Title:

Influence of the final ethanol concentration on the acetification and production rate in the wine vinegar process.

Short title:

Influence of the final ethanol concentration on the acetification rate.

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Abstract

BACKGROUND: The acetification process still needs an overall study of the variables influencing it in order to establish their optimum values. Based on industrial experience and available literature, including a recently proposed model by the authors, amongst the variables most strongly influencing the acetification process are the ethanol concentration at the time the

reactor is unloaded, the unloaded volume and the loading rate. In the scope of ensuring economically efficient industrial production of vinegar, as well as checking the predictions by the aforementioned model, the influence of the final ethanol concentration at unloading time on the mean acetification rate and on productivity has been studied in this work.

RESULTS: An increase in the final ethanol concentration from 0.5 to 3.5 % (v/v) increases the mean overall acetification rate and acetic acid production by 38 and 26 %, respectively. The increase is mainly established during the loading phase.

CONCLUSIONS: The final ethanol concentration is a key variable for the process optimization. If a high rate is desired then a product containing much unused substrate will be obtained, which may be industrially unacceptable. These results suggest the necessity to investigate other possibilities when high values for yield and productivities must to be achieved.

Keywords: Vinegar, wine vinegar, *acetobacter*, acetic acid, fed-batch culture, optimization.

NOTATION

$(-r_E)$	Ethanol uptake rate, mL ethanol · (100 mL medium · h)$^{-1}$ ≡ % (v/v) · h^{-1}
r_A	Acetification rate, g acetic acid · (100 mL medium · h)$^{-1}$ ≡ % (w/v) · h^{-1}
P_A	Acetic acid production, g acetic acid · h^{-1}
$(-r_E)_{LP1}$	Mean ethanol uptake rate during loading phase 1, % (v/v) · h^{-1}
$(-r_E)_{LP2}$	Mean ethanol uptake rate during loading phase 2, % (v/v) · h^{-1}
$(-r_E)_{PP}$	Mean ethanol uptake rate during production phase, % (v/v) · h^{-1}
$(-r_E)_{Global}$	Mean overall ethanol uptake rate, % (v/v) · h^{-1}

INTRODUCTION

Industrially, wine vinegar is obtained mainly by using a semi-continuous process involving submerged acetic acid bacteria[1-4] . Specifically, once the alcohol content falls below a preset level, a portion of the culture medium is unloaded, that remaining in the reactor acting as inoculum for the next load. This allows the production of high-acidity vinegar, facilitates the use of part of the biomass formed in each load to rapidly ferment the next and alters the conditions of the medium in such a way that it can be efficiently used by the most suitable organisms for the intended purpose[5-10].

This operational procedure affords easier control of some variables including the ethanol concentration at unloading time, unloaded volume and loading rate. Such variables influence the concentration and activity of acetic acid bacteria as they act simultaneously on the acidity, ethanol concentration, oxygen supply and even temperature of the medium.

Although a wealth of knowledge currently exists on the vinegar production process, there remains the need to optimize the previous variables, which may have a significant effect on the fermentation rate[4, 11-19].

Recently, a review with previous attempts for modeling the process as well as a new model proposal has been published[4, 17,18]. According to this model, the operational conditions depend of the specific type of product to be obtained. If a high rate is desired, the final ethanol content should not be very low, otherwise the bacterial cells may be severely affected by the scarcity of substrate and the high acidity of the medium, which can seriously hinder their reuse on the next load[18].

This paper reports the results of a study of the influence of the ethanol concentration at unloading time on the productivity and overall rate of the acetification process.

MATERIAL AND METHODS

Raw material

The raw material used was white wine from the Montilla-Moriles region (Córdoba, Spain) with an ethanol content of 12.0 % (v/v) ± 0.3 and an initial acidity of 0.2 % (w/v).

Microorganisms

The inoculum used consisted of a mixed bacterial culture of the genera *Acetobacter* and *Gluconobacter* where *Acetobacter aceti* and *Acetobacter pasteurianus* were the predominant species[20]. The inoculum was obtained from a fully operational industrial fermentation tank of the firm Grupo SOS, in its Alcolea factory (Córdoba, Spain).

Analytical methods

Acidity was determined by acid-base titration and ethanol quantified on-line by means of an Alkosens® probe (Heinrich Frings (http://www.frings.com))

Fermentation conditions

Tests were conducted in a Frings 8 L fermentation tank, operated in a semi-continuous mode consisting of the following steps:

(1) Depletion of ethanol in the medium (production phase, PP) to a concentration of 0.5, 1.5, 2.5 or 3.5 % (v/v) at a constant temperature of 31 ºC and an also constant air flow-rate of 7.5 L air $h^{-1} \cdot L^{-1}$ medium. Once the desired ethanol concentration was reached, 50 % of the tank contents were unloaded.

(2) Regarding the loading phase, several strategies are possible. Nevertheless, the aforementioned model by the authors[18], suggest that the charging step must be carried out in such a way as to keep the ethanol concentration within the

approximate range 5-6 % (v/v). Ethanol levels around and above 6 % (v/v) reduce the proportions of viable cells as well as influence negatively the bacterial activity. So, the tank was slowly loaded (feed rate of 1.2 $L \cdot h^{-1}$) to an ethanol concentration never exceeding 5 % (v/v). This was done in two steps: loading phase 1 (LP1) and loading phase 2 (LP2). In the first, the tank was loaded in a continuous manner to an ethanol concentration of 5 % (v/v); in the second, more wine was added in a semi-continuous manner until the desired final working volume (8 L) was completed.

The bioreactor was fully equipped to operate in an automated mode. Loading, unloading, control and monitoring operations were performed unattended via a previously programmed computer.

Because the primary purpose of this work was to compare the influence of the target operating conditions on the overall rate of the process, the rate had to be previously determined. Provided the total strength remained roughly constant and identical with that of the starting wine throughout the cycle, the ethanol and acetic entrainment losses are negligible, so the mean fermentation rate can be estimated both from the variation of the ethanol concentration during the cycle[21] and from the final acidity.

The fermentation rate determination from the ethanol concentration, $(-r_E)$, allows one to establish the variation of the acetification rate throughout the cycle, so it is possible to assess the influence of the operational variables on the different steps of the process. Details of how estimating the mean acetification rate via on-line monitored changes in ethanol during a semi-continuous vinegar production cycle can be found elsewhere[21].

At the same time, the mean acetification rate, r_A, can be easily calculated from the final acidity in the medium at unloading time, the unloaded volume, the cycle duration and the weighted mean of the fermentation broth volume:

$$r_A = \frac{\text{final acetic acid concentration}\left(\%\ (\text{w/v})\right) \cdot \text{unloaded volume}\left(\text{L medium}\right)}{\text{cycle time (h)} \cdot \text{mean overall volume (L)}} \quad (1)$$

By way of example, eq. (2) shows the calculation of r_A for a final ethanol concentration of 0.5 % (v/v).

$$r_A = \frac{11.0\ \%\ (\text{w/v}) \cdot 4\ \text{L medium}}{31.9\ \text{h} \cdot 7.68\ \text{L}} = 0.18 \frac{\%\ (\text{w/v})}{\text{h}} \quad (2)$$

RESULTS AND DISCUSSION

As stated above, wine vinegar is usually obtained by using a semi-continuous fermentation process involving periodic unloading of the fermentation medium. The ethanol concentration present at the time the reactor is unloaded is one of the variables most strongly influencing the overall fermentation rate. In fact, the more markedly ethanol is depleted, the largest is the amount of acid formed –with which bacteria may eventually react. In this work, the influence of ethanol concentrations of 0.5, 1.5, 2.5 and 3.5 % (v/v) were studied. For instant, Figure 1 shows the variation of the ethanol content, acidity and volume of the medium during the acetification cycle at the ethanol concentration of 1.5 % (v/v). The figure, which shows the results of 8 tests, clearly exposes the steps involved in the fermentation cycle (particularly the ethanol and volume data). Table 1 list the duration of loading and production phases, the time and final acidity values, as well as the average volume for all the experiments; data are accompanied by their respective standard deviations. Table 2 lists the ethanol uptake rate $(-r_E)$ for each phase and global values, the mean acetification rate r_A values, as well as the production of acetic acid, P_A, in g acetic acid $\cdot$ h^{-1}.

Figure 2 shows the acetification rate and acetic acid production percent differences from the lowest levels (viz. those leading to a final ethanol concentration of 0.5 % (v/v)) as well as the mean overall ethanol and acetic acid concentrations for each case. As can be seen, the acetification rate and the acetic acid production increased with increasing ethanol concentration at unloading time by about 38 and 26 %, respectively.

Based on the sensitivity of acetic acid bacteria to both the substrate and product, and on changes in the culture medium, one can expect them to perform disparately under different experimental conditions[4, 22-24]. In fact, our tests exposed differences in mean overall acidity and ethanol concentration (Fig. 2), and also in the highest acidity level reached (Table 1). A high acidity is invariably accompanied by a low ethanol concentration. Both can adversely affect the overall rate of the process, ethanol because it is the limiting substrate and acetic acid because of its inhibitory effect increases with increasing concentration[4]. In this case, the known negative influence that high ethanol concentrations can have on cell viability – demonstrated by authors elsewere[4], it is not a problem because of the followed loading strategy by which the ethanol concentration was never higher than 5 % (v/v). Nevertheless, the acetic acid concentration could be the key factor for explaining the differences observed in this work. Indeed, a decrease in bacterial viability is normally observed as the mean acetic acid concentration increases and, specially, when maxima (final) acidities reach 11 % (w/v)[4].

The process can also be affected by changes occurring between unloading and the end of the loading process; such changes become more marked as the substrate is depleted as well as the loading step is shortened. For instant, Figure 3 shows regression for the experimental variation of ethanol content and volume of the medium during the cycle at each studied final ethanol concentration. From the first, it can be seen that, during the loading phase, bacteria are subjected to important differences in the ethanol concentration (and therefore in acidity) which may have a negative influence in the process. On the other side, from the second, it can be

seen that, from a kinetic point of view, main differences are found in the loading phase 2. During this step, where the ethanol concentration is kept constant, the fermentation rate can be estimated from the temporal variation of the volume[21].

Table 2 lists the ethanol uptake rates for each phase throughout the cycle.

Provide ethanol uptake rates during loading phase 1 (Table 2) have not statistically significant differences (one way ANOVA test), must be in loading phase 2 and production phase where the increase of acetification rate is established. As can be calculated from data listed on Table 2, the uptake ethanol rate for loading phase 2 and production phase increased with final ethanol concentration by about 82 and 13 %, respectively. Nevertheless the overall rate of ethanol uptake increased just by about 40 % since a weighted average as a function of the proportion of time taken by each phase has to be considered. In any case, it is clear that main differences are found in loading phase 2.

Based on the previous results, which contribute to validate the previously proposed model by the authors[18], obtaining a high rate for the process entails ensuring a high ethanol concentration at unloading time; this, however, considerably reduces the acetification yield through the presence of a substantial amount of ethanol in the unloaded liquid. This shortcoming can be circumvented, by using two serially arranged reactors. The two reactors can be optimized in such a way as to ensure that the first is unloaded at a high concentration of ethanol and the second depletes it before it receives a new load from the first; this study is at present going on.

5. Conclusions

The semi-continuous wine acetification process usually employed by the vinegar production industry can be improved as regards overall rate by optimizing some easily adjusted process variables including the ethanol concentration at unloading time, unloaded

volume and loading charge. In this work, the influence of the ethanol concentration on the production and mean acetification rate was studied. Based on the results, the acetification rate and the production of acetic acid increase substantially with increase in the ethanol concentration. Also it is concluded that the increase of the acetification rate is established mainly during the loading phase. However, a product containing much unused substrate is industrially unacceptable. For this reason, two serially arranged reactors, usually available in industry, must be optimized in such a way that the first one must ensure a high acetification rate, but also a final ethanol concentration that can be easily depleted in the second reactor before it receives a new load from the first. This procedure should give mean overall acetification rates higher than those typically obtained when the substrate is depleted much more in the first reactor.

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1

2 Table 1

Final ethanol concentration, % (v/v)	0.5	1.5	2.5	3.5
Number of cycles	6	8	10	29
Duration of loading phase 1, h	2.5 ± 0.1	2.1 ± 0.1	1.4 ± 0.1	0.9 ± 0.1
Duration of loading phase 2, h	6.8 ± 0.4	8.3 ± 0.7	8.9 ± 0.6	11.0 ± 0.8
Duration of production phase, h	22.6 ± 0.9	16.9 ± 0.8	12.0 ± 0.9	6.8 ± 1.2
Cycle duration, h	31.9 ± 0.8	27.3 ± 0.4	22.3 ± 0.5	18.7 ± 0.9
Final acidity (as acetic acid), % (w/v)	11.0 ± 0.2	10.1 ± 0.2	9.2 ± 0.15	8.1 ± 0.1
Mean total volume, L	7.68 ± 0.06	7.54 ± 0.03	7.37 ± 0.02	7.00 ± 0.02

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2 Table 2

Final ethanol concentration, % (v/v)	0.5	1.5	2.5	3.5
$(-r_E)_{LP1}$, % (v/v) · h^{-1}	0.13 ± 0.10	0.23 ± 0.24	0.20 ± 0.17	0.29 ± 0.29
$(-r_E)_{LP2}$, % (v/v) · h^{-1}	0.15 ± 0.01	0.19 ± 0.01	0.25 ± 0.01	0.28 ± 0.01
$(-r_E)_{PP}$, % (v/v) · h^{-1}	0.20 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.23 ± 0.01
$(-r_E)_{Global}$, % (v/v) · h^{-1}	0.19 ± 0.01	0.20 ± 0.02	0.23 ± 0.02	0.26 ± 0.03
r_A , % (w/v) · h^{-1}	0.18 ± 0.01	0.20 ± 0.01	0.22 ± 0.01	0.25 ± 0.01
P_A, g acetic acid · h^{-1}	13.8 ± 0.4	14.8 ± 0.4	16.5 ± 0.5	17.4 ± 0.9

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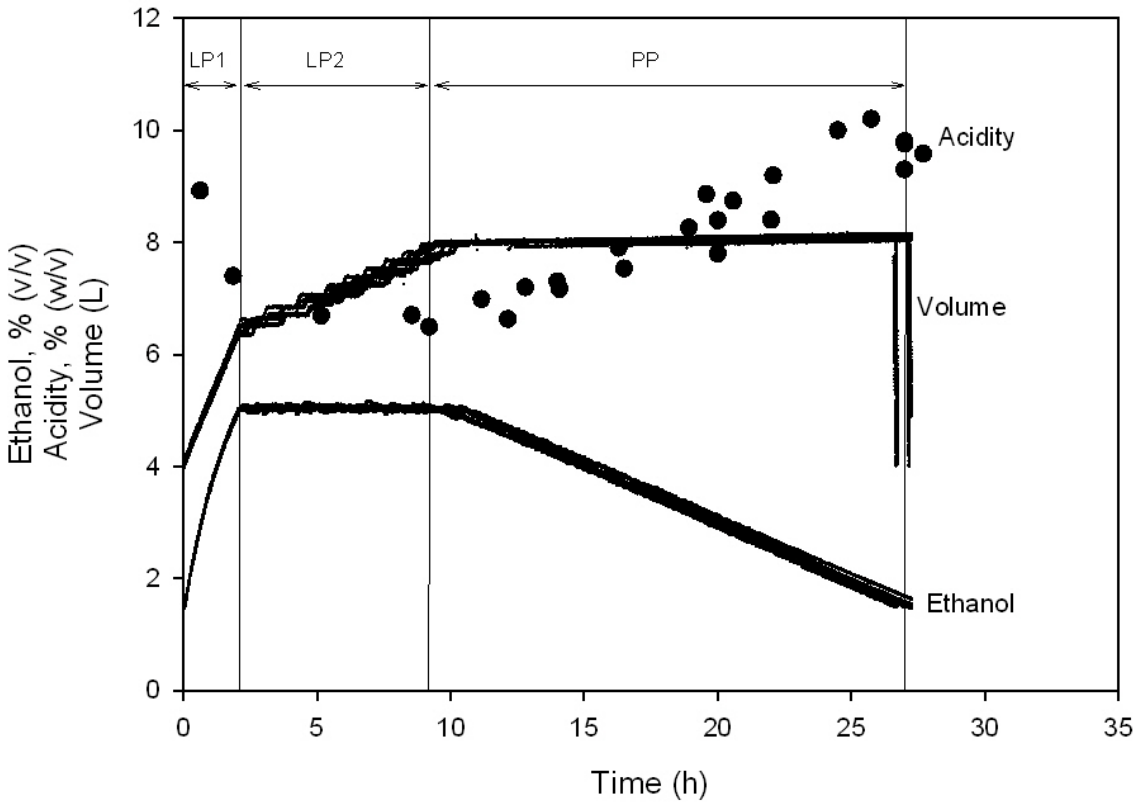
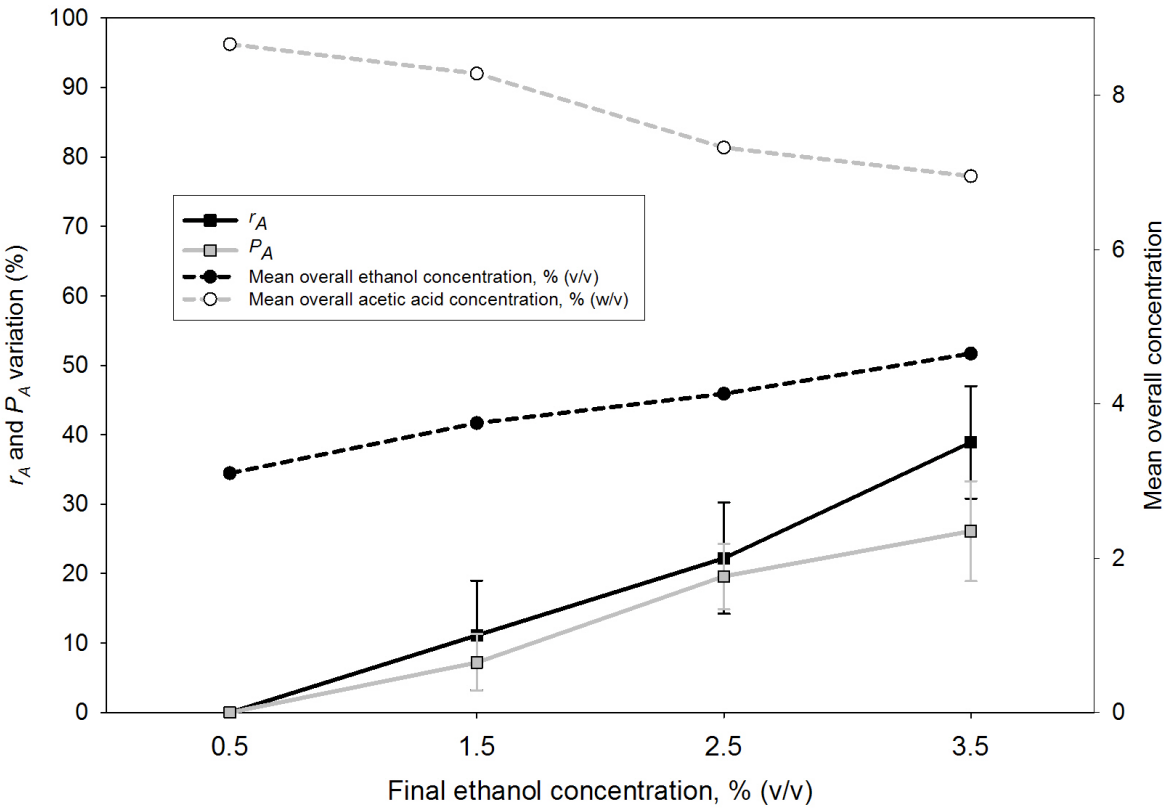
All figures were created by SigmaPlot for Windows Version 11.0

Figure 1



1 Figure 2



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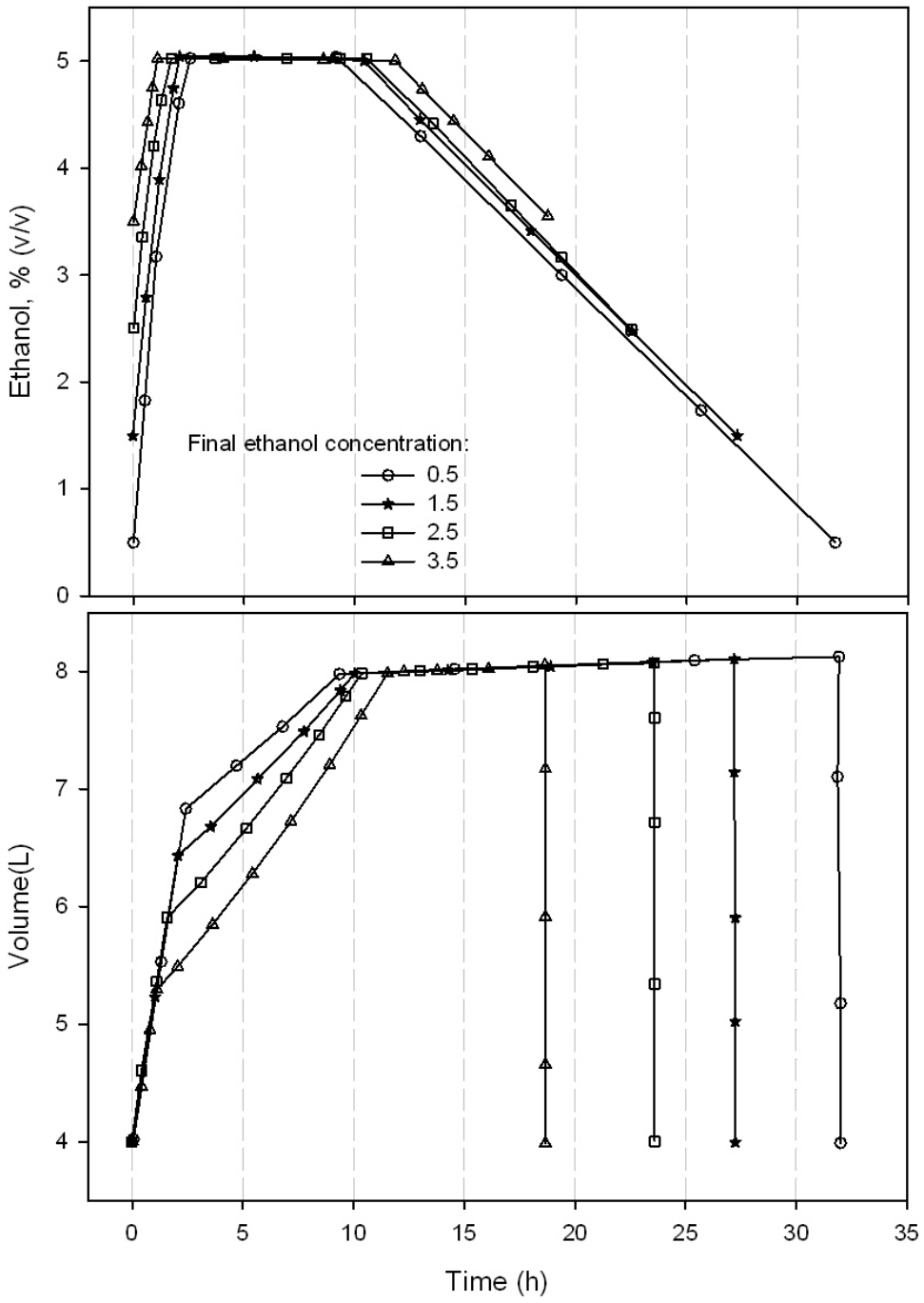
1 Figure 3



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Legends

Table 1

Experimental phase and cycle duration, final acidity and mean overall volume obtained under different experimental conditions plus their standard deviations.

Table 2

Phase and global ethanol uptake rate, mean acetification rate and acetic acid production, accompanied by their standard deviations.

Figure 1

Variation of the ethanol concentration, volume and acidity of the medium during the fermentation cycle. Final ethanol concentration at unloading time: 1.5 % (v/v). (LP1: loading phase 1; LP2: loading phase 2; PP: production phase).

Figure 2

Acetification rate and acetic acid production percent differences from the lowest levels as well as the mean overall ethanol and acetic acid concentrations. Bars represent standard deviations.

Figure 3

Regression for the experimental variation of ethanol content and volume of the medium during the cycle at each studied final ethanol concentration.