

Acetone, Butanol and Ethanol Production from Domestic Organic Waste by Solventogenic Clostridia

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Abstract

Domestic organic waste (DOW) was washed and dried to 85 % dryness by VAM (The Netherlands). This material contained 25.1 g glucose, 8.4 g xylose and 5.8 g other monosaccharides / 100 g dry matter. Using Mansonite steam explosion and enzymatic hydrolysis, a hydrolysate containing 15.4 g glucose, 2.2 g xylose and 0.8 g other monosaccharides per l was made. *Clostridium acetobutylicum* DSM 1731 produced 1.5 and *C. beijerinckii* B-592 0.9 g/l ABE and *Clostridium* LMD 84.48 1.9 g/l IBE, respectively, from this hydrolysate without further supplementation. Incubation with 2 fold concentrated hydrolysate completely impaired ABE production. After removal of unspecific inhibiting components, the yield of ABE production by *Clostridium acetobutylicum* DSM 1731 increased about 3 fold as compared to the nontreated hydrolysate. From 4 fold concentrated, partially purified, hydrolysate containing 34.2 g glucose/l, ABE production was 9.3 g/l after 120 h as compared to 3.2 g ABE/l from non-concentrated hydrolysate which contained 12.0 g glucose/l after elution over the same column. The concentration of butyric acid in the fermented hydrolysates was 2.2 and 0.4 g/l, respectively. This reasonably low amount of butyric acid showed that the fermentation had proceeded quite well.

Introduction

The anaerobic production of acetone, butanol and ethanol (ABE) by solventogenic clostridia has once been the second largest biotechnological industry in the world (Jones and Woods, 1986), initially for production of synthetic rubber precursors but later on for production of solvents in the lacquer industry. As a result of the development of the petrochemical industry and the increase in prices of the agricultural produce, especially molasses, used as substrate for the fermentation, the fermentative ABE production became no longer economically viable and came to a halt in the 1960's (Duerre, 1998). However, interest in the ABE fermentation has resurfaced because of the new global support for the exploitation of biomass as a sustainable source of energy.

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At present, two major bottlenecks still hamper sound economics of the ABE fermentation:

- the cost of the substrates contributing to over 60% of the overall costs and
- the toxic nature of the products prohibiting high concentrations in the fermentation broth.

In the past, ABE fermentation was done employing easily fermentable carbohydrates in mashes derived from maize, grains, beets or potatoes. Because of the saccharolytic property of the solventogenic clostridia these starchy substrates could be converted to ABE without prior pretreatments (Mitchell, 1998). In the present study, an attempt has been made to utilise the abundant supply of cellulose and hemicellulose in domestic organic waste (DOW). As the exoglucanase enzyme, required for cellulase activity, is not present in solventogenic clostridia (in contrast to endoglucanase activity) and as cellulose and hemicellulose are rather unaccessible because of being part of complex structures, this feedstock must be pretreated and hydrolysed in order to liberate fermentable monosaccharides. This paper describes the results obtained with fermentation of hydrolysate from DOW, produced using steam explosion and enzymatic hydrolysis.

Results and Discussion

Composition of Domestic Organic Waste (DOW) and Hydrolysate

The composition of the DOW collected in the spring in the Netherlands and used in this study is given in Table 1. Since in this experiment the DOW had been washed for removal of sand, and dried to 85% dryness for increasing keepability, soluble components like free mono- and oligosaccharides may have been lost from the samples which were passed through the extruder. Notwithstanding, the presence of glucose is prevalent, reflecting the large

Table 1. Composition of domestic organic waste (DOW) in g per 100 g dry matter. Values are means \pm SD (n=3).

Fraction	DOW (g/ 100 g dry matter)
Solvent extractives	5.9 \pm 0.9
Hot water extractives	7.2 \pm 0.6
Acid soluble lignin	1.2 \pm 0.03
Acid insoluble lignin	10.5 \pm 0.8
Uronic acids in polymeric fraction:	4.9 \pm 1.6
Sugars in polymeric fraction:	
Glucose	25.1 \pm 1.7
Xylose	8.4 \pm 0.7
Arabinose	2.3 \pm 0.03
Mannose	1.6 \pm 0.1
Galactose	1.6 \pm 0.1
Rhamnose	0.3 \pm 0.01
Total sugars	39.3
Ashes	15.9 \pm 1.1

Table 2. Composition of hydrolysate, prepared and supplied by CIEMAT (Spain) from domestic organic waste (DOW) provided by VAM. Acknowledgement: The analyses were done by CIEMAT.

Component	Concentration in g/l hydrolysate
Glucose	15.4
Xylose	2.2
Galactose	0.2
Arabinose	0.2
Mannose	0.4
Furfural	0.03
Hydroxymethylfurfural	0.0093

contribution of starch and (ligno)cellulosic compounds to this DOW. The amount of potential fermentable sugars in the studied DOW seems reasonable, whereas the amount of the lignin fraction is fairly low, indicating an interesting applicability for fermentations. As the distribution of the constituents is comparable to some other agrowastes (Gosh and Singh, 1993) this feedstock may serve as a model for studying the utilisation of waste, with low lignocellulose content, for ABE production.

In The Netherlands, DOW is mainly composed of leftovers from fruits and vegetables, and garden waste like weeds and cuttings, and shows a rather constant supply during the year with some increase in the spring and autumn. The composition of the DOW will, to some extent, be affected by the season, since in spring and autumn the contribution from the garden waste is relatively large. The availability of DOW is estimated at 1000 Kton/year (Faaij *et al.*, 1997). Presently, DOW is largely aerobically digested to make compost. The significant amount of polymeric saccharides in the DOW, assumedly cellulose and hemicellulose which yield the fermentable sugars shown in Table 1, amounting to 39% of the dry weight, invite also other conversions which may generate valuable compounds or energy. However, for most of such applications, the mobilisation of the saccharides from the biomass is a prerequisite.

As shown in Table 2, the application of steam explosion followed by enzymatic hydrolysis, has mobilised almost 50% of the saccharides from the polymers. The high ratio of glucose to other sugars, especially xylose which as pentose may confer special demands on the fermentation, is of importance. Even though a simultaneous utilisation of glucose and xylose has been observed in other experiments (results not shown), a high amount of glucose may inhibit xylose consumption in ABE fermentation (Fond *et al.*, 1986). This phenomenon, being dependent on the culture conditions and strain differences has not been the subject of this investigation but is certainly of interest when dealing with such complex substrates. Except for furfural and hydroxymethylfurfural, the hydrolysate has not yet been tested for the presence of other known inhibitory substances, as are phenolic compounds like ferulic acid, vanillin etc. As previous studies (Marchal *et al.*, 1992) have shown an inhibitory effect of 50% by furfurals at concentrations exceeding 2 g/l, the low concentration of furfurals in our hydrolysate seems not significant, although there may be different sensitivities in different strains. Below, the inhibitory effect of concentrated hydrolysate on ABE production is described, indicating that other strong inhibitors are indeed present in the hydrolysate.

At present, the combination of steam explosion and enzymatic hydrolysis for pretreatment of DOW needs further development since on the one hand the efficiency, albeit promising, is only 50% and since on the other hand the concentration of the saccharides in the hydrolysate, until now never exceeding 30 g/l, is low, thus yielding large waste streams. Furthermore, as suggested previously, the cost of the enzymatic hydrolysis is a severe economic burden, even when the enzyme cocktail is being produced on site (Marchal *et al.*, 1992). Therefore, further improvement needs to be achieved by a reduction of enzyme consumption, *e.g.* by recycling or by coupling hydrolysis and fermentation.

Table 3. Production of acetone or isopropanol, butanol and ethanol (ABE or IBE) and butyric acid by *Clostridium* LMD 84.48, *Clostridium acetobutylicum* DSM 1731 and *Clostridium beijerinckii* B-592 from domestic organic waste. For concentration of the hydrolysate, lyophilisation with subsequent dissolving in a 4 fold reduced volume of demineralised water was used. The initial pH was 5.7 .

Strain	LMD84.48				DSM 1731				NRRL B-592			
	Glu	IBE	Butyric acid	pH	Glu	ABE	Butyric acid	pH	Glu	ABE	Butyric acid	pH
[glu] at t=0	Concentration in g/l at the end of the fermentation (180 h)											
Hydrolysate [glu] = 16 g/l	8.7	1.9	1.3	4.8	2.0	1.5	2.1	4.4	5.7	0.9	1.8	4.4
Lyophilised hydrolysate (4:1) in water [glu] = 48 g/l	46.8	0.2	0	5.4	46.4	0.3	0	5.5	44.5	0	1.1	4.9
6% glucose- Gapes [glu] = 69 g/l	39.0	10.4	0.6	5.3	0	20.1	0	4.7	0.4	20.1	0.4	5.0

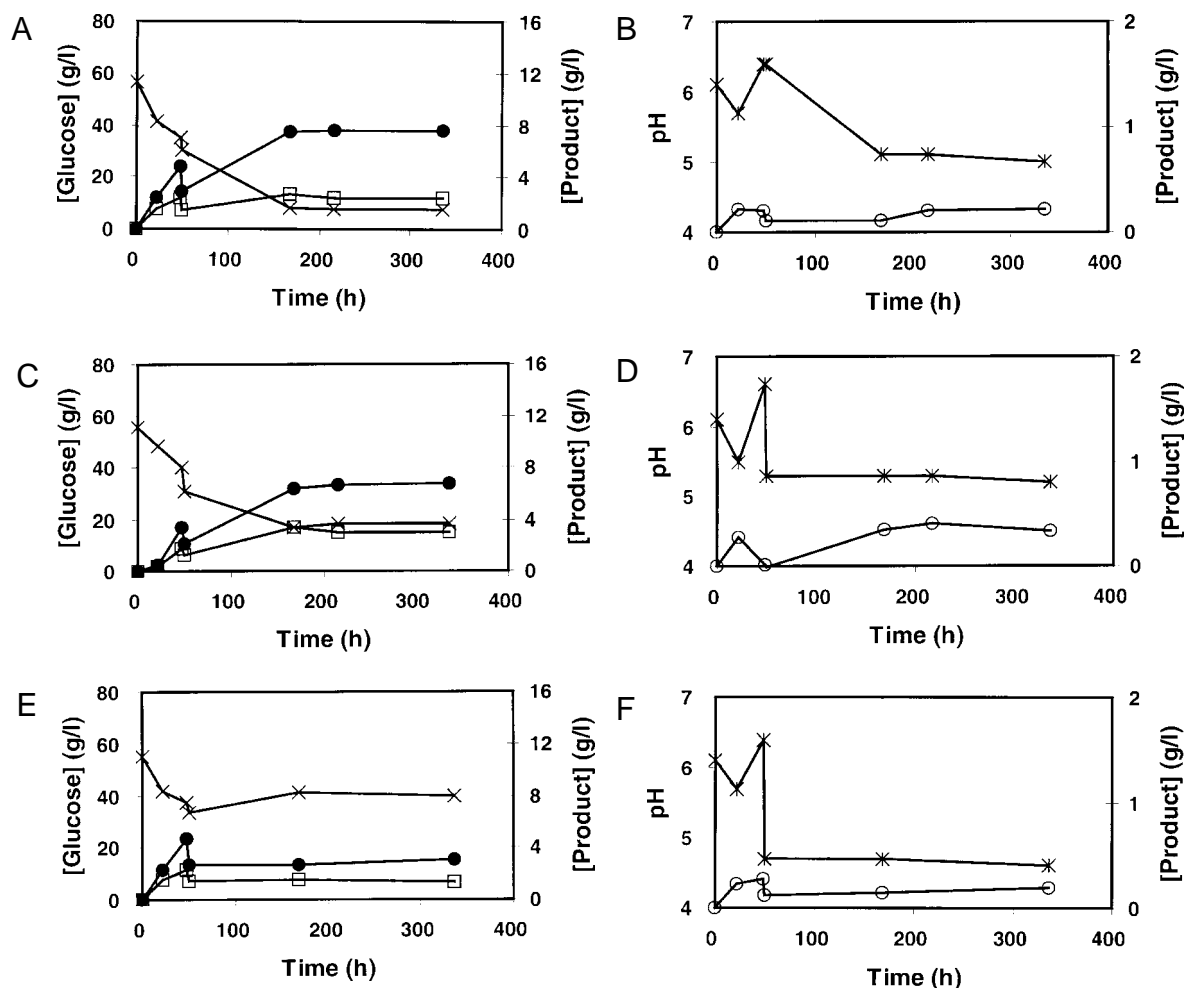


Figure 1. Production of i-propanol and butanol by *Clostridium* LMD 84.48 on glucose and hydrolysate of domestic organic waste. A/B: synthetic medium with 6% (w/v) glucose; C/D: synthetic medium with 6% glucose and addition of concentrated hydrolysate to make the initial hydrolysate concentration at $t = 50$ h and E/F: synthetic medium with 6% glucose and addition of concentrated hydrolysate to make 2 fold the hydrolysate concentration at $t = 50$ h. Controls were done with demineralised water at $t = 50$ h, which showed no effect. In g/l: X glucose; ● butanol; O butyric acid; □ i-propanol; * pH

Fermentation of Hydrolysates by *Clostridium* sp.

For concentrating the amount of fermentable substrate, the hydrolysate was lyophilised and resuspended in a smaller volume of demineralised water and adjusted to pH 7.0. The effect of lyophilisation was checked by monitoring solvent production on nontreated and lyophilised hydrolysate and by recording HPLC profiles (organic acids column, Shodex Ionpack KC-811, with RI detection). There were no differences detected in the chromatograms. The hydrolysate was sterilised in an autoclave and used without removal of the precipitate. After sterilisation the pH had dropped to 5.7. This pH drop was always observed when sterilising DOW and was tentatively attributed to thermal decomposition to acidic compounds. The method of filter sterilisation offered no alternative because filters became clogged within 2 min of usage.

Solvent production from hydrolysate and concentrated hydrolysate was tested using *Clostridium* LMD 84.48, *C. acetobutylicum* DSM 1731 and *C. beijerinckii* B-592 without further supplementation. The results are shown in Table 3. As always observed, strain LMD 84.48 was an IBE

producer, reducing acetone to i-propanol, indicating that this strain should be classified as a *beijerinckii* strain. Besides i-propanol production, strain LMD 84.48 can also be distinguished because of its great sensitivity with respect to butanol concentration which is evident from the large amount of residual substrate in the culture grown on 6% glucose (Table 3). The poor performance of LMD 84.48 on hydrolysate may be explained by the presence of unspecific inhibitors in the hydrolysate, which is supported by the lack of IBE production in concentrated hydrolysate (Table 3). On the other hand, the low initial pH plus the low buffering capacity of the hydrolysate may also have negatively affected IBE production by LMD 84.48 since this strain is, in contrast to other solventogenic clostridia, very sensitive towards pH decreases to below 5.

Solvent production from the hydrolysate by the ABE producing strains, DSM 1731 and B-592, was also quite poor even though substrate consumption was higher as compared to the LMD 84.48 strain (Table 3). As previously no growth inhibition has been observed with these strains at pH's as low as 4.5, the contribution of the initial pH to

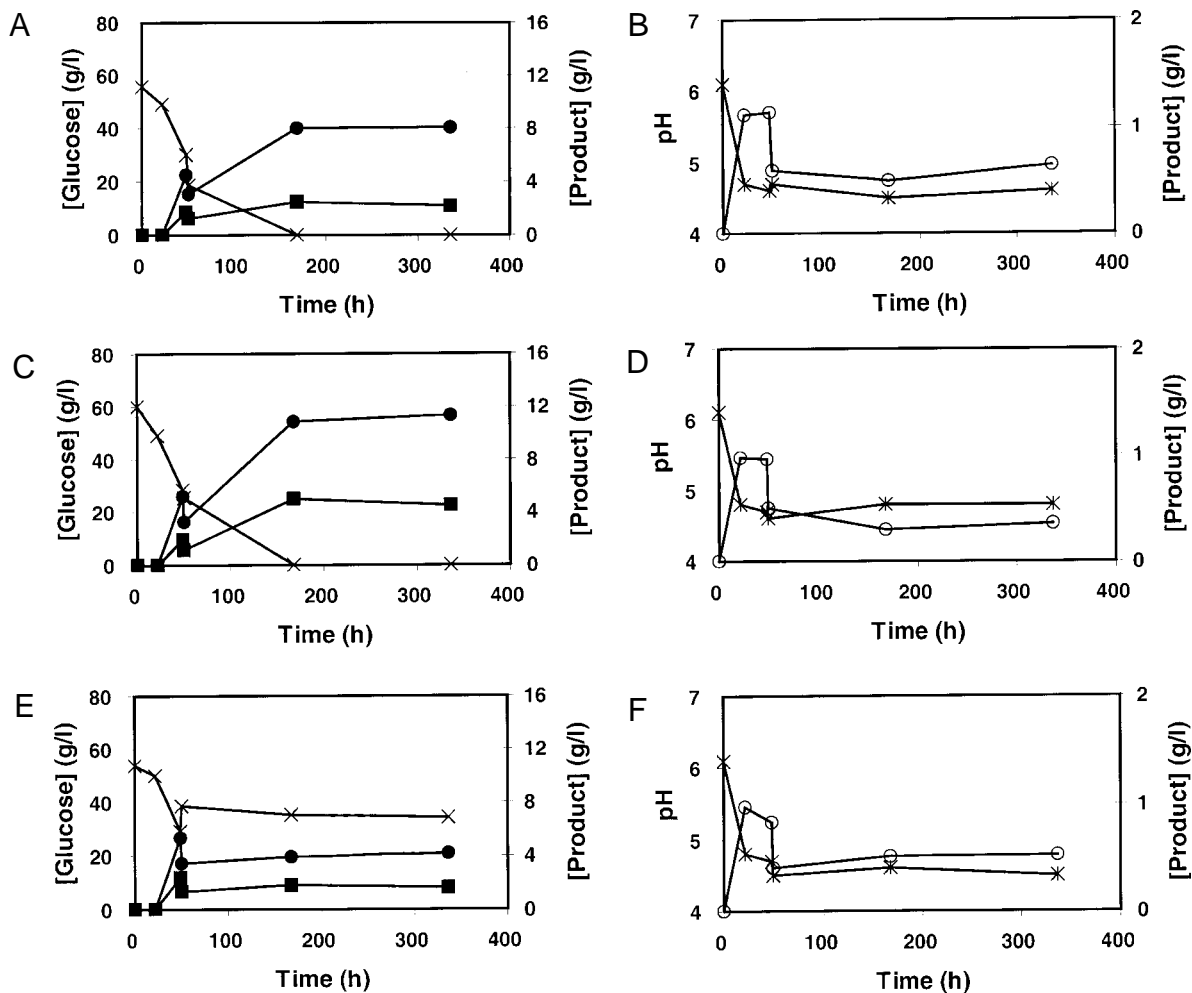


Figure 2. Production of acetone and butanol by *Clostridium acetobutylicum* DSM 1731 on glucose and hydrolysate of domestic organic waste. A/B: synthetic medium with 6% (w/v) glucose; C/D: synthetic medium with 6% glucose and addition of concentrated hydrolysate to make the initial hydrolysate concentration at $t = 50$ h and E/F: synthetic medium with 6% glucose and addition of concentrated hydrolysate to make 2 fold the hydrolysate concentration at $t = 50$ h. Controls were done with demineralised water at $t = 50$ h, which showed no effect. In g/l: X glucose; ● butanol; ○ butyric acid; ■ acetone; * pH

the poor performance seems unlikely. In all cultures, and especially in the culture of B-592, the production of butyric acid was extensive, showing that the bacteria were inhibited in the shift from acidogenesis to solventogenesis. In general, the triggering event for this switch is still obscure although one of the thriving factors may be the ample supply of substrate, necessitating the detoxification of the poisonous organic acids which precede the formation of solvents (Jones and Woods, 1986). This phenomenon, as well as the presence of unspecific inhibitors, illustrate the difficulty of fermenting saccharides in a hydrolysate.

In previous experiments (results not shown) the need for the addition of yeast extract to hydrolysate medium had already been tested. This supplementation was without positive effect. In the experiments presented below (Table 4), it will be shown that absence of nutrients or low buffering capacity of the medium with hydrolysate only, inflict no inhibition, at least as far as the DSM 1731 strain is concerned.

The inhibition by unspecific components in the

hydrolysate was further investigated by adding concentrated hydrolysate to cultures of LMD 84.48, DSM 1731 and NRRL B592 which had been grown on 6% glucose-Gapes medium for 50 h and were producing butanol (Figures 1 and 2). The final concentration of the hydrolysate in the medium was 4 fold, 2 fold and equal to the initial, nonconcentrated, hydrolysate. For a blank the same amount of demineralised water was added to the cultures. In all cases, ABE production came to a halt after the addition of hydrolysate to a final concentration of 4 fold the initial, just as observed in Table 3.

The results with lower final concentrations of hydrolysate are shown in Figures 1B,C and 2B, C for LMD 84.48 and DSM 1731, respectively. As in all fermentations ethanol production was very low, never exceeding 0.5 g/l, this metabolite is not shown in the figures. The data for B-592 were similar to DSM 1731.

The production of (l)ABE was completely impaired in all cultures with 2 fold the initial concentration of hydrolysate. The addition of lyophilised hydrolysate to a

final concentration equal to the nonconcentrated hydrolysate had no effect on IBE production by LMD 84.48 as the IBE production did not increase and as the additionally supplied glucose seemed to remain untouched (Figure 1B). This is probably due to the sensitivity of this strain to butanol. In previous studies, growth of LMD 84.48 halted after the addition of butanol to a final concentration of 8 g/l, despite otherwise favourable growth conditions such as no butyric acid and pH 6. Therefore, it is assumed that the achieved butanol concentration in this culture may have provoked the end of the fermentation. However, as also discussed above, the additional effect of inhibitors on growth inhibition of this strain, can not be excluded. The pH was 5.2 in the blank as well as in the culture to which the hydrolysate had been added. In the cultures with a final hydrolysate concentration of 2 fold the nonconcentrated, the pH had dropped to 4.7 from 6.4 (Figure 1C). Here, the involvement of low pH and/or inhibitors seems clear. However, it remains to be established which of these parameters effects the greatest contribution to the inhibition of ABE production by LMD 84.48.

The cultures of the NRRL B592 and DSM 1731 strains to which concentrated hydrolysate had been added to make a final concentration equal to the nonconcentrated hydrolysate (Figure 2B) showed a better performance as compared to LMD 84.48 and as compared to nonconcentrated hydrolysate (Table 3). In the cultures of Figure 2B, all glucose was consumed, including the additional amount from the hydrolysate, yielding extra revenue in the fermentation due to the sugars from the hydrolysate. Furthermore, the additional glucose from the hydrolysate was converted to ABE with a greater efficiency than after growth on nonconcentrated hydrolysate only. In the cultures, the utilisation of the added hydrolysate yielded an additional production of 5.0 g ABE/l, whereas production of ABE from nonconcentrated hydrolysate (Table 3) was only 1.5 g ABE/l, partially due to the production of butyric acid. Apparently, the 'mixed' substrate system has a beneficial effect with respect to substrate conversion and prevents the production of butyric acid. In the cultures with a final hydrolysate concentration of 2 fold the nonconcentrated (Figure 2C), ABE production was

impaired similar as observed in the cultures of the LMD 84.48 strain (Figure 1C) and also possibly due to either a drop in pH or increase in inhibitor concentration.

The observed phenomena designate an important finding for the application of hydrolysates for solvent production: the drawback of the low fermentable sugar concentration in hydrolysates may be circumvented by offering 'mixed' substrates to the bacteria. These mixtures may be composed out of glucose, just sufficient to trigger the switch to solventogenesis, supplemented by hydrolysates of cheap lignocellulosic wastes for increased revenue. Another approach might be the removal of inhibiting components in order to allow the utilisation of concentrated hydrolysates. This approach will be discussed below.

Fermentation of Partially Purified Hydrolysate by *C. acetobutylicum* DSM 1731

In order to remove unspecific inhibitors, the hydrolysate was first 10 fold concentrated. The precipitate was removed and the concentrated hydrolysate was eluted over a Dowex 1-X8 anionic exchange column, equilibrated with 200 mM Na-acetate buffer, pH 4.8. The fractions containing glucose were pooled and diluted to make 4, 2 and 1 fold concentrated hydrolysate. After adjusting the pH to 6.0, the filter-sterilised hydrolysates were inoculated with a 5% inoculum. Because of an expected long lag phase, glucose consumption and ABE production measurements were started after 120 h of incubation. The results are shown in table 4.

As can be seen in table 4, the removal of inhibiting components from the hydrolysate was quite successful, judged from glucose concentration and ABE production. It was surprising to note that in the medium with the lowest concentration of hydrolysate, in which glucose concentration amounted up to a mere 12 g/L, DSM 1731 showed no sign of remaining acidogenic in contrast to the culture described in table 3 where this strain produced 1.5 and 2.1 g/l of ABE and butyric acid, respectively. Apparently, the triggering event of the switch to solventogenesis can be governed by other constituents in the medium besides the ample supply of glucose.

Table 4. Fermentation of partially purified, concentrated hydrolysate from domestic organic waste by *Clostridium acetobutylicum* DSM 1731. Purification was done by anion exchange. Media were prepared from dilutions of this purified hydrolysate in demineralised water to make 1, 2 or 4 fold the initial concentration. The initial pH was adjusted to 6.0.

	Fermentation time in h								
	Glucose g/l			ABE g/l			Butyric acid g/l		
	0	120	168	0	120	168	0	120	168
Medium									
Hydrolysate 1x concentrated	12.0	0	0	0	3.2	4.5	0	0.4	2.0
Hydrolysate 2x concentrated	21.0	0.2	0	0	6.2	6.7	0	1.4	1.6
Hydrolysate 4x concentrated	34.2	1.6	0.1	0	9.3	10.9	0	2.2	1.8
6%glucose-Gapes	59.9	3.7	1.7	0	17.3	17.9	0	0.7	0.8

In 4 fold concentrated hydrolysate, glucose consumption was virtually complete and production of ABE occurred without any problems. Even though there was some production of butyric acid, the yield of ABE per g of glucose was 38%, which is probably a biased estimate because of the presence of other saccharides in the hydrolysate. If a proportional loss during purification and concentration is assumed, together with a complete utilisation of the other saccharides, the yield on hydrolysate amounts to an average of 28 g ABE per 100 g monosaccharides in the hydrolysate, which is quite in line with the yield of 30% observed on glucose in the synthetic medium (Table 4). As shown in Table 1, the content of saccharides in the polymeric fraction of DOW was 39 g/100 g dry matter. The yield of 28% for ABE from hydrolysate suggests that, on the basis of an average dry matter content of 17% in fresh DOW, there is a potential renewable resource for producing 19 g ABE per kg of fresh weight, provided that all saccharides are made available as fermentable feedstock.

Conclusion

The major drawback of the utilisation of DOW for the production of ABE is the low content of fermentable feedstock in the raw material which results, even after successful pretreatment and hydrolysis, in low substrate concentrations and thus in diluted product streams. There are two strategies optional: either to increase the concentration with a penalty due to the increase of inhibitors, or to use the DOW as a co-substrate, still necessitating the utilisation of more expensive feedstock. A techno-economical analysis is required to evaluate the potential viability of either approach.

Experimental Procedures

Microorganisms

Clostridium acetobutylicum DSM 1731 was obtained from the German culture collection, DSM (Deutsche Sammlung von Mikro-organismen, Braunschweig, Germany), *Clostridium* LMD 84.48 from the Dutch culture collection CBS (Centraal bureau voor Schimmelculturen, Delft, The Netherlands) and *Clostridium beijerinckii* B-592 was kindly supplied by Dr. Gapes (TU Wien, Austria). For standard procedures the strains were grown at 30°C (DSM 1731 and B-592) or 37°C (LMD 84.48) without agitation in medium described by Gapes (1996). For maintenance of the cultures, spores were collected in distilled water from colonies grown on solid medium according to Gapes (1996), supplemented with 2 g/l agar. Spores were stored at -20°C until further use. Spore germination was started by heat shocking 1 ml spore suspension in 5 ml distilled water, 2.5 min at 80°C. For growth of precultures, *i.e.* cultures with most cells in the exponential growth phase, inoculation was done with spore suspensions to a final concentration of 1% (v/v). Inoculation of cultures for experiments was always done at 1% (v/v) from precultures in the exponential growth phase unless stated otherwise. Culture media were made anaerobic by sparging with N₂ prior to sterilisation. After inoculation, valves equipped with sterile 0.2 µm filters were mounted on the flasks in order to prevent building up of pressure. All cultures were done in duplicate.

Pretreatment and Hydrolysis

Hydrolysate from domestic organic waste (DOW; kindly supplied by VAM, The Netherlands) was prepared by Ms. M. Ballesteros (CIEMAT, Madrid) by applying Mansonite steam explosion at 200°C for 6 min at DOW which had been washed and subsequently dried to 85 % dryness prior to steam explosion. The liquid and solid fractions were collected from the cyclone and subsequently filtered. The liquid residue was 2 fold concentrated using a flash evaporator and recombined with the solid residue in order to restore the initial mean solid/liquid ratio of DOW. Enzymatic hydrolysis was carried out with cellulolytic enzymes using a solid/liquid ratio of 1 to 10 (30 FPU (Filter Paper Units)/g substrate at 50°C for 72 hrs). The enzymes used

were Celluclast 1.5L for the breakdown of cellulose into glucose and cellobiose, and Novozym 188 at a loading of 25 IU/g substrate for the conversion of cellobiose into glucose, both enzymes kindly donated by Novo Nordisk (Bagsvaerd, Denmark). Enzyme suspensions were sterilised by passing through a 0.2 µm filter prior to addition to the medium. The solid residue, which is not fermentable, was removed by filtration and after adjusting the pH of the filtrate 4.8–5 in order to prevent contamination, the filtrate was frozen until further use. For concentrating the amount of saccharides in the hydrolysate, the hydrolysate was lyophilised and subsequently resuspended in a reduced amount of demineralised water.

Analytical Methods

For preparing homogenous samples, the DOW was passed through a BC 45 Clextral extruder with the following screw configuration: RSE -25H10/RSE -25H6. Extrusion was done at 120°C with steam. The mean residence time was 1 min. The homogenised samples were lyophilised and stored at room temperature until further analysis.

The analysis of the lyophilised DOW was started after milling and passing through a sieve of 0.5 mm. Solvent and hot water extractives were determined in the DOW using, in succession, ethanol/toluene (2:1) and 95% ethanol, and hot water during 1 h, respectively, (TAPPI (Technical Association of the Pulp and Paper Industry) method T 264 om-88). Chemical hydrolysis of the extractive free material was done in 12 M H₂SO₄ at 30°C during 1 h, followed by 1 M H₂SO₄ at 100°C during 3 h. After centrifugation and filtration, soluble lignin and sugars were determined in the supernatant. The lignin was determined spectrophotometrically at 205 nm. The sugars were derivatised using acetic acid and measured using GLC (CP-SIL 88 WCOT; He as carrier gas) equipped with FID. Acid-insoluble lignin and ash in the pellet were determined gravimetrically. By drying this pellet overnight at 105°C, the amounts of lignin and ash were determined and by heating to 525°C overnight the amount of ash only was determined (TAPPI method T211 om-93). Substraction then gave the amount of insoluble lignin. Dry matter content, also needed for enabling comparisons of efficiencies of conversions, was determined by drying overnight at 105°C.

During fermentation, glucose concentrations were measured either enzymatically using the GOD-PAP kit from Merck (Darmstadt, Germany) or, together with other sugars, by HPLC (CHO-682 carbohydrate column, eluted with distilled water). Concentrations of acetone, butanol and ethanol and organic acids were measured by HPLC (Shodex Ionpak KC-811 column, eluted with 3 mM H₂SO₄). Detection was done with a RI or UV detector (butyric acid at 210 nm).

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