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A B S T R A C T

In order to reduce production costs and environmental impact of bioethanol from sugar beet low purity syrup 2, an intensification of the industrial alcoholic fermentation carried out by *Saccharomyces cerevisiae* is necessary. Two fermentation processes were tested: multi-stage batch and fed-batch fermentations with different operating conditions. It was established that the fed-batch process was the most efficient to reach the highest ethanol concentration. This process allowed to minimize both growth and ethanol production inhibitions by high sugar concentrations or ethanol. Thus, a good management of the operating conditions (initial volume and feeding rate) could produce 15.2% (v/v) ethanol in 53 h without residual sucrose and with an ethanol productivity of 2.3 g L h^{-1}.

Keywords:
Ethanol
Sugar beet low purity syrup
Fed-batch
Very high gravity fermentation
Saccharomyces cerevisiae

1. Introduction

France is currently the largest producer of sugar beet ethanol in the world. From an economic point of view, low purity syrup 2, is a good raw material for ethanol production due to its content of fermentable sugars. Indeed, low purity syrup 2 is obtained from the second thick juice crystallization and still contains 55–60% (w/w) of fermentable sugars and 10–15% (w/w) of non-sugar. The low purity syrup 2 is now commonly used in France for the production of ethanol. However, fermentations carried out at industrial scale usually provide final ethanol concentration between 10 and 12% (v/v) [1,2].

In order to reduce production costs and environmental impacts, it is essential to intensify these fermentations, that is to increase the final ethanol concentration. For the same volume of fermented juice, the increase of final ethanol concentration could permit to reduce both water requirements for the preparation of fermentation media and energy needs for distillation while increasing the production capacity of plants without

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investment [3]. Energy is the second largest cost after raw materials and represents 30% of the ethanol production cost [4,5]. To do this, very high gravity (VHG) fermentation which involves use of medium containing high sugar concentration (>250 g L^{-1}) must be implemented to achieve high ethanol concentration [6]. However, VHG fermentation leads to significant stress for *Saccharomyces cerevisiae* due to osmotic pressure at the beginning of the fermentation and high ethanol content at the end [7,8]. This results in incomplete or stuck fermentation [9–11].

There are three main possibilities to limit drawbacks caused by VHG fermentations [1]. The first one is the improvement of ethanol tolerance by genetic modification of strains but GMO strains of *S. cerevisiae* are banned in France for ethanol production. The second one is the optimization of the fermentation media composition. Many research works studied the role of osmoprotectants or yeast growth factors by supplementing media with various nutrients (available nitrogen sources, protein-lipid complexes, calcium, magnesium, yeast extracts, …) [1,12–14]. However, supplementations should be as limited as possible and low-cost to be realistic from an economic point of view at the industrial scale. Although many studies were conducted on media optimization for VHG fermentation of synthetic medium or starchy substrates, few concerned saccharides substrates [1,15–18]. Therefore optimization of the composition of low purity syrup 2 medium for high gravity fermentation was done in our laboratory [2]. This optimized medium, supplemented with nitrogen, phosphorus, yeast extract, magnesium and antifoam, was used in this study. Finally, the third possibility to limit yeast stress is to improve fermentation process [19–22]. Very few studies are reported in the literature for sugar substrates [23]. Thus investigation on the impact of the process on VHG fermentation of low purity syrup 2 medium is required. Usually, three fermentation processes are used for ethanol production: continuous, multi-stage batch and fed-batch [20]. Continuous processes are difficult to run for VHG fermentation because high ethanol content decreases viability and inhibits yeast growth which could lead to biomass washout [24]. Moreover, oscillatory behaviors have been reported for VHG continuous process [25]. For these reasons, only multi-stage batch and fed-batch processes were investigated in this study. Fed-batch process seems adapted for VHG fermentations because it could minimize inhibition effects of high concentrations of substrate and ethanol, which occur at the beginning and the end of the process.

This paper compares different processes (two-stage batch, three-stage batch and fed-batch with 4 feeding rate strategies) for VHG fermentation of sugar beet low purity syrup. The objective was to reach the highest final ethanol concentration together with the highest ethanol productivity and the lower residual sugars concentration.

2. Materials and methods

2.1. Substrates

Industrial sugar beet low purity syrup 2 and stillage (residue of the distillation step) were provided by a French ethanol producer group. These two industrial products were not sterilized before use. The low purity syrup 2 contained 580 g kg^{-1} of total sugars equivalent sucrose, 720 g_{DM} kg^{-1} of total solids (dried matter) and 494 mg kg^{-1} of assimilable nitrogen (ammonia and amino acids).

2.2. Yeast

The C10 strain of *S. cerevisiae* was provided by UNGDA (Union Nationale des Groupements de Distillateurs d'Alcool) which is the French National Union of Alcohol-Distillers Groups. The starter cultures were carried out in YEPD medium (40 g L^{-1} glucose, 15 g L^{-1} yeast extract and 10 g L^{-1} peptone). They were inoculated from YEPD agar used to store the C10 strain at 4 °C. Media were sterilized at 121 °C for 20 min before use. The starter cultures were incubated at 30 °C on a rotary shaker at 120 rpm (Infors AG CH-4103, Bottingen) for 15 h. The final yeast cells concentration was about 2×10^8 cells mL^{-1}. A sufficient aliquot was centrifuged, then yeast cells were rinsed with sterile water and centrifuged twice to allow the inoculation of fermentation media at 1×10^7 cells mL^{-1}.

2.3. Fermentation media

In order to avoid sugar limitation during the fermentation, media initially contained a total amount of 300 $g_{sucrose}$ L^{-1} (372 $g_{dry\ matter}$ L^{-1}) which has been determined has a large excess of sugar for the fermentation of low purity syrup 2 [2]. The media were made by diluting low purity syrup 2, in a mixture of sterilized water and stillage (66/33% v/v). A supplementation of 31 mg L^{-1} of nitrogen was provided by an ammonia solution 35% (Analytical reagent grade, Fisher Scientific) and a supplementation of 151 mg L^{-1} of phosphorus was provided by a solution of 85% orthophosphoric acid (reagent grade Laboratory, Fisher Scientific). Media were also supplemented with 1 g L^{-1} of yeast extract (Prolabo), 120 mg L^{-1} of magnesium [$MgSO_4$,$7H_2O$] (Prolabo) and 50 μL L^{-1} of an industrial antifoam Ouvrie 922k (PMC Ouvrie SAS).

The acidity of the media was adjusted to 2 g_{H2SO4} L^{-1} with sulfuric acid at 98–100% (Normapur Analytical reagent, Prolabo). The acidity is more relevant in the industry than pH because this parameter does not depend on the dry matter content which varies according to the substrate and its concentration [26]. This acidity corresponded to a pH range from 4.7 to 5.2 according to the dry matter content in the medium.

2.4. Culture conditions

Multi-stage batch fermentations were performed in 1 L Setric reactors at 30 °C with 1 vvh aeration and 120 rpm stirring speed. For the two-stage batch fermentation, first step used for biomass propagation was made in a low gravity medium containing 160 g L^{-1} of sucrose. Then, one third of the vessel content (333 mL) was transferred to another vessel of the same capacity and filled with two-thirds (667 mL) of fresh medium containing 370 g L^{-1} of sugars (Fig. 1a). In this second stage, the medium obtained after mixing contained 240 $g_{sucrose}$ L^{-1} plus the residual sugar from phase 1.

The three-stage batch fermentation (Fig. 1b) was conducted similarly as previously except that the stage 2 consisted in a fermentation in a medium containing 190 $g_{Sucrose}$ L^{-1} plus

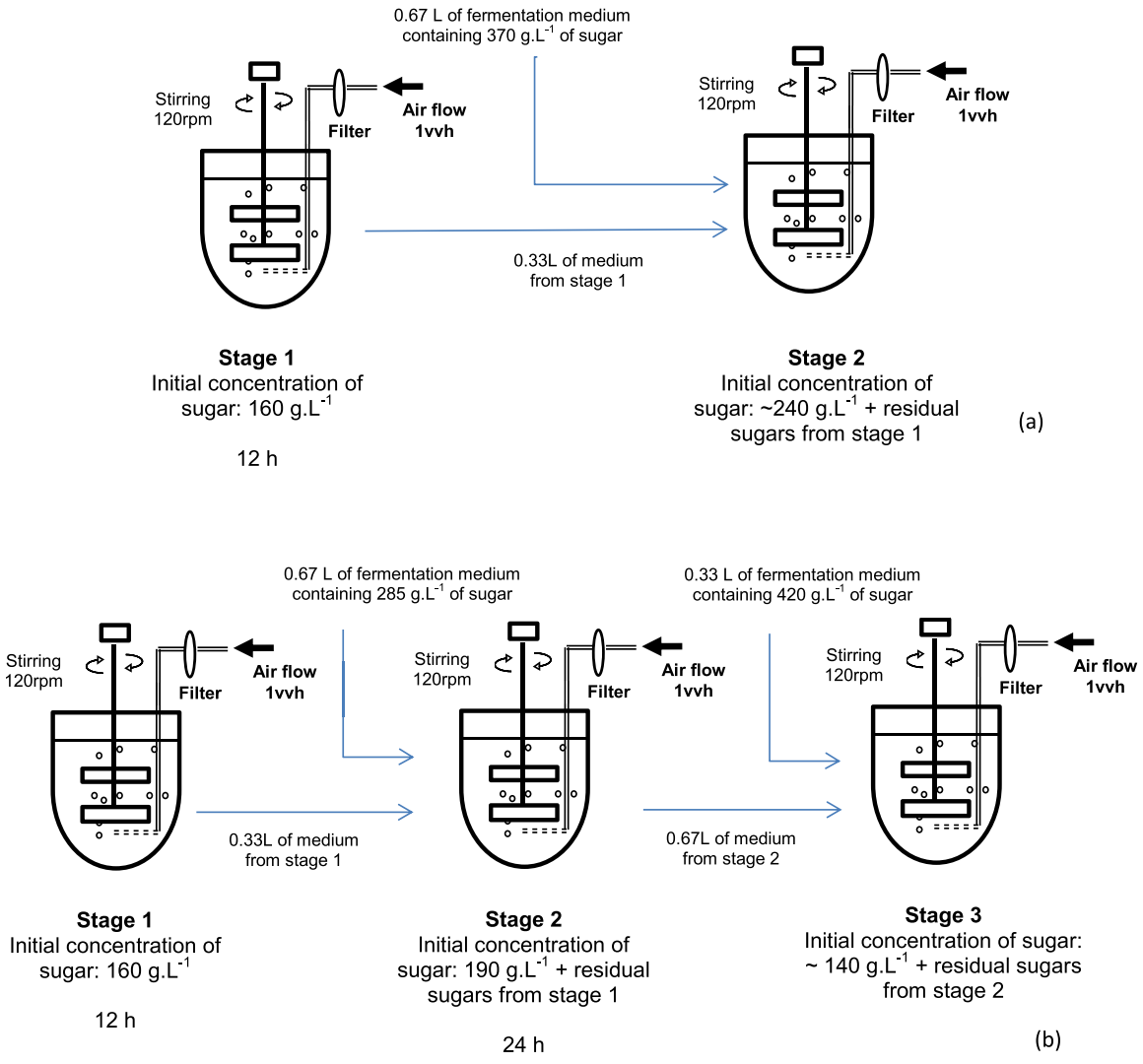

Fig. 1 – Experimental device and culture conditions for (a) two-stage batch fermentation and (b) three-stage batch fermentation.

residual sugars from stage 1 during 24 h. This stage was transferred for two-thirds (667 mL) in another vessel and filled with one-third (333 mL) of fresh medium containing 420 g L^{-1} of sugars. The stage 3 contained after mixing 140 $g_{Sucrose}$ L^{-1} plus residual sugars from phase 2.

Fed-batch fermentations were performed in 4 L (LH Inceltech Series 210) at 30 °C with 1 vvh aeration and 150 rpm stirring speed (Fig. 2). Reactor feeding was made by a peristaltic pump (Gilson). As it is done in the industry, the fed-batch fermentations were divided in three phases: the first one was made in batch mode in a low gravity media for yeast propagation, the second one consisted in reactor feeding and corresponded to ethanol production and the third one was made in batch mode to exhaust as much sugar as possible. These phases are called: propagation phase, feeding phase and exhausting phase respectively (Fig. 2). Concentrations and durations of these different phases are shown in Table 1.

Fermentation kinetics were monitored by measuring viable yeast cells, sugar and ethanol concentrations at 2 h intervals, until two successive samples provided the same ethanol concentration.

2.5. *Analytical methods*

Viable yeast cells concentration was determined by the methylene blue technique. A solution of methylene blue (1 g L^{-1} in 20 g L^{-1} Na_3 citrate) was mixed volume to volume with the diluted yeast suspension, shaken and after 5 min incubation, placed in a Thoma's counting chamber. The number of unstained (active cells) was counted in five different fields. Under these conditions viability was estimated with an accuracy of 12.2% (experimentally measured on five repetitions).

Ethanol, fructose, glucose and sucrose concentrations were determined in the supernatant after centrifugation and filtration at 0.2 μm, by high performance liquid chromatography equipped with a Rezex ROA-H^+ column 300 × 7.8 mm (Phenomenex) maintained at 30 °C and a refractive index detector maintained at 40 °C. The mobile phase was constituted

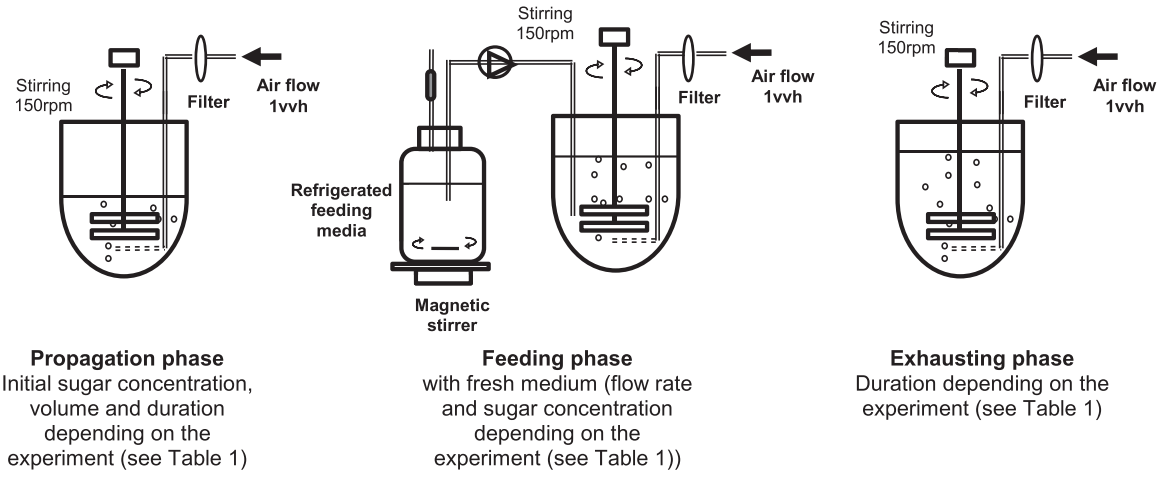

Fig. 2 – Experimental device for fed-batch experiments.

of ultrapure water with 1.9 mmol L^{-1} of sulfuric acid at a flow rate of 170 μL min^{-1}. Sugar concentrations were expressed in sucrose equivalent where 1 g of monosaccharide corresponds to 0.95 g of sucrose. Ethanol and sucrose concentrations were estimated with an experimental error of 1% and 8% respectively.

3. Results and discussion

3.1. *Multi-stage batch fermentations*

The kinetics of ethanol production, sugars consumption and yeast growth are presented on Fig. 3 for both two-stage and three-stage fermentations. The final ethanol concentration, residual sugar and fermentation duration are given in Table 2. One major difference between these two fermentations was the cell growth. During the second stage, the specific growth rate was higher for the three-stage fermentation. It was explained by a lower initial sugar concentration (200 g L^{-1} instead of 270 g L^{-1}) in this fermentation. It is well known that high sugar concentrations cause osmotic pressure stress that leads to growth inhibition and loss of yeast viability, and increases formation of by-products, including glycerol. Then a decrease in ethanol production is usually observed [27,28]. These effects could occur at concentration of 150 g L^{-1} of glucose [29]. In our experiments, substrate inhibition has led to a growth limitation in the two-stage batch fermentation as viable yeast cells concentration was $1.5 \cdot 10^8$ cells mL^{-1} at 30 h against $2.5 \cdot 10^8$ cells ml^{-1} for the three-stage batch fermentation. A decrease in ethanol productivity was also observed between 12 h and 30 h (3.9 g L^{-1} h^{-1} for the three-stage against 3.1 g L^{-1} h^{-1} for the two-stage fermentation). For the two-stage batch fermentation, a drop of viable yeasts and a decrease in ethanol production rate were observed after 30 h. This could be explained by the additional stress caused by high concentration of ethanol. Even if the mechanisms of inhibition of ethanol are not completely elucidated, multiple effects have been reported in the literature as inhibition of cell growth and viability or a decrease in specific rate of fermentation [23]. The inhibition effect of ethanol could occur at relative low concentration (between 8 and 10% v/v) and depends on other parameters of the fermentation (temperature, substrate concentration …). It seemed that a synergetic effect of about 8% (v/v) of ethanol and about 150 g L^{-1} of sugar was inhibitory for yeast viability in the two-stage batch fermentation. These conditions were not met in the three-stage batch fermentation. In the third stage (Fig. 3b), no growth occurred but concentration of viable yeast cells remained constant during 40 h and allowed the transformation of sugar to ethanol. Therefore, it seemed that adding sugar progressively could improve yeast viability and activity as often reported in the literature [19,30,31]. An other major difference between the two fermentations was sugar consumption. The total amount of consumed sugar was higher for the three-stage process. This could be explained by the growth observed during the second

Table 1 – Summary of operating conditions applied in fed-batch fermentations.

Step	Propagation			Feeding				Exhausting sugar	Total sucrose (g L^{-1})	Total duration (h)
	Sucrose (g L^{-1})	Volume (L)	Duration (h)	Sucrose (g L^{-1})	Volume (L)	Flow rate (mL h^{-1})	Duration (h)	Duration (h)		
FB 1	130	1.33	6	385	2.67	55.5	48	20	300	74
FB 2	130	1.33	6	385	2.67	variable	52	17	300	75
FB 3	160	2.67	9	580	1.33	55.5	24	24	300	57
FB 4	160	3.08	9	580	0.92	55.5	16.6	27.4	255	53

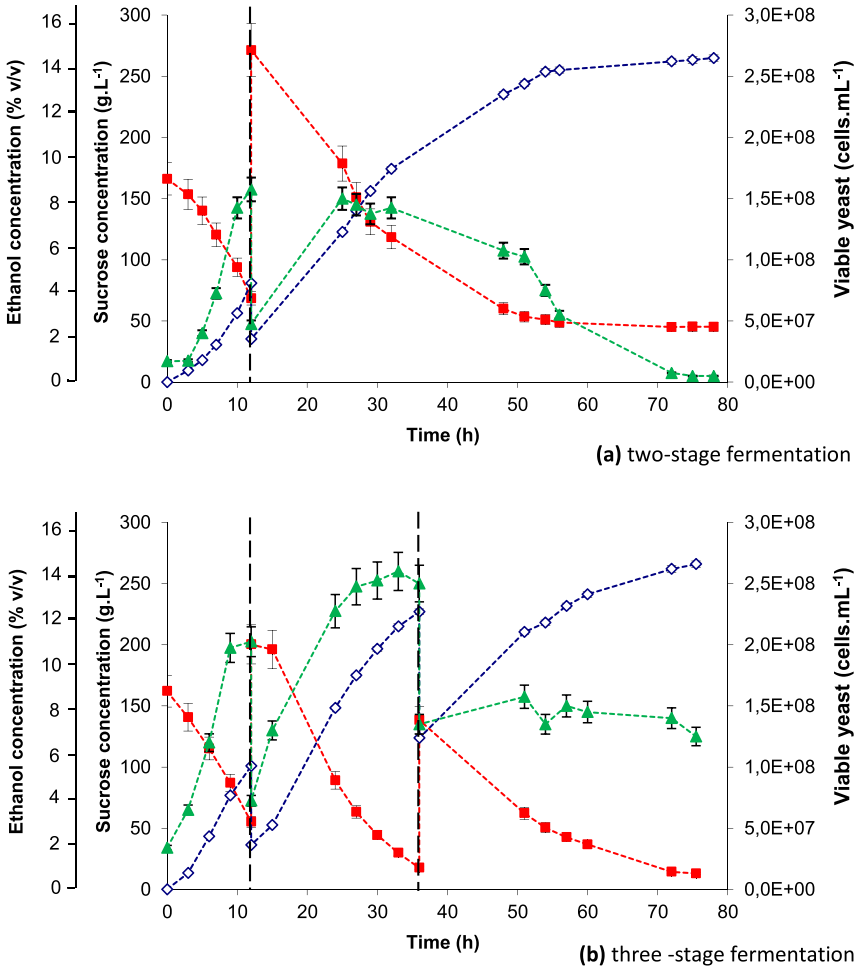

Fig. 3 – Kinetics of sugar (■), ethanol (◇) and viable yeast cells (▲) concentrations during: (a) the two-stage batch fermentation and (b): the three-stage batch fermentation. Dashed lines indicate the transition of one stage to the following.

stage that caused a sucrose use to produce biomass. Indeed, the yield observed was 0.458 $g_{ethanol}\ g_{sugar}^{-1}$ and 0.492 $g_{ethanol}\ g_{sugar}^{-1}$ in the second and the third stage respectively. Yield is an important parameter for the industry, and should be between 85 and 90% of the theoretical yield calculated from sucrose to make the process profitable and avoid residual sugars [1]. In the three-stage batch fermentation, the second growth caused a decrease of the yield from 84 to 76% of the sucrose theoretical yield (Table 2). Nevertheless, there was a slight improvement of 0.2% (v/v) in ethanol production (Table 2). Therefore equilibrium has to be found between ethanol production and yield.

These first results are already very interesting because they demonstrate the feasibility of VHG fermentations from sugar beet substrates with high ethanol concentrations reached (14.9% (v/v)). However, the process could be improved by adding sugar progressively. From the previous experiments, sugar concentration should not exceed 150 g L^{-1}. Therefore fed-batch fermentations were tested, since this process corresponds to fermentation with an infinite number of stages.

Table 2 – Ethanol production for the different operating conditions tested.

	Ethanol (% v/v)	Productivity (g L^{-1} h^{-1})	Residual sucrose (g L^{-1})	Yield[a] (% of theoretical yield)	Total duration (h)
Two-stage batch	14.7 ± 0.1	1.5 ± 0.2	45 ± 4	84	75
Three-stage batch	14.9 ± 0.1	1.6 ± 0.2	13 ± 1	76	75
FB 1	13.4 ± 0.1	1.5 ± 0.2	70 ± 6	85	74
FB 2	14.0 ± 0.1	1.5 ± 0.2	45 ± 4	80	75
FB 3	15.4 ± 0.2	2.1 ± 0.3	46 ± 4	89	57
FB 4	15.2 ± 0.2	2.3 ± 0.3	1 ± 1	88	53

[a] Theoretical yield with sucrose is 0.539 $g_{ethanol}\ g_{sucrose}^{-1}$.

Moreover, fed-batch fermentations could allow to decrease yeast stress during the fermentation by medium feeding management [20].

3.2. *Fed-batch fermentations*

Table 1 shows the operating conditions applied for the different fed-batch processes. For fed-batch 1, 3 and 4, the feeding flow was constant to 55.5 mL h^{-1}. A variable feeding rate was applied for fed-batch 2 according to sugar consumption rate calculated from fed-batch 1 in order to avoid sugar accumulation during the feeding step.

Two strategies were tested. In the first one, the operating conditions were adapted from what is currently done in industry (fed-batches 1 and 2) with a short propagation phase in a small volume and then a long feeding step. The second strategy consisted in a propagation medium in a higher volume for a period of 9 h to obtain at the end of the propagation phase the maximal cell concentration (Fed-batches 3 and 4). For these two experiments, sucrose concentration of the feeding medium was 580 $g_{sucrose}$ L^{-1}, which corresponded to the average low purity syrup 2 sucrose concentration.

For fed-batch 1, the operating conditions caused a significant variation in sugar concentration profile during fermentation (Fig. 4a). After 25 h, sugars accumulated and a slight decrease of ethanol productivity was observed. As previously, inhibition by ethanol explained these results [32]. In this experiment, the inhibition occurred when ethanol concentration was superior to 9% (v/v). After 30 h, the viable biomass concentration decreased. It could be due to a delayed response of yeast cells to ethanol inhibition [23] or a synergetic effect of ethanol concentration and sugar concentration that increased at this time. In consequence, a second fed-batch (fed-batch 2) was carried out with the objective to maintain the sugar concentration during the feeding phase at about 80 g L^{-1} by varying the feeding rate. It could be seen that until 45 h, the viable yeast cells concentration was almost constant even if the ethanol concentration reached 12% (v/v) (Fig. 4b). After 45 h, the cell viability decreased and ethanol production quite stopped. At this moment, the dilution due to the fermentor feeding was too important in comparison with the ethanol production rate. This could be explained by a too long exposure to high ethanol concentration (above 10% (v/v)) and comforted the possibility of a latency time in the response of yeasts to ethanol stress [23]. This latency time was shorter when yeasts were submitted to additional stress as high sugar concentration (fed-batch 1). Thus, controlling the feeding rate to maintain a concentration of sugar below 100 g L^{-1} (fed-batch 2) allowed to increase the final ethanol concentration in comparison to fed-batch 1 (Table 2) even if the ethanol yield decreased from 85 to 80% of the theoretical yield. The yield decrease could be explained by the duration of the stress caused by higher ethanol content in fed-batch 2. However, for these two fed-batch fermentations, ethanol concentration and productivity were lower than for the multi-stage batch fermentations. To improve fed-batch process, it is therefore necessary to limit high ethanol exposure duration while having a sugar concentration not too high. Consequently the feeding phase has been shortened for fed-batches 3 and 4 (Table 1). For that, the volume of the propagation phase was increased and the feeding media was more concentrated in sugar.

Fed batch 3 provided higher ethanol concentration and yield than previous fed-batches (Table 2). During the feeding phase, sucrose concentration was quite constant at 100 g L^{-1} (Fig. 4c). In this phase, ethanol yield was 0.490 $g_{ethanol}$ g_{sugar}^{-1}. That indicates that even if the conditions were stressful (synergy of ethanol and sugar inhibition), the duration was not sufficient to decrease cell activity. Moreover yeast cells concentration was already high at the beginning of the feeding phase ($1.5 \cdot 10^8$ cells mL^{-1}) due to a longer propagation phase. Furthermore, the fermentation duration was the shortest observed in all the experiments so the productivity reached 2.13 g L^{-1}. These results confirmed that the feeding time must be short, since the yeast activity decreased throughout the fermentation due to high ethanol concentration but also to the duration of exposure to ethanol. However, these experimental conditions did not allow the exhaustion of sugars. In the sugar industry, residual reducing sugars and total sugars must be controlled and should not exceed 2 g L^{-1} and 5 g L^{-1} respectively [23]. Therefore, fed-batch 4 was run under similar conditions than fed-batch 3 except a lower total sugar provided (255 instead of 300 g L^{-1}) in order to obtain less than 2 g L^{-1} of residual sugar at the end of fermentation. The total sugar provided has been decreased by reducing the feeding volume and then the feeding duration (Table 1).

As presented in Table 2, fed-batch 4 was the most efficient process for ethanol production due to its highest ethanol final concentration, its shortest fermentation duration, its high ethanol yield and its lowest residual sucrose content. During this fermentation, ethanol productivity stayed almost constant from the beginning of the feeding phase to the end of the fermentation (Fig. 4d). During the feeding phase, yeast viability was maintained. In the exhausting phase, the viable cell concentration decreased but the productivity of ethanol was maintained. It has been reported in the literature that ethanol inhibition could not affect the ethanol yield even if a growth inhibition occurred [32].

Thereby, sugar and ethanol inhibition could be overcome with a good fermentation management of fed-batch process. The propagation phase should provide sufficient quantity of viable yeasts and the feeding phase should be as short as possible to limit synergic inhibition by high concentration of both sugar and ethanol.

The comparison of the fed-batch 4 performances with literature was difficult as few works have been done on VHG fermentation of industrial saccharide substrates and operating conditions, such as temperature could vary [1]. Some studies were carried out at 30 °C in flasks with synthetic medium containing glucose (between 300 and 330 g L^{-1}) supplemented with various compounds (malted cowpea flour, soya flour, yeast extract, corn steep liquor, urea, magnesium sulfate, …) [28,29,33,34]. In these works, final ethanol concentration reached 15–18.6% (v/v) with productivity ranging from 2 to 3.3 g L^{-1} h^{-1} and fermentation durations comprised between 28 and 96 h. Some experiments were carried out in fed-batch process. Alfenore et al. [19] reported a final ethanol concentration of 19% (v/v) and a productivity of 3.3 g L^{-1} h^{-1} using micro-aeration (0.2 vvm) and vitamin supply. Seo et al. [35] reached up to 20% (v/v) of ethanol in 24 h and a

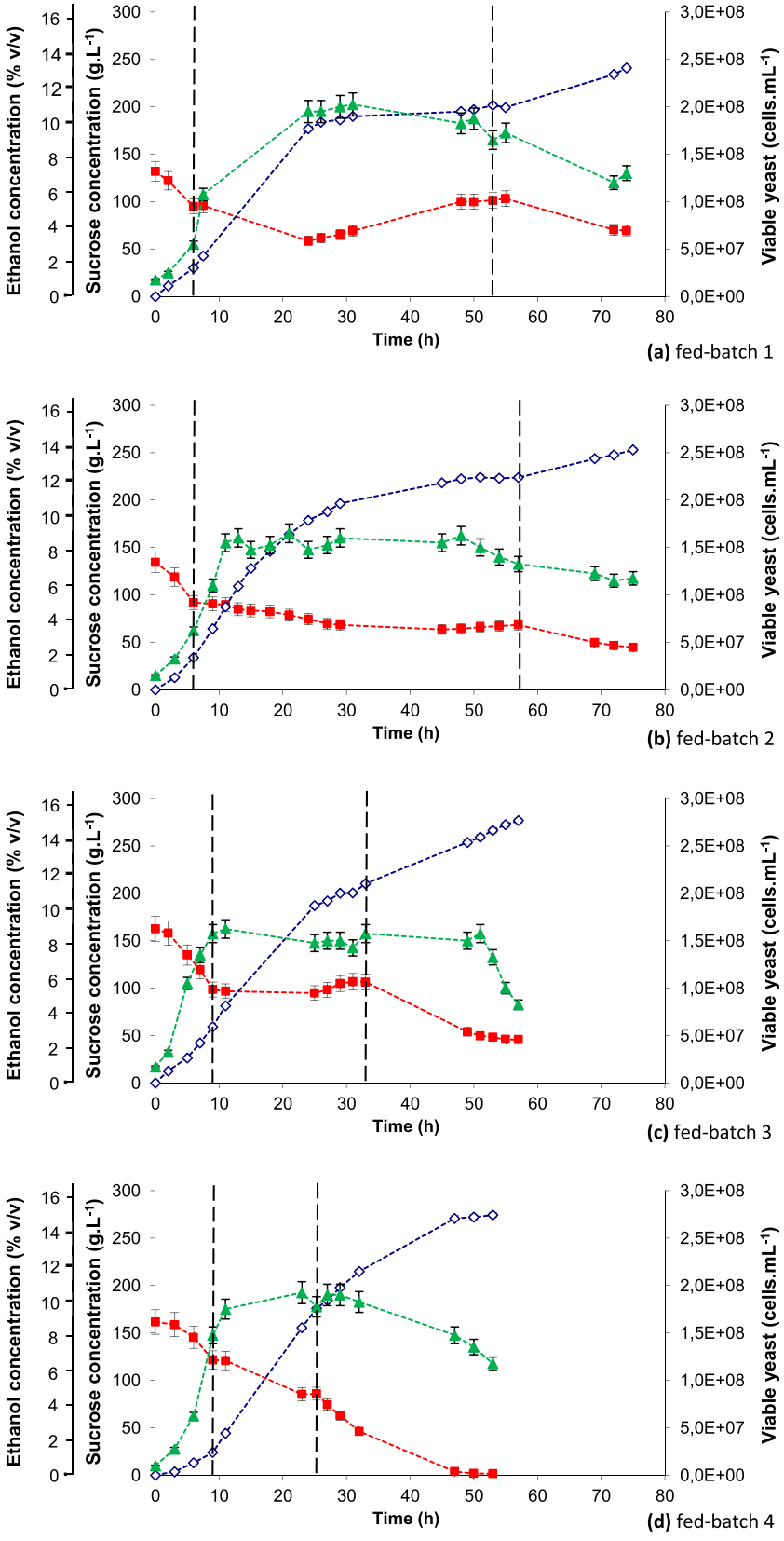

Fig. 4 – Kinetics of sugar (■), ethanol (◇) and viable yeast cells (▲) concentrations during: (a) the fed-batch 1, (b): the fed-batch 2, (c): the fed-batch 3 and (d): the fed-batch 4. Dashed lines indicate the transition of one phase to the following.

productivity of 2 g L^{-1} h^{-1} with micro-aeration (0.13 vvm) and glucose powder and corn-steep liquor addition. Very few works on VHG fermentations in batch laboratory fermentors or flasks of by-products from sugar beet or cane processes are reported. Jones et al. [16] studied fermentation of molasses and sugarcane juice. The best result was obtained for sugarcane juice fortified with wheat hydrolysis: ethanol concentration reached 16.2% (v/v) in 48 h but sugar consumption was incomplete. Pradeep and Reddy [17] reported a final concentration of 13.6% (v/v) and a productivity of 2.2 g L^{-1} h^{-1} without residual sugars, with addition of urea, $MgSO_4$ and soy flour. Finally, a recent study reported a maximal ethanol concentration of 10.6% (v/v) and 16.7% (v/v) with a productivity of 0.74 g L^{-1} h^{-1} and 1.1 g L^{-1} h^{-1} by immobilized cells on maize stem ground tissue for molasses and thick juice from sugar beet process respectively [36].

With regard to these results, the values obtained in this paper are very satisfactory for VHG fermentation. To our knowledge, the ethanol concentration (15.2% v/v) was one of the highest concentration obtained from a real industrial medium with sugar exhaustion. Moreover, the high productivity (2.3 g L^{-1} h^{-1}), the good conversion rate (88% of the theoretical ethanol yield) and the quite short fermentation time (53 h) satisfied the usual constraints of the ethanol industry. Thanks to this work, it is possible to affirm that VHG fermentations of low purity syrup 2 could be achieved at the industrial scale with very little investment. Such fed-batch process could save between 18% and 30% energy per liter of pure ethanol for distillation and between 13% and 16% water for realization of the fermentation media [2].

4. Conclusions

This work demonstrated that fed-batch process with short feeding phase avoided to expose yeasts for a long time to high sugar and high ethanol concentrations simultaneously. The feeding phase should be preceded by a batch growth phase leading to high biomass concentration. With these operating conditions, it was possible to run very high gravity fermentation from low purity syrup 2 and obtain 15.2% (v/v) without residual sugars instead of 10–12% (v/v) currently. These results are directly transferable at the industrial scale and should improve very significantly the environmental impact of bioethanol, while reducing production costs without requiring investment.

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