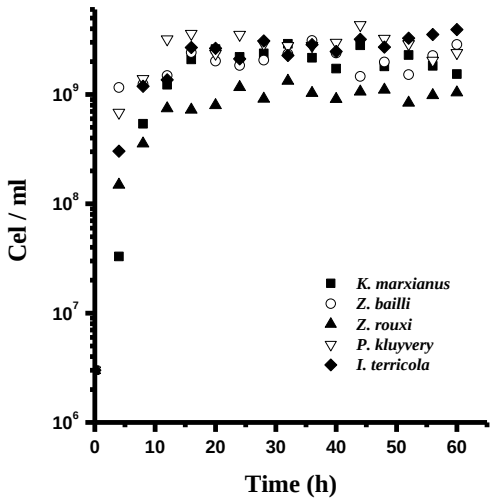

Figure 1: Cell growth with yeasts cultivated in YPD. $3X10^6$ cel/mL were incubated in 1500 mL of *Agave cupreata* juice (initial pH 5, 12°Brix, 28°C, 150 rpm, 0.1933 vvm , n = 3).

Another way of representing population behavior is by means of normal distribution, or the Gauss curve, which is a graphic representation of a continuous, symmetric probability function whose maximum coincides with the average (in this case the inflection point or specific maximum velocity of cell growth).
The average value (μ) influences the graphic location, moving to the right or away from zero when it is greater, and to the left when the value is less. Standard deviation directly influences the shape of the bell curve, less diffusion and the curve steepens, with values greatly concentrated around the average, and with greater deviation the curve levels out.
Below are the cell growth charts adjusted using the logistic equation as well as normal distribution. According to the charts above, for the kinetics performed with the pure strains of each microorganism you can see what real growth should look like according to the logistic equation, where the yeasts *K. marxianus* and *Z. rouxii* are the yeast strains that best adjust or best coincide with the experimental data obtained, while the adjustment of *I. terricola* and *P. kluyvery* range further from the experimental data.

Below are the cell growth charts adjusted using the logistic equation as well as normal distribution. According to the charts above, for the kinetics performed with the pure strains of each microorganism you can see what real growth should look like according to the logistic equation, where the yeasts *K. marxianus* and *Z. rouxii* are the yeast strains that best adjust or best coincide with the experimental data obtained, while the adjustment of *I. terricola* and *P. kluyvery* range further from the experimental data.

Table 2: Tukey-Kramer Analysis

Yeast	Y x/s (g/g)	Y p/s (g/g)	t_d (h)	Ethanol (g/L)	Acetic Acid (g/L)
K. marxianus	0.80^{A}	0.04^{B}	1.340^{D}	50.16^{A}	1.21^{B}
Z. bailli	0.34^{D}	0.02^{B}	1.350^{C}	32.52^{D}	0.76^{C}
Z. rouxi	0.41^{C}	0.11^{A}	1.702^{B}	36.37^{CD}	0.68^{C}
P. kluyveri	0.61^{B}	0.13^{A}	1.824^{A}	41.67^{BC}	0.17^{D}
I. terricola	0.40^{C}	0.03^{B}	1.357^{C}	47.84^{AB}	2.11^{A}

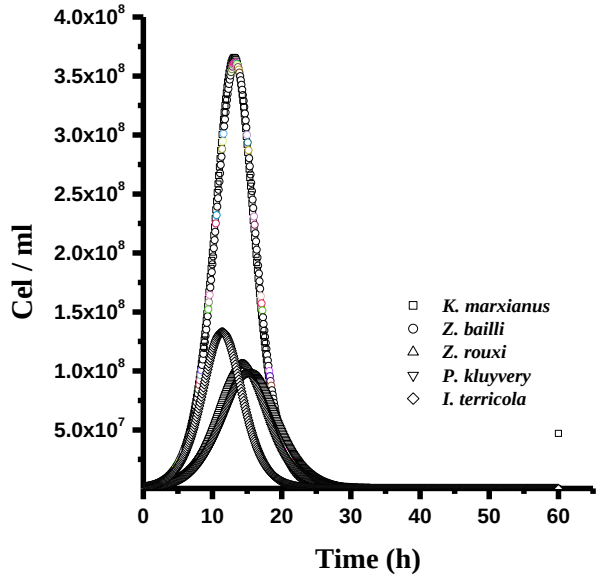

Figure 2.- Representing population behavior by means of normal distribution, or the Gauss curve. Cell growth with yeasts cultivated in YPD. $3X10^6$ cel/mL were incubated in 1500 mL of *Agave cupreata* juice (initial pH 5, 12°Brix, 28°C, 150 rpm, 0.1933 vvm, n = 3).

This can be attributed to experimental errors that cause erroneous values in the kinetic parameters obtained, as derived function requires, for example, the specific growth rate, or directly corresponds to the original and final biomass values, which vary due to error during cell count in the Neubauer chamber. According to the characteristics of the Gauss bell curve, the value of the average or value of the specific growth rate is quite similar for the yeasts *K. marxianus* and *Z. rouxii* (Table 1). Consequently, the position of the graph shows the same movement to the right with times above 10 hours. There are several physicochemical factors that can come to alter the growth of microorganisms during fermentation, among which Togores reports in 2011 are: temperature, aeration, atmospheric pressure, nutrient availability, among many others.

Note that some of these factors may not be taken into account, given that the fermentation trials reported in this paper were conducted under the same conditions in terms of aeration, pressure, temperature, and pH, as can be corroborated in the methodology.

The set of equations was solved using the Euler numeric method, or Tangent method, which makes up the simplest example of the numeric method for solving an original value problem. The known original values, the specific growth rate, and yields were entered. The values obtained using the Euler method were compared with real data and the squares of the differences between the Euler values and the experimental values were obtained to finally give us the sum of the quadratic values.

Finally, by solving in Excel, the sum of squares was taken as objective variable and the variables $kS1$, $kS2$, α and β, were combined in order to minimize the value of the objective variable and so adjust the real data to the Euler data inasmuch as possible.

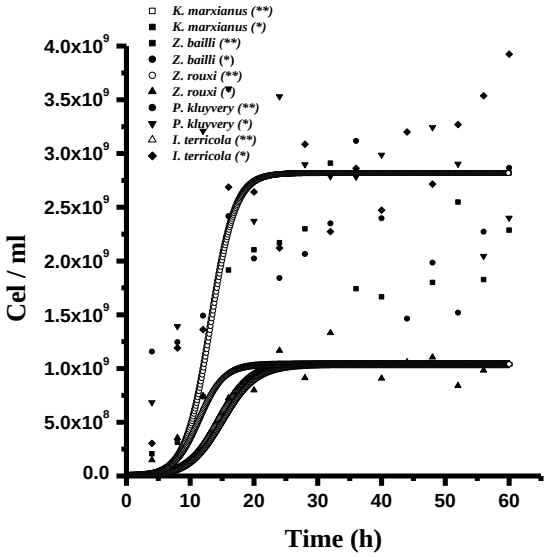

Figure 3.- Cell growth charts adjusted using the logistic equation as well as normal distribution. Cell growth with yeasts cultivated in YPD. $3X10^6$ cel/mL were incubated in 1500 mL of *Agave cupreata* juice (initial pH 5, 12°Brix, 28°C, 150 rpm, 0.1933 vvm, n = 3) (* Experimental results, ** Using logistic equation).

This paper focuses on the unstructured models, being the simplest growth models, which are expressed in terms of abstract life units, generally the term microbial population or "biomass" is used, which completely ignores the internal structure of the cells that make up said biomass, as the population is considered a homogeneously distributed unit. Although unstructured models are a major simplification of the real issue, they tend to be useful for technological purposes, as they provide simple equations with a physical sense, in which the microorganism is treated as a simple reactant species.

In table 3 an example is shown of a comparison of experimental data vs. data obtained using the Euler method, which are modified once variables are adjusted until the objective variable is minimized, which is nothing but the sum of the squared differences. In the ethanol column we can see that once the variables $kS1$, $kS2$, α and β are modified, the Euler values approximate the real data in the given time as much as possible. In this case, and for the example trial, a minimum value for the objective variable of 329.85 was obtained, it being impossible to reduce it any more in accordance with the values given for other parameters that were obtained as yields, and which are not modified. According to the graphs above (data not show), if the objective variable is taken as a reference you can see that the yeast *Z. bailli* best adjusts to the behavior given by Euler, as it has the smallest value compared to the other yeasts.

Discussion

In the Tukey-Kramer analysis shown in Table 2 for the biomass/substrate yield (Yx/s) the *Z. rouxi* and *P. kluyveri* did not differ statistically from each other but differ statistically from the others (*K. marxianus*, *Z bailli* and *I. terricola*). On the

contrary, the latter did not show any statistical differences in the Tukey-Kramer Analysis. In the Tukey-Kramer analysis of the doubling time (td), *Z. bailli* and *I. terricola* were not statistically different from *K. marxianus*, *Z. rouxi* and *P. kluyveri*. However, the latter demonstrated statistical differences among them for this parameter. For the Tukey-Kramer analysis of ethanol production during fermentation, there were no statistical differences between *K. marxianus* and *I. terricola*. Nor were there differences between *P. kluyveri* and *I. terricola*. *Z. rouxi*. In addition, *Z. bailli* did not differ statistically from *P. kluyveri*.

In the Tukey-Kramer analysis of the production of acetic acid, *Z. bailli* and *Z. rouxi* showed statistical differences. On the contrary, *K. marxianus*, *P. kluyveri* and *I. terricola* statistical differences were observed (Table 2).

In the literature, reported maximum ethanol production using *Agave fourcroydes* (Granchi 2003). Their methodology included adjusting the mixture to 12°Brix and allowing it to ferment for 48 hours. To carry out the fermentation they used five different proportions of *K. marxianus* Cicy-Ki and *S. cerevisiae* Safoeno (Safmex SA de CV, Mexico). The ratios implemented were 0/100, 25/75, 50/50, 75/25, 100/0, and at $3X10^{-7}$ cells/mL concentration. The statistical analysis revealed that the 25/75% ratio had a maximum ethanol production of 5.22 ± 1.09 v/v, and the ratio 0/100% resulted in an ethanol production of 4.29 ± 0.14% v/v.

A study reported the biomass yield and volatile compound content produced during Agave fermentation. Yeasts isolated from natural environments, *K. marxianus* and *S. cerevisiae*, were used in the experiment (López-Alvarez 2012., Arellano-Plaza et al., 2017). These yeasts are commonly implemented in the fermentation of Agave species for tequila production. The results obtained and reported in numerous industrial fermentation experiments, *K. marxianus* has demonstrated production of 54.2 g of ethanol / L of extract after 72 h of fermentation. On the other hand, *S. cerevisiae*, produced 39.4 g of ethanol / L of extract. In the study carried out with *A. cupreata* juice, ethanol production using *K. marxianus* after 40 hours was 50.16 g ethanol / L of juice, followed by *I. terricola* at hour 40 showing an ethanol production of 47.84 G ethanol / L and *P. kluyveri* at hour 28 with 41.67 g ethanol / L. In contrast is reported, the difference in fermentation time for ethanol production is significant compared to each of the yeasts studied (Moo-Young 1994). In order to optimize fermentation conditions in mezcal production processes from the fructans contained in the heart (stem and leaf base of the plant) of *A. salmiana*, is reported a study that demonstrated that the fermentation process conditions affect the quality of the mezcal as well as the amount of ethanol produced. The highest production of ethanol obtained was (37.7 g / L) at a temperature of 28°C. The initial sugar concentration was 105 g / L, however, the maximum productivity of the process was obtained at 34.6 ° C with a sugar concentration of 90 g / L. However, the combination that resulted in the maximum yield of the product and the highest quality mezcal was obtained when the fermentation was carried out at a temperature of 28 ° C with an initial sugar concentration of 77 g / L. This is in contrast to the results found using *A. cupreata*. It was shown that the highest ethanol production regardless of the yeast used was 50.16 g ethanol / L at a temperature of 28 ° C and with a reducing sugar concentration of approximately 120 g / L. The organoleptic quality of the distilled liquors produced by the fermentation of sweetened juices is one of the most important parameters in the quality of the formulations (Pérez et al., 2013., Pérez et al., 2016). The production of acetic acid significantly affects the quality of the mezcal. The results in the study obtained demonstrate that the concentrations of this by-product are relatively low using the parameters previously mentioned.

While *P. kluyveri* has an adjustment due to the Euler method considerably removed from real values, showing the greatest objective variable value among the yeasts studied.

Table 3 lists the kinetic parameters for the yeast trials, where the value for each variable and their kinetic parameters can be seen. If we emphasize saturation constants ($kS1$, $kS2$) for each substrate (glucose and fructose), we see that the saturation constant for fructose ($kS2$) produces values of zero or near zero in every trial, a characteristic that can be attributed to the fact that fructose, unlike glucose, is found in much higher amounts (a 5:1 ratio, approximately), which means that it is not a limiting substrate as such. Furthermore, as Dumont et al., 2008, said, in enological conditions, the main sugars that can be fermented by *S. cerevisiae* are glucose and fructose. These two hexoses found in agave musts can vary the proportion of some musts. *S. cerevisiae* prefers to consume glucose, which explains why, when fermentation stops, the remaining sugars are made up primarily of fructose, which poses the question of the yeast's capacity for consuming this hexose.

Table 3: Kinetic parameters obtained with the application of unstructured model.

Parameter	*K. marxianus*	*Z. bailli*	*Z. rouxii*	*I. terricola*	*P. kluyveri*
µmáx (h^{-1})	0.519	0.513	0.407	0.511	0.38
Ks1 (mM)	5.563	13.622	2.411	4.816	3.312
Ks2 (mM)	0	0.001	0.0013	0.001	0.0003
Ys1/x (g/g)	0.423	1.566	0.7363	0.736	0.4077
Ys2/x (g/g)	12.769	9.269	8.6929	8.692	8.3241
Cs10 (g/L)	2.587	7.562	4.0799	4.079	5.5725
Cs20 (g/L)	63.03	46.375	44.869	44.869	88.535
Cx0 (g/L)	0.2	0.2	0.2	0.2	0.2
Cp0 (g/g)	2.581	2.740	2.4958	2.495	3.9348
Cpmáx (g/g)	50	50	50	50	50
YpEt-OH/x (g/g)	7.998	3.906	4.7725	4.772	2.6634
β	0.095	1.611	11.595	10.590	18.421
α	9.881	4.544	2.4890	2.5539	1.5849
n	1	1	1	1	1
h (h^{-1})	0.01	0.01	0.01	0.01	0.01
Objective Variable	914.8860	329.85	616.51	661.155	2303.80

Sugar utilization kinetics by *S. cerevisiae* during fermentation are carried out mostly through the transportation of sugars, and, as a general rule, glucose is consumed at a faster rate than fructose. In slow fermentation, the maximum fermentation rate is reduced when most of the glucose has been consumed, and fermentation can stop when a significant concentration of fructose remains.

If values are compared for the saturation constant for glucose ($kS1$) we see that higher values were obtained for two pure yeasts *Z. bailli* and *K. marxianus* (13.56 and 5.56 mm, respectively). Zapata et al., 2005, assessed the kinetic growth parameters of *S. cerevisiae* in presence of a low-intensity, high-frequency variable magnetic field in molasses (raw honey), in which they obtained constant saturation values between 34 and 74 g/L. Furthermore, Postma et al., 1989, assessed glucose competence between the yeasts *S. cerevisiae* and *C. utilis*, obtaining constant saturation values of up to 110 g/L. Consequently, compared to the experimental values obtained for this project, it can be said that the values are all within acceptable range. Furthermore, the studies mark constant saturation values for glucose from 5 to 50 g/L. Note that the smaller or nearer the constant saturation value is to

zero, the greater the microorganism's affinity for the substrate. The substrate value depends in large part on, and will vary according to, the conditions in which the microorganism grows, given that different factors can improve or impede their growth. Another interesting data point in the table 3 is that biomass product yield (YP Et-OHX) compared to the value of α (which is, in the end, the biomass-product yield associated with growth), are practically the same, given that the value obtained experimentally was not taken, rather the mean of the Excel program iterations.

Agave juice is characterized as having large quantities of fructose, less glucose, and sucrose in much lower concentrations. The sugar content of the agave must used for making tequila usually ranges between 40 and 140 g/L of fermentable sugars (containing around 9-15% glucose, 80-92% fructose, and 2-5% other sugars) (Mellado and López, 2013). *S. cerevisiae* consumes glucose easier than it does fructose. However, in conditions in which glucose is scarce, it will consume fructose, although at a slower rate.

In table 3 an example is shown of a comparison of experimental data vs. data obtained using the Euler method, which are modified once variables are adjusted until the objective variable is minimized, which is nothing but the sum of the squared differences. In the ethanol column we can see that once the variables $kS1$, $kS2$, α and β are modified, the Euler values approximate, as much as possible, the real data in the given time. In this case and for the example trial, a minimum value for the objective variable of 329.85 was obtained, it being impossible to reduce it any more. With regards to saturation constants, a zero or near-zero value was obtained for fructose given that it is found in elevated quantities as opposed to glucose. Consequently, it is not considered a limiting substrate. Saturation constant values for glucose are within range compared to values reported in the literature.

Conclusions

Due to its high content of total reducing sugars and the high production volumes, *A. cupreata* leaves are a promising raw material for the industrial production of mezcal. In the process of ethanol production by fermentation of the *A. cupreata* fructans, the *K. marxianus* presented certain advantages over the other yeasts studied. For example, it demonstrated higher ethanol yields and shorter duplication times. However, *P. kluyveri*, had a significant ethanol yield, the shortest fermentation time (28 h), the maximum yield of biomass / substrate in addition to demonstrating lower production of acetic acid. For this reason, *P. kluyveri* has too promising potential for the industrial production of mezcal. While *P. kluyveri* has an adjustment due to the Euler method considerably removed from real values, showing the greatest objective variable value among the yeasts studied.

The present comparative analysis of fermentation at the bioreactor level allows us to establish statistical differences in the ethanol and biomass production of the five yeasts isolated from industrial mezcal consortia. These results will allow for further study using the three highest performing strains to evaluate ethanol production, biomass, and organoleptic properties in order to improve and standardize the production of mezcal in the state of Michoacán. This study was carried out with the purpose of explaining the problems faced by the agave-mezcal production chain and to outline scientific strategies to solve them. By providing solutions to these issues, the goal is to achieve a position in the national and international market by producing a product that complies with the chemical characteristics established by Mexican Mezcal Regulatory Council's standards.

Acknowledgements

The authors would like to thank Tecnológico Nacional de México (**Proyecto 5368.14-P**) for the funding that they provided (Arcos de Belén Núm. 79, Colonia Centro, Delegación Cuauhtémoc, C.P. 06010, México, D.F.).

References

Arrizon J, Concetta F, Acosta G, Romano P, Gschaedler A (2006) Fermentation behaviour and volatile compound production by agave and grape must yeasts in high sugar and grape must fermentations. Anton Leeuw 89 (1):181-189.

Arellano-Plaza M, Noriega-Cisneros R, Clemente-Guerrero M, González-Hernández JC, Robles-Herrera PD, Manzo-Ávalos S, Saavedra-Molina A, Gschaedler-Mathis A (2017) Fermentative capacity of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* after oxidative stress. J Inst Brew 123(4): 519-526.

Cáceres M, Lappe Oliveras P, Larqué Saavedra S, Magdub Méndez A, Barahona Pérez L (2008) Producción de etanol a partir de jugo de henequén (*Agave fourcroydes* Lem.) y melaza utilizando una mezcla de levaduras. Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México.

Damián Robles RM, Pérez E C (2010) Caracterización molecular y fermentativa de una levadura utilizada para la producción de mezcal en la región de Etúcuaro, Michoacán. Departamento de Ingeniería Bioquímica, Instituto Tecnológico de Morelia: Morelia, Michoacán, México

De León R, Escalante M, Barba R, Blaschek P (2008) Optimization of fermentation conditions for the production of the mezcal from *Agave salmiana* using response surface methodology. Chem Eng Process 47: 76-82

Dumont A, Raynal C, Raginel F, Ortiz-Julien A, Suarez C, Heras JM (2008) Capacidad de las levaduras enológicas de consumir fructosa. La semana vitivinícola 3237: 2502-2507

Durán H, Pulido J (2007) Análisis de la Molienda en el Proceso de Elaboración de Mezcal. Información Tecnológica 18: 47-52

González-Hernández JC, Farías Rosales L, Vera Villa JC, Martínez Corona R. Álvarez-Navarrete M, Zamudio-Jaramillo MA, Chávez-Parga MC, Peña, A (2012) Chemical hydrolysis of the polysaccharides of the tamarind seed. J. Mex. Chem. Soc 56: 395-401

Granchi L, Guerrinni S, Bastianini A, Blalotta G, Moschetti P, Vincenzini, M (2003) Phenotypic and genotypic characterization of *Oenococcus oeni* strains isolated from Italian wines. Int. J. Food Microbiol 83: 1-14.

López-Alvarez JA, Díaz-Pérez AL, Campos-García J (2012) Estudio comparativo de rendimiento y contenido de compuestos volátiles en un proceso de elaboración de Tequila a nivel industrial. Ciencia Nicolaita 57: 41-52

Martínez-Corona R, Cortés Penagos C, Chávez-Parga MC, Alvarez-Navarrete M, and González-Hernández JC (2016) Analysis of the Effect of Agitation and Aeration on Xylitol Production by Fermentation in Bioreactor with *Kluyveromyces marxianus* Using Hydrolized Tamarind Seed as Substrate Int Journal of Current Microbiol App Sciences 5(6): 479-499

Martínez J, Wrent P, Quirós M, Socota R, Silóniz M, Valderrama M (2004) Alteración de embutidos por levaduras productoras de gas. Alimentación, equipos y tecnología, 72-78

Mellado-Mojica E, and López-Pérez M (2013) Análsis comparativo entre jarabe de agave azul (*Agave tequila* Weber var. azul) y otros jarabes naturales. Agrociencia 47: 233-244

Moo-Young M, Chisti Y (1994) Biochemical engineering in biotechnology. Pure and Appl. Chem 66(1):117-136

Pérez E, González-Hernández JC, Chávez-Parga MC, Cortés-Penagos C (2013) Fermentative characterization of producers ethanol yeast from *Agave cupreata* juice in mezcal elaboration. Rev. Mex. Ing. Quim 12(3): 451-461

Pérez Hernández E, Chávez Parga Ma. del C, González Hernández JC (2016) Review of agave and mezcal. Rev. Colomb. Biotecnol XVIII (1) 148-164

Postma E, Kuiper A, Tomasouw F, Scheffers A, Van DijKen JP (1989) Competition for glucose between the yeast *Saccharomyces cerevisiae* and *Candida utilis*. Applied and Envirom Microbiol 55(12): 3214-3220

Togores JH (2011) Tratado de enología I Vol. I Ediciones Mundi-Prensa