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Clostridial conversion of corn syrup to Acetone-Butanol-Ethanol (ABE) via batch and fed-batch fermentation

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Abstract

Corn syrup - a commercial product derived from saccharification of corn starch - was used to produce acetone-butanol-ethanol (ABE) by *Clostridium* spp. Screening of commercial *Clostridium* spp., substrate inhibition tests and fed-batch experiments were carried out to improve ABE production using corn syrup as only carbon source. The screening tests carried out in batch mode using a production media containing 50 g/L corn syrup revealed that *C. saccharobutylicum* was the best performer in terms of total solvent concentration (12.46 g/L), yield (0.30 g/g) and productivity (0.19 g/L/h) and it was selected for successive experiments. Concentration of corn syrup higher than 50 g/L resulted in no solvents production. Fed-batch fermentation improved ABE production with respect to batch fermentation: the butanol and solvent concentration increased up to 8.70 and 16.68 g/L, respectively. The study demonstrated the feasibility of producing solvents via ABE fermentation using corn syrup as a model substrate of concentrated sugar mixtures.

Keywords: Bioengineering, Chemical engineering

1. Introduction

Among biofuels, butanol from Acetone-Butanol-Ethanol (ABE) fermentation is one of the oldest solvents produced biochemically at industrial scale which can be traced to early 1900s [1, 2, 3].

Butanol is a four-carbon alcohol used in many different industrial processes as a: solvent in rubber production; extractant in pharmaceuticals; supplement in polishes and cleaners; precursor for the production of chemicals (acrylic esters, glycol ethers, butyl acetate, butyl amines) [4, 5]. In addition to these industrial applications, butanol is also considered a superior biofuel candidate with respect to ethanol [6]. Indeed, butanol has interesting features compared to ethanol: higher energy content, lower vapor pressure and a similar air-to-fuel ratio to gasoline [6, 7]. More importantly, butanol can be blended with either gasoline or diesel at any fraction and is compatible with the current engines without any retrofitting [8, 9]. Thanks to its physical/chemical properties, butanol is an ideal candidate to replace gasoline.

ABE fermentation is typically carried out by solventogenic *Clostridium* species under appropriate operating conditions [10]. *Clostridium* species are saccharolytic butyric acid-producing bacteria able to ferment pentoses, hexoses, mono-, di- and polysaccharides [9, 10]. Batch ABE fermentation can be divided into two distinctive phases: acidogenic phase and solventogenic phase. During the first phase (acidogenesis), the cells grow rapidly and produce carboxylic acids, like acetate and butyrate; the secretion in the medium of these acids lowers the external pH. The increasing concentration of acids causes cell metabolism shifts to solvent production (solventogenesis). A morphological change of acidogenic cells is also observed during the shift: active cells become endospores unable to reproduce themselves. At this point acids formed earlier re-enter the cells and are used as substrate for solvents production [11]. In a typical butanol fermentation, the solvents produced are butanol, acetone and ethanol, with an ABE ratio 3:6:1. A maximum concentration of total solvents of ~20 g/L is observed when traditional species and traditional batch processes are employed [4].

Clostridium spp. can ferment both simple and complex carbon sources. *C. acetobutylicum* was originally isolated and grown on starch, while other species like *C. beijerinckii* and *C. saccharobutylicum* were isolated that performed better on molasses-based feedstock [3]. The fermentation substrate directly affects the cost of the process to produce bio-butanol.

ABE fermentation can be performed starting from first- or second-generation feedstock. First-generation biomass includes sugarcane, corn and cereals grains; while second-generation biomass includes lignocellulosic material like agriculture residues [12]. Both kind of feedstock have advantages and drawbacks: first-

generation biomass are highly available, rich in sugars, easily hydrolysable and fermentable and do not require any pretreatment process, but they are in competition with human/animal food, that causes an increase of their price [13]; second-generation biomass are lignocellulosic biomass that due to their complex composition require pretreatment/hydrolysis steps before fermentation. These processes increase the price of the final ABE production cost. However, these feedstock are highly available during all year and their price is lower than that of first-generation biomass; and in the last years great efforts have been carrying out to improve the efficiency of pretreatment processes, even using new "kind" of biomass (agro-food wastes) or novel pretreatment processes [14, 15].

The entire process could be made more economically feasible if cheaper and rich-in-sugars commercial substrates are used [16, 17]. Possible candidates could be corn industrial products (starch and/or syrup).

Corn starch was the first substrate used of ABE production during Weizmann process era; later, several other starchy materials such as cassava, sweet potato, wheat starch have been used as substrates for ABE fermentation [5]. Corn syrup is a sweetener widely applied in both the food and pharmaceutical industries: it is an ingredient in canned fruits, chemicals, medicines, ice cream, and beverages [18]. In food industries, corn syrup is mainly used as sweetener and preservative agent due to its acidity, reducing the use of other preservatives. In pharmaceutical industries, corn syrup is used as flavouring agent and humectant, especially for cosmetics preparations. Corn syrup is produced starting from corn grain: it undergoes several unit processes that include steeping, wet milling, starch and bran separation, saccharification of starchy material. Three industrial enzymes (α-amylase, glucoamylase, glucose isomerase) are employed to carry out the saccharification step. At the end of the process, a very concentrated sugar solution mainly composed of glucose, fructose and maltose is obtained is obtained [19].

Due to the very high sugars concentration, corn syrup requires a dilution to be used as fermentation substrate, as well as for molasses fermentation [4]. Few papers about ABE fermentation of very concentrated sugar solution (or molasses) are reported in literature. Qureshi et al. (2001) [20] obtained a total ABE production up to~ 23 g/L with a mutant strain *C. beijerinckii* BA101 when 80 g/L of soy molasses supplemented with 25 g/L glucose was used. Kim and Day (2010) [21] utilized 6% cane molasses for ABE fermentation by *C. beijerinckii*, and a total solvent production of 9 g/L was attained. Ni et al. (2012) [22] carried out ABE fermentation starting from 60 g/L of sugarcane molasses using *C. saccharobutylicum* DSM 13864, obtaining a total ABE of~ 18 g/L. More recently, Moon et al. (2015) [23] produced 8 g/L butanol from fermentation of 30 g/L sugarcane molasses by *C. beijerinckii optinoii*.

This study evaluates ABE fermentation by *Clostridium spp*. using corn syrup — a corn industrial product — as feedstock. The activity was focused on the fermentation

of the monosaccharides (glucose and fructose) and disaccharides (maltose) present in corn syrup. Among the three solvents produced, the attention was focused on butanol since it can be used directly as biofuel. The aim of the work was threefold: i) the screening of four commercial *Clostridium* spp. to select the best for ABE fermentation of corn syrup; ii) the assessment of the effect of high corn syrup concentration during batch fermentation; iii) the fed-batch fermentation to increase butanol production from corn syrup. Time resolved data of biomass, acids and solvents concentrations as well as pH values were considered to characterize the conversion process.

2. Materials and methods

2.1. Raw materials

Corn syrup (Versatose® 36) used in this study was kindly provided by Ingredion Incorporated (London, Ontario, Canada). The main sugars present in this commercial product were: glucose (43.46 %w/w), maltose (17.69 %w/w) and fructose (11.33 %w/w). Corn syrup was stored at room temperature in a plastic container and used as received.

2.2. Microorganisms and growth conditions

In this study four *Clostridium* species were used: *C. acetobutylicum* DSM 1731, *C. pasteurianum* DSM 525, *C. saccharobutylicum* DSM 13864, *C. beijeirinckii* DSM 6422. All species were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures). The following reinforced *Clostridium* media (RCM) was used for all seed cultures: peptone, 10.0 g/L; beef extract, 10.0 g/L; yeast extract, 3.0 g/L; glucose, 5 g/L; NaCl, 5 g/L; soluble starch, 1.0 g/L; sodium acetate, 3 g/L; pH adjusted to 6.8. The following producing medium was used for all fermentation tests: KH_2PO_4, 0.5 g/L; K_2HPO_4, 0.5 g/L; $(NH_4)_2SO_4$, 5 g/L; $MgSO_4 \bullet 7H_2O$, 0.2 g/L; $CaCl_2 \bullet 2H_2O$, 0.02 g/L; $FeSO_4$, 0.05 g/L; yeast extract, 1 g/L; $CaCO_3$, 2 g/L. pH was adjusted to 6.8 and nitrogen was bubbled (while heating) to drive off oxygen. Then, media was autoclaved for 20 min at 121 °C. After autoclave, media was transferred into an anaerobic chamber (Model 855-ACB, Plas-Labs, Inc., Lansing, MI) and 2 mL of trace element solution (SL7) were added through a 0.2-μm membrane filter. The following trace element solution (SL7) was used: $FeCl_2 \bullet 4H_2O$, 1.5 g/L, dissolved in 10 ml HCl solution (25% solution); $CoCl_2 \bullet 6H_2O$, 0.19 g/L; $MnCl_2 \bullet 4H_2O$, 0.1 g/L; $ZnCl_2$, 0.07 g/L; H_3BO_3, 0.062 g/L; $Na_2MoO_4 \bullet 2H_2O$, 0.036 g/L; $NiCl_2 \bullet 6H_2O$, 0.024 g/L; $CuCl_2 \bullet 2H_2O$, 0.017 g/L. A medium composed only by corn syrup and distilled water was also prepared to carry out fermentation tests aimed at checking the influence of external nutrients (including trace element solution) added to corn syrup. Only $CaCO_3$ (2 g/L) was added to the diluted solution

of corn syrup as buffering agent of pH. Medium was autoclaved for 20 min at 121 °C. After autoclave, medium was transferred into an anaerobic chamber. No further additions of nutrients or trace element solution were operated.

2.3. ABE fermentation

Fermentation medium was prepared adding corn syrup to the producing medium along with the other chemicals (as described above) to obtain a final sugar concentration of 50 g/L, representing the sum of glucose, maltose and fructose concentrations (hereinafter referred as GMF). All fermentations were carried out on an orbital shaker in anaerobic chamber (Model 855-ACB, Plas-Labs, Inc., Lansing, MI) using 125 mL flasks containing 50 mL of fermentation medium.

The screening of the four *Clostridium* species was carried out in batch conditions using the producing media containing a final sugar concentration of 50 g/L (GMF).

To test the effect of increased corn syrup concentration on ABE fermentation, producing media with final corn syrup concentrations of 50, 75, 100 and 125 g/L (GMF) were prepared.

For fed-batch fermentation, a producing media containing 50 g/L total sugars (GMF) was used to initiate the fermentation. When sugars concentration decreased, concentrated corn syrup solution (400 g/L GMF) was manually added (by means of pipette) to the shaking flasks to increase again sugars concentration. The feeding was continued until no more sugar consumption or solvent production was observed.

All experiments were performed in triplicate under anaerobic conditions. Five millilitres of actively growing cells were inoculated into 50 mL of fermentation medium. Fermentation cultures were kept in an anaerobic chamber at 200 rpm and 37 °C for at least 72 h. Sampling was performed periodically. The samples were centrifuged and filtered using 0.2-μm grade filters. The filtered liquid phase was stored in a freezer (-20 °C) for acids, solvents and sugars analysis. The product yield and productivity calculated for each fermentation were estimated considering the total sugar consumption and products present in the fermentation broth.

2.4. Analytical methods

The batch fermentations were characterized in terms of pH, sugars conversion, and acids and solvents production.

Bacterial growth was monitored by measuring the optical density at 600 nm using an UV-Visible spectrophotometer (Evolution 60S, Thermo Scientific). Concentration of sugars and fermentation products (acids and solvents) was determined by means of high-performance liquid chromatography (HPLC) on an Agilent 1260 infinity (Agilent USA, Santa Clara) equipped with an Agilent Hi-Plex H (7.7 × 300 mm)

column and Cation H+ guard column (Agilent USA, Santa Clara). A refractive index detector (RID) was used for sugars and metabolites detection. Sulphuric acid (5 mM) was used as the mobile phase in isocratic mode at a constant flow rate of 0.6 mL/min. Before the analysis, samples were diluted to the opportune concentration with mobile phase and filtered through a 0.2-µm grade filter. The analytes were quantified using pure chemicals as standards.

The total amount of solvents (ABE, g_{ABE}/L) was divided by the concentration of sugar (g_{Sugars}/L) consumed during the fermentation to calculate the total solvent yield ($Y_{ABE/Sugars}$, g_{ABE}/g_{Sugars}). Similar calculation was carried out to determine the butanol yield ($Y_{B/Sugars}$, g_B/g_{Sugars}). The maximum concentration of ABE (g/L) obtained during fermentation was divided by the time (h) necessary to reach that concentration to calculate the ABE productivity (g_{ABE}/L/h).

3. Results and discussion

3.1. Screening of *Clostridium* spp. in medium supplemented with corn syrup as only carbon source

Clostridium spp. are known to be able to ferment different kinds of carbon source, from simple monosaccharides (both hexoses and pentoses) to complex polysaccharides e.g. starch [24]. Four commercial *Clostridium* species were screened for their ability to convert corn syrup to solvents. Comparative batch experiments among *C. acetobutylicum* DSM 1731, *C. pasteurianum* DSM 525, *C. saccharobutylicum* DSM 13864, *C. beijeirinckii* DSM 6422 were performed in 125 mL flasks containing 50 mL of synthetic medium supplemented with corn syrup to reach a total initial GMF concentration of about 50 g/L. Fig. 1 reports the time-resolved profiles of the concentrations of cells, GMF (initial concentration of 25.3, 9.0, 9.6 g/L for glucose, maltose and fructose respectively), and metabolites (acetic acid, butyric acid, acetone, butanol, ethanol and 1,3-propanediol) as well as of pH, measured during a batch culture. Among the species screened, only for two of them, *C. saccharobutylicum* and *C. beijerinckii*, the data analysis confirmed the usual two-phases behaviour of fermentation: acidogenesis and solventogenesis. During acidogenic phase, increase of cell and acids concentration was observed, as well as a decrease of pH. As the pH reached 4.5 (around 12h), the solventogenesis started. In this phase decrease of cell (due to cell lysis), acids and sugars concentration and steady increase in the concentration of solvents was observed. As reported in Fig. 1a, for all species no lag phase was observed. This was probably due to the presence of rapidly metabolizing sugars, i.e. GMF. Moon et al. (2015) [23] and Ni et al. (2012) [22] found similar behaviour during ABE fermentation of the complex sugars mixture of sugarcane molasses by *C. beijerinckii optinoii* and *C. saccharobutylicum* DSM 13864, respectively. Similar observation was also reported by Raganati *et al.*
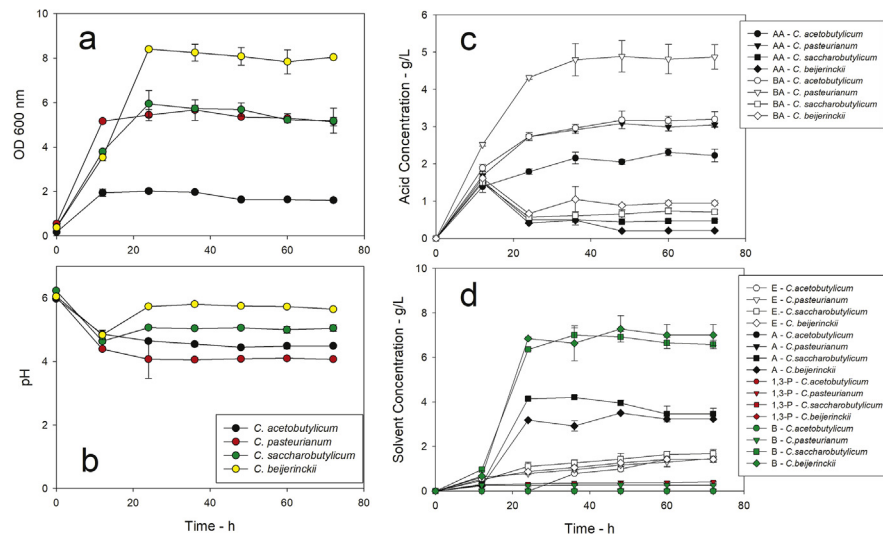
Fig. 1. Time-resolved profiles of data measured during *Clostridium* species batch fermentation in medium supplemented with corn syrup as only carbon source (GMF initial concentration: 50 g L^{-1}). a) microbial growth; b) pH; c) acid concentration; d) solvent concentration. AA: acetic acid; BA: butyric acid; E: ethanol; A: acetone; 1,3-P: 1,3-propanediol; B: butanol.

(2015) [10] for ABE fermentation of a synthetic mixture of glucose, mannose, arabinose and xylose by *C. acetobutylicum* DSM 792. From Fig. 1a, *C. beijerinckii* gave the best performance in terms of microbial growth, reaching an optical density of about 8 at 600 nm. The growth of *C. pasteurianum* and *C. saccharobutylicum* was comparable: both approached an optical density of 6 after 24h. Among the four species, *C. acetobutylicum* gave the worst results in terms of microbial growth on corn syrup solution. These results agree with that reported by Shaheen et al. (2000) [25]: during their experiments of ABE fermentation on sugarcane molasses by different *Clostridium* spp. the authors demonstrated that both *C. saccharobutylicum* and *C. beijerinckii* were able to grow and produce solvents on these feedstocks, while *C. acetobutylicum* did not perform well on these kind of carbon sources.

The performances of ABE fermentations can also be inferred by looking at the time-solved pH profile. All the four species started their fermentation with a pH value of the medium of about 6 (Fig. 1b). During the first hours of fermentation, the simple sugars of corn syrup (GMF) present in the medium were metabolized for both cellular growth and acids production: acetic acid and butyric acid were produced and secreted in the medium, causing a decrease of pH. When the pH values approached ∼4.5, solventogenic phase was expected to start, but the metabolic switch from acidogenesis to solventogenesis was observed only for *C. saccharobutylicum* and *C. beijerinckii*. Only for these two species a pH increase was measured, indicating that the other two saccharolytic species (*C. acetobutylicum* and *C. pasteurianum*) were not able to tolerate such low pH value without affecting solvent production. For the species in solventogenic phase a final pH value comprised

between 5 and 6 was attained. The acidogenic phase lasted less than 20 h (Fig. 1). During this time all species produced both acetic and butyric acid. After about 12 h of fermentation, *C. saccharobutylicum* and *C. beijerinckii* switched to solventogenic phase, stopping acids production and starting solvents production. The other two species continued their acidogenic phase until the end of fermentation (48 h) (Fig. 1c), without ever switching to solventogenesis. After 48 h of fermentation, the total amount of acids produced by *C. acetobutylicum* and *C. pasteurianum* were 2.05 and 3.18 g/L acetic acid and 3.09 and 4.89 g/L butyric acid, respectively. Solventogenic phase of *C. saccharobutylicum* and *C. beijerinckii* was characterized by re-assimilation of acids into the cells. These molecules were used as intermediate to produce acetone, butanol and ethanol [11]. The batch fermentations were carried out for 72 h, and ABE accumulation reached its highest level at 36 h (Fig. 1d). The residual acid concentration at the end of fermentation was about 1 g/L for both species.

A total solvent of 12.46 g/L was reached after 36 h for *C. saccharobutylicum*, including acetone 4.20 g/L, butanol 7.00 g/L, ethanol 1.26 g/L. Butanol and ABE productivity was 0.19 and 0.35 g/L/h; butanol and ABE yield was 0.17 and 0.30 g/g. A total solvent of 10.61 g/L was achieved after 36 h for *C. beijerinckii*, including acetone 2.92 g/L, butanol 6.64 g/L, ethanol 1.06 g/L. Butanol and ABE productivity was 0.18 and 0.29 g/L/h; butanol and ABE yield was 0.17 and 0.27 g/g.

The summary of the fermentative processes in terms of solvents production, yield and productivity is reported in Table 1 for all the species tested.

Fig. 2 reports the time profile of glucose, maltose and fructose concentrations during the 72 h of fermentation. Both *C. saccharobutylicum* and *C. beijerinckii* were able to ferment all the three sugars: the total sugar (GMF) consumption attained was 96 and 90 %, respectively. Only maltose was not completely utilized (Fig. 2b): a residual maltose concentration of 1.10 and 1.41 g/L was measured at the end of batch fermentation, respectively. Although *C. saccharobutylicum* started to use maltose after 12 h of fermentation, suggesting a lagged metabolism of maltose than that of glucose and

Table 1. Summary of fermentative performances of the four *Clostridium* species.

Species (strain)	A^a	B^a	E^a	ABE^a	Y_B^b	Y_{ABE}^b	B productivity^c	ABE productivity^c
C. acetobutylicum (DSM 1731)	0.08	/	0.98	1.06	/	0.10	/	0.03 (36 h)
C. pasteurianum (DSM 525)		/	0.25	0.96	1.23	0.01	0.07	0.01	0.03 (36 h)
C. saccharobutylicum (DSM 13864)	4.20	7.00	1.26	12.46	0.17	0.30	0.19	0.35 (36 h)
C. beijerinckii (DSM 6422)	2.92	6.64	1.06	10.61	0.17	0.27	0.18	0.29 (36 h)

A = Acetone; B = Butanol; E = Ethanol.
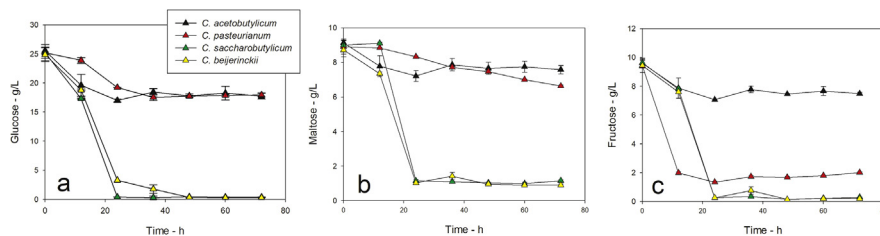a [=] g/L; b [=] $g/g_{total\ sugars\ consumed}$; c [=] g/L/h.

Fig. 2. Time-resolved profiles of sugar consumption during batch fermentation of corn syrup (GMF initial concentration: 50 g L^{-1}) by different *Clostridium* species. a) glucose; b) maltose; c) fructose.

fructose, all the sugars were utilized almost at the same time, indicating that both bacteria can metabolize maltose, glucose and fructose without catabolic repression [23]. *C. acetobutylicum* attained a total sugar (GMF) consumption of 26 %, allowing the microorganism to start acidogenic phase (Fig. 1a-c), but not enough biomass was produced to switch to solventogenesis, indeed negligible amount of solvents was produced (Fig. 1d and Table 1). *C. pasteurianum* was screened because unlike the other saccharolytic *Clostridium* spp., its metabolic pathway allows the bacterium to produce 1,3-propanediol (PDO) - instead of acetone - along with butanol and ethanol [26]. As shown in Fig. 1, *C. pasteurianum* was able to grow and produce acids when corn syrup was used as only carbon source, but as seen for *C. acetobutylicum*, also in this case no significant amount of solvents was measured (Fig. 1d and Table 1): only 0.40 g/L 1,3-propanediol and 0.25 g/L butanol were produced. The total sugars consumption was 38 %. Interestingly, the main sugar consumed during fermentation of *C. pasteurianum* was fructose (50 % of total GMF consumed) (Fig. 2c). If glucose, maltose and fructose concentrations are compared, the utilization of glucose — as well as maltose - does not start until the fructose is almost completely exhausted (12 h). This result seems to indicate that fructose prevents glucose fermentation in these fermentative conditions, i.e. carbon source, sugar concentration, medium composition. Similar behaviour was observed by Andreesen et al. (1973) [27] when fermenting *C. thermoaceticum* on a mixture of xylose, glucose and fructose. The authors hypothesized a possible repression by fructose of an enzyme needed for glucose fermentation. Our data don't allow us to confirm or deny the hypothesis of Andreesen et al. (1973) [27], but it is evident that an interesting relationship exists between fructose and glucose (and maltose) when a mixture of these sugars is used for fermentative processes by *C. pasteurianum*. In this work, no further experiments were carried out with this microorganism due to the negligible solvents production during the screening tests.

From the screening of the four microorganisms on corn syrup, *C. saccharobutylicum* and *C. beijerinckii* were the only two species to reach yields and productivities comparable to others reported in literature referring to fermentation of *Clostridium* spp. on complex sugars mixtures, e.g. molasses (Table 1 and Table 2) [20−23]. The comparison of the performances of the two species highlighted that *C.*

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Table 2. Summary of ABE fermentation from concentrated sugars solutions.

Substrate	Species (strain)	Fermentation method	A^a	B^a	E^a	ABE^a	Y_B^b	Y_{ABE}^b	B productivity^c	ABE productivity^c	References
Soy molasse	C. beijerinckii (BA101)	Batch	2.0	7.5	1.2	10.7	0.22	0.31	0.08	0.11 (96 h)	[20]
Sugarcane molasse	C. beijerinckii	Batch	2.5*	6.5	/	9.0	0.27	0.36	0.07	0.09 (90 h)	[21]
Sugarcane molasse	C. saccharobutylicum (DSM 13864)	Batch	4.6	11.9	1.4	17.9	0.22	0.33	0.33	0.50 (36 h)	[22]
Sugarcane molasse	C. beijerinckii optinoii	Batch	4.4*	8.0	/	12.4	0.27	0.41	0.09	0.14 (90 h)	[23]
Corn syrup	C. saccharobutylicum (DSM 13864)	Batch	4.21	7.00	1.26	12.47	0.17	0.30	0.20	0.35 (36 h)	This study
Corn syrup	C. saccharobutylicum (DSM 13864)	Fed-batch	5.44	8.70	2.54	16.68	0.18	0.34	0.24	0.47 (36 h)	This study
Switchgrass	C. saccharobutylicum (DSM 13864)	Batch	/	/	/	22.7	/	0.40	/	0.63 (36 h)	[37]
Corncob	C. saccharobutylicum (DSM 13864)	Batch	/	/	/	19.4	/	0.35	/	0.54 (36 h)	[38]
Lettuce residues	C. acetobutylicum (DSM 792)	Batch	/	/	/	1.5	/	0.07	/	0.03 (56 h)	[39]
Corn stach	C. beijerinckii (BA101)	Batch	/	/	/	18.6	/	0.31	/	0.27 (68 h)	[16]

A = Acetone; B = Butanol; E = Ethanol.

a [=] g/L; b [=] $g/g_{total\ sugars\ consumed}$; c [=] g/L/h.

* isopropanol concentration [=] g/L.

saccharobutylicum performed a better fermentation of corn syrup solution in terms of total solvent concentration, yield and productivity with respect to *C. beijerinckii*. Considering what has been said so far, *C. saccharobutylicum* was selected for successive experiments of substrate inhibition and fed-batch fermentation.

A fermentation of corn syrup solution by *C. saccharobutylicum* was carried out on media prepared without adding nutrients (only $CaCO_3$ was added to buffer the pH as reported in Materials and methods). This fermentation was performed to determine if minerals present in corn syrup [19] are sufficient to allow fermentation to start. In this fermentative conditions *C. saccharobutylicum* was not able to perform well as on media enriched with nutrients. The initial GMF concentration was the same used for the screening tests (about 50 g/L), but in this case the total sugar consumption was only 12 %, a very low percentage if compared with the consumption in presence of nutrients (96 %). Moreover, negligible concentration of acids and solvents was measured after 72 h of fermentation (data not shown). These results suggest that the addition of nutrients is mandatory to start the fermentation of diluted solutions of corn syrup by *C. saccharobutylicum* DSM 13864.

Since the aim of this work was to assess the possibility of using corn syrup for biofuels production, the effect of each nutrient added to corn syrup solution was not investigated. The data collected here are just preliminary results useful for successive works about media optimization of corn syrup solution for ABE production.

3.2. Effect of corn syrup concentration on ABE fermentation

One of the main factors influencing the performances of a fermentative process is the amount of fermentable sugars present in the medium. Usually, increasing the sugars concentration causes an increase of final products concentration [28, 29]. This is true until a limit value of sugars concentration is approached: inhibition of fermentation is observed when sugars concentration exceeds the maximum value that can be handle by the specific microorganism. In order to increase the amount of ABE produced by *C. saccharobutylicum* DSM 13864, the concentration of corn syrup in the medium was increased from 50 up to 125 g/L (GMF concentration). Fig. 3 reports the time-resolved profile of the concentrations of cells, GMF concentration and metabolites (acetic acid, butyric acid, acetone, butanol, and ethanol) quantified during the batch fermentation. The pH values measured for each sampling are also reported. As shown in Fig. 3a, increase of the sugars concentration produced a negative effect on microbial growth: the higher sugars concentration, the slower grow rate. At 75, 100 and 125 g/L GMF the maximum optical density attained was less than half of that measured with 50 g/L GMF. pH values also indicated that solventogenic phase started only with an initial corn syrup concentration of 50 g/L (GMF). For the other three concentrations, no increase of pH was measured after reaching a pH value of 4.5 (Fig. 3b). During acidogenic phase of fermentation at 75, 100 and 125 g/L GMF
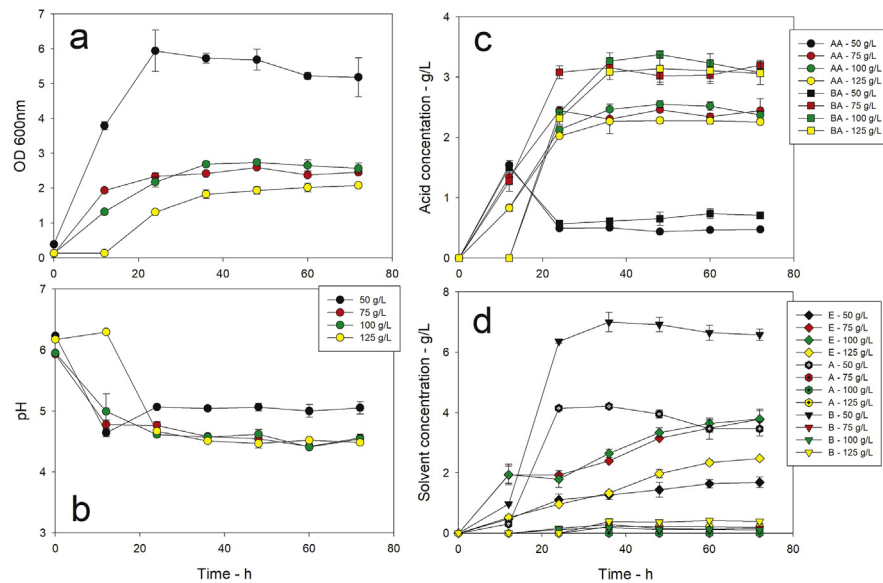
Fig. 3. Time-resolved profiles of data measured during *C. saccharobutylicum* batch fermentation in medium supplemented with different concentrations of corn syrup (GMF initial concentrations: 50, 75, 100 and 125 g L^{-1}). a) microbial growth; b) pH; c) acid concentration; d) solvent concentration. AA: acetic acid; BA: butyric acid; E: ethanol; A: acetone; B: butanol.

the total amount of acids produced was about 6 g/L for all the three conditions (Fig. 3c). Again, only for 50 g/L GMF a decrease of acids concentration was observed: these acids were used as intermediates to generate solvents during solventogenesis (Fig. 3c-d). Interestingly, even if a solventogenic phase was not observed from pH and acids profile, at 75, 100 and 125 g/L GMF, ethanol production was detected for all sugars concentrations, while negligible amount of butanol and acetone were produced. About 4.5 g/L ethanol were produced with 75 and 100 g/L GMF and 2 g/L with 125 g/L GMF (Fig. 3d).

Fig. 4 shows the sugars consumption for all the initial corn syrup concentrations investigated. The highest sugars conversion was obtained with 50 g/L GMF. In this case almost all sugars (96%) were consumed during ABE fermentation. For the other concentrations, the total consumption ranged from 15 to 18%, indicating that inhibition is occurring when GMF concentration is higher than 50 g/L. Generally, inhibition of fermentative processes might be due to: substrate inhibition, end product inhibition and presence of inhibitory molecules in the growth medium. Product inhibition is caused by the high toxicity of butanol to the cells of *Clostridium* spp. that produce it [30]. Usually, ~20 g/L of ABE is the limit concentration beyond which product inhibition is observed in batch fermentation [30]. From the results reported in this work, negligible concentration of butanol was attained when GMF concentration was higher than 50 g/L (Fig. 3d): this implies that product inhibition is not the cause that can explain the interruption of fermentative process for GMF concentrations above 50 g/L. Sugar inhibition could be a probable reason for the
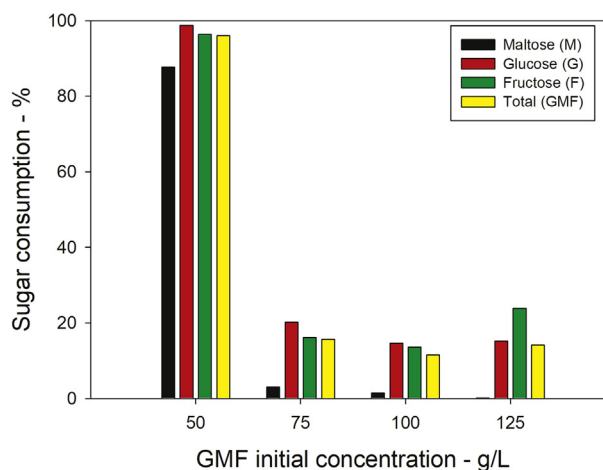
Fig. 4. Sugar consumption during *Clostridium saccharobutylicum* fermentation of medium supplemented with different concentrations of corn syrup (GMF initial concentrations: 50, 75, 100 and 125 g L^{-1}).

drastic decrease of metabolic activity of *C. saccharobutylicum* for GMF concentrations higher than 50 g/L. Similar behaviour is found in literature when very high sugar concentrations were used for batch ABE fermentation. For example, Ni et al. (2012) [22] observed a decrease in butanol and total ABE production when *C. saccharobutylicum* was employed to ferment sugarcane molasses at sugar concentrations higher than 6 %w/w; at the same time the residual sugar concentration increased with the increase of sugar concentration. Qureshi et al. (2013) [31] found that increasing sugars concentration above 60 g/L caused a decrease of ABE concentration, yield and productivity when *C. beijerinckii* P260 was used to ferment concentrated lignocellulosic hydrolysates. The authors explained these results declaring that high sugar levels were inhibitory to fermentation. In this study a dramatic decrease of cell growth and ABE production was measured when GMF concentration was increased from 50 to 75 g/L: 12.5 g/L versus 5.07 g/L ABE. Qureshi et al. (2013) [31] obtained similar rapid decrease in ABE production when sugar concentration in corn stover hydrolysates was increased from 60 to 80 g/L; but only a slow decrease was found when sugar concentration was increased in barley straw hydrolysates. The different behaviour among the two hydrolysates could be due to the presence of different inhibitory molecules produced by the pretreatment of lignocellulosic biomass. It is well known that inhibitory molecules of different nature (e.g. phenolic compounds, salts, heavy metals) are able to stop ABE fermentation [20, 32]. The presence of inhibitory compounds in the corn syrup could be another useful factor to explain why the increase of corn syrup concentration in the medium causes an evident decrease of metabolic activity of *C. saccharobutylicum*. In this work no inhibitor analysis was carried out, but the corn syrup composition reported in literature confirm the presence of molecules that could act as inhibitors for ABE fermentation, e.g. heavy metals (Zn, Fe), minerals (Na, P,

Mg), and salts [19]. Several dilution steps are required to reduce the inhibitory effect of these molecules. For experiments here reported, corn syrup was highly diluted to be used as carbon source for ABE fermentation; in fact, no inhibition was observed during fermentation of 50 g/L GMF. Conversely, inhibition was found for the successive tests for which also the concentration of inhibitory molecules was increased along with sugar concentration. As described above, a test with only corn syrup without additional nutrients confirmed that the presence of nutrients is fundamental to carry out fermentation of corn syrup. Considering the hypothesis of sugar and other molecules inhibition, and comparing the results of this work with other present in literature, it can be said that both inhibition processes can be taken in account as reason for ABE fermentation inhibition when corn syrup concentration was higher than 50 g/L (GMF). The aim of this study was not to assess the effect of each nutrient used, but the information reported here can be used for successive experiments more specific about medium composition for ABE fermentation with corn syrup.

3.3. Fed-batch fermentation of medium supplemented with corn syrup as only carbon source

Fed-batch fermentation is a fermentative strategy usually considered when substrate inhibition or other phenomena like catabolite repression might occur. This process is started with a low substrate concentration: when almost all substrate concentration is consumed, more substrate is added to maintain the fermentation process [33]. Fed-batch fermentation presents some advantages that make it a strong alternative to other fermentation strategies: with respect to batch fermentation (close system), fed-batch process (open system) allows to add fresh medium when the depletion of nutrients is occurring so that fermentation can continue; with respect to continuous fermentation (open system), fed-batch process does not need a strictly control of the feeding flow because there is no risk of wash-out (loss of biomass). To increase the solvents production from fermentation of corn syrup by *C. saccharobutylicum*, an ABE fermentation was carried out in fed-batch mode. Fermentation culture was first operated in batch mode: a diluted corn syrup solution of 50 g/L GMF was used to start the batch fermentation. When switching to fed-batch mode highly concentrated media (containing 400 g/L GMF) was manually fed (4 mL via pipette) to the fermentation broth when sugar depletion was detected. Volume and concentration were choses to set the sugars concentration as close as possible to their starting values. Fig. 5 reports the time-solved profile of the concentrations of cells, GMF concentration and metabolites (acetic acid, butyric acid, acetone, butanol, and ethanol) quantified during the fed-batch culture. The pH values measured for each sampling are also reported. In the first 12 h of fermentation, the microorganism showed the same behaviour reported for the screening and inhibition tests (Figs. 1, 2, and 3): no lag phase was observed, indeed *C. saccharobutylicum* started the acidogenesis from the beginning of fermentation. This is highlighted by the pH
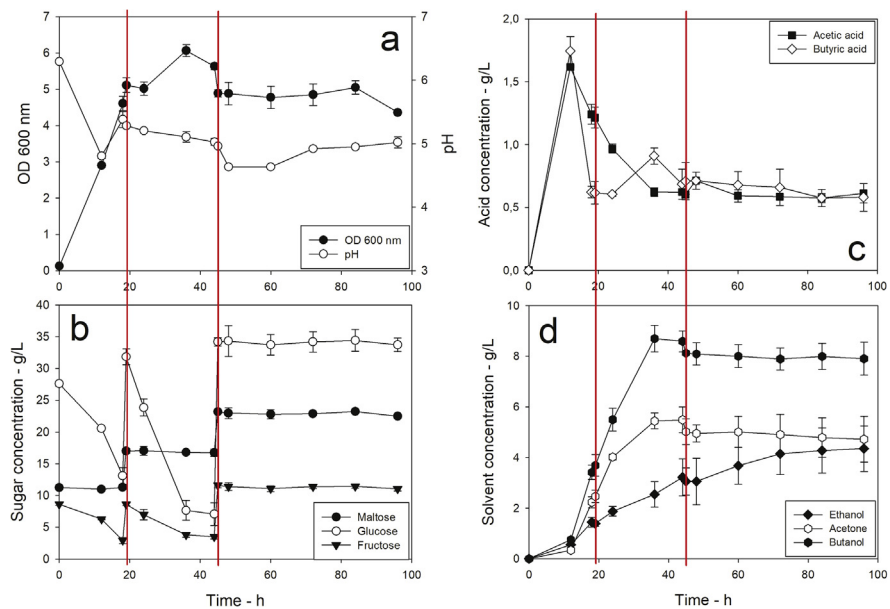
Fig. 5. Time-resolved profiles of data measured during fed-batch fermentation of corn syrup (GMF initial concentration: 50 g L^{-1}) by *Clostridium saccharobutylicum*. Red lines represent the fermentation time at which fresh medium (GMF concentration: 400 g L^{-1}) was added to the fermenting broth.

decrease and cell and acids concentration increase (Fig. 5a and c). After 12 h, the metabolic activity of *C. saccharobutylicum* shifted from acidogenesis to solvento-genesis. At this point acids concentration rapidly decreased (Fig. 5c), while solvents were produced (Fig. 5d). At 18 h, the fermentation culture was fed with fresh media, so that glucose and fructose concentration was increased again to their initial value (Fig. 5b). As reported in Fig. 2, *C. saccharobutylicum* starts to use maltose only after the concentration decrease of glucose and fructose. Since glucose and fructose amount are not limited during fed-batch fermentation, the anaerobic bacterium did not need to metabolize maltose for its growth. After the first feeding at 18 h, biomass concentration first decreased slightly, but then increased rapidly as shown in Fig. 5a, reaching an optical density of 6, that was the same reached during the batch fermentation with 50 g/L GMF (Fig. 1a). From 18 to 44 h, acetic acid concentration decreased until reaching a constant value (0.60 g/L), while butyric acid concentration increased up to 0.90 g/L before start to decrease again.

Within this time range, the pH value was almost constant (Fig. 5a), due to the contemporary production of acids and solvents (Fig. 5c and d). A higher butanol concentration was found during the fed-batch process compared to the batch process (Fig. 5d; Table 2), implying that more butyric acid was produced and utilized to produce butanol in fed-batch system can be supposed.

After 44 h, the glucose and fructose concentration decreased again, then fresh media was added to the fermentation culture. At this point no more sugars consumption or

acids/solvents production was observed (only a slight ethanol production was still measured). Biomass concentration remained constant until 84 h; after that it started to decrease (Fig. 5a), indicating the end of fermentation due to the cell death [10]. The stop of sugars consumption and acids/solvents production implies that an inhibition of fermentative process is occurring. Indeed, after the second feeding with fresh media at 44 h, the total GMF concentration rose up to 70 g/L. In the previous experiments regarding the effects of substrate concentration increase, the results indicated that GMF concentrations higher than 50 g/L cause inhibition of ABE fermentation by *C. saccharobutylicum* in the range of GMF concentrations investigated. For this reason, it could be supposed that a substrate inhibition occurred for the fed-batch process. The same behaviour about ethanol production with high GMF concentrations is also observed in fed-batch mode with respect to batch process (Figs. 5d and 3d). In case of fed-batch fermentation, product inhibition could also affect the performances of the process. As reported in previous works, butanol at 5−10 g/L could be cytotoxic to Clostridia, reducing cells metabolic activity and solvent production [6, 34]. In this study, the maximum amount of butanol produced in fed-batch fermentation was 8.70 g/L. This butanol concentration can be considered as possible inhibitory factor for the ABE fermentation of corn syrup in fed-batch mode. This examination is also confirmed by the fact the substrate consumption appears to have stopped in the last 5 h before the addition of new medium. At this points the cells where not substrate limited, hence product inhibition is a likely explanation. It is worthy to note that residual metabolic activity is still going. After 44 h the pH slowly increased due to the slow ethanol production (Fig. 5a). The comparison of these results (Fig. 5d) with that obtained from inhibition experiments (Fig. 3d), allows to hypothesize that substrate and product inhibition directly affect the metabolic pathways responsible for acetone and butanol production, while ethanol can be produced until cell death. This conclusion refers to the results reported in this work for *C. saccharobutylicum* DSM 13864. The use of different species or different medium composition could produce contradictory results.

In the last years several attempts at the ABE fermentation of very concentrated sugars solution (or molasses) have been reported. The results of these researches are summarized and compared to the results obtained in this study in Table 2. Most of these studies investigate ABE fermentation using molasses from soy or sugarcane as substrate. No papers are present in literature about ABE fermentation of commercial corn syrup. Considering the batch fermentation, ABE yield achieved in this study was comparable to those obtained from fermentation of molasses. In particular, Ni et al. (2012) [22] achieved an ABE yield of 0.33 g/g using the same *Clostridium* species employed in this study to ferment sugarcane molasse. ABE productivity achieved in this study is among the highest reported thus far for the ABE fermentation of this kind of substrates. This result can be partly explained considering that in the present study the microorganism used (*C. saccharobutylicum*)

can complete the fermentation process in less time (36 h) with respect to the species used in the other studies (90−96 h). Ni et al. (2012) [22] achieved a higher ABE productivity because high amount of solvents (17.9 g/L) was produced from sugarcane molasse with respect to corn syrup (12.47 g/L). The difference in performances could be due to the lower sugar concentration used in this study and the difference in sugar composition of the two substrates (50 g/L of glucose, maltose and fructose for corn syrup vs. 60 g/L glucose, sucrose and fructose for sugarcane molasse). Moreover, differences in concentrations of heavy metals, minerals and salts in the two substrates could also affect fermentation processes. The solvents production from corn syrup was increased when ABE fermentation was carried out in fed-batch mode. Comparing the results of both batch and fed-batch fermentation, an overall improve of solvents concentration, yield and productivity was observed for the fed-batch process. Total ABE production and ABE productivity were increased of 34 % (16.68 g/L fed-batch vs 12.47 g/L batch and 0.47 g/L/h fed-batch vs. 0.35 g/L/h batch, respectively). Both butanol and ABE yields were improved, but butanol yield was still lower than those obtained from fermentation of molasses. This is due to the higher amount of sugars metabolized in fed-batch fermentation with respect to batch fermentation. However, the use of a fed-batch process allowed to obtain ABE concentration, yield and productivity more like that obtained by Ni et al. (2012) [22]. These results indicate that *C. saccharobutylicum* can be employed to efficiently ferment corn syrup for ABE production; especially because of its very fast fermentation time with respect to other *Clostridium* spp.

Since 20 g/L of ABE (5−10 g/L butanol) is the limit concentration beyond which product inhibition is observed in batch (or fed-batch) fermentation [6, 30, 34], to improve the amount of solvents that can be achieved from ABE fermentation with *Clostridium* spp., two paths can be investigated: the use of engineered strains for higher butanol tolerance and production [35] and/or the use of solvent recovery systems to couple with bioreactor during solvents production phase [9, 36]. The recovery system is a good option to try to increase the solvents production when *C. saccharobutylicum* is used to ferment corn syrup. Scale up of the process from the flask to a bench bioreactor should be first considered, as well as the possibility of a continuous process. At this point, the coupling with a recovery system (contemporarily to production) could be very useful to improve the overall performances of the process. Successive experiments will be carried out using *C. saccharobutylicum* as fermenting microorganism using corn syrup (or similar molasses) as feedstock to test the above-mentioned theories.

Table 2 also reports ABE yield from fermentation based on other kinds of feedstock: switchgrass, corncob and lettuce residues as lignocellulosic material; corn starch as starch-based material. The fermentation carried out using hydrolysate from switchgrass, corncob and corn starch gave ABE yield comparable (or slightly

higher) to that obtained in the present study. The differences could be due to the carbon source used to carry out the fermentation, indeed the switchgrass, corncob and corn starch hydrolysate are mainly composed of simple sugars (glucose and xylose for the lignocellulosic biomass; glucose for corn starch) that can be metabolized faster than disaccharides like maltose present in corn syrup. However, even if a comparable ABE yield was obtained with lignocellulosic biomass, it is important to stress that this kind of biomass often requires expensive steps of pretreatment to facilitate the enzymatic hydrolysis of the carbohydrates present in the biomass, that can also be partly lost during the pretreatment process. Lettuce residues are also reported in Table 2 as lignocellulosic feedstock for the ABE production, but the ABE yield from this biomass is very low due to the high water and low carbohydrates content.

4. Conclusions

Corn syrup was assessed as ABE fermentation substrate. The fermentation tests with different *Clostridium* spp. were successful. *C. saccharoburylicum* metabolized all glucose, maltose and fructose (GMF) present in corn syrup for solvents production. Substrate inhibition was suggested the likely explanation for low solvent production with initial corn syrup concentration higher than 50 g/L. The maximum amount of butanol and ABE produced were 8.70 and 16.68 g/L, respectively, obtained with a fed-batch process. The results of this work were comparable with that of other studies about ABE fermentation of molasses, indicating that corn syrup can be considered as a good substrate for solvents production.

Declarations

Author contribution statement

Lars Rehmann: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Saverio Niglio: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Antonio Marzocchella: Analyzed and interpreted the data.

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