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Optimum starting-up protocol of a pilot plant scale acetifier for vinegar production

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Abstract

In the present work, the influences of ethanol (fermentation substrate) and acetic acid (fermentation product) concentrations on the starting-up of a pilot plant acetifier are evaluated. Some effects of activation and inhibition acting on bacterial growth of acetic acid bacteria are registered as a function of the concentrations of substrate and product. As a consequence of the related studies, an optimum starting-up protocol has been proposed. In this, the volumes of wine and inoculum that must be added at the beginning of the process, together with the appropriate instant to make the additions of fresh wine, are established. This protocol is perfectly scalable to other industrial acetifiers, following the proposed stages and addition sequences. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Vinegar; Acetic acid fermentation; Acetifier; Starting-up

1. Introduction

Vinegar has been traditionally considered as a secondary industrial product with no relevant commercial interest. However, the wide diversity of products containing vinegar (sauces, ketchup, mayonnaise, etc.) and the current fall in wine consumption (its main substrate all over the world) have favoured the resurgence of this product. The main biotechnological process involved in vinegar making is *acetic acid fermentation*. It consists of a biological oxidation (strictly aerobic and thermodynamically favoured), in which a substrate with a low content of alcohol (50–100 g/l) is partially oxidised by means of acetic acid bacteria to produce acetic acid and water (Suárez-Lepe, 1990). The result of the biological transformation of the ethanol contained in the initial medium is a solution called *vinegar*, with a high content of acetic acid – the stoichiometry for the conversion of substrate into product is 1:1, and low residual quantities of non-converted ethanol and, moreover, a wide variety of secondary compounds (co-generics).

The most common technology in the actual vinegar industry is based on the submerged cultures (Hromatka & Ebner, 1951). The typical operation mode in industrial submerged cultures is the semi-continuous one. So, once the desired acidity is reached by fermentation, a percentage of volume of the reactor is discharged – near to 50%, and then replenished with an identical volume of fresh wine, beginning a new cycle. The possibilities of this system have been demonstrated versus other alternatives, such as continuous operation mode (de Ory, Romero, & Cantero, 1999).

Nevertheless, operation on semi-continuous acetifiers presents a key aspect that is decisive for the whole production process: the starting-up of the reactor. This is the process by which, beginning with a small amount of a starting culture (a medium with a high number of viable cells in their exponential growth phase), a progressive filling-up of the reactor is carried out, up to its final volume. That is the moment for the beginning of production cycles. The influence of the time employed for this start-up step on the final cost of the overall process is very significant so good determination and control of this step will determine the consequent productivity. So, improving the rate of this initial process – and thus decreasing the time of this non-productive stage, needs an exhaustive knowledge of the key factors that affect the viability of bacteria during the start-up. In this, the concentrations of ethanol and acetic acid in the fermentative medium play the major role. The successive

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charges of volume in the reactor produce significant alterations to the metabolic state of the bacteria, due to changes in the concentration of both compounds. It is very important to note that, both ethanol and acetic acid, can have an activating and an inhibiting effect on the bacterial growth, depending on the concentrations in the medium.

1.1. Influence of ethanol concentration

In laboratory scale discontinuous cultures it has been found that the specific growth rate of the microorganism (μ_c) increases from 0.13 to 0.21 h^{-1} within the interval between 0.5 and 6 g/l of initial concentration of ethanol in the medium (Nanba, Tamura, & Shiro, 1984). From this value and up to 30 g/l the growth rate remains almost constant, and over 40 g/l the growth is seriously inhibited and the growth rate decreases. At the same time, it has been proven that when ethanol concentration is 120 g/l it is very unlikely that any growth of bacterial is seen (Drysdale & Fleet, 1988). In addition, the lag phase increases proportionally to the ethanol concentration. These results were proved later by Soo Park, Ohtake, Fukaya, Kawamura, and Toda (1989), who established 40 g/l as the beginning of the linear decrease for the oxidation ability of the microorganism. Taking into account the kinetic models proposed for the specific growth rate of *Acetobacter aceti* (Gómez, Romero, Caro, & Cantero, 1994) it is possible to conclude that the optimum value for ethanol concentration in the fermentative medium would be about 13 g/l, even though this value strongly depends on the concentration of other toxic compounds like acetic acid.

1.2. Influence of acetic acid concentration

The activating–inhibiting character of acetic acid on the activity of the microorganism *Acetobacter* has been also widely reported in the literature. So, different authors (Bar, Gainer, & Kirwan, 1987) have proved that an activating effect on the growth (and then on the ethanol consumption) is observed for product concentrations below 10 g/l. The studies of Nanba et al. (1984) on this activating effect in discontinuous fermentation show a significant decrease on the lag phase at low concentrations. However, when the acetic acid concentration in the medium increases over 20 g/l, an inhibiting effect begins to be registered and, when the concentration is over 40 g/l, this effect opposes bacterial growth. Thus, the inhibition exceeds 70% at acid concentrations of 60 g/l (Soo Park et al., 1989). Taking into account the kinetic equations which consider the activating-inhibiting effects on the bacterial growth (Gómez et al., 1994) it is possible to establish 10 g/l as the optimum acetic acid concentration for the bacterial metabolism.

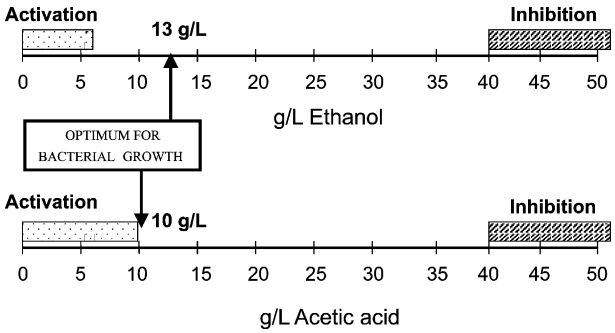


Fig. 1. Influence of ethanol and acetic acid concentrations on bacterial growth and optimum values proposed in the literature.

Fig. 1 shows the ranges of ethanol and acetic acid concentrations, their influence on the bacterial growth and the optimums proposed in the literature.

The synergistic effect of ethanol and acetic acid acting together was studied by Nanba et al. (1984) who proposed a mechanism of enzyme kinetics with co-operative effects

$$\mu = \mu_0 \left(\frac{1 + \alpha_0 P}{\beta_0 + \beta_1 P + \beta_2 P^2 + \beta_3 P^3} \right),$$

where P is the product concentration, $\beta_0 = 1, \beta_1 = \beta_2 = 0$ and α_0 and β_3 are expressed as a function of substrate concentration (E).

The sharing effect can also be expressed as the product of the individual influences (equation from Romero, Gómez, Caro, & Cantero, 1994).

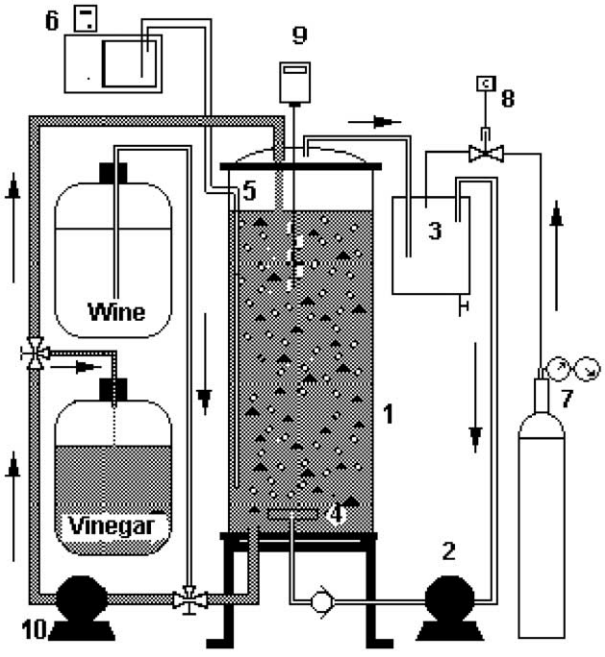
In this experimental work, a study of the effect of ethanol and acetic acid concentrations on the start-up process of a pilot plant acetifier is carried out. It is important to point that, even though the proposed optimum values for ethanol and acetic acid concentrations are very low (13 and 10 g/l, respectively), in actual pilot plant scale experiments higher concentrations have been used in order to reproduce the usual industrial conditions. Once the influence of ethanol and acetic acid concentrations is studied, a standard starting-up protocol, useful for the most common industrial reactors, is proposed.

2. Materials and methods

2.1. Acetic acid fermenter

The pilot plant fermentation equipment was described before in a previous paper (de Ory et al., 1999). In summary, it consists of a stainless steel cylinder-shaped acetic acid fermenter – 0.47 m internal diameter and 1.48 m height. An internal heat exchanger connected to a thermostatic bath controls the temperature of operation within the range 30–31°C, since these have

been considered the optimum values (de Ory, Romero, & Cantero, 1998). The equipment was designed including a closed gas recycling system: the volatile compounds in the gas stream are re-circulated to the bottom of the fermenter by an air pump, after passing through an expansion chamber. Then, they are injected again into the medium through two sinterised stainless steel diffusers, also providing the necessary stirring rate (maximum air flow = 150 l/min). Then, the reactor is fully closed to the atmosphere and the oxygen required for cellular metabolic and fermentative functions is supplied from an industrial oxygen cylinder using an ON/OFF electrovalve controlled by time. The optimum dissolved oxygen concentration (2 mg/l) can be assured, avoiding unnecessary oxygen supplies. The equipment is also fitted with safety valves and various gauges for liquid level control. A diagram of the maximum yield fermenter is shown in Fig. 2.



1.Reactor.
2.Gas recycling pump.
3.Expansion tank.
4. Air diffusers.
5.Heat exchanger.
6.Thermostatic bath.
7.Oxygen cylinder N-50.
8.Electrovalve ON/OFF
9.Dissolved oxygen sensor.
10.Feed inlet and effluent outlet pump.

Fig. 2. Industrial acetification equipment used in the experimental work (de Ory et al., 1999).

2.2. Fermentative medium and inoculum

The medium used for acetic acid fermentation is a young wine from the Jerez-Xèrés-Sherry area (Spain), with an ethanol concentration of 70–80 g/l and a low acetic acid concentration (2.5–5 g/l). These ranges obey certain seasonal variability, usual in this kind of natural substrates.

The starting inoculum, offered by a collaborating vinegar cellar, is a selected culture of acetic acid bacteria, which was previously adapted to our reactor environmental conditions after carrying out several trials. The taxonomical classification corresponds to a mixed strain, with *A. aceti* prevailing (Swings, DeLey, & Gillis, 1984). This is a powerful inoculum with a total biomass concentration of $300–500 \times 10^6$ cells/ml in their exponential growth phase, and variable concentrations of ethanol and acetic acid.

2.3. Analytical methods

Several analytical determinations were developed:

- *Ethanol*; by gas chromatography (Hewlett–Packard 5890 Series II) with a flame ionisation detector and a Carbowax 20 M Capillary column.
- *Acetic acid*; by titration with a 0.3M NaOH solution, and phenolphthalein as indicator. Other organic acids were considered non-relevant for titration.
- *Dissolved oxygen*; by a polarographic electrode OXI-92 (Crison).
- *Total biomass*; by counting in an optical microscope with a Neubauer Chamber.

2.4. Operation procedure

The objective of a starting-up cycle is the filling-up of the reactor, beginning with a given percentage of inoculum and filling to its final working volume with fresh wine in successive stages.

In the first stage of a start-up cycle, the reactor contains a given proportion of wine and inoculum (vinegar) and, as a consequence, a particular set of initial ethanol and acetic acid concentrations. When the inoculated biomass begins its metabolic activity showing a maximum growth rate, a new volume of fresh wine is added to the fermenter. The process continues in successive stages and finishes when the desired working volume is reached. Then, the number of total stages will depend on the percentage of initial inoculum and the reactor working volume.

The developed experiments have been classified according to the ethanol and acetic acid concentrations at the beginning or the end of every single step concerning the start-up cycle. Therefore, three series of experiments are included (see Table 1): the first, GROUP I, corresponds to those start-up cycles carried out in different

Table 1
Initial conditions for the experiments

	Inoculum (l)	Wine (l)	Initial mixture acidity (g/l)
GROUP I (*initial acetic acid concentration* $\leqslant 20$ g/l) (*initial ethanol concentration* $\geqslant 55$ g/l)			
1	40	40	20
2	50	50	12
3	30	30	19
GROUP II (*initial acetic acid concentration* = 30 g/l) (*initial ethanol concentration* = 47 g/l)			
4	30	20	30
5	37	75	30
GROUP III (*final acetic acid concentration* = 80 g/l)			
6	50	50	20

stages with an initial acetic acid concentration either equal to or lower than 20 g/l. The second, GROUP II, concerns those cycles beginning with an acetic acid concentration near to 30 g/l. In these two experimental series, the most influential parameter for the optimisation of the start-up rate is the initial ethanol concentration. Such initial concentrations are in the range of values described in the literature as *critic* (GROUP I $\geqslant$ 55 g/l; GROUP II = 47 g/l).

On the other hand, the third series (GROUP III) includes a single starting-up experiment and tries to evaluate the influence of the final acetic acid concentration on every step. In this case, the medium was fermented up to acidity values clearly above those recommended in bibliography (more than 80 g/l).

The trials included six start-up experiments, working with initial proportions of inoculum between 10% and 23% of the total working volume. In all the experiments, a temperature of 30–31°C and a dissolved oxygen concentration of 2 mg/l were used, as these are considered optimum conditions.

3. Results and discussion

Figs. 3–5 show the variation in the experimental values of acetic acid concentrations in the medium (g/l) versus time (days) for each group of experiments indicated in Table 1. Such figures show the the various stages from the start (inoculation) to the end of the whole operation. Experimental data on ethanol concentration and total biomass have been omitted from these figures because they do not contribute significantly to a better understanding of the process.

Before the initial mixing of inoculum and fresh wine (time = 0 h), a lag phase with no significant acid production was observed in every experiment, even though with different time intervals. This was due to the sudden

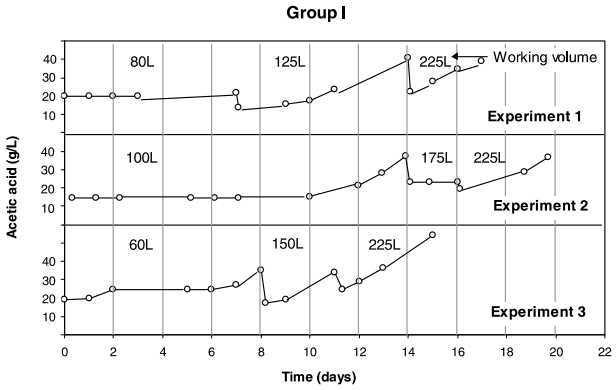


Fig. 3. Experimental data on acetic acid concentration during start-up experiments of Group I (1, 2 and 3).

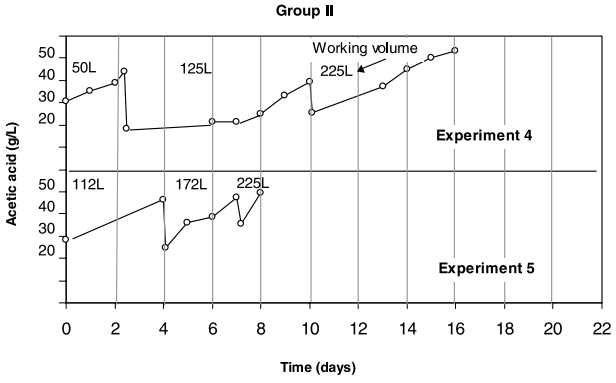


Fig. 4. Experimental data on acetic acid concentration during start-up experiments of Group II (4 and 5).

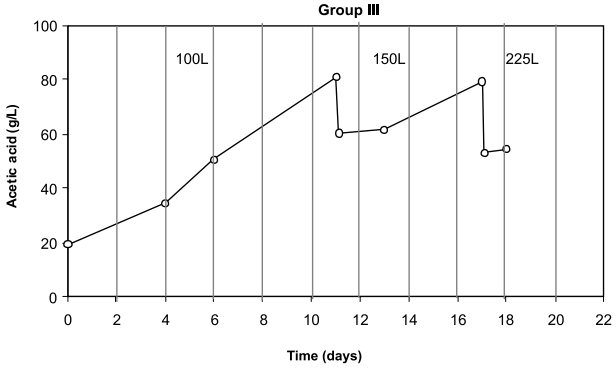


Fig. 5. Experimental data on acetic acid concentration during start-up experiments of Group III (6).

change in the medium conditions before the initial mixing. Consequently, the microorganisms' response is an adaptation phase in which the required enzymes for substrate degradation are synthesised (Brock & Madigan, 1991). Then, during the lag phase, acetic acid bacteria use the main proportion of their energy

resources in this synthesis. Consequently, no net production of acetic acid is observed.

When the lag phase finishes, the beginning of acid formation can be observed, and this is related to a maximum cellular growth. So, high production rates (increasing slopes of the acidity graphs) can be interpreted as the best conditions for the start of a new step in the start-up process. At that time, an amount of fresh wine is added and, as a consequence, a new lag phase appears. Then, every new step in the start-up has a new lag phase.

3.1. Study of the influence of initial ethanol and acetic acid concentrations

In Group I experiments, in which the initial mixing of inoculum and fresh wine is done in such proportions that the medium acidity becomes approximately 20 g/l, a very long-lasting lag phase is observed. In these trials, ethanol concentration at time = 0 is over 55 g/l. Related literature (Soo Park et al., 1989) points out that, at these values, a toxic effect on the acetic acid bacterial growth occurs. This explains the difficulties of cells in overcoming this initial lag phase.

On the other hand, in Group II experiments the initial mixing has been done in such proportions that the acidity is set to 30 g/l. Fig. 4 shows that the length of the lag phase considerably decreases. In practice, an immediate biomass growth (with instant formation of fermentation product) is seen. Initial acidity is higher than in Group I, but not so high as to cause a significant toxic effect on the bacteria. However, ethanol concentration is lower (in this case 47 g/l), substantially decreasing its inhibitory effect on the growth. A direct consequence of these concentrations is a significant decrease in the length of the lag phase and, thus, a shortening in the total start-up time.

The decisive influence of the alcohol concentration on the length of the lag phase is also clearly shown in the second step of experiment 4. In the first step of this trial, acetic acid concentration is over 30 g/l and product formation begins rapidly. But in the second step, the acidity is below 20 g/l because of the high dilution applied, so the following lag phase considerably lengthens.

3.2. Study of the influence of the final acetic acid concentration

In the experiment of Group III, the effect of an excess of final acidity on the start-up was studied. For this purpose, the upper limit of acetic acid concentration up to which fermentation occurs was significantly increased, beginning with an initial mix of 20 g/l and developing the fermentation possibilities to the maximum, finally reaching 80 g/l. In this case, the ethanol toxic effect seen at the beginning of the experiment (about 55 g/l of ethanol) is added to the acetic acid toxic effect at the end of every step. As a consequence, a synergistic effect is produced, promoting a general deceleration in the process. This corroborates the operation limits advised in the specialised literature references.

From the exhaustive analysis of the obtained results in these three groups of experiments, it is possible to establish an optimum protocol for the start-up operation, with the aim of decreasing the process time.

3.3. Optimum starting-up protocol

An optimised protocol for the starting-up of a pilot plant acetifier (including a closed aeration system) is shown here. This is based on a series of consecutive stages by which, beginning with a proper inoculum, it is possible to obtain a full reactor with its final working volume and with the biomass in the optimum activity state, within the shortest time.

Such a protocol takes into account the different conditions in which the inoculum can appear and requires a prior analysis of ethanol and acetic acid concentrations. On the other hand, it is necessary to confirm that current biomass is abundant and has a good viability. Fresh wine will have an alcohol concentration of about 72 g/l (usual concentration in the vinegarmaking industry) and residual concentrations of acetic acid. Optimum operation temperature will be about 30–31°C and the precise oxygen required will be pumped in to bring the oxygen concentration in the medium to the optimum of 2 mg/l. The proposed protocol has been determined for a 225 l reactor but is easily applicable for every working volume and particular conditions (Fig. 6). As is shown, several stages are needed to achieve a full reactor with optimum conditions.

1. *Reactor inoculation*: inoculum and fresh wine are mixed in such a proportion that a total acetic acid concentration of 30 g/l is reached, since the ethanol concentration is limited to a maximum of 47 g/l. The volume added to the acetifier is 67 l – 30% of the total working volume. In this situation, the medium will have suitable initial values of ethanol and acetic acid concentrations to get immediate biomass growth. In this step, only a low amount of oxygen need be added to the medium to reach the optimum value of dissolved oxygen concentration (2 mg/l). This is due to the previous aeration of substrates during pumping operations.
2. *First stage*: when acetic acid concentration reaches 45 g/l (and ethanol concentration is then about 35.5 g/l), 33 L of fresh wine are added (15% of final volume), giving a partially filled volume of 100 l (45%). This again brings the medium to 30 and 47 g/l of acetic acid and ethanol, respectively.
3. *Second stage*: when the medium concentration reaches 45 g/l of acetic acid, 50 l of fresh wine are added (22%) bringing the volume to 150 l (67%)

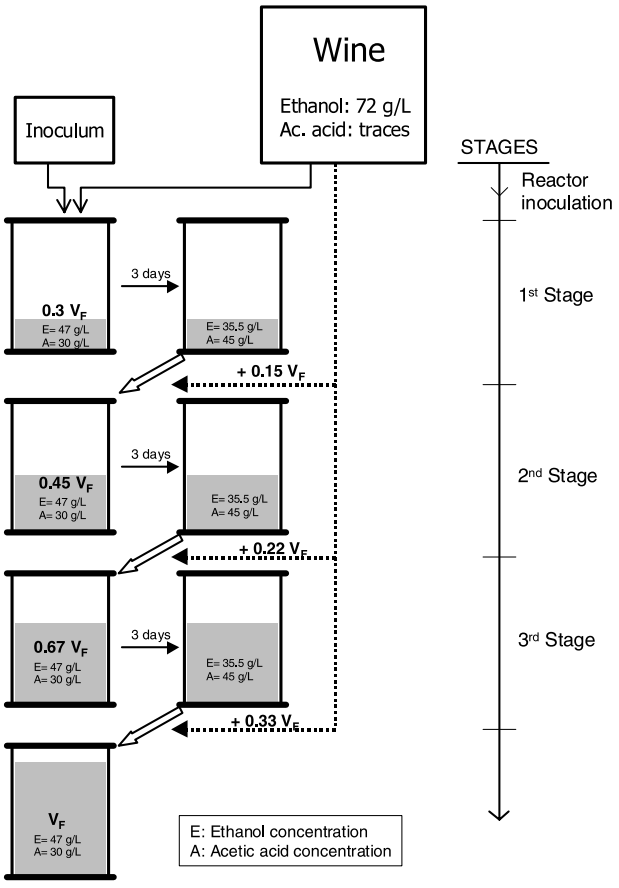


Fig. 6. Optimum start-up protocol of the fermenter.

and then the concentration values again come to the optima.

4. *Third stage*: once again, when an increase from 30 to 45 g/l of acetic acid has taken place, the last 75 l of fresh wine are added. Then, the reactor contains 225 l of medium (100% of total working volume) with an acetic acid concentration of 30 g/l and an ethanol concentration of 47 g/l, with a high biomass population – around 500×10^6 cells/ml, in the maximum activity stage, ready for the beginning of productive cycles.

In the proposed protocol it is clearly stated that the main interest lies in achieving values of acetic acid concentration that changes between 30 and 45 g/l, and ethanol concentrations between 47 and 35.5 g/l.

By using this procedure, the duration of the start-up process reduced to 8 days under the current conditions and equipment, as opposed to the 15 or 20 days registered in other experiments that did not follow this protocol. By applying this protocol it is possible to reduce the total time of this non-productive phase to one-third, with the subsequent increase in profits.

It is essential to point out that the total number of stages in the process will depend on the initial volume of inoculum in the medium. As an example, if the process runs with an initial volume of 100 l (inoculum + wine) – 45% of the total reactor volume, only two stages would be necessary for the optimum starting-up. However, in the industry it is not easy to make use of large inoculum volumes for the start-up, as a consequence of the large working volumes of industrial acetifiers. So this possibility would be impractical in the majority of cases.

4. Conclusions

The duration of the start-up process in a pilot plant scale acetifier mainly depends on the ethanol and acetic acid concentrations within the successive stages that are developed. So, when the substrate is wine with an ethanol concentration of 72 g/l, their toxic effect is smaller when the stages are developed in ethanol concentration values below 47 g/l (in comparison with trails over 55 g/l), and also smaller with an acetic acid concentration upper than 30 g/l (in comparison with trails below 20 g/l). Taking into consideration these trails and the literature cited, we can point that the toxic effects would be smaller in ranges between 47 and 35.5 g/l of ethanol concentration and ranges between 30 and 45 g/l of acetic acid concentration.

An optimum start-up protocol for industrial acetifiers has been established. This consists in filling up the reactor in several successive steps, in which the established ethanol and acetic acid concentration ranges are maintained.

The application of the described optimum protocol allows a reduction in start-up time to 1/3 of normal values and, as a consequence, the minimisation of overall process costs.

Acknowledgements

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