Sequential fed batch extractive fermentation for enhanced bioethanol production using recycled Spathaspora passalidarum and mixed sugar composition

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Sequential fed batch extractive fermentation for enhanced bioethanol production using recycled *Spathaspora passalidarum* and mixed sugar composition.

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ABSTRACT

The simultaneous ethanol production and removal during sequential cell recycle fed batch fermentation provides a complementary route to produce this biofuel from sugar mixtures, which may greatly improve yields and productivity from lignocellulosic hydrolysates. *Spathaspora passalidarum* is a wild-type strain able to naturally convert glucose, fructose, xylose and arabinose into ethanol. Therefore, the present work has focused on 2G bioethanol production by *S. passalidarum* aiming at the consumption of all sugars released after pre-treatment and enzymatic hydrolysis of sugarcane bagasse in a single fermentation step. The fermentation strategy with sequential cell recycle, fed-batch mode and ethanol removal in situ was performed on a hemicellulosic hydrolysate medium supplemented with molasses. This strategy gave improved fermentation performance and enabled the co-fermention of all sugars under microaerobic conditions. The maximum ethanol yield and productivity was 0.482 $g.g^{-1}$ and 9.5 $g.L^{-1}.h^{-1}$, respectively, showing a process efficiency of 94.3%. The selective ethanol removal enables the operation of the bioreactor at low levels of ethanol (20-30 $g.L^{-1}$), even with

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high sugar concentration inputs, accelerating the fermentation performance and avoiding inhibitory effects on yeast metabolism. Applying the cell recycle strategy, *S. passalidarum* was able to increase its robustness, as shown by a 10-fold increase in ethanol productivity, and it was also able to tolerate a high acetic acid concentration (4.5 $g.L^{-1}$) during long-term fermentations. These results demonstrate that the bioprocess strategy has a strong potential to improve bioethanol production of rich mixed sugar from lignocellulosic hydrolysates in a single fermentation step.

Keywords: *Spathaspora passalidarum*, cell recycle, extractive fermentation, product recovery, hemicellulosic hydrolysate, molasses, biofuel.

1. Introduction

Considering today's global energy situation, the need for energy security and environmental safety has intensified the demand for an alternative and eco-friendly energy source [1]. Bioethanol is one of the most promising alternatives for renewable fuels, since this fuel may be produced from a wide range of renewable sources (rich in fermentable sugars) [2]. According to the Paris Agreement, many countries and several EU member states (around 195) have already proclaimed commitments to bioethanol programs as part of efforts to reduce dependence on fossil fuel, as well as reduce greenhouse gas emissions [3].

Industrial bioethanol production by Brazilian biorefineries is a well establish process, using sugarcane molasses, enabling the achievements of high yields (90-95%) and productivities (10-15 $g.L^{-1}.h^{-1}$), with a technology known as first generation bioethanol (1G) [4]. On the other hand, second generation bioethanol (2G) production still needs process improvements. Lignocellulosic biomass appears to be the most

abundant and promising agro-industrial raw material around the world, which may be used to produce bioethanol and others bio-based products [2]. The biomass-based bioethanol industry in Brazil utilizes sugarcane bagasse and straw, which is considered the cheapest waste raw material, derived from sugarcane processing [5]. On average, one ton of sugarcane used in sugar and bioethanol production can generate a mixture of around 50/50% bagasse (250 – 270 kg) and straw (more than 200 kg). Considering the 2018/2019 harvest, Brazil produced 620.8 million tons of sugarcane, resulting in 29 million tons of sugar, 33.1 billion liters of bioethanol and around 310-380 million tons of bagasse [6]. In order to generate bioethanol from sugarcane bagasse (2G), the biomass essentially needs to be degraded into its individual polymers: cellulose (42-48%), hemicellulose (19-25%) and lignin (20-42%), using an appropriate pre-treatment method [7]. In this way, the released cellulose and hemicellulose molecules are then hydrolysed into soluble sugars (by chemical or enzymatic procedures). Finally, after biomass pre-treatment and hydrolyses steps have been performed, this mixed soluble sugar composition (composed essentially of hexoses and pentoses) is then converted into bioethanol using microbial fermentation strategies [8].

Simultaneous co-fermentation of mixed sugar (xylose, glucose, arabinose and cellobiose), mainly glucose and xylose (two main sugars released after bagasse hydrolyses and pre-treatment), is still problematic for most microorganisms because the presence of glucose represses the utilization of the other saccharides [9]. For most microorganisms, the consumption of glucose (or other readily metabolizable carbon sources) represses the expression of genes encoding enzymes responsible for the metabolization of other carbon sources, a phenomenon known as catabolic repression [10]. During consumption of mixed sugars, a lot of enzymes, especially those engulfed

68 in catabolic pathways, are subject to such repressive regulation, what represents one of
69 the major bottlenecks for 2G bioethanol fermentation processes, preventing the
70 achievement of high yields and productivities at the industrial scale. In order to tackle
71 this challenge, an important technique for overcoming catabolic repression in enzyme
72 biosynthesis is the use of fed batch cultures. Fed batch fermentations allows the
73 concentration of sugars in the fermenting medium to be kept at low levels, the cell
74 growth restricted, and the biosynthesis of specific enzymes depressed [11,12].
75 Remarkably, the ascomycetous, beetle-associated yeast *S. passlidarum*, can co-ferment
76 a wide variety of sugars simultaneously, showing high ethanol yield [9,13-15]. This
77 strain exhibits rapid D-xylose consumption and the ability to ferment glucose,
78 arabinose, fructose and cellobiose, simultaneously, but at different sugar uptake rates,
79 allowing the possibility to consume all mixed sugar released after bagasse pre-treatment
80 for 2G bioethanol production [15].

81 One of the other possibilities to reduce capital costs for 2G bioethanol
82 production is process integration [16]. Until this date, there isn't any report in the
83 literature considering process integration for bioethanol production by *S. passalidarum.*
84 However, integration of 1G and 2G bioethanol production may provide solutions for
85 several challenges in standalone 2G process, showing the opportunity to convert the
86 whole agricultural crops into ethanol, which consequently will increase ethanol yield
87 per hectare of cultivated land disposable for feedstocks production, such as sugarcane
88 [17]. Considering this aspect, simultaneous ethanol production and removal during
89 microbial fermentation has been studied with the aim of developing more cost-effective
90 processes for bioethanol production [18,19]. The conceptual idea is the use of fed batch
91 extractive fermentation with cell recycle, which combines mixed sugar feeding at

92 controlled levels, cell adaptation due to sequential recycling, fermentation processes and
93 ethanol extraction within a single bioreactor. This fermentation technology is an
94 attractive strategy due to the possibility of reducing operation costs, increasing
95 conversion efficiencies, and reducing products and by-products inhibition. However,
96 this technology is still far from being suitable for industrial application. Despite all
97 these features, this study investigates the fermentation performance of a *Spathaspora*
98 *passalidarum* strain, using fed batch extractive fermentations with cell recycle and
99 simultaneous ethanol removal in situ, under conditions that are relevant to the 2G
100 bioethanol production process (high cell concentrations, mixed sugar composition, high
101 yields and process efficiency).

102 **2. Materials and Methods**

103 **2.1. Agro-industrial raw-materials**

104 **2.1.1. Sugarcane molasses**

105 Sugarcane molasses was obtained from Costa Pinto Sugar Mill (Piracicaba, SP,
106 Brazil), containing around 65% total reducing sugar (TRS). Because *S. passalidarum* is
107 unable to produce ethanol from sucrose, the sugarcane molasses was hydrolysed
108 beforehand (to glucose and fructose) using a commercial *Saccharomyces cerevisiae*
109 baker's yeast strain. The sucrose hydrolysis was held in a rotatory shaker at 45 °C, at
110 200 rpm for 12 h, using 10% of inoculum size. This strategy achieved a fermenting
111 media with 100% of hexoses. The sugarcane molasses was used in the experiments as a
112 strategy to dilute the hemicellulosic hydrolysate, reducing the effect of inhibitors,
113 mainly acetic acid, on yeast metabolism. Furthermore, it also works as a source of
114 nutrient supplementation as well as having a buffering effect, keeping the pH stable

during the fermentations. Sugarcane molasses has an acid pH, usually around 5, and its salt content (2 – 8%) has great buffering capacity, which contributes to stabilize pH during fermentation [20]. The composition of hydrolysed molasses is presented on Table 1.

2.2.2. Sugarcane bagasse: hemicellulose hydrolysate

Diluted acid pre-treatment was carried out in order to release a high concentration of pentoses. Reactions were performed in a 350 L reactor (Pope Scientific Inc, Saukville, WI) equipped with a stirrer and heated through an oil thermal jacket. 25 kg of dry sugarcane bagasse (Serrana Mill, São Paulo) were processed at 145°C, for 12 min, with H_2SO_4 diluted solution (0.5% w/v), and a final solid-to-liquid ratio of 1:10. At the end of this reaction time, solid-liquid separation was carried out through a filtration step, in a Nutshe filter with 100 L capacity (Pope Scientific Inc). To alleviate the inhibitory effects to microbes as well as to promote sugar concentration, a detoxification process (by evaporation) was carried out. The liquid fraction (hemicellulose hydrolysate) was concentrated (from 5 to 19.5 °Brix) using an evaporation step (50 $L.h^{-1}$ of hemicellulosic hydrolysate, 475 mbar, 80 °C) [15]. The hemicellulose hydrolysate was stored at 4°C for later use in fermentation processes. Table 1 shown the hemicellulosic hydrolysate composition, before and after the detoxification step been perfomed.

2.2. Fermentation

2.2.1. Microorganism and propagation step

Spathaspora passalidarum NRRL Y-27907 was obtained from the ARS Culture Collection (National Center of Agricultural Utilization Research, Peoria, IL) and was

used to ferment the mixed sugar composition (hexose and xylose). Stocks were kept at -80 °C in XYMP with 20% of glycerol. *S. passalidarum* cultures were streaked from -80°C stocks and transferred to XYPM media containing ($g.L^{-1}$): xylose (10); yeast extract (5); malt extract (20); and monobasic sodium phosphate (2) and incubated in a rotatory shaker for 24 h at 28°C and 150 rpm. A two-step propagation process was standardized: the first step was used to obtain exponential cell growth with defined media (XYMP), and the second one was for cell adaptation in a complex medium with a carbon source from an agro-industrial raw material, in order to mimic an industrial process. These procedures were carried out to adapt yeast cells to an industrial medium.

After the first step had been performed, cells were centrifuged at 10.000 rpm (19.762 xg) at 4 °C for 10 min in a Sorvall centrifuge, and suspended in an appropriate volume of sterile water. The suspensions with microorganism were transferred to inoculum media containing ($g.L^{-1}$): yeast extract (3), malt extract (3), $MgSO_4.7H_2O$ (0.5), $(NH_4)_2HPO_4$ (2), KH_2PO_4 (4), xylose (20) from hemicellulosic hydrolysate and glucose and fructose (20) from sugarcane molasses. The inoculum was kept in a rotatory shaker for 24 h, 28°C and 150 rpm. After this time, cells were centrifuged again (with the same conditions as described above) and re-suspended in an appropriate volume of sterile water to follow the next steps of propagation in the bioreactor.

2.2.2. Fed batch fermentation

A *S. passalidarum* suspension was used to perform fed batch fermentations in the bioreactor. Cells were transferred to the bioreactor, (Bioflo III, 2 L, New Brunswick Scientific Co., Inc., Edison, NJ) with 0.5 L of initial working volume (1.5 L). Agitation was kept at 150 rpm, temperature at 28°C and aeration at 0.05 vvm. The growth media had the following composition ($g.L^{-1}$): yeast extract (3); malt extract (3); $MgSO_4.7H_2O$

(0.5); $(NH_4)_2HPO_4$ (2) and KH_2PO_4 (4). The initial concentration of mixed sugar composition inside the bioreactor vessel was zero and biomass was inoculated to give an initial cell concentration of 10 $g.L^{-1}$.

The fermentation feed medium with mixed sugar composition was composed of hemicellulosic hydrolysate diluted in sugarcane molasses, resulting in about 50.0 $g.L^{-1}$ of pentoses (xylose and arabinose) and around 50.0 $g.L^{-1}$ of hexoses (fructose and glucose). In order to avoid nutrient limitation, the feed medium was also supplemented with a nutrient solution at concentration levels as described above. The feed rate was ranged from 30 to 60 $mL.h^{-1}$ to evaluate fermentation kinetics, and the feed was carried out until the maximum reactor working volume was achieved. These values were defined based on our previous study, regarding the use of mixed sugar composition by *S. passalidarum* during batch runs [15]. These feed rates were based on xylose consumption rate by this strain, because this sugar is consumed slowly compared to glucose, when mixed sugar concentration is target. So, in order to avoid xylose accumulation in fermentation medium, we set up these different flow rates to identify the best fermentation performance during fed batch runs. The fermentation was extended until total sugar consumption. Samples were taken periodically for measurements of cell, ethanol and sugar concentrations. The best fed batch fermentation performance as evaluated in this step was used for the subsequent steps.

2.2.3. Fed batch fermentation with ethanol removal and cell recycling

In order to reduce ethanol toxicity in the fermenting medium, as well as to improve yeast activity, fed batch experiments with simultaneous ethanol removal and repeated recycle of cells were performed. For this, the cells obtained in the propagation

185 step were inoculated (1 L) in a proportion of 1/3 (v/v) of the system work volume (3 L)
186 and supplemented with nutrients, similarly to previous steps. Agitation was kept at 150
187 rpm, temperature at 28°C, and aeration at 0.1 vvm. The reaction system is represented
188 in Fig. 1.

189 The composition of the fermentation feed medium was similar to that described
190 in Table 2. The feed rate was 57.2 $mL.h^{-1}$, based on *S. passalidarum* kinetic
191 performance during fed batch fermentations as evaluated previously. The reactor
192 feeding was carried out until the system working volume was reached, and fermentation
193 was run until sugar depletion. At the end of fermentation, the yeast cells were recycled
194 to a subsequent fed batch fermentation step. The cell recycle was performed using a
195 micro-filter, coupled to a bioreactor vessel, so that cells could be completely retained
196 and accumulated in the system, and the fermented broth was drained from the
197 bioreactor. In this way, cell recycling was promoted by continuous pumping through the
198 micro-filter (tangential filtration) and the cell free liquid effluent (permeate) was
199 removed by a peristaltic pump coupled to the output of the filtering system (see Fig. 1).
200 The yeast cells were concentrated until achieving the initial inoculum condition (1/3 of
201 the working volume system). The micro-filtration system was composed of a crossflow
202 micro-filtration type, which was designed by Millipore Corporation (Ceraflow model:
203 pore size 0.22 μm, filtration area of 0.0372 m^2, length of 22.8 cm). It consisted of an
204 external inorganic tubular filter made of alumina (high purity) and mounted inside a
205 stainless-steel housing.

206 Ethanol removal was performed intermittently, every 6 h, aiming at a constant
207 and low ethanol concentration inside the bioreactor vessel (lower than 40 $g.L^{-1}$, below
208 toxic concentration for the yeast). Each ethanol extraction cycle was about 1 h. The

fermentation system was totally controlled and monitored on line, through the use of a supervisory system, using the software LabVIEW 10.0. To promote ethanol removal, a flash tank was coupled to the bioreactor vessel (see Fig. 1). The flash tank operated under vacuum conditions (100-150 mmHg). The extraction of ethanol from the fermented medium is possible due to the volatility difference between the molecules. So, an enriched vapour mixture of ethanol and water evaporates and passes through a condenser (5°C) and the condensed alcoholic solution was collected in another vessel. After each ethanol removal cycle, the fermenting medium was sent back to the bioreactor. A more detailed description regarding the fermentative ethanol extraction system can be found in Farias et al. [15].

2.3. Sterilization

In order to minimize variations in medium composition, fermentation medium was cold sterilized separately using a sterile system of cellulose ester membrane, 0.2 mm of pore diameter (model Minikap HF Filter MK2M-512-V6S, Sprectum Laboratories, Inc., Fl, USA) and aseptically added to the system. The sterilization of the entire vacuum extractive fermentation prototype (Fig. 1) was performed in an autoclave at 121 °C for 30 min.

2.4. Analytical methodology

The optical cell density was determined using a spectrophotometer detector at 600 nm. Yeast concentrations were measured by gravimetrical analysis in triplicate, and 2 mL of each sample was centrifuged (13.000 rpm, 4 °C for 10 min), re-suspended twice in distilled water in order to remove soluble components and dried in an oven at 70 °C.

The cell free supernatant was used after dilution to quantify sugars, alcohols, glycerol and organic acids using a HPLC apparatus (Varian Inc. Scientific Instruments, Palo Alto, CA), equipped with a refractive index (RI) and an Aminex HPX-87H column (Bio-Rad, 300 x 7.8 mm), at 35 °C, with eluent flow rate 0.6 $mL.min^{-1}$ (degassed and ultrapure water, pH adjusted to 2.6 with H_2SO_4). Furfural and hydroxymethylfurfural were separated by a Nova-Pack C18 column (Water Co., Milford, MA), with an effluent flow rate of 0.8 $mL.min^{-1}$ (acetonitrile: water / 1:8) and detected by UV at 276 nm. The column was placed in an oven at 30°C. The system was equipped with guard cartridges to avoid contaminant interference in the analysis.

Sucrose and monomeric sugars (arabinose, fructose, glucose and xylose) were analyzed using a HPAEC-PAD DX-500 system (Dionex, USA) equipped with a CarboPac PA-1 column (0.4x25 cm; Dionex, USA). Elution was performed at 1 $mL.min^{-1}$ and involved an isocratic step of 1mM NaOH for 8 min followed by a linear gradient of 1-5 mM NaOH for 8 min and an isocratic step of 5 mM NaOH for 14 min. The column was cleaned between runs for 5 min with 150 mM NaOH and equilibrated for 5 min with 1 mM NaOH.

2.5. Kinetic performance

In order to access the fermentation performance of *S. passalidarum* using a mixed sugar composition, the kinetic parameters were calculated based on the fermentation strategy adopted. The ethanol yield (Y_{ps}) was calculated based upon the ratio of maximum product titer and consumed sugars, as described by Eq. 1 and Eq. 3, for fed batch and fed batch extractive fermentation, respectively. The consumption of mixed sugar was based on the total sugar concentration (glucose, xylose, fructose and

arabinose) at the beginning, minus the residual sugar remaining at the end of the fermentation process. Ethanol productivity (Q_p) was calculated considering the ratio of ethanol produced and fermentation time, as described by Eq. 2 and Eq. 4. The list of symbol is provided as supplementary material.

In fed-batch fermentation:

$$Y_{ps} = \frac{P_f - P_i}{S_a - S_f} \quad (1)$$

$$Q_p = \frac{P_f - P_i}{t_f} \quad (2)$$

In fed-batch extractive fermentation:

$$Y_{ps} = \frac{\Delta P_{FBE}}{\Delta S_{BAE}} \quad (3)$$

$$Q_p = \frac{\Delta P_{FBE}}{t_f} \quad (4)$$

Where:

$$\Delta P_{FBE} = \left[\left(P_f - P_i\right)V_r\right]_{\ bioreactor} + \left[\left(P_{extracted}\right)V_{cond}\right]_{\ flash} \quad (5)$$

$$\Delta S_{FBE} = \left[\left(S_f - S_i\right)V_r\right]_{\ bioreactor} + \left[F_a * S_a * (t_a)\right]_{\ feeding} \quad (6)$$

Ethanol efficiency (Eq. 7) was calculated based on ethanol yield (Y_{ps}) described by Eq. (1) and Eq. (3) and compared to the maximum theoretical ethanol yield ($Y_{ps}^{max} = 0.511\ g.g^{-1}$). This value corresponds to the stoichiometric conversion of xylose and glucose into ethanol by *S. passalidarum*. The values reported in the tables are the average of two independent experiments.

$$E(\%) = \frac{Y_{ps}}{Y_{ps}^{max}} \quad (7)$$

The substrate consumption rate (r_S) and specific consumption rate (q_S) for each individual sugar were calculated using Eq. (8) and Eq. (9), respectively:

$$r_s = \frac{F_a}{V_r}(S_a - S) - \frac{dS}{dt} \quad (8)$$

$$q_S = r_S \frac{1}{X} \quad (9)$$

3. Results and Discussion

3.1. Raw material compositions

Table 1 shows the composition of hemicellulose hydrolysate and molasses used as a carbon source during fermentation experiments. According to Table 1, after the diluted acid pre-treatment and detoxification steps (by evaporation) been performed, an enriched pentose hydrolysate was obtained. The evaporation step enables to concentrate 4.5 times the hemicellulosic hydrolysate in terms of xylose concentration (from 21 – 95.0 $g.L^{-1}$). A diluted acid pre-treatment was chosen because it shows high specificity for pentose removal and allows for better consumption of sugars, when ethanol is concerned, due to low generation of furfural and HMF. During bagasse pre-treatment, short-chain aliphatic acids can be formed, including formic acid, acetic acid and levulinic acid [21]. Acetic acid is formed mainly from the hydrolysis of acetyl groups of hemicellulose, while formic acid and levulinic acid are formed from 5-hydroxymethylfurfural (HMF) degradation [21]. The current work presents low concentrations of formic acid (0.282 $g.L^{-1}$) and levulinic acid (0.007 $g.L^{-1}$). On the other hand, significant concentrations of acetic acid was detected (5.5 $g.L^{-1}$). The acetic acid

294 in fermentation media is responsible for the reduction of specific growth rate, sugar
295 consumption uptake, cell yield and it prolongs lag phase, decreasing the overall
296 fermentation performance [15]. Crude lignocellulosic hydrolysates generally contain
297 acetic acid concentrations around 1 – 8 $g.L^{-1}$ [23,24]. Considering this aspect, the
298 concentration used in this work (hemicellulosic hydrolysate diluted with molasses)
299 implies that the suggested fermenting media are of practical relevance for
300 lignocellulose-based bioethanol production processes.

301 According to previous works, *S. passalidarum* can consume sucrose, although
302 this sugar is used for cell growth instead of producing ethanol. On the other hand, this
303 yeast is able to convert hexoses into ethanol. In this way, the molasses used to
304 supplement the fermenting medium was hydrolysed in order to improve product
305 accumulation from mixed sugars [15]. The enzymatic hydrolysis of molasses enabled
306 100% of sucrose conversion into glucose and fructose. Finally, in order to prepare a
307 mixed fermenting medium composed of pentoses and hexoses, a mixture of
308 hemicellulosic hydrolysate and hydrolysed molasses (50:50) was carried out. This
309 strategy enabled a low acetic acid concentration, as well as to supplement the
310 fermentation medium with nutrients from molasses. The final composition of the
311 fermentation culture medium used in all experiments is shown in Table 2.

312 **3.2. *S. passalidarum*: fed batch fermentations**

313 Figure 2 shows the fermentation results of *S. passalidarum* growing at different
314 flow rates (from 30 $mL.h^{-1}$ until 60 $mL.h^{-1}$). The dashed line identifies the fermentation
315 time when the feeding of fresh culture medium was interrupted. The feeding was
316 stopped when the maximum working volume of the bioreactor was achieved, and this

317 time varied according to the flow rate. It can be observed in Figure 2 that glucose was
318 quickly and completely consumed in all cases, even with changes in flow rate. In fact,
319 all sugars were consumed simultaneously, but the substrate uptake rate of each
320 individual sugar was different. This simultaneous sugar consumption occurs probably
321 because the glucose content was always kept low during fermentation, and the diauxic
322 phenomenon was not verified. At higher flow rates, it was also possible to verify that
323 xylose and fructose started to accumulate in the bioreactor, and the higher the flow rate
324 the higher the residual sugar concentration.

325 Table 3 shows the fermentation performance of *S. passalidarum* during fed
326 batch runs using mixed sugar composition. According to Table 3, the time required for
327 the total consumption of sugars increased with a higher flow rate. During the fed batch
328 performed at flow rate of 30 $mL.h^{-1}$ (Fig. 2a), all sugars were depleted at around 34 h.
329 At this condition, the highest ethanol productivity (2.4 $g.L^{-1}.h^{-1}$) was achieved. On the
330 other hand, in order to avoid sugar starvation (and consequently reduced ethanol
331 accumulation), it is desirable to keep the sugar concentration inside bioreactor at around
332 10 $g.L^{-1}$ [11]. It is important to emphasize that if sugar concentrations are kept next to
333 zero during fed batch fermentations, the yeast starts to assimilate ethanol as a carbon
334 source, consequently reducing the process yield [15].

335 The maximum ethanol titer (30.3 $g.L^{-1}$) was achieved at flow rate of 34.2 $mL.h^{-1}$,
336 with ethanol yield of 0.470 $g.g^{-1}$ and productivity of 2.04 $g.L^{-1}.h^{-1}$. On the other hand,
337 the highest ethanol yield (0.501) and efficiency (98.0 %) was achieved for fed batch
338 fermentation performed at a flow rate of 37.2 $mL.min^{-1}$. This experiment reached the
339 highest process yield and efficiency, and demanded 90 h of fermentation. However,
340 there were still small amounts of residual sugars (17 $g.L^{-1}$) at the end of the process,

which were responsible for a drop in ethanol productivity (1.92 $g.L^{-1}.h^{-1}$) and titer (26.3 $g.L^{-1}$). At higher flow rates the fermentation performance was reduced. The ethanol titer, yield and productivity were also reduced.

Regarding by-products, acetic acid, glycerol and xylitol were detected during fed batches runs. Glycerol production was very low and reached a maximum concentration around 0.3 $g.L^{-1}$. The maximum acetic acid and xylitol concentrations at the end of experiments are shown on Table 3. As mentioned previously, acetic acid is produced during the pre-treatment and accumulated in the bioreactor according to the flow rate, reaching maximum concentration levels of about 2.3 $g.L^{-1}$, as shown on Fig. 2. Xylitol accumulation changed accordingly to the flow rate and reached levels around 2.6 $g.L^{-1}$. According to Table 3, it can be noted that the higher the flow rate the higher the xylitol accumulation.

The sugar consumption rates (r_S) and specific sugar consumption rates (q_S) for each individual substrate during fed batches fermentations are shown in Figure 3. Virtually, all sugars were consumed simultaneously, but at different uptake rates. It is also possible to verify that xylose was consumed at the highest consumption rate, followed by glucose, fructose and arabinose. As the flow rate increases (from 30 up to 60 $mL.h^{-1}$), higher rate values were observed. The highest xylose consumption rate (r_S) was of the order of 3.4 $g.L^{-1}.h^{-1}$ achieved at a flow rate of 60 $mL.h^{-1}$. However, higher flow rates resulted in higher residual sugar concentrations, reducing the overall fermentation performance. For glucose, a low substrate consumption rate was observed compared to xylose, with values around 2 $g.L^{-1}.h^{-1}$. Therefore, this fermentation strategy led to a xylose uptake rate higher than that of glucose, indicating that *S. passalidarum* directed its metabolism to the xylose consumption while maintaining glucose, fructose

365 and arabinose at low rates. This behaviour is probably due to a higher expression of
366 specific enzymes necessary to promote the xylose uptake - xylose reductase (XR) and
367 xylitol dehydrogenase (XDH). Considering the xylose assimilation pathway reported to
368 be express for *S. passalidarum*, the xylose is firstly reduced to xylitol due the activity of
369 enzyme XR, and then this molecule is oxidized to D-xylulose promoted by activity of
370 XDH. The activity of XDH is exclusively dependent of coenzyme NAD^+. However, it
371 has been reported that *S. passalidarum* has two different active genes that encode XR
372 (genes XYL1.1 and XYL1.2); the first one is strictly dependent on NADH, while the
373 second one is capable to use both coenzymes (NADH or NAPH), but it presents a
374 higher affinity for NADH [25]. Regarding these aspects, the excessive utilization of
375 NAPDH leads to insufficient regeneration of NAD^+, producing a redox imbalance and,
376 consequently the xylose consumption will be interrupted, leading to a xylitol
377 accumulation instead of ethanol [26]. Adding to this, it is well established that a low
378 concentration of glucose in the fermenting media promotes higher xylose conversion
379 into ethanol [27]. Nevertheless, the sequential utilization of these sugars extends
380 fermentations times, resulting in an incomplete substrate consumption. These
381 behaviours are due to the product accumulation, which achieves inhibitory levels before
382 sugars are depleted, and the complete consumption of sugar is delayed [28].

383 The specific sugar consumption rate exhibited similar trends. The maximum
384 xylose specific consumption rate was 0.180 $g_{consumed\ xylose}.g_{dry\ cell}^{-1}.h^{-1}$ at a flow rate of 30
385 $mL.h^{-1}$ and 0.356 $g_{consumed\ xylose}.g_{dry\ cell}^{-1}.h^{-1}$ at flow rate of 60 $mL.h^{-1}$. Despite the fact
386 that sugar consumption rates increased with increasing flow rates, from the fed batch
387 performed at 37.2 $mL.h^{-1}$, the sugar content began to accumulate in the bioreactor,
388 hindering the accumulation of ethanol. The glucose specific consumption rate varied

389 from 0.08 to 0.258 $g_{consumed\ glucose}.g_{dry\ cell}^{-1}.h^{-1}$, proving that *S. passalidarum* directed its
390 metabolism to xylose consumption when a mixed sugar substrate was used. For
391 fructose and arabinose specific consumption rates, a lower conversion was also
392 observed. The use of mixed sugar in fed batches runs showed better results at an
393 intermediate flow rate, when no residual sugar was detected. Therefore, the results
394 suggest that the combined mixed sugar composition favoured the simultaneous
395 utilization of xylose, glucose, fructose and arabinose. The positive and expressive effect
396 of fed batches runs at high mixed sugar concentrations, is a relevant point in driving
397 further advances in 2G bioethanol production from sugarcane bagasse and molasses.

398 Despite acetic acid concentration being 2.8 $g.L^{-1}$ in the feed medium, *S.*
399 *passalidarum* was able to grow, to produce ethanol and efficiently consume all mixed
400 sugars. *S. passalidarum* was even able to degrade acetic acid, as reported in a previous
401 study using mixed sugar in mono and co-cultures batch runs [15]. However, the
402 mechanisms involved in this degradation have not been completely elucidated [29].

403 The data from this work may represent a good prospect for improved ethanol
404 production from lignocellulosic sugars. On the other hand, the ethanol produced during
405 fermentation can be inhibitory leading to drops in the yeast performance. In order to
406 promote a significant effect on ethanol production, improvements in ethanol-fermenting
407 microorganisms as well as bioreactor design and fermentations techniques have been
408 developed. In this sense, the selective ethanol production and removal using sequential
409 cell recycles and mixed sugar composition were carried out to investigate the
410 improvements in process yields and productivities.

3.3. Sequential fed batch extractive fermentation: a high cell density of *Spasthaspora passilidarum* with bioethanol removal in situ

The results achieved during fed batch extractive fermentation are shown in Figure 4, regarding the investigation of the capability of *S. passalidarum* to tolerate five repeated cell recycles with intermittent bioethanol removal in situ. The dashed lines define the end of each fed batch run, when the cell recycle for the next stage was performed. The fermentation was carried out for around 400 h at a flow rate of 57.2 $mL.h^{-1}$, considering all five sequential steps. Bioethanol removal was carried out with a flash tank, every 6 h, during the entire process through the application of vacuum conditions at around 150 mmHg. Each bioethanol removal cycle was about 1 h, and with this strategy it was possible to maintain a low bioethanol concentration inside the bioreactor, around 20-30 $g.L^{-1}$ (below toxic concentrations for *S. passalidarum* strain). Regarding the bioethanol condensed in the flash tank, it was possible to achieve a maximum ethanolic concentration of 25°GL, reached at the end of the second cell recycle.

The cell concentration was 56.92 $g.L^{-1}$ at the beginning of the process and approximately 36.5 $g.L^{-1}$ at the end of the first fed batch fermentation (Fig. 4). Until the third sequential cell recycle, the cell concentration showed similar performance. At the beginning of the fourth cell recycle, the fermentation achieved the highest cell concentration of 105.1 $g.L^{-1}$ and around 33.8 $g.L^{-1}$at the end of this step. This behaviour is in agreement with the concept of sequential fed batch cell recycling, widely known as Melle-Boinot [4]. The strategy of this process was to start fermentation at high cell density (60 – 90 $g.L^{-1}$, dry mass), which was used as inoculum for each fermentation recycling, corresponding to approximately 1/3 of total bioreactor working volume. After

435 that, the fresh culture medium was gradually fed into bioreactor until reaching the total
436 working volume of the system. At this point, yeast concentrations were supposed to
437 achieve approximately 30-40 $g.L^{-1}$ at the end of each fermentation step [4,11].

438 The major advantage of applying cell recycling is that it is possible to provide
439 high cell concentrations, minimizing the consumption of sugars for yeast growth, what
440 consequently reduce the fermentation time and others costs related to cell propagation
441 steps, and consequently will increase the overall fermentation performance [11]. The
442 sequential cell recycling allowed the starting of each subsequent fermentation with high
443 cell density, and with the additional strategy of ethanol removal it was possible to
444 achieve impressive values for ethanol productivity. Accordingly in Table 4, under these
445 conditions, the maximum ethanol productivity using sugar mixture reaches 9.5 $g.L^{-1}.h^{-1}$,
446 showing an ethanol yield of 0.482 $g.g^{-1}$ and a process efficiency of 94.3%, achieved
447 during second cell recycle of fed batch extractive fermentation. This ethanol
448 productivity was around 10-fold higher than conventional processes, which is the
449 highest 2G ethanol productivity reported to date. The ethanol productivity declined
450 significantly after the forth cell recycle (200 h), but cell growth continued. The
451 adaptation of *S. passalidarum* strains during sequential cell recycle also allowed the
452 simultaneous consumption of all sugars, under microaerobic conditions. On the other
453 hand, it is well known that sugar uptake rate depends on the initial cell concentration
454 and the nutrient supplementation. The glucose in the medium was promptly metabolized
455 (100%), even after prolonged fermentation. Regarding xylose content, the time required
456 for complete consumption increased at each cell recycle. Reduced values were observed
457 after the third cell recycle, delaying the fermentation performance. The main reason for
458 this behaviour is probably the accumulation of acetic acid, which is due to the ethanol

459 removal. It may be possible that the increased concentration of this inhibitor compound
460 led to the deficient xylose bioconversion.

461 Despite of the increasing values of residual xylose over time, at the end of each
462 cell recycling, both ethanol yield and productivity increased compared to the first cycle.
463 These increasing values reinforce the two main ideas proposed by this work: i) the
464 benefits of cellular recycling during sequential fed batch fermentation, which provided
465 energy-saving proprieties (cells converted all carbon content into ethanol instead of
466 shifting for the metabolism of cell growth); and ii) the intermittently ethanol removal,
467 what will avoid inhibition of cells metabolism by the ethanol accumulated. Considering
468 these aspects, a physiological adaptive effect of cell recycling was still observed cycle-
469 to-cycle, implying that ethanol removal was essential to improve yeast metabolism and
470 that the adaptation of cells after third-cycle was necessary for the cell population to
471 thrive at high acetic acid concentration.

472 Figure 5 shows the sugar consumption rates (r_S) and specific sugar consumption
473 rates (q_S) for each individual substrate. It is possible to see the interference of glucose
474 on the xylose consumption and it is also possible to observe the difference between
475 mixed sugars consumption profiles. At the first fed batch fermentation, xylose was
476 consumed at a higher substrate consumption rate (2.208 $g.L^{-1}.h^{-1}$), followed by glucose
477 (1.752 $g.L^{-1}.h^{-1}$), fructose (1.112 $g.L^{-1}.h^{-1}$) and arabinose (0.115 $g.L^{-1}.h^{-1}$), respectively.
478 Interestingly, the xylose consumption rate decreased from the second cell recycle (1.795
479 $g.L^{-1}.h^{-1}$) and became similar to glucose (1.789 $g.L^{-1}.h^{-1}$). Considering this scenario, it
480 can be noted that the cell population of *S. passalidarum* was able to adapt itself to the
481 mixed sugar composition, showing similar consumption rates for glucose and xylose
482 after sequential cell recycles. Remarkably, concerning fructose and arabinose contents,

483 the substrate uptake rate became higher during the sequential recycles, showing that the
484 strategies of ethanol removal and cell recycling improved the overall fermentation
485 performance.

486 Concerning by-products accumulation, acetic acid, glycerol and xylitol were
487 detected during sequential fed batch runs. Figure 6 shows the profile of by-products
488 accumulation during fermentation time. The glycerol concentration remained low all
489 along the sequential cycles performed (0.14 – 0.75 $g.L^{-1}$). Regarding xylitol
490 accumulation, it is possible to note that at the end of the third fed batch (around 200 h),
491 xylitol started to be accumulated. *S. passalidarum* produced 4.8 $g.L^{-1}$ of xylitol in the
492 last fed batch (fifth recycle), however this accumulation may probably be avoided by
493 controlling the oxygen levels during the fermentation. Precisely controlled micro-
494 aeration is crucial during xylose fermentation by wild-type strains, in order to
495 regenerate the NAD^+ cofactor required by xylitol dehydrogenase enzyme (XDH) on
496 xylose metabolism [30]. Considering this aspect, if an insufficient oxygen level is
497 provided during fermentation runs, it will lead to an insufficient amount of NAD^+
498 regenerated and an increased xylitol accumulation, blocking the xylose metabolism as
499 described previously [31].

500 The accumulation of acetic acid during fermentation is related to two main
501 factors. Firstly, this inhibitor is current in hemicellulosic hydrolysate and the final
502 concentration of the culture medium is of the order of 2.89 $g.L^{-1}$. Adding to this, during
503 the ethanol removal (by evaporation), there is also the concentration of acetic acid
504 inside the bioreactor. Although acetic acid is also a volatile substance, the concentration
505 of this inhibitor kept increasing during ethanol removal, probably because the
506 conditions of vacuum x temperature adopted were favourable to the extraction of the

ethanol-water mixture. Besides the fact that selective ethanol removal during fermentation decreases the alcohols toxicity, the presence of inhibitors generated during the pre-treatment increased, which affects the microbial growth and fermentation performance.

It is possible to note that after the third fed batch cell recycle, the acetic acid concentration is about 4.5 $g.L^{-1}$. Despite the high concentration of this compound, there is still a fraction of highly resistant cells able to resume growth after prolonged exposure to this inhibitor, and probably this seems to be related to a superior phenotype for acetic acid tolerance compared to the strains in the first fed batch cycle. The low reproducibility during sequential fed batch with cell recycle led us to hypothesize that for the *S. passalidarum* strain, only a small portion of cells are capable of keeping their metabolic activities. Accordingly, any small variation in this cell population along the sequential cell recycles, due to the higher concentration of acetic acid, would significantly decrease the xylose uptake rate, ethanol yield, productivity and consequently the overall fermentation performance. The fact that only a subpopulation of cells resume growth after dealing with prolonged stress conditions, can be attributed to cell-to-cell heterogeneity, or even to mutations that have naturally increased in these cells [32]. The current study shows that acetic acid resistance affects the performance of microorganism in industrial fermentation. Once the high acetic acid tolerance of *S. passalidarum* is accepted as a desirable phenotype in industrial 2G bioethanol, it is possible to screen these portions of yeasts capable of growth after repeated cell recycling and investigate their genetics in order to construct more robust strains. Yeast screening could be used to guide further studies related to strain-specific differences that

530 are genetically determined as well as to be used as a valuable starting point to identify
531 genetic targets for yeast improvements.

532 There is not any other report in the literature considering the use of sequential
533 fed batch extractive fermentation with cell recycle and ethanol removal. Nevertheless,
534 the benefits of sequential fed batch fermentation using *S. passalidarum* was also
535 reported by other researchers. Nakanihi et al. [13] investigated the performance of fed
536 batch cell recycle by the same strain using a fermentation medium composed by
537 sugarcane bagasse hydrolysate, however, unlike this study, with a higher concentration
538 of hexoses instead pentoses (Glucose: 42.9 $g.L^{-1}$; Xylose: 14.9 $g.L^{-1}$). The authors
539 achieved improvements in ethanol productivity and process efficiency, from the first to
540 the fourth cell recycle (0.38 - 0.81 $g.L^{-1}.h^{-1}$ and 62 – 80%, respectively). Recently,
541 Neitzel et al. (2020) [33] also reported the development of sequential fed batch cell
542 recycle fermentation by *S. passalidarum*, using a synthetic fermentation medium with a
543 similar composition applied in our work (Xylose: 63 $g.L^{-1}$; Glucose: 27 $g.L^{-1}$), but once
544 again without the extractive fermentation and sugarcane biomass. As a result,
545 researchers reported improvements in xylose consumption (from 84.2 to 99.5%),
546 ethanol efficiency (from 68.9 to 90.8%) and productivity (from 1.34 to 1.79 $g.L^{-1}.h^{-1}$)
547 and they linked the better fermentation performance mainly due the physiological
548 adaption through cellular recycling. Anyway, with the application of ethanol removal
549 techniques together with sequential fed bath cell recycle we successfully achieved the
550 highest productivity (9.5 $g.L^{-1}.h^{-1}$) reported in the literature, proving that the elimination
551 of inhibition promoted by product accumulation was crucial in order to achieved higher
552 fermentation performance.

553 The results from this study showed that high yields and productivities were
554 achieved during sequential cell recycle with ethanol removal in situ, suggesting that this
555 method can be a suitable strategy to improve the process. Additionally, what is believed
556 to be the first report has been given, regarding an organism capable of fermenting a
557 wide range of sugars (xylose, arabinose and fructose) in the presence of glucose. This
558 ability makes *S. passalidarum* a potentially useful yeast strain for 2G ethanol
559 production, from mixed sugars released by lignocellulosic biomass, in a single reactor.
560 Furthermore, *S. passalidarum* is an interesting microorganism for unravelling the
561 regulatory mechanisms involving bioconversion of lignocellulosic materials by yeasts
562 and it is also possible to access this knowledge as a source of new D-xylose metabolism
563 genes for robust recombinant industrial strains of *Saccharomyces cerevisiae*. The results
564 presented may establish a baseline for further improvements when mixed sugar
565 fermentation is the target.

566 **4. Conclusions**

567 The simultaneous consumption of mixed sugar by *S. passalidarum* increases the
568 bioethanol yield and productivity during sequential cell recycling with simultaneous
569 ethanol removal. Ethanol productivity reached values around 9.5 $g.L^{-1}.h^{-1}$, with mixed
570 sugar concentration of 100 $g.L^{-1}$ in a feed medium composed of hemicellulosic
571 hydrolysate supplemented with molasses. The selective ethanol removal was
572 responsible for a reduced ethanol concentration inside the bioreactor (20-30 $g.L^{-1}$),
573 achieving high concentrations, condensed from the flash tank, with a titer around 192.4
574 $g.L^{-1}$. The long-term adaptation of *S. passalidarum* cells through sequential
575 fermentation in hemicellulosic hydrolysate media was successfully established.

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Figures Caption

Fig. 1. Schematic illustration of the fed batch extractive fermentation with sequential cell recycling. This fermentation scheme was designed to obtain a high cell density of *Spathaspora passalidarum* in cell recycle fed batch fermentations and to promote cell adaptation in mixed sugar rich-media, with ethanol removal in situ.

Fig. 2. Fermentation performances of *Spathaspora passalidarum* during fed batch runs in a mixed sugar fermenting medium composed by 50% hemicellulosic hydrolysate enriched with 50% molasses. Symbols: (▲) Xylose, (□) Glucose, (■) Fructose, (◊) Arabinose, (○) Ethanol, (●) Acetic acid. The dashed lines identifies the fermentation time when feeding of fresh medium was interrupted.

Figure 3. Sugar consumption rate (r_s) and specific consumption rate (q_s) by *Spathaspora passalidarum* for each individual sugar from the mixture of hemicellulosic hydrolysate and sugarcane molasses in fed batch fermentations according to the flow rate.

Fig. 4. Time course for *Spathaspora passalidarum* during five sequential cellular recycle through fed batch extractive fermentation runs with selective bioethanol removal in situ. Symbols: (▲) Xylose, (□) Glucose, (■) Fructose, (◊) Arabinose, (○) Ethanol inside bioreactor, (X) Ethanol extracted, (●) Acetic acid, (Δ) DCW. The dashed lines identifies the end of each fed batch run, when the cells were recycled to the next fermentation step.

Fig. 5. Sugar consumption rate (r_s) and specific consumption rate (q_s) by *Spathaspora passalidarum* for each individual sugar, during five sequential fed batch steps of the extractive fermentations with cell recycling.

Fig. 6. By-product accumulation profile during five sequential cellular recycle fed batch extractive fermentation by *Spathaspora passalidarum* using mixed sugar composition as substrate.

Figure 1.

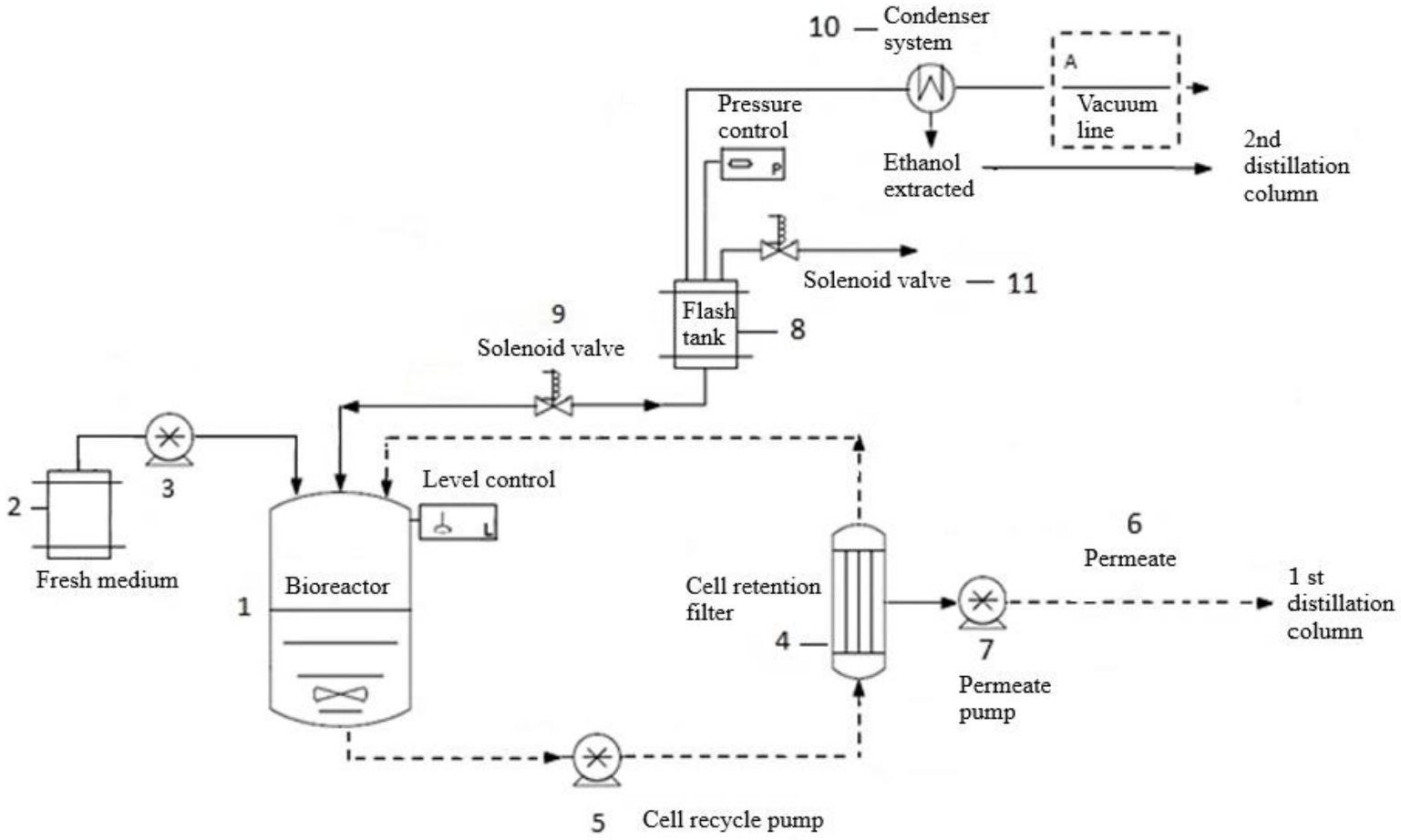
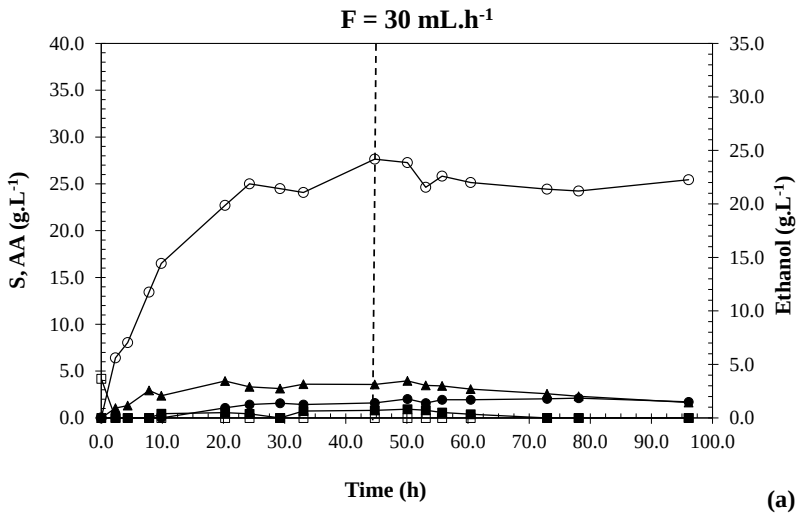
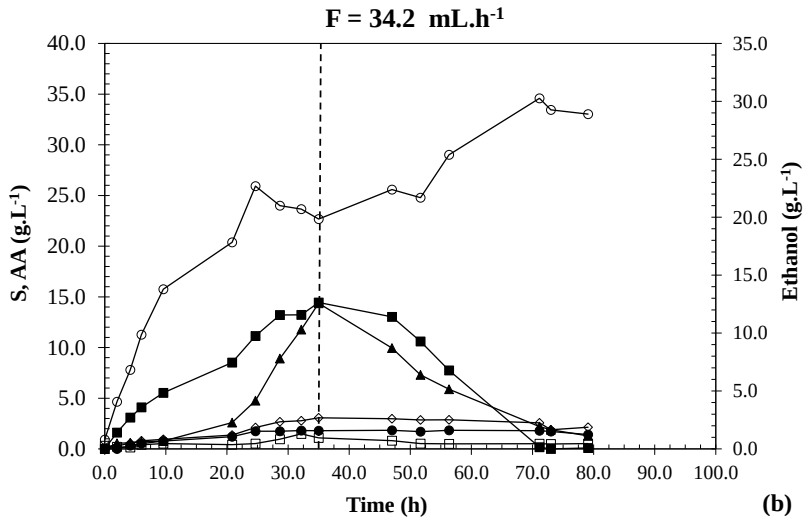
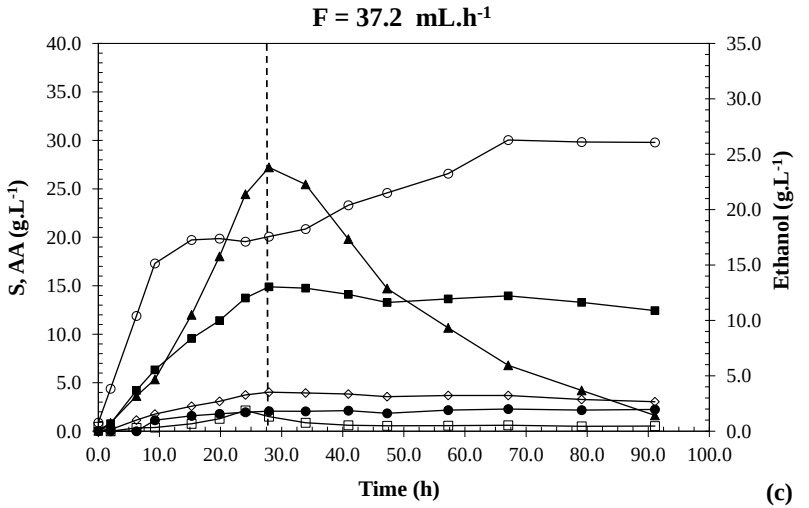
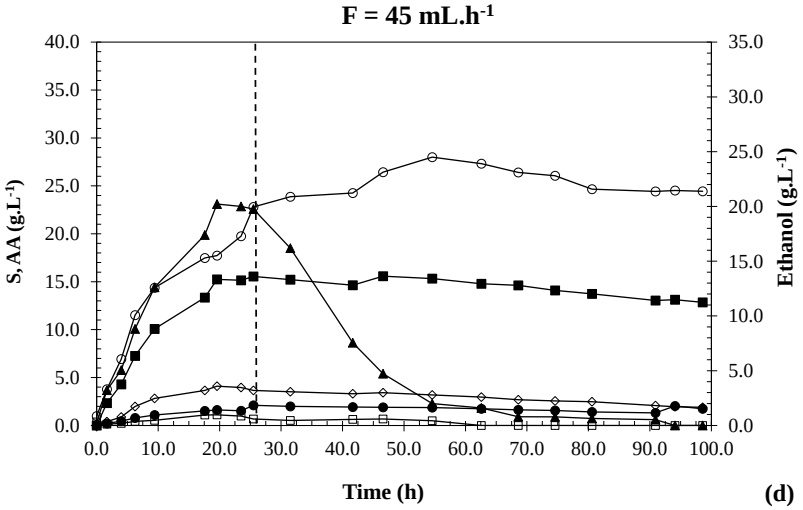

10
Condenser system
A
Vacuum line
Pressure control
P
Ethanol extracted
2nd distillation column
Solenoid valve
11
9
Solenoid valve
Flash tank
8
3
2
Fresh medium
Level control
1
Bioreactor
Cell retention filter
4
6
Permeate
1 st distillation column
7
Permeate pump
5
Cell recycle pump

Figure 2


F = 30 mL.h-1
S, AA (g.L-1)
Ethanol (g.L-1)
Time (h)
(a)


F = 34.2 mL.h-1
S, AA (g.L-1)
Ethanol (g.L-1)
Time (h)
(b)


F = 37.2 mL.h-1
S, AA (g.L-1)
Ethanol (g.L-1)
Time (h)
(c)


F = 45 mL.h-1
S, AA (g.L-1)
Ethanol (g.L-1)
Time (h)
(d)

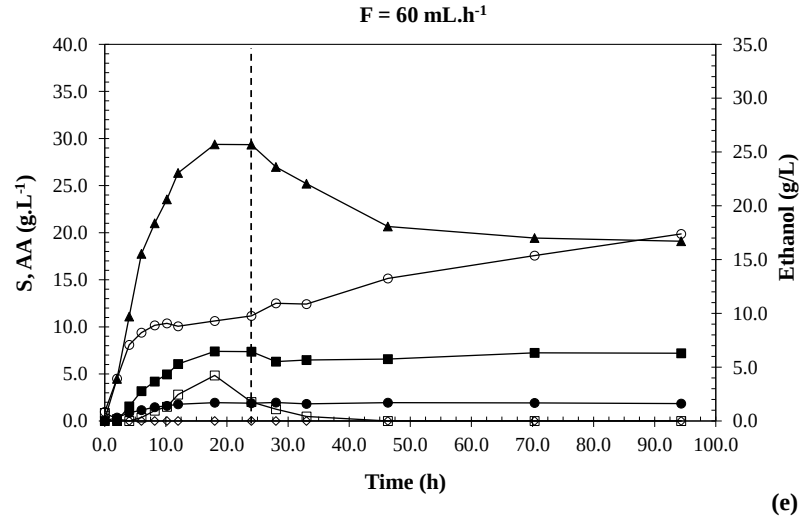
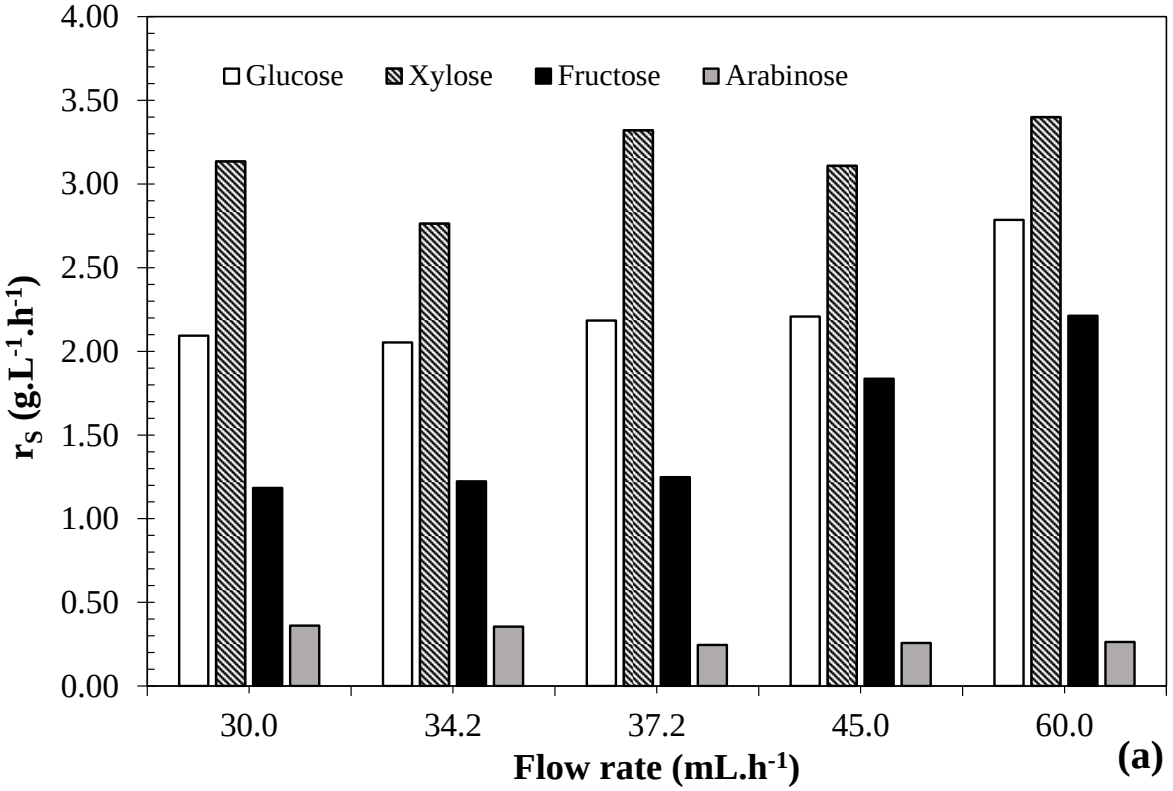

F = 60 mL.h-1
S, AA (g.L-1)
Ethanol (g/L)
Time (h)
(e)

Figure 3.


Glucose
Xylose
Fructose
Arabinose
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
r_S (g.L^{-1}.h^{-1})
30.0
34.2
37.2
45.0
60.0
Flow rate (mL.h^{-1})
(a)

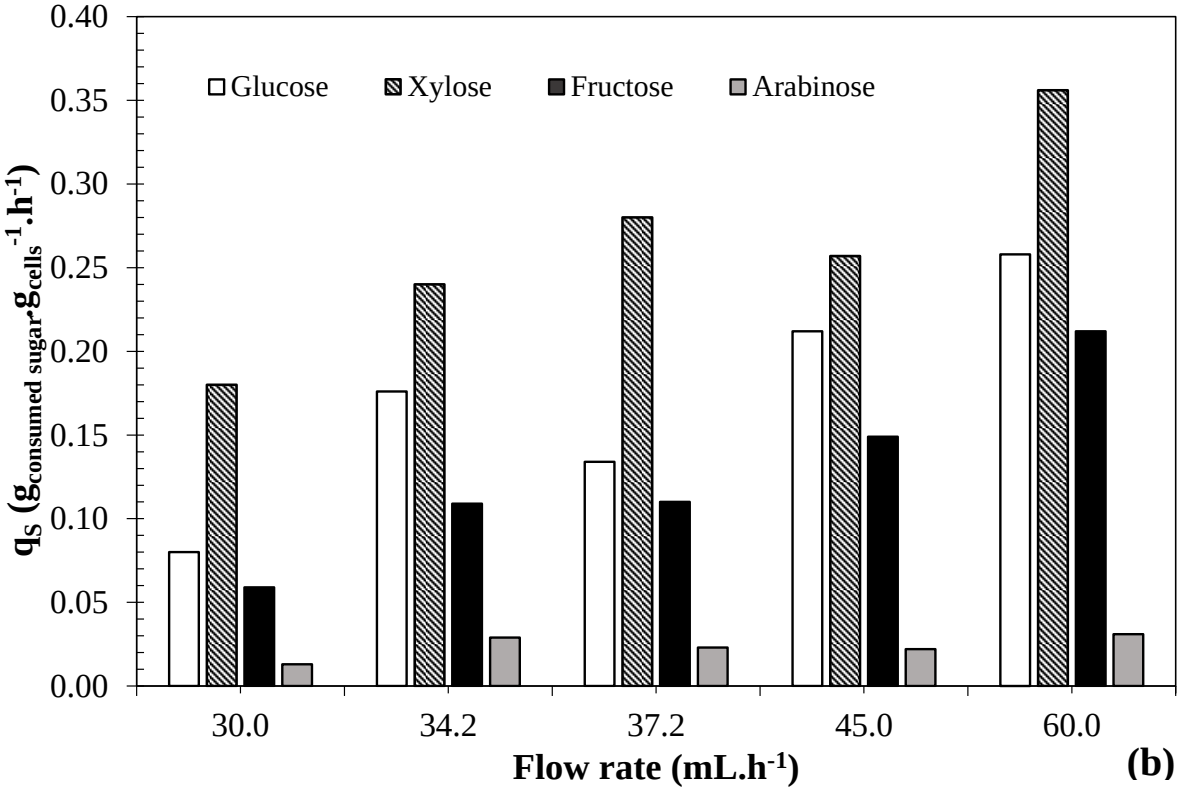

Glucose
Xylose
Fructose
Arabinose
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
q_S ($g_{consumed\ sugar}$.g_{cells}^{-1}.h^{-1})
30.0
34.2
37.2
45.0
60.0
Flow rate (mL.h^{-1})
(b)

Figure 4

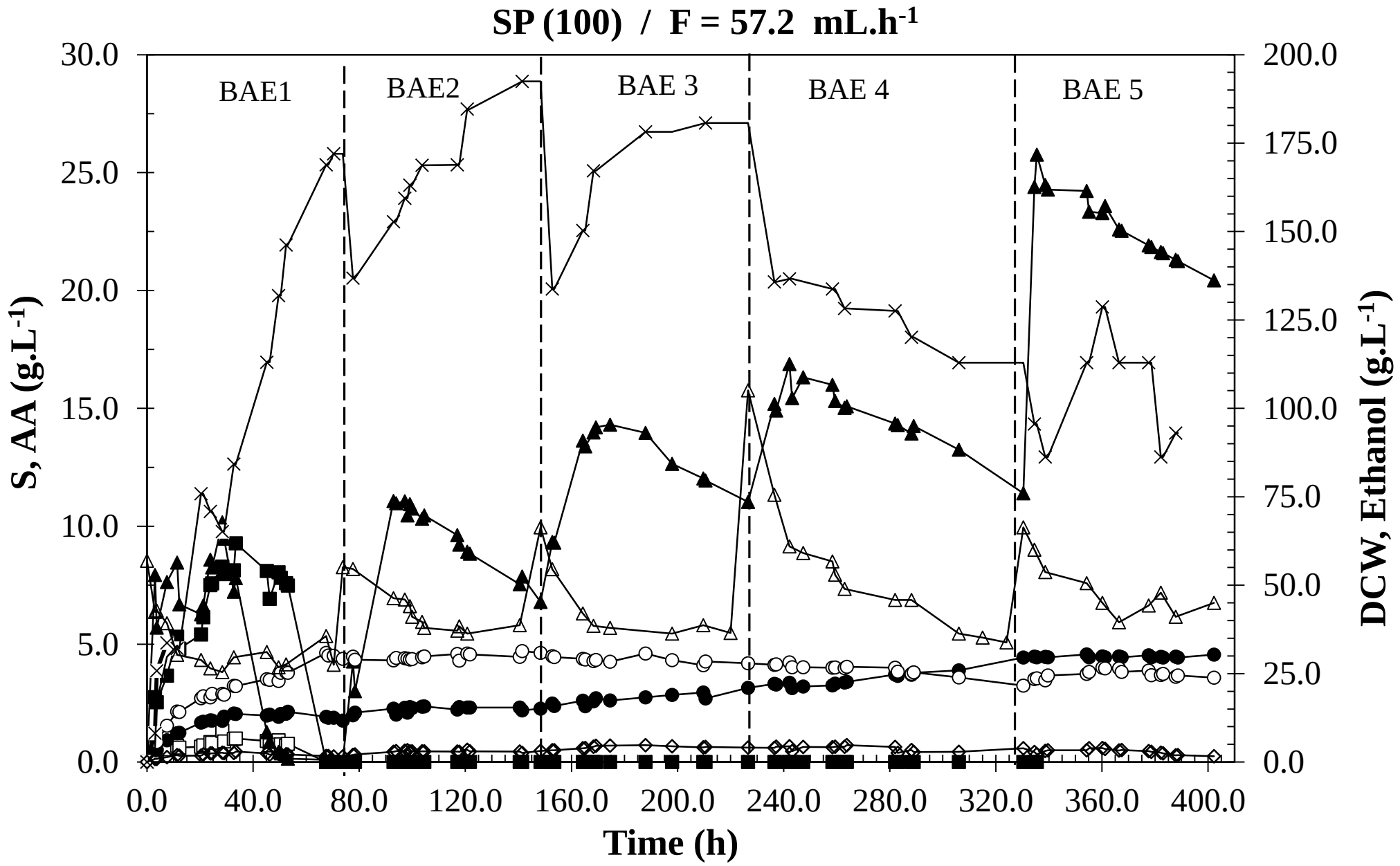
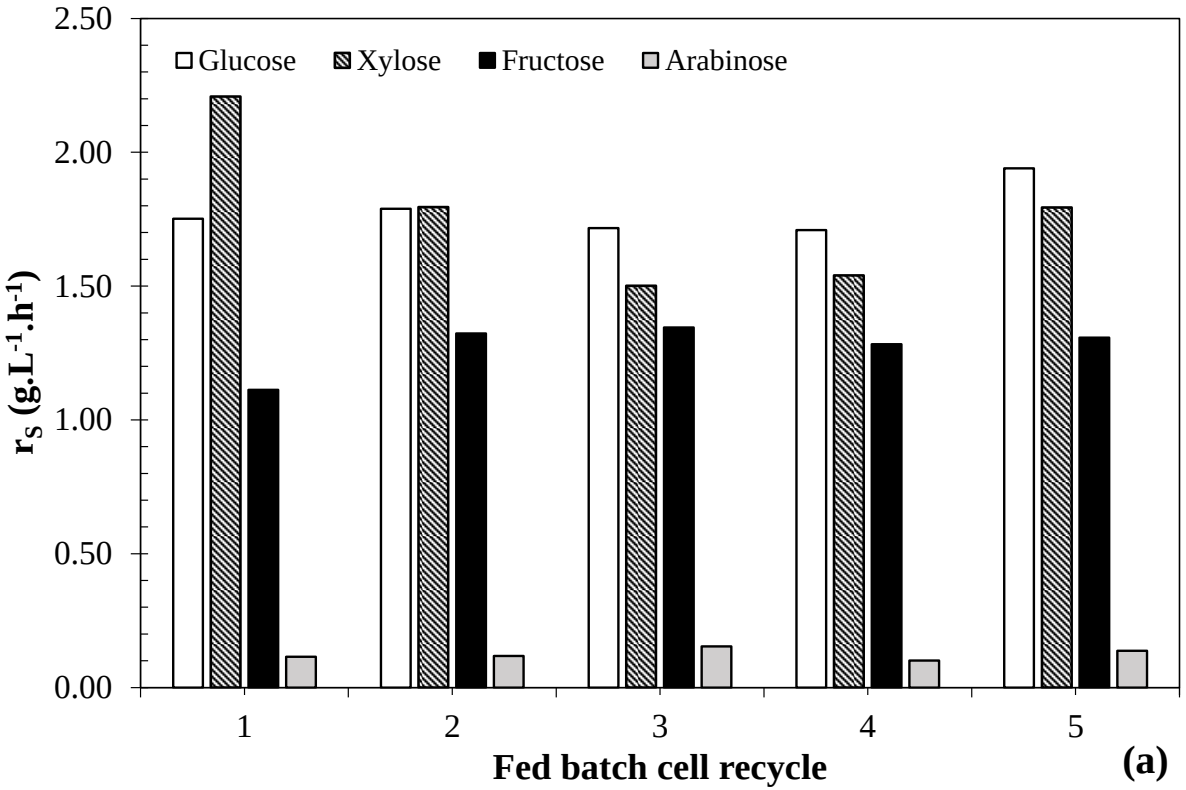

SP (100) / F = 57.2 mL.h-1
BAE1
BAE2
BAE 3
BAE 4
BAE 5
S, AA (g.L-1)
DCW, Ethanol (g.L-1)
Time (h)
0.0
5.0
10.0
15.0
20.0
25.0
30.0
0.0
25.0
50.0
75.0
100.0
125.0
150.0
175.0
200.0
0.0
40.0
80.0
120.0
160.0
200.0
240.0
280.0
320.0
360.0
400.0

Figure 5


Glucose
Xylose
Fructose
Arabinose
2.50
2.00
1.50
1.00
0.50
0.00
r_S (g.L^{-1}.h^{-1})
1
2
3
4
5
Fed batch cell recycle
(a)

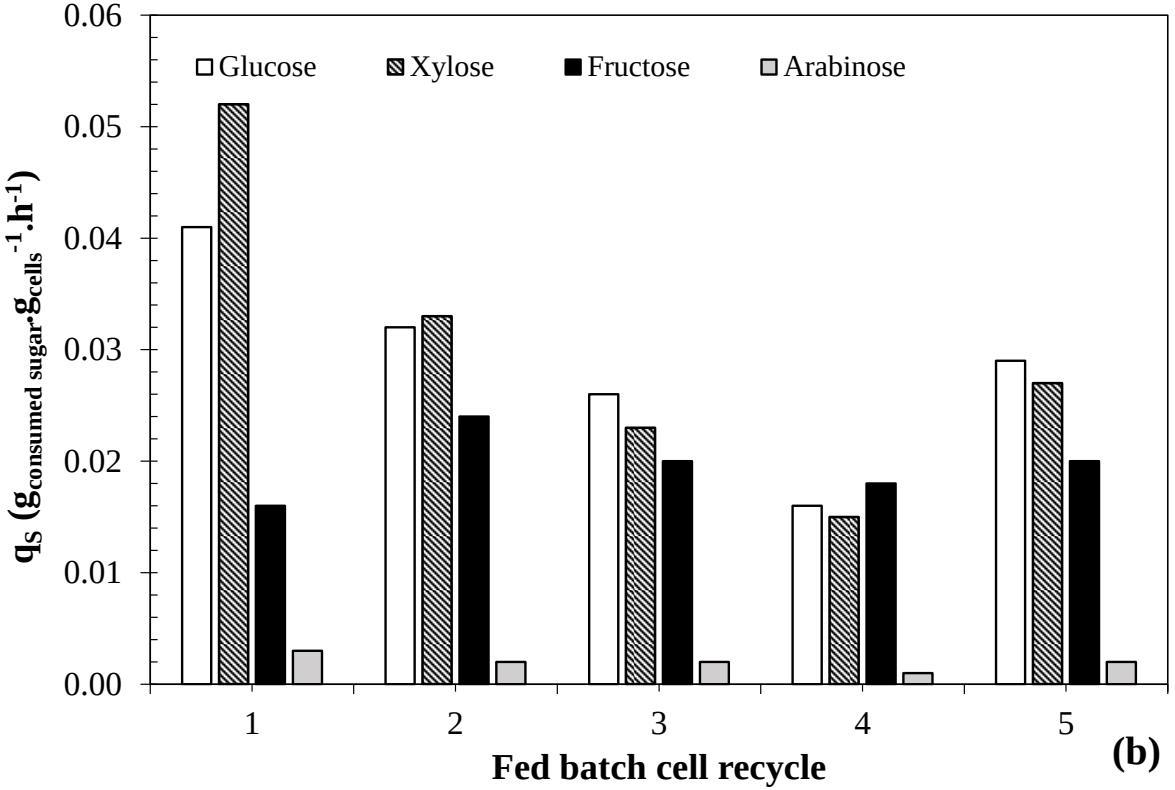

Glucose
Xylose
Fructose
Arabinose
0.06
0.05
0.04
0.03
0.02
0.01
0.00
q_S ($g_{consumed\ sugar}$.g_{cells}^{-1}.h^{-1})
1
2
3
4
5
Fed batch cell recycle
(b)

Figure 6

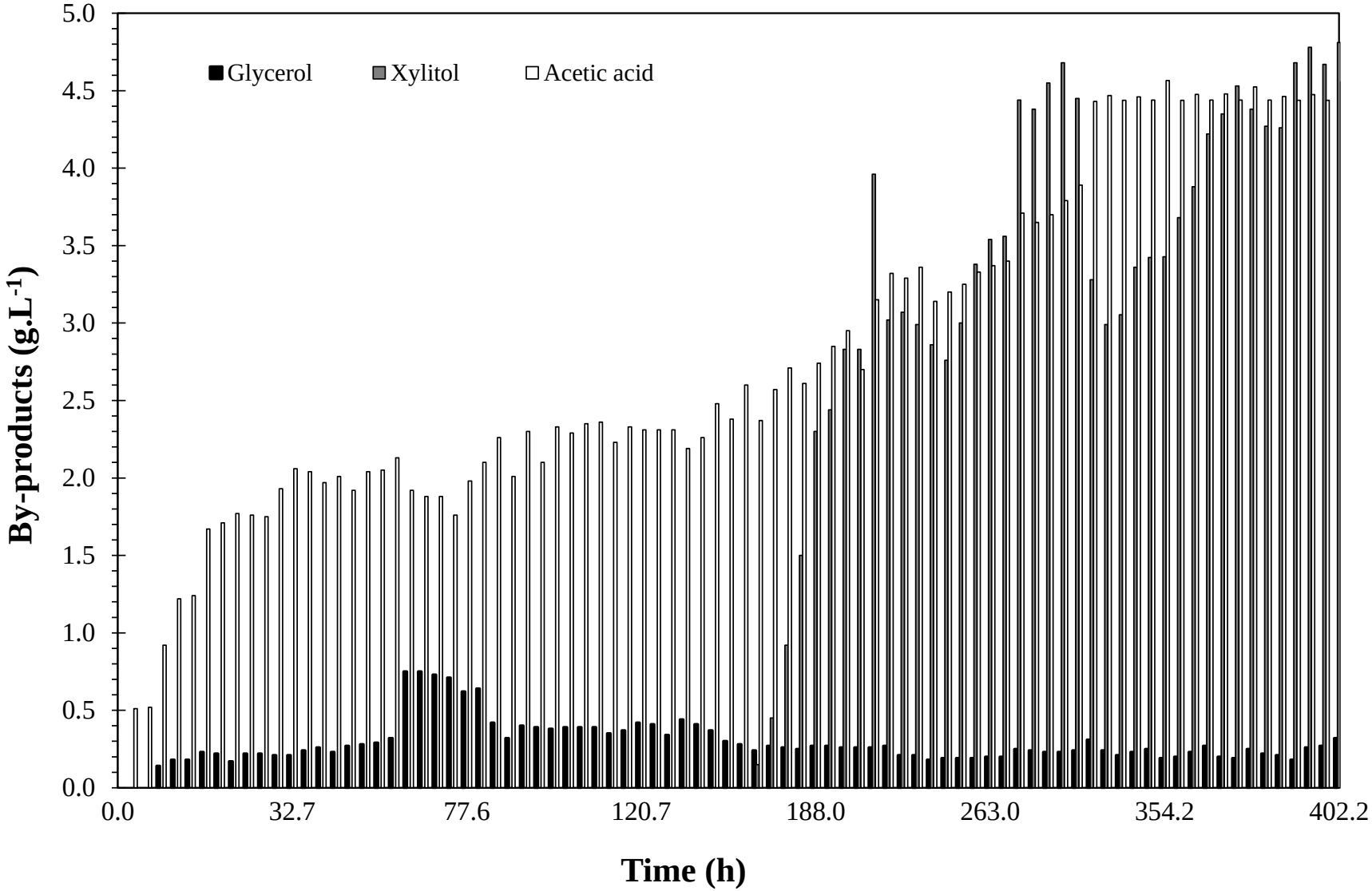

Glycerol
Xylitol
Acetic acid
By-products (g.L-1)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
32.7
77.6
120.7
188.0
263.0
354.2
402.2
Time (h)

Table 1. Hemicellulosic hydrolysate and sugarcane molasses used in medium composition.

Hemicellulosic hydrolysate Before Detoxification (BD) / After Detoxification (Evaporation) (AD)					
	Sugar ($g.L^{-1}$)			**Inhibitors ($g.L^{-1}$)**	
	BD	AD		BD	AD
Xylose	21.0	95.0	Acetic acid	3.46	5.83
Glucose	2.0	15.2	Formic acid	0.31	0.28
Arabinose	3.8	8.5	Levulinic acid	0.02	0.01
Cellobiose	0.4	2.1	HMF	0.11	0.07
			Furfural	0.25	0.05

Sugarcane Molasses		
Sugar ($g.L^{-1}$)	**In natura**	**Hydrolysed**
Sucrose	393.59	----
Fructose	143.41	322.16
Glucose	79.68	287.15
ART	637.40	609.31

Table 2. Final fermentation composition used in fresh medium.

Components	**Concentration ($g.L^{-1}$)**
Xylose	47.3
Glucose	25.9
Fructose	22.8
Arabinose	5.2
Acetic acid	2.8
Final sugar concentration	**101.2**

Table 3. Fermentations parameters for *S. passalidarum* in fed batches runs using mixed sugar fermenting medium composed of hemicellulosic hydrolysate and sugarcane molasses (50:50).

Flow rate ($mL.h^{-1}$)	**Y_{ps} ($g.g^{-1}$)**	**Q_{pmax} ($g.L^{-1}.h^{-1}$)**	**Titer ($g.L^{-1}$)**	**$S_{residual}$ ($g.L^{-1}$)**	**Xy ($g.L^{-1}$)**	**AA ($g.L^{-1}$)**	**Time (h)**	**E (%)**
30.0	0.430	2.40	24.2	0.0	0.0	2.0	34	84.1
34.2	0.470	2.04	30.3	2.7[a]	1.7	1.8	70	92.0
37.2	0.501	1.92	26.3	17.0[b]	2.0	2.3	90	98.0
45.0	0.493	1.99	23.9	12.9[c]	2.4	2.1	100	96.5
60.0	0.473	1.97	17.4	29.0[d]	2.6	2.0	100	92.6

Residual sugars during fermentation:
[a]Arabinose: 2.1; Xylose: 1.3.
[b]Arabinose: 3.0; Fructose: 12.4; Xylose: 1.6.
[c]Arabinose: 1.9; Fructose: 11.0;.
[d]Arabinose: 2.7; Fructose: 7.2; Xylose: 19.1.

Table 4. Fermentation parameters for sequential cell recycle by *S. passalidarum* extractive fed batch runs using mixed sugar fermenting medium composed of hemicellulosic hydrolysate and sugarcane molasses (50:50).

Fed batch	Y_{ps} ($g.g^{-1}$)	Qp_{max} ($g.h^{-1}$)	Ethanol (Bioreactor) ($g.L^{-1}$)	Ethanol (Condenser) ($g.L^{-1}$)	$S_{residual}$ ($g.L^{-1}$)	Xy ($g.L^{-1}$)	Acetic Acid ($g.L^{-1}$)	E (%)
1	0.383	2.2	30.9	168.8	0.0	0.0	1.9	75.0
2	0.482	9.5	31.4	192.4	3.8[a]	0.0	2.2	94.3
3	0.427	8.8	30.7	180.7	18.1[b]	2.8	2.7	83.6
4	0.424	4.3	28.2	136.7	17.3[c]	4.5	3.9	82.9
5	0.468	7.0	26.7	128.7	22.8[d]	4.8	4.5	91.6

Residual sugars during fermentation:
[a]Arabinose: 2.5; Xylose: 1.3.
[b]Arabinose: 4.3; Fructose: 1.9; Xylose: 11.9.
[c]Arabinose: 2.9; Fructose: 1.2;.Xylose: 13.2.
[d]Arabinose: 1.7; Fructose: 0.7; Xylose: 20.4.

1 - Bioreactor
2 - Feeding medium
3 - Peristaltic pump for feeding
4 - Filter system for cell recycling
5 - Peristaltic pump for cell recycling
6 - Solenoid valve
7 - Flash tank
8 - Solenoid valve to atmosphere
9 - Condenser
A - Vacuum system