

# Butanol production from inulin-rich chicory and *Taraxacum kok-saghyz* extracts: Determination of sugar utilization profile of *Clostridium saccharobutylicum* P262



Victor Ujor<sup>a,1</sup>, Ashok K. Bharathidasan<sup>b,1</sup>, Frederick C. Michel Jr.<sup>b</sup>, Thaddeus C. Ezeji<sup>a,\*</sup>, Katrina Cornish<sup>b,c,\*\*</sup>

<sup>a</sup> Department of Animal Sciences, The Ohio State University and Ohio Agricultural Research and Development Center (OARDC), Wooster, OH 44691, United States

<sup>b</sup> Department of Food, Agricultural and Biological Engineering, The Ohio State University and Ohio Agricultural Research and Development Center (OARDC), Wooster, OH 44691, United States

<sup>c</sup> Department of Horticulture & Crop Sciences, The Ohio State University and Ohio Agricultural Research and Development Center (OARDC), Wooster, OH 44691, United States

## ARTICLE INFO

### Article history:

Received 1 April 2015

Received in revised form 28 June 2015

Accepted 21 July 2015

Available online 15 August 2015

### Keywords:

*Clostridium saccharobutylicum*

Butanol

Chicory

*Taraxacum kok-saghyz*

*C. beijerinckii*

## ABSTRACT

Developing alternative applications for wastes stemming from the processing of industrially applicable crops adds value to industrial crops and limits agricultural land use. Kazak dandelion (*Taraxacum kok-saghyz*; TKS) and Chicory are rich in inulin. In this study, unhydrolyzed inulin-rich chicory and TKS extracts were assessed as substrates for acetone–butanol–ethanol (ABE) production, using different solventogenic *Clostridium* species/strains. Extracts from chicory and TKS were rich in inulin (~42 and 36 g/L respectively). In addition to inulin, the chicory extract also contained glucose (3.5 g/L), sucrose (6.2 g/L), fructose (19.5 g/L), and kestose (6.0 g/L), while the TKS extract contained fructose (28.3 g/L), kestose (2.7 g/L) and an unidentified sugar (2.0 g/L). Among the species/strains tested, *Clostridium saccharobutylicum* P262 demonstrated superior ability to utilize inulin, with at least 86% and 153% higher cell growth and ABE concentration, respectively, on inulin relative to the other species/strains. With pure commercial inulin, TKS and chicory extracts, *C. saccharobutylicum* P262 utilized 29.0, 25.2 and 40.5 g/L total sugars respectively, and produced 9.7, 8.5 and 12.5 g/L ABE, respectively. However, when fructose, the major product of inulin hydrolysis was used as the sole substrate, cell growth and ABE production by *C. saccharobutylicum* P262 were at least 74% and 83% lower, respectively, relative to the other species tested. These results demonstrate the suitability of *C. saccharobutylicum* P262 for butanol production from unhydrolyzed plant inulin and also identify limited fructose utilization as a potential metabolic engineering target for improving the fermentability of unhydrolyzed inulin-rich plant extracts to butanol.

© 2015 Elsevier B.V. All rights reserved.

## 1. Introduction

Plant-derived wastes are particularly attractive as substrates for industrial fermentation (Dale et al., 2010). Therefore, development of new multipurpose industrial crops, which serve as sources of both valuable products and fermentable wastes for industrial

fermentation, has become a focus of bioenergy researchers and plant breeders. The world chicory production, which is estimated to be approximately one million metric tons annually, is produced in Belgium, France, Netherlands, South Africa, Poland, and Puerto Rico (<http://faostat3.fao.org/search/chicory/E>). *Taraxacum kok-saghyz* also known as "Buckeye Gold" or the Kazak dandelion (TKS) is currently being developed by multiple groups as an alternative source of rubber (van Beilen and Poirier, 2007; Cornish et al., 2013). Interest in TKS and other alternative plants for the production of natural rubber derives from prolonged and complicated breeding cycles, and enormous labor demands associated with natural rubber (*Hevea brasiliensis*) production (van Beilen and Poirier, 2007; Cornish et al., 2013).

TKS roots contain 2–20% rubber, and 25–60% inulin (a naturally-occurring polysaccharide of fructose), on a dry weight basis

\* Corresponding author at: Department of Animal Sciences, The Ohio State University and Ohio Agricultural Research and Development Center (OARDC), Wooster, OH 44691, United States

\*\* Corresponding author at: Department of Food, Agricultural and Biological Engineering, The Ohio State University and Ohio Agricultural Research and Development Center (OARDC), Wooster, OH 44691, United States.

E-mail addresses: [ezeji.1@osu.edu](mailto:ezeji.1@osu.edu) (T.C. Ezeji), [cornish.19@osu.edu](mailto:cornish.19@osu.edu) (K. Cornish).

<sup>1</sup> These authors contributed equally to this article.

(Buranov and Elmurodov, 2010; Schütz et al., 2006; van Beilen and Poirier, 2007). Likewise, the root of Chicory (*Cichorium intybus* L.), which is grown largely as a forage crop, is a rich source of inulin – approximately 70% of dry matter (Quanzen and Jian, 2011; Gupta et al., 2003). Therefore, TKS is an ideal source of inulin, a byproduct of rubber production as a fermentation substrate. Hence, use of inulin from rubber production as fermentation substrate does not exert further pressure on agricultural resources. Similarly, the residual root bagasse and the leaves of TKS could also be used for biofuel production. Although inulin from Jerusalem artichoke has previously been used as a substrate in acetone–butanol–ethanol (ABE) fermentations, these involved pre- or *in situ* hydrolysis of inulin during fermentation (Marchal et al., 1985; Oiwa et al., 1987; Sarchemi and Rehmann, 2014), which add to the overall process cost. Fermentation of unhydrolyzed inulin may be a more cost effective approach for low cost ABE fermentation.

Although solventogenic *Clostridia* are capable of utilizing a wide range of substrates, inulin has proven to be fairly intractable warranting inulin hydrolysis pre- or during fermentation (Marchal et al., 1985; Oiwa et al., 1987; Sarchemi and Rehmann, 2014). Identification of an inulin-hydrolyzing solventogenic *Clostridium* species/strain may allow inulin to realize its enormous potential as a substrate for ABE fermentation. Further, such species/strain may contribute to our understanding of the enzyme repertoire involved in inulin metabolism, which could provide a roadmap for metabolic engineering-driven strain improvement leading to ABE-producing *Clostridium* species that are able to efficiently utilize unhydrolyzed inulin as a substrate. More importantly, efficient conversion of inulin-rich crops such as TKS and Chicory to ABE will significantly add economic values to and industrial applicability of these crops.

In the current study, we screened four solventogenic *Clostridium* species for ABE production using unhydrolyzed pure commercial inulin as a substrate. *Clostridium saccharobutylicum* P262 which exhibited the best ABE production on pure unhydrolyzed commercial inulin was used in ABE fermentation of inulin-rich extracts of chicory and TKS. HPLC analysis was used to determine the pattern of inulin utilization by *C. saccharobutylicum* P262 and likely limitations to enhanced inulin consumption by this strain were determined. Overall, this investigation explores the use of unhydrolyzed inulin-rich extracts from chicory and TKS for the production of butanol, a next-generation biofuel and an important industrial base chemical. It is expected that the findings of this study will help to advance efforts aimed at developing Chicory and TKS cultivation for industrial applications.

## 2. Materials and methods

### 2.1. Microorganisms

Four strains were used in this study. *Clostridium beijerinckii* NCIMB 8052, *Clostridium beijerinckii* NRRL B-592, *Clostridium acetobutylicum* ATCC 824 and *C. saccharobutylicum* P262. *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 (*C. beijerinckii* ATCC 51743) were procured from the American Type Culture Collection, Manassas, VA. *C. saccharobutylicum* 262 was kindly provided by Professor David Jones of University of Otago Dunedin, New Zealand. *C. beijerinckii* NRRL B-592 (formerly *C. butylicum* NRRL B-592) was obtained from Agricultural Research Service (NRRL) Culture Collection, Peoria, Illinois. Spore stocks were stored in sterile double-distilled water at 4 °C.

### 2.2. Inulin extraction

Given that the particle size of ground TKS and chicory roots, the extraction solvent ratio, and extraction temperature are

important factors that influence inulin extraction efficiency, the dried TKS and Chicory roots were roller-milled to average particle size of 0.25 mm. Inulin was extracted by mixing them with the extraction solvent (0.1% Na<sub>2</sub>CO<sub>3</sub> in water) at the ratio of 1:6 (w/v), at ~90 °C for 30 min (Eskew, 1946). The supernatant was collected by screening, and the extracted chicory or TKS materials were extracted 2 more times (a total of 3 extractions) using fresh extraction solvent. The extracts were then combined, resulting in a final inulin concentration of ~28–30 g/L for both chicory and TKS. The extracts were placed in a hot air oven (45 °C) for ~36 h to evaporate excess water, thereby concentrating the inulin. Afterwards, inulin concentration in both extracts increased to ~40 g/L. In addition to inulin, the TKS and chicory extracts contained fructose, glucose, sucrose and kestose at different concentrations.

### 2.3. Inoculum preparation and batch fermentation

For *C. beijerinckii* NCIMB 8052, *C. beijerinckii* NRRL B-592, *C. acetobutylicum* ATCC 824, 200 µL of spore suspensions were heat-shocked at 75 °C for 10 min followed by cooling in ice for 3 min. *C. saccharobutylicum* P262 spores (200 µL) were heat-shocked for 3 min at 70 °C and then cooled in ice for 1 min (Ezeji and Blaschek, 2008). The heat-shocked spores (with the exception of *C. saccharobutylicum* P262) were inoculated into 10 mL of tryptone–glucose–yeast extract (TGY) medium and incubated anaerobically for 12–14 h at 35 ± 1 °C. Heat-shocked spores of *C. saccharobutylicum* P262 were incubated anaerobically for 16–18 h (Qureshi and Maddox, 2005). When the preculture reached an optical density (OD<sub>600nm</sub>) of 0.9–1.1, 8 mL of actively growing preculture was transferred into 92 mL of anoxic pre-sterilized TGY medium and incubated for another 3–4 h until OD<sub>600nm</sub> reached 0.9–1.1, and then they were transferred to production media (Ezeji and Blaschek, 2008). Production media were prepared using the extracts from Chicory and TKS roots. Additionally, prior to fermentation TKS extract was supplemented with glucose (3.5 g/L) and sucrose (6.2 g/L) to mimic the levels of glucose and sucrose present in the chicory extract. Commercially available inulin (Alfa Aesar, Heysham, England) was used as control (~55 g/L). All media were supplemented with yeast extract (1 g/L) and calcium carbonate (4 g/L) as previously described (Han et al., 2013). Pyrex screw-capped bottles (250 mL) containing 100 mL of fermentation medium were used for fermentation. Bottles containing 91 mL of the fermentation media were sterilized for 15 min at 121 °C, cooled to 40 °C and then transferred to an anaerobic chamber for 14–16 h prior to inoculation to attain a completely anaerobic condition. The anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI) was maintained at a modified atmosphere of 82% N<sub>2</sub>, 15% CO<sub>2</sub>, and 3% H<sub>2</sub> (Ujor et al., 2014). This was followed by addition of 1 mL of filter-sterilized P2 stocks, namely vitamin (0.1 g/L para-amino-benzoic acid; 0.1 g/L thiamine; 0.001 g/L biotin), buffer (50 g/L KH<sub>2</sub>PO<sub>4</sub>; 50 g/L K<sub>2</sub>HPO<sub>4</sub>; 220 g/L ammonium acetate), and mineral (20 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O; 1 g/L MnSO<sub>4</sub>·H<sub>2</sub>O; 1 g/L FeSO<sub>4</sub>·7H<sub>2</sub>O; 1 g/L NaCl) stock solutions to the fermentation medium. Actively growing preculture in TGY medium was used as inoculum, and each bottle was inoculated with 6% (v/v) inoculum from the preculture (Ujor et al., 2014). Since fructose is the major monomer of inulin, we investigated the ABE profiles of *C. beijerinckii* NCIMB 8052, *C. beijerinckii* NRRL B592, *C. acetobutylicum* ATCC 824, and *C. saccharobutylicum* P262 grown on fructose (60 g/L) as the sole carbon source. The fructose medium was supplemented with yeast extract, calcium carbonate, vitamin, mineral and buffer stocks, as described above, and each culture was inoculated with 6% (v/v) inoculum. All fermentations were conducted in triplicate and incubated statically at 35 ± 1 °C.

## 2.4. Analytical methods

Cell growth was quantified by measuring optical density at 600 nm ( $OD_{600}$ ) using a DU® 800 spectrophotometer (Beckman Coulter Inc., Brea, CA). The concentrations of acetic and butyric acids, acetone, butanol, and ethanol (ABE) were determined using a gas chromatography system (Agilent Technologies 7890A, Agilent Technologies Inc., Wilmington, DE, USA), equipped with a flame ionization detector (FID) and a J × W 19091N-213 capillary column [30 m (length), 320  $\mu$ m (internal diameter), and 0.50  $\mu$ m (HP-Innowax film thickness)]. Nitrogen was used as the carrier gas. The inlet and detector temperatures of the gas chromatograph were maintained at 250 °C and 300 °C, respectively, and the oven was set to span from 60 to 200 °C with increments of 20 °C per minute, with a 5-min hold at 200 °C. Each filtered fermentation sample (1  $\mu$ L) was injected into the gas chromatograph with a split ratio of 10:1 (Ujor et al., 2015). The concentrations of glucose, sucrose, fructose, kestose and inulin [with a degree of polymerization (DP) of 4+] were determined using an Agilent 1200 Series high performance liquid chromatography (HPLC) system, equipped with a refractive index (RI) detector (Agilent Technologies, Santa Clara, CA), and a Rezex organic acid column (Rezex ROA-Organic Acid H<sup>+</sup> column) whose length and internal diameter are 300 mm × 7.8 mm, respectively (Phenomenex, Torrance, CA). The Rezex organic acid column is an ion-exclusion column packed with 8% cross-linked sulfonated styrene-divinylbenzene (SDVB) adducts, which distinguishes oligosaccharides up to DP 3, while compounds greater than DP 3 co-elute. Sulfuric acid (Sigma-Aldrich, St. Louis, MO) diluted to 0.0025 M with sterile distilled water was used as mobile phase (pH ~2) at a flow rate of 0.6 mL/min. Samples (10  $\mu$ L) were injected automatically, and the column and the detector temperatures were set at 25 °C.

## 3. Results

### 3.1. Screening of select solventogenic Clostridium species for ABE production on pure inulin and fructose as sole sources of carbon

To determine the best species/strain for the fermentation of chicory and TKS root extracts, *C. beijerinckii* NCIMB 8052, *C. beijerinckii* NRRL B592, *C. acetobutylicum* ATCC 824, and *C. saccharobutylicum* P262 were grown on inulin (55 g/L) or fructose (60 g/L). When grown on pure inulin as the sole source of carbon, *C. saccharobutylicum* P262 clearly out-performed the other *Clostridium* species with at least 88% higher cell density ( $OD_{600}$ ), and ~109% and 152% higher butanol and ABE concentrations, respectively (Fig. 1A–C), reaching  $OD_{600}$  up to ~4.7 and butanol and ABE concentrations of 7.1 and 10.3 g/L respectively. However, when fructose the major product of inulin hydrolysis was the sole carbon source, *C. saccharobutylicum* P262 grew poorly compared to the other species studied. With fructose, the maximum cell density measured for *C. saccharobutylicum* P262 was ~43% lower than that observed for *C. beijerinckii* NRRL B592, and at least 145% lower than the peak cell densities achieved by the cultures of *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824 (Fig. 1D).

Additionally, with fructose substrate *C. saccharobutylicum* P262 produced 47% less butanol than *C. beijerinckii* NRRL B592, and about 94% less than *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824 (Fig. 1E). Similarly, the maximum ABE produced by *C. saccharobutylicum* P262 was at least 58% less than the peak ABE concentrations produced by the other three species (Fig. 1F). Fermentation of fructose by *C. saccharobutylicum* P262 was not as efficient as that observed with *C. beijerinckii* NRRL B592, *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824, but it is worth mentioning that *C. saccharobutylicum* P262 produced similar

solvent profiles when grown on inulin or fructose, with butanol and ABE titers of ~7 and ~10 g/L, respectively (Fig. 1). However, *C. saccharobutylicum* showed 51% higher growth on inulin relative to fructose (Fig. 1A and D).

### 3.2. Sugar composition of chicory and TKS root extracts

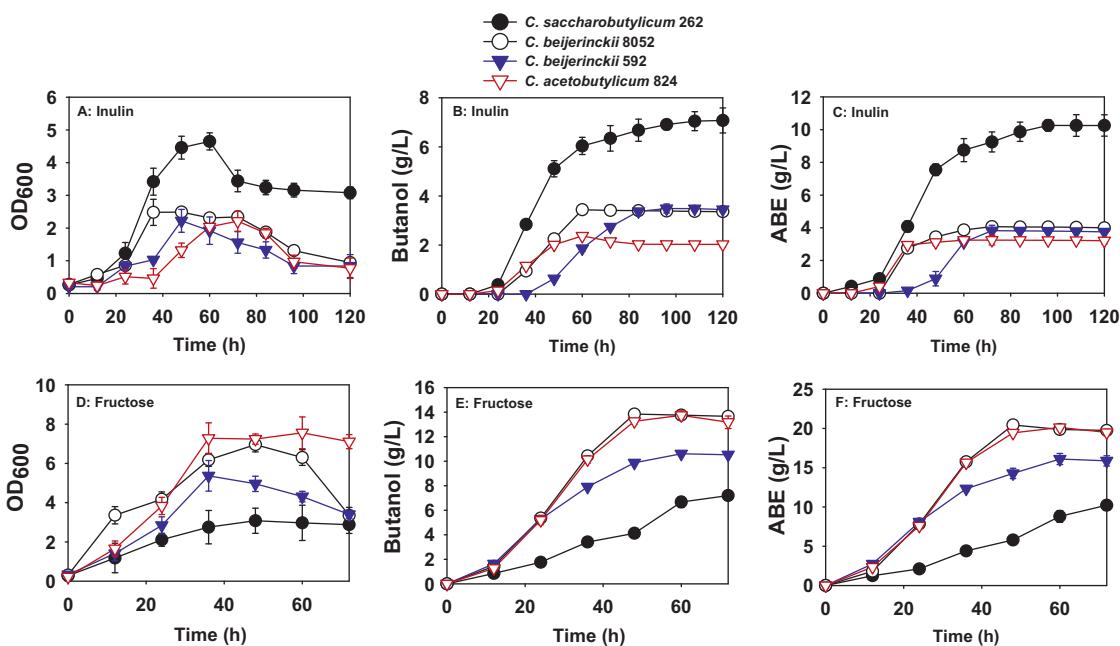
The sugar compositions of chicory and TKS extracts (Table 1) indicate that inulin was the predominant sugar in both extracts, with the chicory extract showing a 16% higher inulin content than the extract from TKS. Similarly, kestose and fructose were present in both extracts, with the chicory extract containing higher amounts of kestose (~125% higher) than the TKS extract, while fructose was more abundant in the TKS extract (~46% higher). Also, while 6.2 g/L sucrose and 3.5 g/L glucose were detected in the chicory extract, neither was found in the TKS extract.

### 3.3. ABE production from unhydrolyzed chicory and TKS extracts by *C. saccharobutylicum* P262

Given the ability of *C. saccharobutylicum* P262 (hereafter referred to as *C. saccharobutylicum*) to utilize unhydrolyzed inulin relative to the other species studied, *C. saccharobutylicum* was used in subsequent experiments to ferment inulin-rich chicory and TKS extracts. *C. saccharobutylicum* was able to ferment chicory and TKS extracts, as well as commercial pure inulin (control) to ABE (Fig. 2). Solvent production was considerably higher with the unhydrolyzed chicory extract than with unhydrolyzed TKS extract or pure inulin (Fig. 2). With the chicory extract, butanol and ABE concentrations reached 8.6 and 12.5 g/L respectively. These are 30% and 28% higher, respectively, than the butanol and ABE titers obtained with pure inulin, and 75% and 47% higher than the maximum concentrations observed with the TKS extract, respectively (Fig. 2). Notably, butanol and ABE concentrations obtained with chicory extract were comparable to concentrations typically obtained with glucose (9–10 g/L butanol; 12.5–14.5 g/L ABE). The preference of *C. saccharobutylicum* for the three inulin-based substrates was as follows: Chicory extract > pure inulin > TKS extract. With the TKS extract, butanol production was not observed until 48 h post inoculation, resulting in the lowest butanol titer obtained among the three inulin-based substrates.

Butanol and ABE production from pure inulin was noticeably higher than the titers obtained with TKS extract. On pure inulin, butanol and ABE concentrations were 35% and 15% greater, respectively, relative to the TKS extract. However, despite the considerably greater ABE titer obtained with the chicory extract, ABE yield for this extract (0.32 g/g of substrate) was marginally less than that obtained with pure inulin and TKS extract (0.33 g/g; Table 1). This is attributable to utilization of the considerably greater amounts of sugars in the chicory medium – ~40 g as compared to the 25 g and ~29 g used in the TKS and pure inulin media, respectively (Table 1). Conversely, enhanced utilization of sugars in the chicory medium led to a much higher ABE productivity (0.21 g/L·h), than the productivities observed with pure inulin and TKS; 0.1 and 0.12 g/L·h, respectively (Table 1). This represents up to 52% increase in productivity when chicory extract was used as substrate.

To better understand the poor and delayed ABE production observed with TKS extract, we supplemented the TKS extract-based cultures with glucose (3.5 g/L) and sucrose (6.2 g/L), representing the levels of glucose and sucrose found in the chicory extract. Glucose and sucrose supplementation of the TKS extract resulted in a more rapid accumulation of ABE, particularly within the first 36 h of fermentation when ABE production was significantly delayed in the unsupplemented extract (Fig. 2). Consequently, with glucose and sucrose supplementation of the TKS extract, butanol and ABE



**Fig. 1.** Growth (A), butanol (B), and ABE (C) profiles of *C. beijerinckii* NCIMB 8052, *C. beijerinckii* NRRL B592, *C. acetobutylicum* ATCC 824, and *C. saccharobutylicum* P262 grown on commercial inulin and fructose as the sole sources of carbon.

**Table 1**

Performance and kinetic parameters of ABE production from unhydrolyzed inulin media by *C. saccharobutylicum*.

Parameters	Pure inulin (control)	Raw TKS	Raw chicory
Initial inulin (g/L)	55.78 ± 0.34	35.98 ± 0.01	41.83 ± 0.34
Initial kestose (g/L)	0.00 ± 0.00	2.65 ± 0.07	5.96 ± 0.03
Initial sucrose (g/L)	0.00 ± 0.00	0.00 ± 0.00	6.21 ± 0.02
Initial glucose (g/L)	0.00 ± 0.00	0.00 ± 0.00 <sup>a</sup>	3.53 ± 0.00
Initial fructose (g/L)	0.00 ± 0.00	28.32 ± 0.29	19.45 ± 0.08
Initial amount of total sugars (g/L)	55.00 ± 0.34	69.03 ± 0.47	76.97 ± 0.47
Final inulin (g/L)	21.86 ± 4.20	31.55 ± 4.15	28.66 ± 3.22
Final kestose (g/L)	0.00 ± 0.00	2.57 ± 0.13	4.14 ± 0.00
Final sucrose (g/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Final glucose (g/L)	0.00 ± 0.00	1.97 ± 0.09	0.00 ± 0.00
Final fructose (g/L)	4.80 ± 1.86	7.97 ± 0.25	3.65 ± 0.01
Final amount of total sugars (g/L)	26.82 ± 2.22	44.07 ± 2.36	36.45 ± 3.21
Total sugars utilized (g)	28.96 ± 2.22	25.20 ± 0.17	40.52 ± 3.67
Acetone (g/L)	1.71 ± 0.41	1.64 ± 0.06	2.80 ± 0.05
Ethanol (g/L)	1.67 ± 0.44	2.14 ± 0.04	1.36 ± 0.15
Butanol (g/L)	6.56 ± 0.50	4.88 ± 0.28	8.58 ± 0.11
Total ABE (g/L)	9.71 ± 0.57	8.48 ± 0.38	12.50 ± 0.13
ABE yield (g/g of substrate)	0.33 ± 0.04	0.33 ± 0.01	0.32 ± 0.03
ABE productivity (g/L/hour)	0.10 ± 0.01	0.12 ± 0.01	0.21 ± 0.01

<sup>a</sup> An unknown peak (2 g/L) which appeared to be glucose was detected in the TKS extract. However, the sugar with this peak was not utilized during fermentation suggesting that it is not glucose, which solventogenic *Clostridium* species as well as other bacteria utilize rapidly.

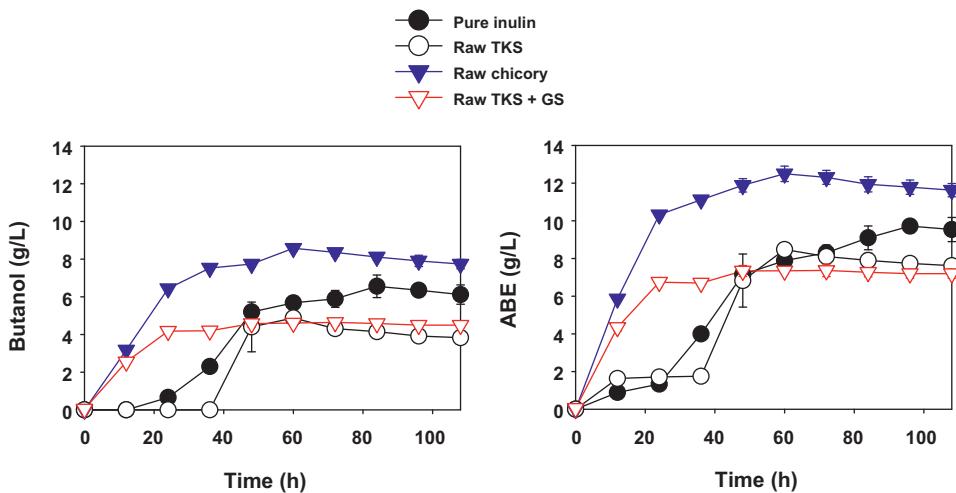
productivities increased by ~2.0-folds (Fig. 2). However, the maximum butanol and ABE titers achieved in the supplemented and unsupplemented TKS extracts did not vary substantially.

#### 3.4. Sugar utilization profiles of *C. saccharobutylicum* in pure inulin, chicory and TKS media

Quantification of initial and residual sugars in the pure inulin, chicory and TKS media during fermentation revealed the patterns of sugar utilization by *C. saccharobutylicum* in all three inulin-based media. The sugar profile of *C. saccharobutylicum* cultures in pure inulin medium clearly demonstrates its inherent inulinase activity. In this medium, *C. saccharobutylicum* utilized ~29 g/L of inulin, and only inulin and fructose were detected in the broth throughout fermentation, with the exception of 0 h when only inulin was present in the broth (Fig. 3A–C). The pattern of fructose accumulation in

the pure inulin medium suggests that *C. saccharobutylicum* first hydrolyses inulin to fructose up to a certain level before utilizing the accumulated fructose for growth and ABE production. For instance, fructose concentration in the pure inulin medium increased from 0 to 5.73 g/L from 0 h to 36 h, followed by decreases in fructose concentration up until 96 h when another increase was observed (Fig. 3A). Interestingly, the period of fructose accumulation (36 h post inoculation) in the pure inulin medium coincides with a lag in growth, particularly in the first 24 h (Fig. 1A), and apparent delay of butanol and ABE accumulation (Figs. 1B–C, and 2A–B).

When TKS extract was used as substrate, only a small amount of inulin was utilized by *C. saccharobutylicum* (Fig. 4A–C). This can be ascribed to the high concentration of fructose (28.3 g/L) in this extract, which is preferentially utilized over inulin, likely due to catabolite repression. A decrease in inulin concentration was not observed until 48 h into the fermentation, after most of the fructose



**Fig. 2.** Butanol (A) and ABE (B) concentrations in cultures of *C. saccharobutylicum* with pure inulin and inulin-rich unhydrolyzed chicory and TKS extracts, and TKS extract supplemented with glucose and sucrose. GS – glucose (3.5 g/L); S – sucrose (6.2 g/L).

had been utilized. *C. saccharobutylicum* utilized 20.2 g/L of fructose out of an initial fructose concentration of 28.32 g/L (Fig. 4A). Conversely, only 4.3 g/L of the initial ~36 g/L inulin was utilized during fermentation. As depicted in Fig. 4A–C, a peak which eluted as glucose during HPLC analysis was not utilized during fermentation. Since glucose is the preferred sugar by solvent-producing clostridia, and results presented below further demonstrate that *C. saccharobutylicum* preferentially consumes glucose over other sugars in a mixed-sugar medium, we concluded that this peak which eluted with a retention time similar to glucose was in fact not glucose. The kestose present in the TKS extract was not utilized by *C. saccharobutylicum*.

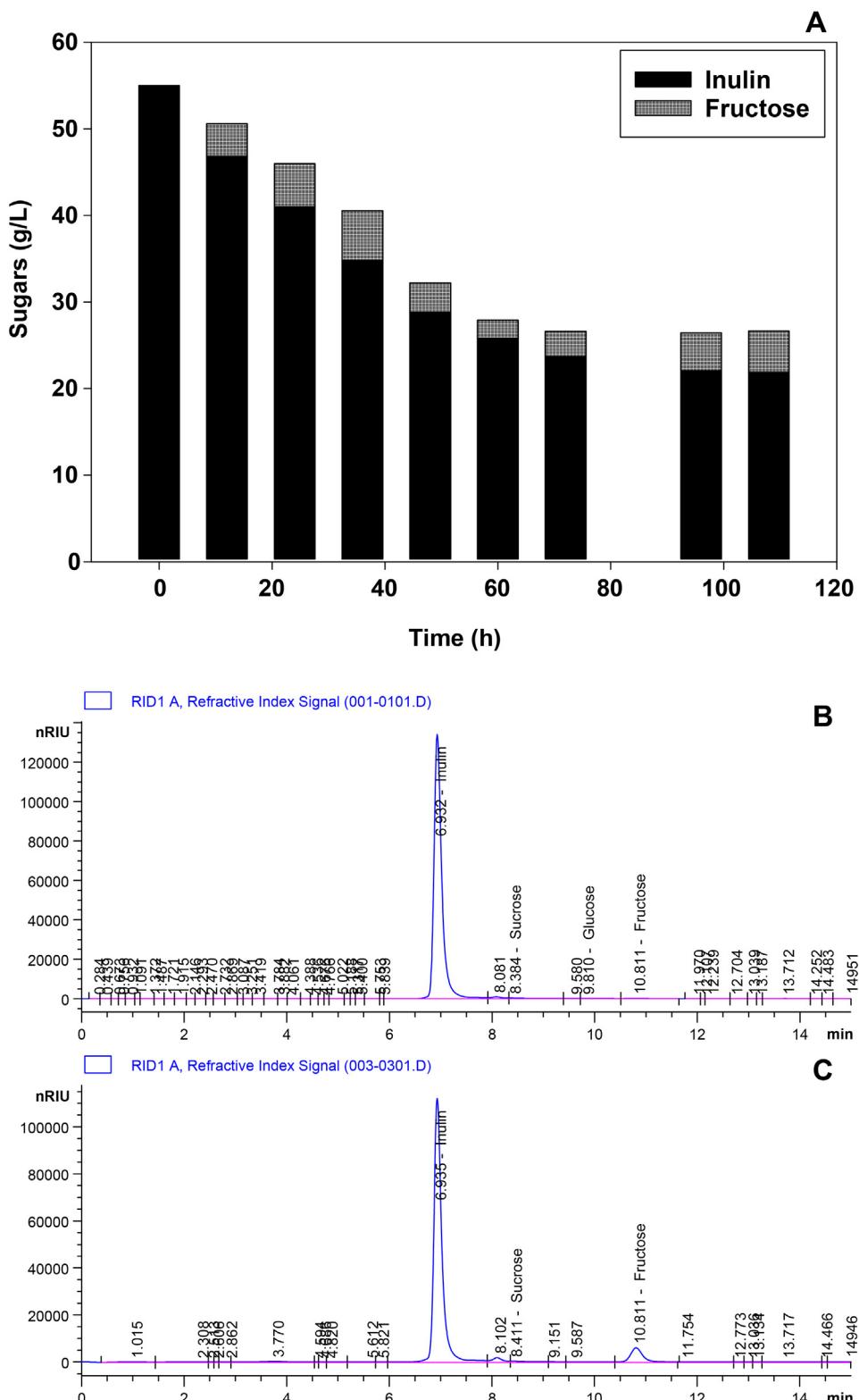
In the chicory-based medium, glucose and sucrose were rapidly utilized by *C. saccharobutylicum* (Fig. 5A–C). At 12 h, 75% (2.7 g/L) of glucose in the medium had been used, while 48% (~3 g/L) of the sucrose was utilized, and at 24 h all the glucose and sucrose in the medium had been used up. Similarly, fructose was utilized rapidly during the first 24 h of fermentation, during which 92% (18 g/L) of the fructose was utilized by *C. saccharobutylicum*. Inulin consumption was not observed until nearly all the glucose, sucrose and fructose in the medium had been utilized (24 h; Fig. 5A). Overall, ~207% more inulin (13.2 g/L) was utilized in the chicory medium than in the TKS medium (Figs. 4 and 5). While kestose was not utilized by *C. saccharobutylicum* in the TKS medium, 30.5% (1.8 g/L) of the kestose in the chicory extract was utilized. As with ABE production, sugar utilization was poorest in the TKS medium. While 24.5 g/L of total sugars was utilized by *C. saccharobutylicum* in the TKS medium, 38.7 g/L, 37% more sugars was expended when chicory extract was used as the substrate. A similar trend was observed with the pure inulin medium (relative to the TKS medium) in which a slightly higher amount of sugars was utilized by *C. saccharobutylicum* than in the TKS extract, despite the presence of a high initial concentration of fructose in the TKS extract (Figs. 3 and 4). In the pure inulin medium, *C. saccharobutylicum* hydrolyzed a total of 33.1 g/L of inulin (Fig. 3). However, at the end of fermentation, residual 4.8 g/L of fructose was present in the broth; hence a total of 28.3 g/L of inulin was utilized by *C. saccharobutylicum* in the pure inulin medium, which is ~16% higher than the total sugars utilized in the TKS medium.

#### 4. Discussion

In this study, we evaluated the use of unhydrolyzed inulin-rich root extracts of chicory and TKS for butanol production. To this

end, we screened notable solventogenic *Clostridium* species/strains (*C. beijerinckii* NCIMB 8052, *C. beijerinckii* B-592, *C. acetobutylicum* ATCC 824 and *C. saccharobutylicum* P262) for ABE production on unhydrolyzed inulin and its major hydrolysis product, fructose. Notably, all the species/strains studied demonstrated an ability to utilize inulin for ABE production, albeit to varying degrees. This is in contrast to previous studies by Montoya et al. (2000, 2001) who did not observe inulin utilization by *C. beijerinckii* NCIMB 8052 or *C. acetobutylicum* ATCC 824. A possible reason for the ability of these species to utilize unhydrolyzed inulin in this study may have been the presence of CaCO<sub>3</sub> in the growth medium. Previous studies in our laboratory have shown that calcium ions (Ca<sup>2+</sup>), supplied in the form of CaCO<sub>3</sub> or CaCl<sub>2</sub>, exert a pleiotropic effect on solventogenic *Clostridium* species, which results in increases in the levels various proteins, including those involved in sugar transport and utilization (Richmond et al., 2011; Han et al., 2013). Others have demonstrated that the presence of CaCO<sub>3</sub> and CaCl<sub>2</sub> in the fermentation medium abolished carbon catabolite repression thereby allowing concomitant utilization of glucose and xylose by *C. acetobutylicum* (El Kanouni et al., 1998; Raganati et al., 2012). Therefore, it is possible that the presence of CaCO<sub>3</sub> in the medium elicited inulin-metabolizing enzymes in the organisms studied, thereby explaining consumption of unhydrolyzed inulin by *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824 in this study. More importantly, these results demonstrate that inulolytic activity is not completely absent in both species.

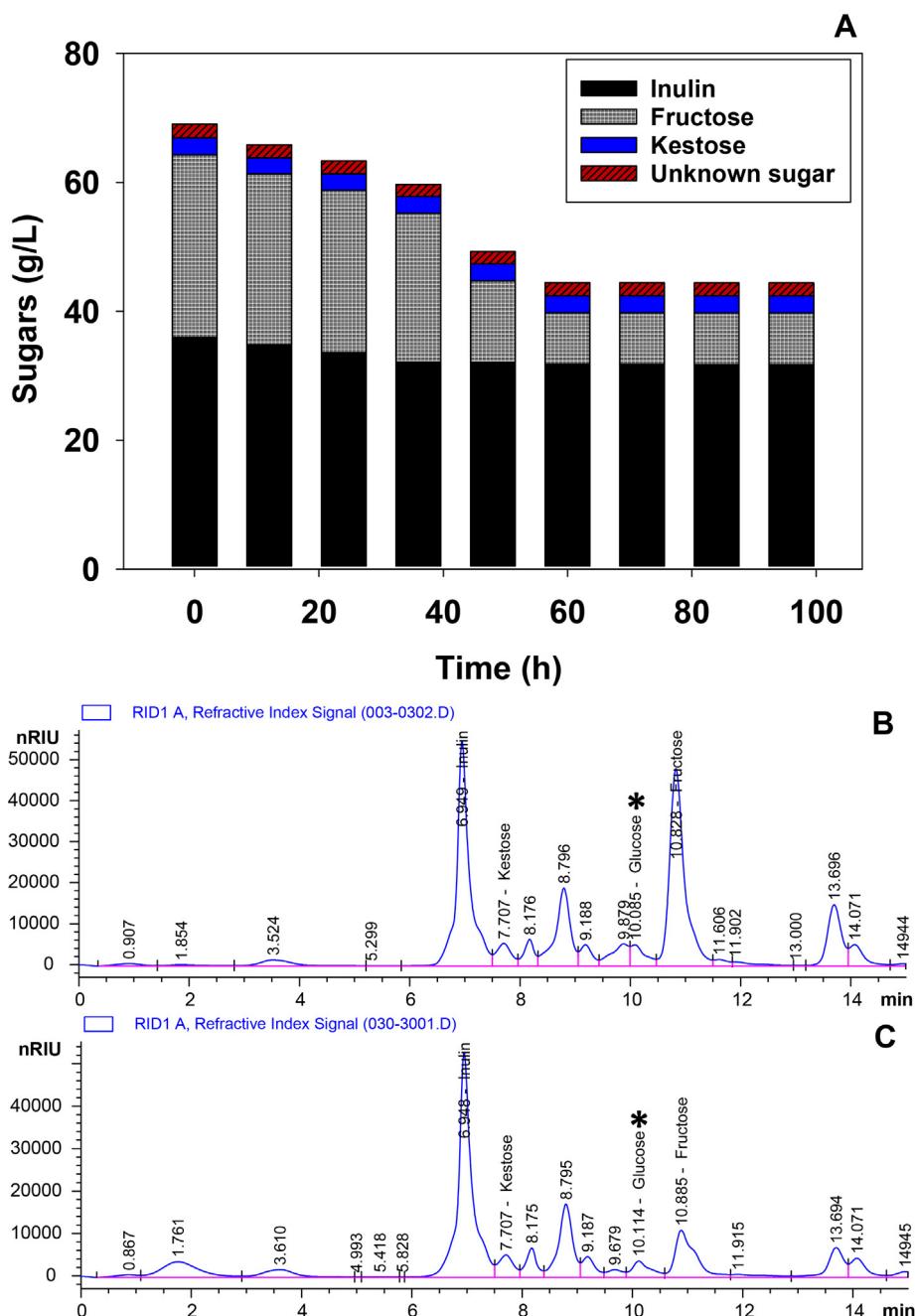
*C. saccharobutylicum* demonstrated a superior inulolytic activity relative to the other species studied, and so it was used in the ABE fermentation of unhydrolyzed chicory and TKS extracts. Intriguingly, the ABE profiles of *C. saccharobutylicum* on fructose and inulin were similar (although fructose was preferentially utilized by *C. saccharobutylicum* over inulin), while the growth on inulin was considerably higher than that observed on fructose (Fig. 1). Considering the metabolic cost of hydrolyzing a polymer such as inulin prior to consumption, we anticipated higher *C. saccharobutylicum* growth and ABE profiles on fructose than on inulin. However, inulin hydrolysis yields small amounts of glucose, the sugar most preferred by *C. saccharobutylicum* (Ezeji and Blaschek, 2008), which may in part account for the higher growth observed on inulin than on fructose. Conversely, *C. beijerinckii* NCIMB 8052, *C. beijerinckii* B-592, *C. acetobutylicum* ATCC 824 did grow better on fructose than *C. saccharobutylicum*, while their growth and ABE production on inulin, which was better utilized by *C. saccharobutylicum*, were largely poor. Hence, it can be deduced that *C. beijerinckii* NCIMB



**Fig. 3.** Sugar profile of *C. saccharobutylicum* culture in pure inulin medium: (A) Inulin and fructose concentrations during fermentation; HPLC chromatograms showing sugar compositions before (B) and after (C) fermentation. With pure inulin as substrate, fermentation proceeded beyond 96 h and terminated after 108 h.

8052, *C. beijerinckii* B-592, *C. acetobutylicum* ATCC 824 efficiently ferment fructose, the major product of inulin hydrolysis, however they possess poor inulolytic ability. The preferential utilization of fructose over inulin by *C. saccharobutylicum* is likely due to carbon catabolite repression, which inhibits inulin-hydrolyzing enzymes in the presence of fructose. Therefore, among the strains studied,

*C. saccharobutylicum* is clearly the superior strain for fermenting unhydrolyzed inulin to butanol. These results therefore, suggest that a likely roadblock to enhanced inulin utilization by *C. saccharobutylicum* is poor fructose utilization upon inulin hydrolysis. In view of this, we infer that *C. saccharobutylicum* possesses the requisite level of inulolytic activity to serve as a basis for genetically

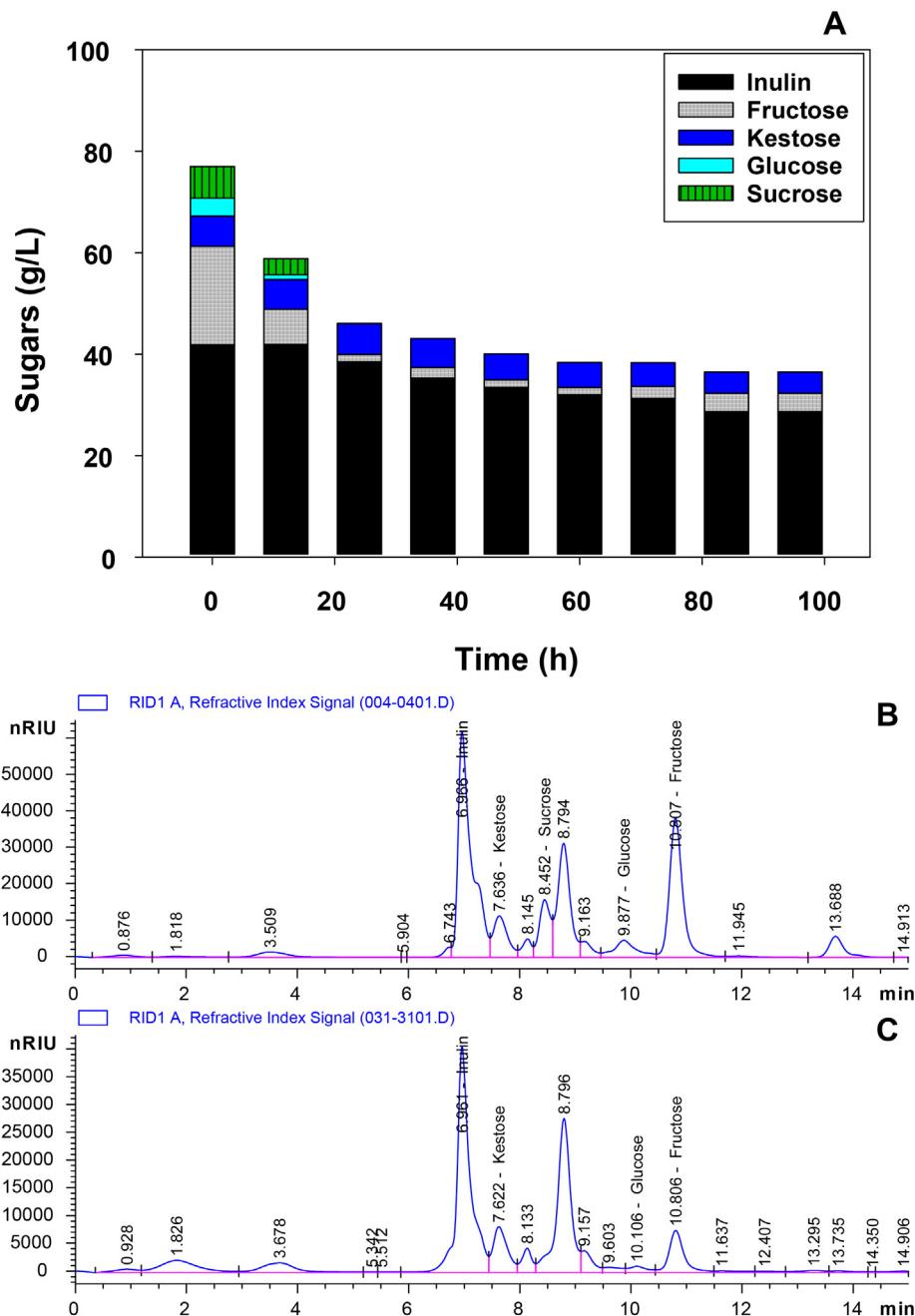


**Fig. 4.** Sugar profile of *C. saccharobutylicum* grown in TKS medium: (A) sugar concentrations during fermentation; HPLC chromatograms showing sugar compositions before (B) and after (C) fermentation. \*Sugar with peak similar to that of glucose, but was not utilized during fermentation. Hence, this peak is labeled unknown sugar in panel A.

engineering a more robust strain for ABE fermentation of unhydrolyzed plant-derived inulin, possibly by heterologous expression of transport and/or catabolic enzymes with higher affinities for fructose.

The chicory and TKS extracts yielded different sugar compositions (Table 1; Figs. 4 and 5), which is the likely basis (at least in part) of the varying ABE profiles obtained with these substrates (Fig. 2). Since inulin is the major sugar found in chicory and TKS, we infer that conditions of extraction employed in this study – alkaline solution (0.1% Na<sub>2</sub>CO<sub>3</sub>) at high temperature (90 °C) – caused the degradation of both chicory and TKS inulin. Hydrolysis of inulin from chicory produced glucose, sucrose, fructose and kestose, while an unknown sugar, fructose and kestose were produced from the

hydrolysis of TKS inulin (Table 1; Figs. 4 and 5). Of these two substrates, chicory extract is the more promising for ABE fermentation, with considerably higher butanol and ABE titers than the TKS extract (Fig. 2). In the same vein, ABE yield (Table 1) obtained with the chicory root extract compares favorably with the theoretical yield (80% of the theoretical yield; Gapes, 2000; Yerushami et al., 1983). This might be explained in part by the presence of glucose (3.5 g/L) and sucrose (6.2) g/L in the chicory extract (Table 1; Fig. 5), both of which were rapidly utilized by *C. saccharobutylicum*. However, glucose and sucrose were not present in the TKS extract (Table 1; Fig. 4). Although a HPLC peak appeared in the TKS extract with a retention time similar to glucose, the sugar accounting for this peak was not utilized during fermentation (Fig. 4), leading



**Fig. 5.** The sugar profile of *C. saccharobutylicum* grown in chicory medium: (A) sugar concentrations during fermentation; HPLC chromatograms showing sugar compositions before (B) and after (C) fermentation.

us to conclude that this in fact, is not glucose. We suspect that this peak might be a disaccharide of fructose, possibly inulobiose (a difructose) or difructose anhydride (DFA), a cyclic disaccharide composed of two fructose molecules (Zittan, 1981). Identification of the sugar responsible for this peak would shed more light on the characteristics of TKS-derived inulin as well as the reactions that take place during extraction, and why *C. saccharobutylicum* did not utilize TKS-derived inulin as much as it did chicory-derived inulin.

It is plausible that the rapid consumption of glucose and sucrose (sugars more readily utilized by *C. saccharobutylicum*) in the chicory extract, which resulted in enhanced growth particularly in the early exponential phase (data not shown), led to the higher ABE production relative to the TKS extract. By rapidly accumulating a higher cell

density, cultures grown on chicory extract subsequently achieved higher fructose and inulin utilization, which in turn translated to higher ABE production. This is supported by the ~61%, ~76%, and 47% increases in total sugar utilization, butanol and ABE concentrations respectively, in the chicory extract relative to the TKS extract. However, additional factors other than absence of glucose and sucrose clearly affected the butanol and ABE profiles obtained with the TKS extract in relation to those obtained with the chicory extract. By supplementing the TKS extract with glucose and sucrose to the same concentrations observed in the chicory extract, butanol and ABE productivities increased considerably in the TKS extract-based medium, but not the maximum concentrations (Fig. 2). While this suggests that the presence of glucose and sucrose was involved

in earlier accumulation of solvents in the chicory extract, it also reveals that other factors impeded solvent production in the TKS. Apparently, supplementation of the TKS extract with glucose and sucrose abolished the delay in ABE production observed in the unsupplemented TKS extract. It is likely therefore that inulin from TKS is not efficiently utilized by *C. saccharobutylicum* due to the presence of some compounds in the crude TKS extract. Perhaps, the TKS contains some compounds that inhibit ABE fermentation or some specific changes/reactions occurred during the extraction and thus led to poor inulin utilization in this extract, hence, the ABE profile obtained. This calls for further study targeted at understanding chemical composition of TKS extract and chemicals that may be generated during inulin extraction from TKS that likely interfere with the ABE production by *C. saccharobutylicum*.

While kestose was not utilized in the TKS extract, ~44% was utilized in the chicory extract (Figs. 4 and 5). The higher concentration of kestose in the chicory extract may have elicited its utilization in this medium. Kestose was utilized by *C. saccharobutylicum* in the chicory extract, albeit poorly (Fig. 5). It could be that kestose utilization is more severely repressed by fructose, relative to inulin. Alternatively, other factors such as poor transport may have contributed to this pattern. Specific evaluation of kestose consumption by *C. saccharobutylicum* would help to better understand this trend. Asides the inhibition of inulin in the presence of glucose, inulin utilization by *C. saccharobutylicum* is clearly subject to catabolite repression in the presence of more readily utilizable monosaccharide and disaccharide sugars (Grimmeler et al., 2010). For instance, inulin concentrations in the chicory and TKS media were largely unchanged until the concentrations of the other sugars (the mono- and disaccharides, and not kestose, a trisaccharide) reduced considerably, or completely disappeared from the media (Figs. 3–5).

During inulin utilization, *C. saccharobutylicum* appears to accumulate fructose via inulin hydrolysis, which is then followed by fructose utilization. This is particularly striking with commercial pure inulin utilization (Fig. 3), where fructose concentration clearly rises, falls and rises again during fermentation. This is a likely reflection of inulolytic enzyme repression and fructose utilization, which is relieved at lower fructose concentrations thereby allowing inulin hydrolysis to fructose, which in turn represses the inulolytic enzymes after increasing to a certain concentration threshold. Only fructose and inulin were detected in the commercial pure inulin medium throughout fermentation. This indicates that *C. saccharobutylicum* produces an exo-inulinase, which terminally cleaves inulin resulting in the release of monomeric sugars (predominantly fructose and small amounts of glucose at the terminal end). Since glucose is rapidly metabolized by solventogenic clostridia (Ezeji and Blaschek, 2008), and commercial pure inulin is thought to have a glucose to fructose ratio as low as 1:32 (Zittan, 1981), we conclude that glucose molecules arising from inulin hydrolysis by *C. saccharobutylicum* were small and rapidly utilized. The genome of *C. saccharobutylicum* was recently sequenced and annotated, albeit a different strain (DSM 13864; Poehlein et al., 2013). A search of the *C. saccharobutylicum* DSM 13864 genome on the National Center for Biotechnology Information (NCBI) database shows the presence of a fructan beta-fructosidase FruA (AGX43051.1) with an exo-inulinase conserved domain (EC 3.2.1.80). It is likely that a similar enzyme functions in *C. saccharobutylicum* P262.

## 5. Conclusions

Fermentation of unhydrolyzed inulin-rich chicory and TKS extracts and pure inulin by *C. saccharobutylicum* demonstrates the capacity of this microorganism for ABE production from unhydrolyzed biomass-derived inulin. Supplementation of the growth medium with CaCO<sub>3</sub> revealed inherent inulolytic activity in

*C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824, which were previously thought to completely lack the ability to utilize inulin. This underscores the role of Ca<sup>2+</sup> in modulating the metabolism of solvent-producing clostridia. Elimination of metabolic roadblocks to fructose utilization by *C. saccharobutylicum* would likely enhance the use of unhydrolyzed plant-derived inulin, a cheaper alternative to pre- or *in situ* hydrolysis and fermentation in butanol production. The possibility of fermenting plant-derived inulin to butanol, devoid of an additional hydrolysis step which abolishes the inherent cost adds weight to efforts targeted at commercializing Chicory and TKS cultivation for industrial applications.

## Acknowledgements

Salaries and research support were provided in part by State funds appropriated to the Ohio Plant Biotechnology Consortium by The Ohio State University, Ohio Agricultural Research and Development Center (OARDC), and the Hatch grant (Project No. OHO01333).

## References

- Buranov, A.U., Elmuradov, B.J., 2010. Extraction and characterization of latex and natural rubber from rubber-bearing plants. *J. Agric. Food Chem.* 58, 734–743.
- Cornish, K., Bates, G.M., McNulty, S.K., Kopicky, S.E., Grewal, S., Rossington, J., Michel Jr., F.C., Walker, S., Kleinhenz, M.D., 2013. "Buckeye Gold" storage: a study into rubber production in *Taraxacum kok-saghyz* with an emphasis on post-harvest storage. *USA Tech. Int.* 10, 36–38.
- Dale, V.H., Kline, K.L., Wiens, J., Fargione, J., 2010. Biofuels: Implications for Land Use and Biodiversity. *Ecological Society of America*. Washington, DC, pp. 1–13.
- El Kanouni, A., Zerdani, I., Zaafa, S., Znassi, M., Loufti, M., Boudouma, M., 1998. The improvement of glucose/xylose fermentation by *Clostridium acetobutylicum* using calcium carbonate. *World J. Microbiol. Biotechnol.* 14, 431–435.
- Eskew, R.K., 1946. Process for recovering rubber from fleshy plants. U.S. Patent No. 2,393,035.
- Ezeji, T., Blaschek, H.P., 2008. Fermentation of dried distillers' grains and solubles (DDGS) hydrolysates to solvents and value-added products by solventogenic Clostridia. *Bioresour. Technol.* 99, 5232–5242.
- Gapes, J.R., 2000. The economics of acetone–butanol fermentation: theoretical and market considerations. *J. Mol. Microbiol. Biotechnol.* 2, 27–32.
- Grimmeler, C., Held, C., Liebl, W., Ehrenreich, A., 2010. Transcriptional analysis of catabolite repression in *Clostridium acetobutylicum* growing on mixtures of D-glucose and D-xylose. *J. Biotechnol.* 150, 315–323.
- Gupta, A.K., Kaur, N., Kaur, N., 2003. Preparation of inulin from chicory roots. *J. Sci. Ind. Res.* 62, 916–920.
- Han, B., Ujor, V., Lai, L.B., Gopalan, V., Ezeji, T.C., 2013. Use of proteomic analysis to elucidate the role of calcium on acetone–butanol–ethanol (ABE) fermentation in *Clostridium beijerinckii* NCIMB 8052. *Appl. Environ. Microbiol.* 79, 282–293.
- Food and Agriculture Organization of the United Nations Statistics Division Data. <http://faostat3.fao.org/search/chicory/E> (accessed on 28.06.15).
- Marchal, R., Blanchet, D., Vandecasteele, J., 1985. Industrial optimization of acetone–butanol fermentation: a study of the utilization of Jerusalem artichokes. *Appl. Microbiol. Biotechnol.* 23, 92–98.
- Montoya, D., Spitia, S., Silva, E., Schwarz, W.H., 2000. Isolation of mesophilic solvent-producing Clostridia from Colombian sources: physiological characterization, solvent production and polysaccharide hydrolysis. *J. Biotechnol.* 79, 117–126.
- Montoya, D., Arévalo, C., Gonzales, S., Aristizabal, F., Schwarz, W.H., 2001. New solvent-producing *Clostridium* sp. strains, hydrolyzing a wide range of polysaccharides, are closely related to *Clostridium butyricum*. *J. Ind. Microbiol. Biotechnol.* 27, 329–335.
- Oiwa, H., Naganuma, M., Ohnuma, S., 1987. Acetone–butanol production from dahlia inulin by *Clostridium pasteurianum* var I-53. *Agr. Biol. Chem. Tokyo* 51, 2819–2820.
- Poehlein, A., Hartwich, K., Krabben, P., Ehrenreich, A., Liebl, W., Dürre, P., Gottschalk, G., Daniela, R., 2013. Complete genome sequence of the solvent producer *Clostridium saccharobutylicum* (DSM 13864). *GenomeA* 1 (6), e00997–e1013.
- Quanzhen, W., Jian, C., 2011. Perspectives and utilization technologies of chicory (*Cichorium intybus* L.): a review. *Afr. J. Biotechnol.* 10, 1966–1977.
- Qureshi, N., Maddox, I., 2005. Reduction in butanol inhibition by perstraction: utilization of concentrated lactose/whey permeate by *Clostridium acetobutylicum* to enhance butanol fermentation economics. *Food Bioprod. Process.* 83, 43–52.
- Raganati, F., Curthb, S., Götzb, P., Oliviera, G., Marzocchella, A., 2012. Butanol production from lignocellulosic-based hexoses and pentoses by fermentation of *Clostridium acetobutylicum*. *Chem. Eng. Trans.* 27, 91–96.
- Richmond, C., Han, B., Ezeji, T.C., 2011. Stimulatory effects of calcium carbonate on butanol production by solventogenic *Clostridium* species. *Cont. J. Microbiol.* 5, 18–28.
- Sarchemi, T., Rehmann, L., 2014. Optimizing enzymatic hydrolysis of inulin from Jerusalem artichoke tubers for fermentative butanol production. *Biomass Bioenergy* 69, 175–182.

- Schütz, K., Carle, R., Schieber, A., 2006. *Taraxacum – a review on its phytochemical and pharmacological profile*. *J. Ethnopharmacol.* 107, 313–323.
- Ujor, V., Bharathidasan, A.K., Cornish, K., Ezeji, T.C., 2014. Feasibility of producing butanol from industrial starchy food wastes. *Appl. Energy* 136, 590–598.
- Ujor, V., Agu, C.V., Gopalan, V., Ezeji, T.C., 2015. Allopurinol-mediated lignocellulose-derived microbial inhibitor tolerance by *Clostridium beijerinckii* during acetone–butanol–ethanol (ABE) fermentation. *Appl. Microbiol. Biotechnol.* 99, 3729–3740.
- van Beilen, J.B., Poirier, Y., 2007. *Guayule and Russian dandelion as alternative sources of natural rubber*. *Crit. Rev. Biotechnol.* 27, 217–231.
- Yerushami, L., Volesky, B., Leung, W.K., Neufeld, R.J., 1983. Variations of solvent yield in acetone-butanol fermentation. *Eur. J. Appl. Microbiol. Biotechnol.* 18, 279–286.
- Zittan, L., 1981. Enzymatic hydrolysis of inulin – an alternative way to fructose production. *Starch/Stärke* 33, 373–377.