Chapter 8

Continuous Agave Juice Fermentation for Producing Bioethanol

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Additional information is available at the end of the chapter

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1. Introduction

The production and utilization of fossil fuels introduce several negative environmental impacts. Bioenergy and biobased products are not a panacea for these problems. However, the environmental burden from use of biorenewable resources is generally much less than from the use of fossil resources. Biofuels include fuels derived from biomass conversion, as well as solid biomass, liquid fuels and various biogases. Forest biomass, agricultural residues and energy crops constitute the three major sources of biomass for energy, with the latter developing into probably the most important source in the 21st century. Land use and the changes thereof is a key issue in sustainable bioenergy production as land availability is ultimately a limiting factor [1]. Biodiesel and bioethanol are the main biofuel. Biodiesel can be made from vegetable oils, microalgae, and animal fats; on the other hand, bioethanol is an alcohol made by fermentation, mostly from carbohydrates produced by sugar or starch crops such as corn or sugarcane, as well as from non-food sources such as agricultural residues. Nevertheless, these processes require as an additional step, prior to saccharification, making production a difficult and expensive. Using agave plants as raw material could be a viable alternative to bioethanol production.

2. Microorganisms involved in the bioethanol production

There is an ever-growing demand for new and improved bioethanol production microorganism strains. Desirable characteristics of bioethanol production microorganisms are listed in Table 1.

Ethanol production microorganisms, mainly *Zymomonas mobilis* and *Saccharomyces cerevisiae,* are potential candidates for bioethanol productions because they showed many of the characteristics presented in the table 1. However, *Zymomonas mobilis* strains have attracted much attention because their growth rate is higher than that of *Saccharomyces cerevisiae*,

conventionally used microorganisms for commercial bioethanol production. *Zymomonas mobilis* has been used in tropical areas for making alcoholic beverages from plant sap [2], but its narrow spectrum of fermentable carbohydrates has hampered its industrial exploitation [3]. Several researchers have taken on the challenger on developing recombinant organisms, including: *S. cerevisiae*, *Z. mobilis*, *Escherichia coli*, *Klebsiella oxytoca* and *Erwinia herbicola* [4-5], but the bioethanol production from biomass materials by genetically engineered strains has not yet reached a sufficient level for commercial application [6]. *Zymomonas* cells are gram-negative rods; a minority of the strains are motile, with 1 to 4 polar flagella. These organisms need glucose, fructose, or (for some strains) sucrose in the growth medium. They are very unusual microorganisms since they ferment these sugars anaerobically by way of the Entner-Doudoroff mechanism, followed by pyruvate decarboxylation. The oxidation-reduction balance between G6P dehydrogenase and triosephosphate dehydrogenase on one hand and ethanol dehydrogenase on the other, is mediated through NAD^+. Sugar fermentation is accompanied by formation of a small amount of lactic acid, with traces of acetaldehyde and acetoin [2].

Fermentation Properties	Technological Properties
• Rapid initiation of fermentation • High fermentation efficiency • High ethanol tolerance • High osmotolerance • Low temperature optimum • Moderate biomass production	• High genetic stability • Low foam formation • Flocculation properties • Compacts sediment • Low nitrogen demand

Table 1. Desirable characteristics of bioethanol production microorganisms

The simplified fermentation process is:

$$C_6H_{12}O_6 + (\text{carbon source}) \rightarrow 1.8\ CH_3CH_2OH\ +\ 1.8\ CO_2\ + \\ +\ 0.2CH_3CH(OH)COOH + \ 0.22\ CH_2O\ +\ ATP + 32.7\ \text{kcal} \quad (1)$$

The molar growth yield indicates that *Zymomonas* is only about 50% efficient in converting its carbon and energy sources. Growth is partially uncoupled. About 2% of the glucose substrate is the source of about half of the cellular carbon. Several amino acids also serve as carbon sources. Some strains grow only anaerobically; others display various degrees of microaerophily. Apparently, the main effect of oxygen is the oxidation of part of the ethanol which converts into acetic acid. Most strains are alcohol tolerant (10%) and grow in up to 40% glucose. The wide pH for growth range from 3.5 to 7.5, and acid tolerance are quite typical. This bacterium has been isolated from fermenting agave sap in Mexico, from fermenting palm saps in Zaire, Nigeria, and Indonesia, from fermenting sugarcane juice in Northeastern Brazil. Undoubtedly, they are important contributors to the fermentation of plant saps in many tropical areas of the America, the Africa, and Asia.

Saccharomyces cerevisiae is a eukaryotic microorganism classified in the fungi kingdom. This yeast is a unicellular microorganism and is defined as basidiomycetes or ascomycetes. *S.*

cerevisiae cells measure 3-7 microns wide and 5-12 microns long. It has elliptic, round and oval shapes and reproduces is by a division process known as budding [7]. It is believed that *S. cerevisiae* was originally isolated from the skin of grapes [8]. Its optimum temperature growth range is 30° C [9]. *S. cerevisiae* is tolerant of a wide pH range (2.4-8.2), being the optimum pH for growth between values of 3.5 to 3.8 [10]. In addition, *S. cerevisiae* is high growth rate (0.5 h-1) in the yeast group. With respect to *S. cerevisiae* nutritional requirements, all strains can grow aerobically on glucose, fructose, sucrose, and maltose and fail to grow on lactose and cellobiose. Also, all strains of *S. cerevisiae* can use ammonia and urea as the sole nitrogen source, but cannot use nitrate since they lack the ability to reduce them to ammonium ions. They can also use most amino acids, small peptides and nitrogen bases as a nitrogen sources [11]. *S. cerevisiae* have a phosphorus requirement, assimilated as a dihydrogen phosphate ion, and sulfur, which can be assimilated as a sulfate ion or as organic sulfur compounds, such as the amino acids: methionine and cysteine. Some metals, such as magnesium, iron, calcium and zinc are also required for good growth of this yeast.

Alcoholic fermentation by yeast consists of three main stages: (1) transporting sugars within the cell, (2) transforming sugars into pyruvate through glycolysis pathway and finally (3) converting acetaldehyde to ethanol.

The simplified fermentation process is:

$$C_6H_{12}O_6 + \left(\text{carbon source}\right) \rightarrow 2CH_3CH_2OH + 2\,CO_2 + 2\,\text{ATP} + 25.5\,\text{kcal} \qquad (2)$$

3. Modes of fermentation process

There are basically three modes of fermentation process: (1) Batch fermentation process. (2) Fed batch fermentation process and (3) Continuous fermentation process (Figure 1).

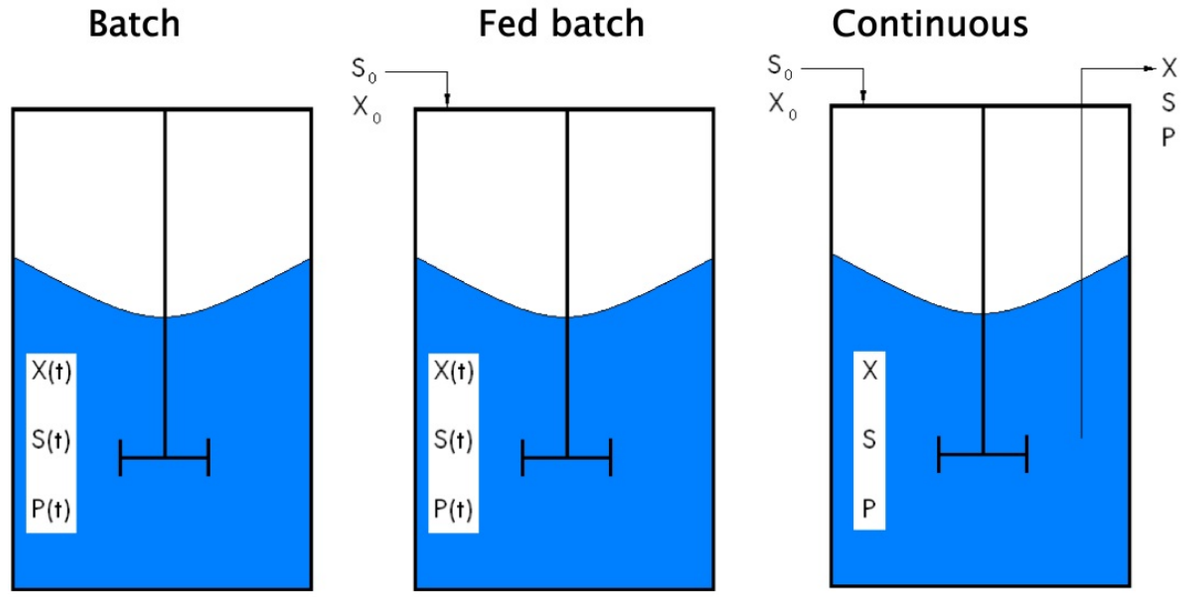

Figure 1. Fermentation process; x: biomass, s: sustrate, p: product, t: time

The mode of operation is dictated by the type of product being produced.

The fermentation process may be divided into six phases:

a. The formulation of media to be used in culturing the process organism during the development of the inoculum and in the production fermenter.
b. The sterilization of the medium, fermenters and ancillary equipment.
c. The production of an active, pure culture in sufficient quantity for inoculating the production vessel.
d. The growth of the microorganism in the production fermenter under optimum conditions for product formation.
e. The extraction of the product and its purification.
f. The disposal of effluents produced by the process.

The interrelationships between the six phases are illustrated in Figure 2.

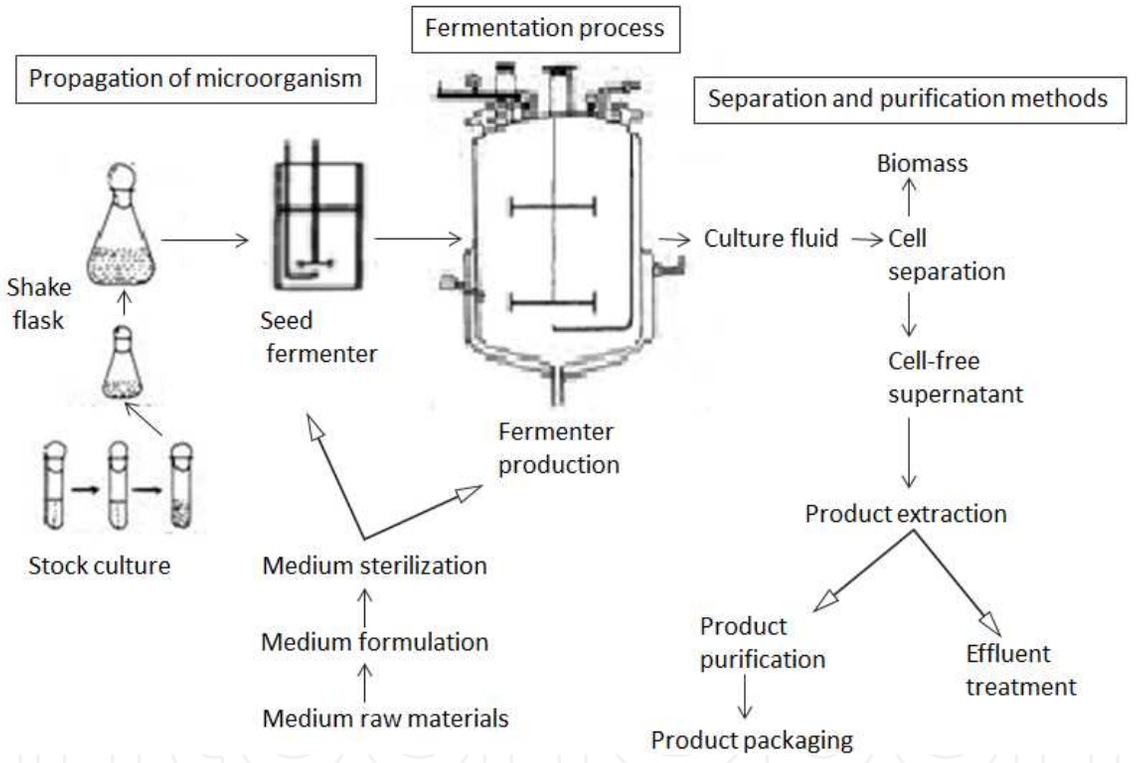

Figure 2. A schematic representation of a typical fermentation process

3.1. Batch fermentation

In the batch fermentation process, the entire medium is removed from the fermentation vessel. The vessel is then thoroughly washed, cleaned and the new batch is started only thereafter. The bioreactor is initially loaded with fresh medium and inoculated with selected microorganism.

During the growth period, no medium is added or removed. The Biomass, nutrients and products concentrations change continuously in time [12].

During the batch fermentation process, various physiological states of the microorganism are observed (Figure 3):

a. Lag phase - Period where microorganisms adapt to the new environment.
b. Positive acceleration phase - Period of slow increase in the population
c. Logarithmic or exponential phase - Period of rapid rise in population due to availability of nutrients. The exponential phase may be described by the following equation:

$$dx/_{dt} = \mu x$$

Where x is the concentration of microbial biomass
t is time, in hours
and μ is the specific growth rate in hours^{-1}

d. Negative acceleration phase - Period in which there is a slow rise in population as the environmental resistance increases.
e. Stationary phase - Finally, growth rate becomes stable because mortality and natality rates become equal. During the stationary phase, the organism is still maintaining a certain metabolic activity, while some secondary metabolites are formed (products not associated with microbial growth).
f. Death phase - Finally, environmental stress causes a decrease in metabolic activity of yeast and autolysis.

3.2. Fed batch fermentation

Fed-batch fermentation is described as the type of system where nutrients are added when their concentration falls. In the absence of outlet flow, the volume in the bioreactor will increase linearly. The nutrients are added in several doses to ensure that there are not surplus nutrients in the fermenter at any time. Surplus nutrients may inhibit microorganism growth. By adding nutrients little by little, the reaction can proceed at a high production rate without getting overloaded. The best way to control the addition of the feed is monitoring the concentration of the nutrient itself in the fermenter or reactor vessel.

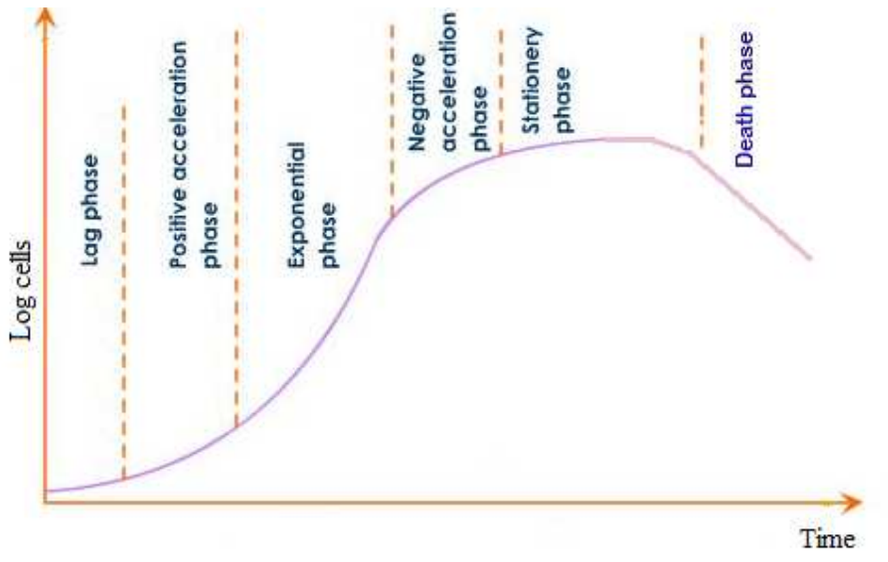

Figure 3. Growth curve of microorganism

The main advantages of the fed batch fermenter are:

a. The extension of the exponential growth phase and production of metabolites of interest.
b. The production of high biomass and product concentrations.
c. The reduced inhibition by the substrate.

However, accumulations of toxic products to the microorganism in the medium and downtime due to charging and discharging (which also occur in batch fermentations) are the main disadvantages of Fed batch fermentation [12].

3.3. Continuous fermentation

Exponential growth in batch fermentation may be prolonged by adding of fresh medium to the vessel. In the continuous fermentation process, the added medium displaced an equal volume of culture from the vessel. Thus, the process of continuous fermentation non-stop and the exponential growth will proceed until the substrate is exhausted. By using proper technique, the desired products are obtained from the removed medium [13].

If medium is fed continuously to such a culture at a suitable rate, a steady state is eventually achieved i. e., the formation of new biomass by the culture is balanced by the loss of cells from the vessel. The flow medium into the vessel is related to the volume of the vessel by the term dilution rate, *D*, defined as:

$$D = {}^{F}/_{V}$$

Where F is the flow rate (volume units/time) and V is the volume (volume units).

The net change in cell concentration over a time period may be expressed as:

$${}^{dx}/_{dt} = \ growth \ - output$$

$${}^{dx}/_{dt} = \mu x - Dx$$

Under steady state conditions the cell concentration remains constant, thus ${}^{dx}/_{dt} = 0$ and:

$$\mu = D$$

Thus, under steady state conditions, the specific growth rate is controlled by the dilution rate, which is an experimental variable. It is recalled that under batch culture conditions, an organism will grow at its maximum specific growth rate and, therefore, continuous culture may be operated only at dilution rates below the maximum specific growth rate.

4. Agaves species in the Americas, characteristics and uses

Chiefly Mexican, agaves are also native to the southern and western United States and central and tropical South America. They are succulents with a large rosette of thick, fleshy

leaves, each ending generally in a sharp point and with a spiny margin; the stout stem is usually short, the leaves apparently springing from the root. Agave taxa give particulars for all 197 taxa in the two subgenera, Littaea and Agave. The first of a slender form with high in saponin concentration is intended as ornament mainly, except *Dasylirion spp*. Species, which is the raw material to produce Sotol (a Mexican distilled alcoholic beverage). Also the Littaea is used as raw material producing medicinal steroids, since contains smilagenin. In the other hand, the species in the subgenus Agave have been exploited since the ancient pre-Columbian civilization mainly for producing: fiber, fodder, food and alcoholic beverage (Table 2) [14].

5. Alcoholic fermentation process of agave juice

Agave juice bioethanol production from involves multiple steps: at harvest, fermentable sugars are obtained from heads of the agave plant by steaming, milling and pressing. During the steaming process, the polysaccharides (fructans) are hydrolyzed into a mixture of sugars consisting of fructose mainly. After fermentation, the alcohol from the must is purified by distillation and dehydration for obtaining anhydrous ethanol.

Agave species	**Main State of Production**	**Uses**	**Characteristic**
Agave *tequilana* Weber	Jalisco, regions of the states of Nayarit, Michoacán, Tamaulipas, Guanajuato.	Tequila industry	High sugar content
Agave *angustifolia* Haw. Agave *rhodacantha* Trel. Agave *shrevei* Gentry Agave *wocomahi* Gentry Agave *durangensis* Agave *palmeri* Engelm. Agave *zebra* Gentry Agave *asperrima* Jacobi Agave *potatorum* Zucc. Agave *weberi* Cels Agave *tequilana* Weber	Oaxaca, San Luis Potosí, Durango, Jalisco,	Mezcal industry	High sugar content
Agave *angustifolia* Haw.	Sonora	Bacanora Industry	High sugar content
Agave *atrovírens* Kawr Agave *lehmannii* Agave *cochlearís* Agave *lattísíma* Jacobí Agave *mapisaga* Agave *salmiana*	Distrito Federal, Tlaxcala, Hidalgo, Querétaro, Puebla, Morelos, San Luis Potosí	Pulque industry	High sugar content

Agave species	Main State of Production	Uses	Characteristic
Agave *angustifolia* Agave *inaequidens* Agave *maximiliana*	Jalisco	Raicilla industry	
Agave *lechuguilla* Agave *striata* Agave *sisalana*	Yucatan	Fiber industry	Obtained from leaf
Agave *lechuguilla*	Jalisco	Cleaning cloth product	Obtained from agave pulp
Agave *salmiana*	San Luis Potosí	Food and fodder	Obtained from leaf
Agave *sisalana* Agave *fourcroydes*	Yucatan	Paper source	Obtained from leaf
Agave *salmiana* Agave *fourcroydes* Agave *agustifolia* Agave *deweyana*	San Luis Potosi, Jalisco, Yucatan, Sonora	Medicinal uses: steroid drugs	Obtained from leaf High sapogenins concentration

Table 2. Main species of agave with economic importance in México

Alcoholic Fermentation is one of the most important stages in the bioethanol process, as sugars (mainly fructose) are transformed into ethanol and CO_2. Agave juice can be fermented by inoculation (with selected microorganisms) or spontaneously (without inoculums). Significant differences were observed between fermentation conducted with controlled microorganism or inoculated media and spontaneous or no inoculated media. The introduction of selected strains allows fermentation to be regulated and accelerated. Inoculation of culture media with starter cultures allows a high population of selected strain, thereby assuring it dominance. The results are quicker ethanol synthesis, shorter fermentation time, and higher productivity.

Knowledge of physiological behavior of indigenous tequila yeast used in the agave juice alcoholic fermentation process for obtaining bioethanol is still limited. The raw material and physiochemical and biological conditions have significant impact on the productivity fermentation process. For these reasons, a better knowledge of the physiological and metabolic features of these yeasts in agave juice fermentation is required. A study of bioethanol production from *Agave tequilana* Weber var. azul juice fermentations is presented below. For this, the alcoholic fermentation of *Agave tequilana* Weber var. azul juice was carried out in batch and continuous modes of fermentation process.

a. *Agave tequilana* Weber var. azul juice characterization
   The *Agave tequilana* Weber juice used in the experimentation was supplied by a distillery. The sugar concentration of the agave juice was 20 °Bx and pH was 4.0. In the distillery, the agave plants are cooked in an autoclave at 95 to 100°C for 4 hours.

The analysis of agave juice amino acids of and of its hydrolyzate was performed and compared to grape juice (Table 3). These results show that agave juice is naturally amino acid poor, even when hydrolyzed [15].

Amino acid (mg/L)	Grape juice[1]	Agave juice[2]	Hydrolyzate Agave juice[2]
L- alanine	58.5*	0.72±0.005	20.98±0.153
L-arginine	255.9±182.3	5.76±0.030	38.68±0.676
L-aspartate	46.4± 22.9	0.41±0.018	25.51±0.322
L-glutamate	91.2± 37.7	0.12±0.001	42.12±0.117
L-glutamine	122.9± 93.9	nq	nq
L-glycine	4.1± 3.1	0.44±0.016	21.75±0.526
L-histidine	103.9± 85.9	0.19±0.008	10.09±0.301
L-isoleucine	13.4*	0.06±0.003	11.70±0.196
L-leucine	13.4*	0.14±0.003	21.28±0.524
L-lysine	7.6± 6.67	0.06±0.002	6.59±0.150
L-metionine	24.2± 13.9	nd	4.10±0.126
L-phenylalanine	16.9± 11.3	0.06±0.003	12.44±0.100
L-serine	53.1± 23.4	1.34±0.024	32.52±0.306
L-threonine	51.6± 25.1	0.32±0.014	18.54±0.270
L-tyrosine	13.3*	0.22±0.010	13.97±0.109
L-valine	17.7*	0.14±0.004	21.49±1.058

[1]: amino acid concentration of 11 grape varieties must [16]; [2]: Each value represents the average ± standard deviation of duplicate determinations, the method limited detection is 1 pmols/mL; *: amino acid concentration constant in the 11 varieties of grape [16]; nd: not detected; nq: not quantified.

Amino acid analyses were determined by HPLC [17]. The acid hydrolysis of agave juice was performed as reported by Umagath et al. [18].

Table 3. Amino acid composition of grape and agave juices.

b. Batch fermentation process

The bioethanol production from agave juice batch fermentation process is shown. For this work, three yeast strains isolated from agave juice were studied for their fermentative capacity. The strains (S1, S2 and S3) were identified by biochemical and molecular tests [15]. The experiments were performed using agave juice supplemented with sufficient ammonium sulphate, for maintaining a good performance of the yeast strains. For fermentation medium, sugar concentration of the agave juice was adjusted to 12 ºBrix (95±5 g/L reducing sugar) and then supplemented with 1g/L of ammonium sulphate. Culture media were sterilized at 121 °C for 15 min. The pH of the unadjusted juice was 4.2. This fermentation medium was similar to the must typically used in industrial distilleries for obtain alcoholic beverage. The fermentations were carried out under anaerobic conditions at 35 °C and 250 rpm in a 3 L bioreactor (Applikon, Netherlands). The inoculation level was 20 million cells/mL. Two fermentations were performed with each yeast.

Each must was fermented for 72 h, and sampling was performed every 2 h during the first 12 h of fermentation, then every 4 h during the following 48 h, until the last sampling event at 72 h. Biomass concentration was obtained by dry weight measurement. Reducing sugar concentration was determined by the DNS method modified and glucose, fructose and

glycerol concentration was determined by HPLC [15]. Samples were micro-distilled and ethanol concentration was determined in distillates by using the potassium dichromate method [19].

Fermentation Kinetic Analysis **-** The evolution of biomass, sugar consumption and ethanol production versus time were plotted in Fig. 1 and Table 1, showing the kinetic parameters of each strain. All *Saccharomyces* strains grew faster reaching a biomass concentration level of 4-5.3 g/L by approximately 12 h and sugar was completely depleted by 18-24 h of the fermentation (Figure 4). The S1 and S2 strains showed a higher ethanol concentration and sugar consumption than S3 (Figure 4 and Table 4).

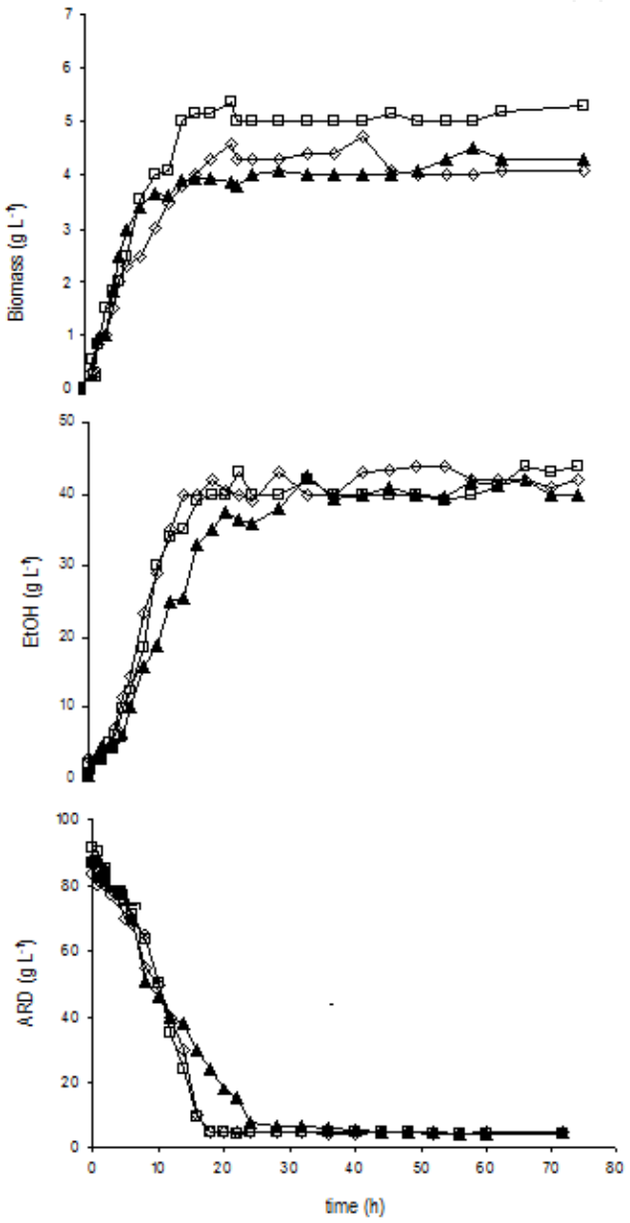

Figure 4. Kinetic profiles of the fermentation of S1(◊), S2(□) and S3(▯) strains in a *Agave tequilana* Weber blue variety juice medium at 12 ºBx, supplemented with ammonium sulfate (1g/L). Biomass: biomass concentration profile; ARD: reduction sugar concentration profile; ETOH: ethanol concentration profile.

Growth and ethanol yields were different: 0.046-0.059 g/g and 0.47-0.49 g/g, respectively (Table 4). Statistical analysis (95% LSD) showed significant differences between yeast strains in all kinetic parameters (Table 4). *S. cerevisiae* S1 strain presented a higher value of maximum specific growth and sugar consumption than S2 and S3 strains. Likewise, S1 and S3 strains showed a high maximum specific ethanol rate (Table 4).

Strain	Kinetics parameters							
	μ_{max} (h^{-1})	q_{smax} (g/gh^{-1})	q_{pmax} ($g/g\ h^{-1}$)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)	X_f (g/L)	S_c (g/L)	$Etoh_f$ (g/L)
S1	0.43±.016	4.28±.27	1.56±.12	0.050±.004	0.49±.027	4.34±.26	86.7±2.0	42.6±1.0
S2	0.33±.030	2.85±.15	1.34±.06	0.055±.004	0.49±.001	4.86±.44	87.4±1.2	43.5±.55
S3	0.35±.020	3.74±.27	1.52±.06	0.052±.001	0.47±.015	4.35±.10	83.9±.30	39.9±1.4

μ_{max}: maximum specific growth rate; q_{smax}: maximum specific sugar consumption rate; q_{pmax}: maximum specific ethanol production rate; $Y_{x/s}$ and $Y_{p/s}$: yields of biomass and ethanol; S_c: consumed substrate concentration; X_f: final biomass concentration; $Etoh_f$: final ethanol concentration. Each value represents the average ± standard deviation of duplicate determinations of two fermentations.

Table 4. Comparison of kinetic parameters and final concentration of biomass, consumed substrate and ethanol for the different strains.

c. Continuous fermentation process

Bioethanol production from agave juice continuous fermentation process is shown below. In continuous fermentation process, the effects of dilution rate, nitrogen and phosphorus source addition and micro-aeration on growth, and synthesis of ethanol of two native *Saccharomyces cerevisiae* S1 and S2 strains were studied.

Continuous cultures were carried out in a 3 L bioreactor (Applikon, The Netherlands) with a 2 L working volume. Cultures were started in a batch mode, by inoculating fermentation medium with 3.5 x 10^6 cells/mL (97±2 %. initial viability) and incubating at 30 °C and 250 rpm for 12 h. Afterwards, the culture was fed with fermentation medium (12 °Brix = 95 ± 5 g/L reducing sugar and 1 g/L of ammonium sulfate). Culture media were sterilized at 121 °C for 15 min.

To reach the steady state in each studied condition, the culture was maintained during five residence times and samples were taken every 6 h. A steady state was reached, when the variation in the concentrations of biomass, residual sugars and ethanol were less than 5%. Data presented on tables and figures are the mean ± standard deviation of three assays at the steady state.

Effect of the dilution rate on S. cerevisiae strains fermentative capability in continuous cultures

Both yeast strains (S1 and S2) were used and fermentation medium was fed at different D (0.04, 0.08, 0.12 and 0.16 h^{-1}) for studying the effect of dilution rate (D) on the kinetic

parameters and concentrations of biomass, residual reducing sugar and ethanol at a steady state of agave juice continuous fermentation process (Table 5 and Figure 5).

Concentrations of biomass and ethanol decreased as D increased for both strains cultures while residual reducing sugars increased parallel with the increase of D (Figure 5).

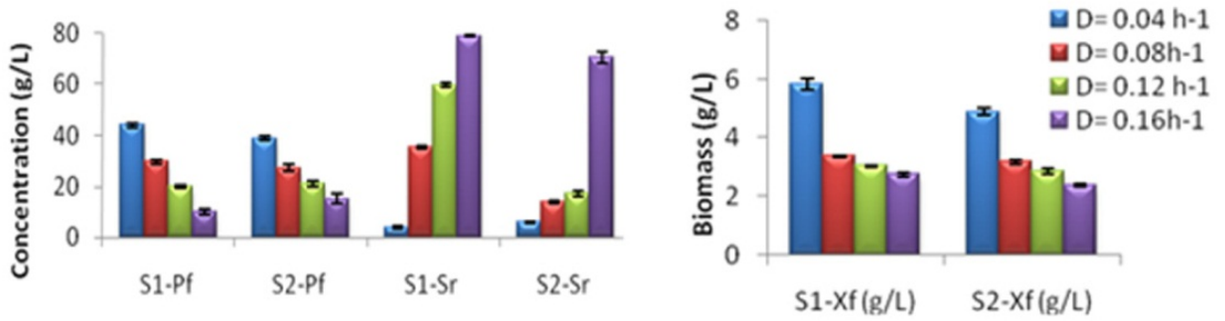

Figure 5. Concentration of Residual reducing sugar (Sr), Ethanol (Pf) and Biomass (Xf) at the steady state of continuous culture of two strains of S. cerevisiae (S1 and S2) fed with agave juice at different dilution rate (D). Data are presented as mean ± standard deviation of four assays at the steady state.

Although, *S. cerevisiae* S2 consumed more reducing sugars than S1 for each D, ethanol yields reached by S1 were higher than those obtained by S2, which were near the theoretical value (0.51) with no significant differences among the different D tested ($p>0.05$) (Table 5).

At D = 0.04 h^{-1}, S1 and S2 strains reached the highest ethanol productions (43.92 and 38.71 g/L, respectively) and sugar consumptions (96.06 and 94.07 g/L, respectively) which were similar to those obtained using batch fermentations (see *Batch fermentation process* section). The low fermentative capacities displayed by both strains at higher D than 0.04 h^{-1} could be due to a low content of nutrients and/or toxic compounds in agave juice cooked [15].

Both strain cultures reached maximal ethanol production rates at 0.12 h^{-1} (2.37 and 2.53 g/L·h, respectively for S1 and S2), maximal growth rates were achieved at 0.16 h^{-1} (0.44 and 0.38 g/L·h, respectively for S1 and S2) and maximal sugar consumption rates were obtained at 0.08 h^{-1} (5.08 g/L·h) for S1 and at 0.12 h^{-1} (9.96 g/L·h) for S2 (Table 5 and Figure 6).

Effect of the pH value on the fermentative capacity of S1 and S2 strains - The effect of pH was observed, switching from a controlled pH (at 4) to an uncontrolled pH (naturally set at 2.5±0.3). Figure 7 shows biomass and ethanol productions for strain S1, in non-aerated or aerated (0.01 vvm) systems fed with sterilized medium. Results did not show significant differences on the biomass or ethanol productions ($P > 0.05$) between the fermentations with control (4) and with no control (2.5) of pH. Conversely, biomass and ethanol productions increased on aerated culture compared to that non aerated, for both pH levels studied. These results agreed with those reported by Díaz-Montaño et al. [20]. These results are important, since the operation of a continuous culture naturally adjusted to a low pH would limit the growth of other yeasts [21, 22] or bacteria [23, 24], indicating the feasibility of working with non-sterilized media on an industrial scale. Another advantage of not controlling the pH is that instrumentation for this operation is not required, thus removing it from the initial investment [25].

Parameter	Strain	*D* (h^{-1})			
		0.04	0.08	0.12	0.16
Biomass (g/L)	S1	5.83 ± 0.21	3.38 ± 0.03	3.04 ± 0.04	2.75 ± 0.07
	S2	4.89 ± 0.12	3.18 ± 0.08	2.86 ± 0.08	2.39 ± 0.06
Ethanol (g/L)	S1	43.92 ± 0.81	29.63 ± 0.79	19.76 ± 0.32	9.95 ± 0.39
	S2	38.71 ± 0.74	27.33 ± 1.60	21.10 ± 0.48	15.20 ± 0.51
RS (g/L)	S1	3.94 ± 0.53	35.34 ± 0.94	59.75 ± 0.81	79.08 ± 1.08
	S2	5.93 ± 1.16	13.69 ± 1.70	16.96 ± 0.43	70.70 ± 2.17
Glucose (g/L)	S1	nd	1.41 ± 0.06	2.32 ± 0.06	3.07 ± 0.16
	S2	nd	0.43 ± 0.03	0.65 ± 0.04	3.46 ± 0.48
Fructose (g/L)	S1	2.79 ± 0.57	32.12 ± 0.85	51.48 ± 0.28	65.94 ± 1.39
	S2	2.14 ± 0.05	10.54 ± 0.37	15.74 ± 0.50	63.10 ± 2.82
Glycerol (g/L)	S1	2.44 ± 0.28	1.94 ± 0.04	1.70 ± 0.03	1.86 ± 0.26
	S2	2.09 ± 0.09	2.34 ± 0.07	2.54 ± 0.08	1.32 ± 0.05
$Y_{X/S}$ (g/g)	S1	0.06 ± 0.00	0.05 ± 0.00	0.08 ± 0.00	0.17 ± 0.01
	S2	0.05 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.07 ± 0.01
$Y_{P/S}$ (g/g)	S1	0.46 ± 0.01	0.47 ± 0.02	0.49 ± 0.01	0.47 ± 0.01
	S2	0.39 ± 0.01	0.30 ± 0.02	0.24 ± 0.00	0.44 ± 0.04
r_X (g/Lh)	S1	0.23 ± 0.01	0.27 ± 0.00	0.36 ± 0.01	0.44 ± 0.01
	S2	0.19 ± 0.01	0.25 ± 0.01	0.34 ± 0.01	0.38 ± 0.01
r_S (g/Lh)	S1	3.80 ± 0.02	5.08 ± 0.08	4.69 ± 0.10	2.52 ± 0.17
	S2	3.96 ± 0.05	6.91 ± 0.14	9.96 ± 0.05	4.69 ± 0.35
r_P (g/Lh)	S1	1.76 ± 0.03	2.37 ± 0.06	2.37 ± 0.04	1.59 ± 0.06
	S2	1.55 ± 0.03	2.19 ± 0.13	2.53 ± 0.06	2.43 ± 0.08

RS: Residual reducing sugar concentration, $Y_{X/S}$: yield of biomass, $Y_{P/S}$: yield of ethanol, r_X: growth rate, r_S: reducing sugars consumption rate, r_P: ethanol production rate, nd: not detected at the assayed conditions. Data are presented as mean ± standard deviation of four assays at the steady state.

Table 5. Kinetic parameters at the steady state of continuous cultures of two strains of *S. cerevisiae* (S1 and S2) fed with agave juice at different dilution rates (*D*).

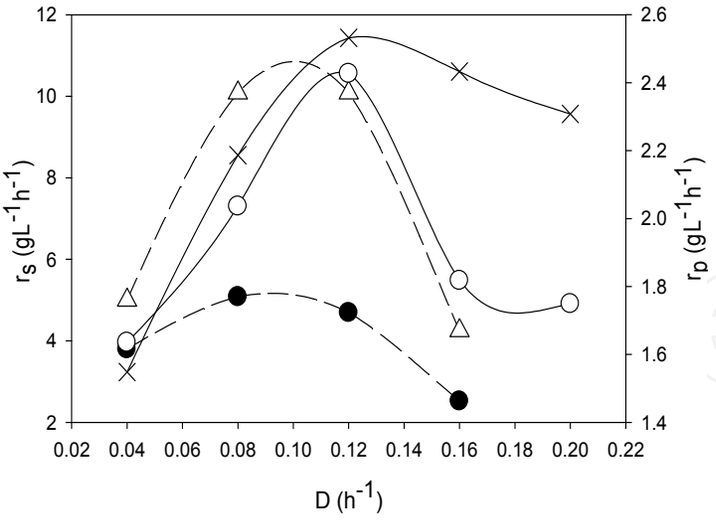

Figure 6. Ethanol production and reducing sugars consumption rates at different dilution rates for *S. cerevisiae* S1 (r_p -Δ- and r_s -•-) and S2 (r_p -x- and r_s -o-).

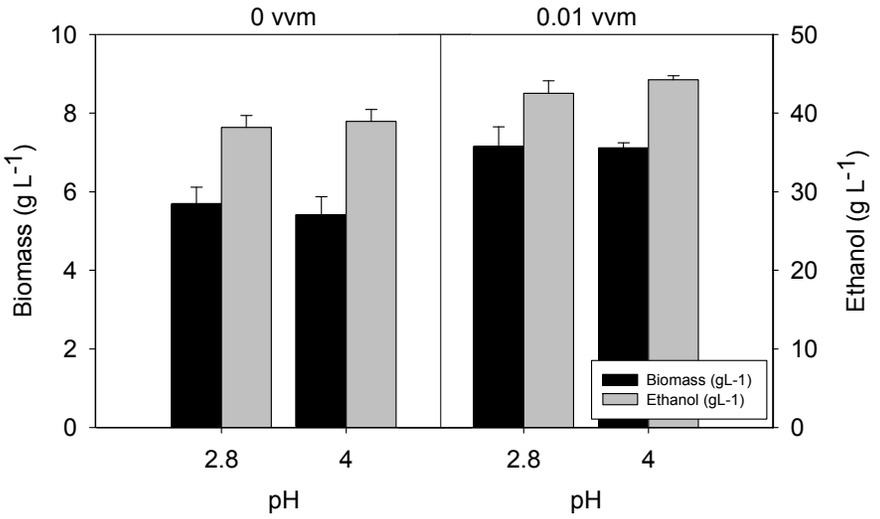

Figure 7. Effect of controlling (at 4) or not controlling (2.5 ± 0.3) pH, in the production of biomass and ethanol at aeration rates of 0 or 0.01 vvm during the culture of S1 strain.

Effect of the nitrogen and phosphorus supplementation on S. cerevisiae S1 sugar consumption

Since both *S. cerevisiae* strains were unable to consume sugars efficiently in cultures fed at D higher than 0.04 h^{-1}, a nutritional limitation and/or some inhibitory substances formed in the agave cooking step (Maillard compounds), which can act on *S. cerevisiae* strain activity. In fact, *Agave tequilana* juice is deficient in nitrogen sources (Table 3). Amino acids are the most important nitrogen source in agave juice; however, their natural concentrations (0.02 mg N/L) are not enough to support balanced yeast growth and the complete fermentation of sugars [26]. Therefore, agave juice supplemented with ammonium sulfate at 1 g/L could be insufficient. Several authors point out the importance of nitrogen sources (type and

concentration) for achieving a complete fermentation, since they improve cell viability, yeast growth rate, sugar consumption and ethanol production (11; 20). It is worth noting that ammonium phosphate (AP) was chosen as a nitrogen source, since the two macronutrientes frequently implied in the causes of stuck fermentation when present in small quantities are nitrogen and phosphate (see the reviews by Bisson [11]).

Therefore, the effect of the ammonium phosphate (AP) addition on *S. cerevisiae* S1 sugar consumption was studied in a continuous culture (Figure 8). To study the effect of nitrogen and phosphorus source addition on the agave juice fermentation by *S. cerevisiae*, S1 strain was used and fermentation medium was fed at D of 0.08 h^{-1}, while after the steady state was reached, the ammonium phosphate (AP) concentration was gradually increased, as follows: 1g/L (first addition), 2 g/L (second addition), 3 g/L (third addition) and 4 g/L (fourth addition).

The fermentation was started in batch mode using the fermentation medium. After 12 h, the culture was fed using medium supplemented with 1 g/L of AP (first addition). At the steady state, residual concentrations of sugars and ammonium nitrogen were 29.42 and 0.08 g/L, respectively. These results were not significantly different ($p>0.05$) from the condition previously tested for the same strain (at D = 0.08 h^{-1}), feeding an unsupplemented fermentation medium (Figure 5).

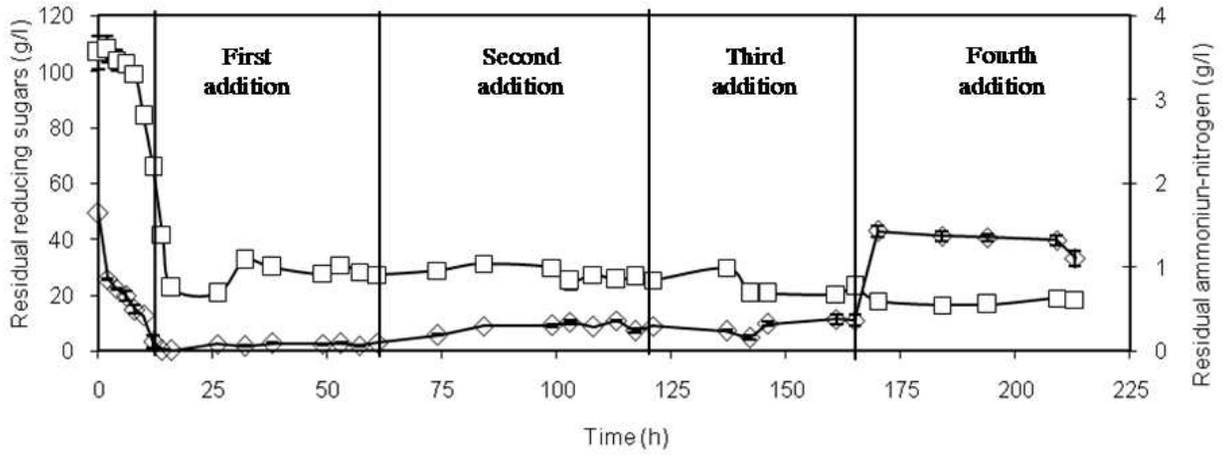

Figure 8. Effect of the addition of ammonium phosphate to the agave juice fed to *S. cerevisiae* S1 chemostat culture (at D=0.08 h^{-1}), on the consumptions of reducing sugars (□) and ammonium-nitrogen (◊). First addition: 1 g/L; Second addition: 2 g/L; Third addition: 3 g/L; Fourth addition: 4 g/L.

Those residual concentrations of reducing sugars (high) and ammonium nitrogen (low) indicate the necessity of adding more AP. At the steady states of the second (2 g/L), third (3 g/L) and fourth (4 g/L) additions of AP, the residual sugars concentrations were 25.96, 21.25 and 17.60 g/L, respectively. This indicates that the residual ammonium nitrogen concentrations were 0.31, 0.36 and 1.29 g/L, respectively; indicating that the AP addition improved *S. cerevisiae* S1 fermentative capability, but other nutritional deficiencies still existed [27].

Effect of the micro-aeration rate on S. cerevisiae S1 fermentative capability **-** Lack of oxygen has proved to be a main limiting factor to fermentation [11], since yeasts require low amounts of oxygen for synthesizing some essential lipids to assure cell membrane integrity [28]. Because *S. cerevisiae* is Crabtree-positive, alcoholic fermentation is privileged in culture media containing high sugars concentrations, even in the presence of oxygen [29]. The effect of the micro-aeration rate (0, 0.01 and 0.02 vvm) on the fermentative capacity of *S. cerevisiae* S1 (at D = 0.08 h^{-1}) was studied for investigating the yeast oxygen requirement during the continuous fermentation, using the last fermentation medium supplemented with 4 g/L of AP for feeding at D of 0.08 h^{-1}. Biomass and ethanol concentrations increased as air flow increased, reaching at the steady state, 5.66, 7.18 and 8.04 g/L, and 40.08, 44.00 and 45.91 g/L, respectively for 0, 0.01 and 0.02 vvm (Figure 9).

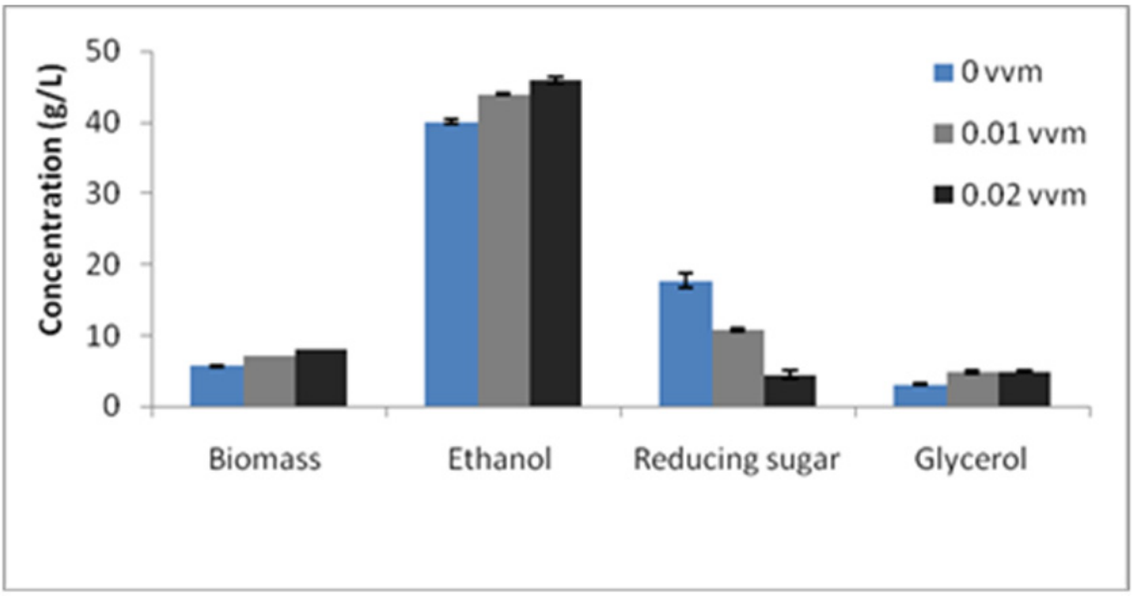

Figure 9. Concentration of Residual reducing sugar, Ethanol and Biomass at the steady state of continuous culture of two strains of S. cerevisiae S1 fed with agave juice (*D* = 0.08 h-1) at different micro-aeration rates. Data are presented as mean ± standard deviation of four assays at the steady state.

Meanwhile, residual sugars decreased as micro-aeration increased, reaching 17.67, 10.71 and 4.48 g/L, respectively for 0, 0.01 and 0.02 vvm; showing an improvement in the fermentation process due the dissolved oxygen in the must. However, statistical differences were not found in biomass and ethanol yields at the different tested aeration rates ($p>.05$) (Table 6). In addition, sugars consumption rates and ethanol and biomass productions increased as micro-aeration increased, achieving a faster fermentation (Table 6). These results were in accordance to those reported by Díaz-Montaño [20]. Viability of the S1 strain was 100% in aeration experiments.

Glycerol is a metabolite providing yeast metabolic activity information. In fact, yeasts produce glycerol mainly for reoxidating the NADH generated by glycolysis. Since the citric acid cycle and the respiratory chain are slightly activated by micro-aeration, NAD might be partially regenerated, and consequently, glycerol concentration decreases [30]. However, in this work, glycerol concentration increased as aeration increased (Table 6). Given that

biomass concentration and fermentation efficiency also increase as aeration increases, glycerol production could contribute to faster NAD regeneration.

Parameter	**Micro-aeration rates (vvm)**		
	0.00	0.01	0.02
$Y_{X/S}$ (g/g)	0.06 ± 0.00	0.07 ± 0.00	0.08 ± 0.00
$Y_{P/S}$ (g/g)	0.48 ± 0.01	0.49 ± 0.00	0.48 ± 0.01
r_X (g/Lh)	0.45 ± 0.01	0.57 ± 0.00	0.64 ± 0.01
r_S (g/Lh)	6.55 ± 0.08	7.14± 0.02	7.64 ± 0.05
r_P (g/Lh)	3.21 ± 0.03	3.52 ± 0.02	3.67 ± 0.04

$Y_{X/S}$: yield of biomass, $Y_{P/S}$: yield of ethanol, r_X: growth rate, r_S: reducing sugars consumption rate, r_P: ethanol production rate. Data are presented as the mean ± standard deviation of four assays at each steady state.

Table 6. Kinetic parameters of *S. cerevisiae* S1 continuous cultures at steady state fed with agave juice (D = 0.08 h^{-1}) at different micro-aeration rates.

Effect of feeding non-sterilized medium on the fermentative capability of S. cerevisiae strains

Non-sterilized medium (NSM) was fed to S1 and S2 continuous cultures and the aeration rate was gradually increased from 0 to 0.02 vvm. For these experiments, pH was controlled at 4 for S2 strain and not controlled for S1 strain. Ethanol production increased significantly ($P < 0.05$) as the aeration rate increased during S1 fermentations fed with SM or NSM. In contrast, aeration did not have any effect on ethanol or biomass production during the S2 fermentation fed with NSM (Figure 10-B). For S1 continuous fermentation, medium type (SM or NSM) did not show a significant difference in the production of ethanol ($P > 0.05$), but it had a significant difference in the production of biomass ($P < 0.05$). Multiple range tests divided S1 fermentations in aerated (0.01 and 0.02 vvm) and non-aerated systems, indicating higher biomass and ethanol productions in aerated cultures. Nevertheless, no significant difference was found in the productions of biomass or ethanol ($P > 0.05$) between experiments aerated at 0.01 and those aerated at 0.02 vvm. These results could be attributed to the lower pH (2.3) observed at 0.02 vvm, which could have reduced cell viability. Interestingly, S1 strain flocculation was not observed for 0.02 vvm and biomass retention time was lowered, decreasing the cell population (Figure 10-B).

For all the fermentation conditions, the consumption of reducing sugars was significantly augmented ($P < 0.05$) as aeration rate increased, reaching 4 ± 2 g L^{-1} of residual reducing sugars at 0.02 vvm for both medium types. It has been reported that more than 12% of total sugars contained in agave juice are non-fermentable, since fructans hydrolysis is not complete during the cooking step. In this study, oligosaccharides might be taken into account as residual reducing sugars, because they are difficult to degrade by *S. cerevisiae*.

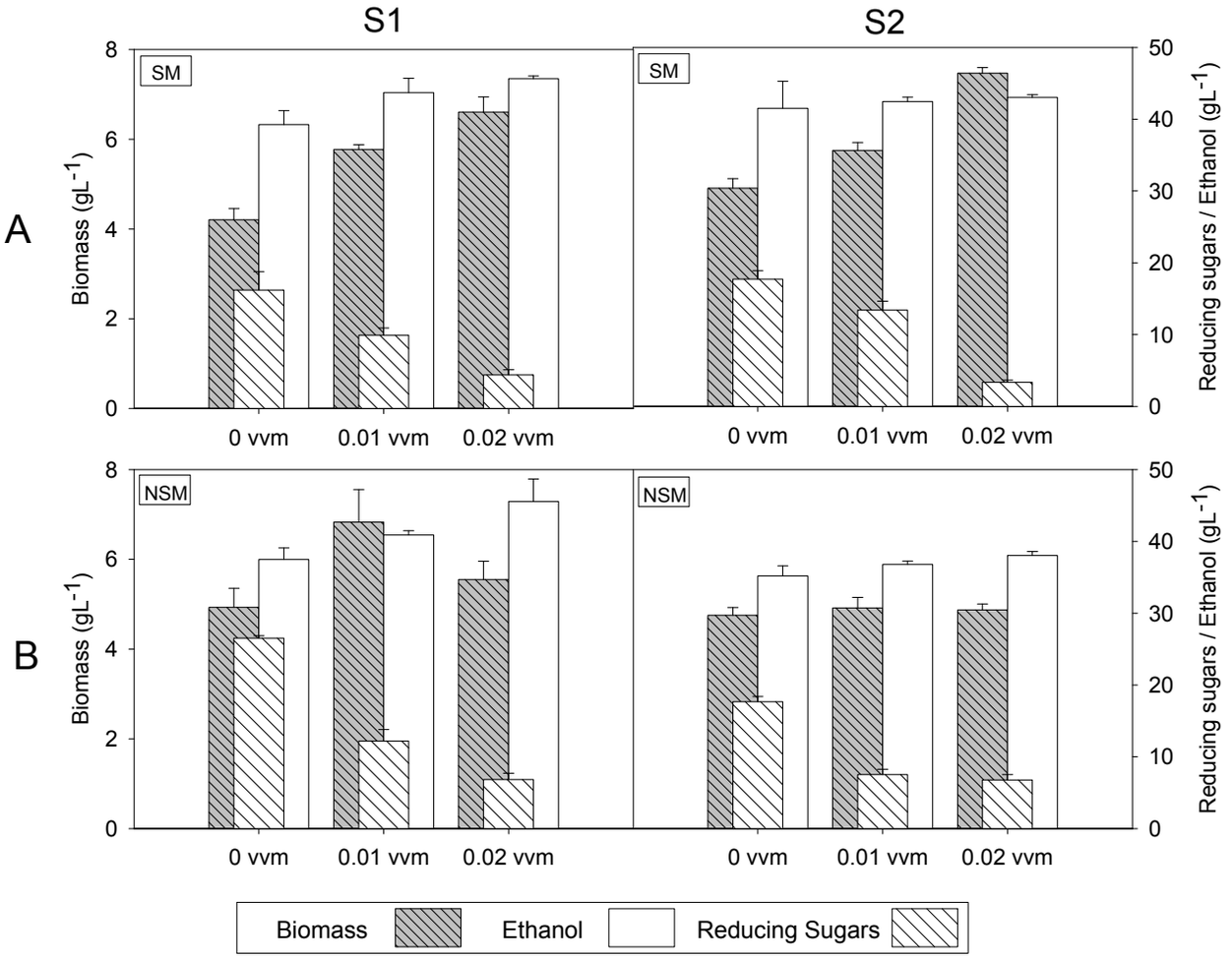

Figure 10. Effect of the aeration on the productions of biomass and ethanol of two *S. cerevisiae* strains (S1 and S2) using the continuous addition of A) sterilized (SM) and B) non-sterilized (NSM) media, pH was 4 and 2.5 ± 0.3 for S1 and S2 strain cultures.

S2 continuous fermentations were divided by the multiple range test, according to the aeration rates (0, 0.01 and 0.02 vvm), showing an increase in the fermentative capability of the S2 strain as aeration increased. The type of medium led to a significant difference ($P < 0.05$) in ethanol and biomass production. Nevertheless, no significant differences ($P > 0.05$) were found in the consumption of reducing sugars between both types of medium. Higher biomass and ethanol production was observed during SM fermentations. Differences between cultures with different types of medium (NSM and SM) could not be attributed to changes in medium composition during sterilization (121 .C, 15 min), since the cooking of agave heads is a more aggressive treatment (100 .C, 36 h). Furthermore, Maillard reactions during the heating are not favored since agave juice nitrogen source content is low (Table 3). Work is ongoing to answer this phenomenon; however, those changes could be attributed to a possible contamination of wild yeast carried by the non-sterilized agave juice. Nevertheless, microscopy did not show any bacterial contamination for fermentation of either strain. Moreover, the pH during S2 continuous fermentation was controlled at 4 for all the experimental conditions in comparison to S1 fermentation, which was not controlled

and reached lowered pH values, which could have limited the microbial contamination. In addition, compared to S2, the capacity of S1 to flocculate could be an advantage for this strain to be retained longer inside the bioreactor. Several studies have proved the capability of inoculated *S. cerevisiae* strains in continuous fermentations to resist contamination by wild yeast. Cocolin *et al*. showed by molecular methods that the starters strain was able to drive the fermentation until the end of the process (12 days). On the other hand, de Souza Liberal *et al*. identified *Dekkera bruxellensis* as the major contaminant yeast, even though its growth rate is lower than that of *S. cerevisiae* in batch fermentations. They indicated the possibility that *D. bruxellensis* grows faster than *S. cerevisiae* in a continuous culture under certain conditions.

6. Conclusion

Agave plants could be a viable alternative as an accessible raw material for bioethanol production, since high concentration of fermentable sugar is released when agave plant fructans is cooked and/or hydrolyzed. This mixture of sugars, mainly fructose, could be converted into ethanol by microorganism action.

The present study examined the use of batch and continuous fermentation processes for investigating bioethanol production from *Agave tequilana* Weber var. azul. juice.

The fermentable sugars of agave juice fermentation in batch culture were depleted between 18-24 hours by indigenous tequila *S. cerevisiae* strains. The ethanol productivity obtained in batch fermentation was 2.36, 2.42 and 1.66 g/Lh for S1, S2 and S3 yeast strains respectively. Agave juice continuous fermentation was examined for increasing ethanol productivity in the fermentation process. For this, a chemostat system was used for investigating the impact of the dilution rate, pH value, nitrogen and phosphorus source addition, micro-aeration and non-sterilized medium on growth, sugar consumption and ethanol production of two *S. cerevisiae* strains. The dilution rate and nutrient addition have a significant impact on the physiology of the *S. cerevisiae* yeast strains. When S1 and S2 yeast strains are used in continuous cultures, they show low sugar consumption at $D \geq 0.08h^{-1}$. The study revealed a nutritional limitation on the agave juice, which was corrected by adding of nitrogen sources and oxygen, achieving *S. cerevisiae* S1 strain complete sugar consumption with high ethanol conversion at $0.08h^{-1}$. The pH did not have a significant effect on the fermentative capability of *S. cerevisiae* S1 strain at the levels studied. Uncontrolled pH fermentations naturally reached acid values (pH ≅2.5 ± 0.3), which is advisable, since bacteria or yeasts contamination could be limited. The type of agave juice tested (SM and NSM) did not have a significant effect on ethanol production in S1 cultures, but did have an effect on ethanol production in S2 cultures. These results could be attributed to the higher pH fermentation during S2 continuous cultures, which could have favored the proliferation of contaminant wild yeasts. The ethanol productivity obtained in S1 strain agave juice continuous fermentation process was 3.6 g/Lh. Thus, the ethanol productivity in continuous fermentation is higher, 34.4% more than in S1 strain batch fermentation.

These results showed the possibility of performing agave juice fermentations in continuous culture feeding non-sterilized medium and taking advantage of the possible improvements that continuous fermentations and agave plant could offer to the bioethanol industry, such as high productivity with full sugar consumption.

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