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BIOBUTANOL PRODUCTION FROM AGRICULTURAL WASTE: A SIMPLE APPROACH FOR PRE-TREATMENT AND HYDROLYSIS

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One of the main concerns regarding extensive production of bio-butanol has been associated with the high costs of the substrate (preparation of fermentable sugars) and the relatively low tolerance of *Clostridia* to butanol. In this study a simple, mild approach was tested to obtain fermentable sugars from agricultural waste. Giant hogweed and hay was pre-treated with simple boiling and enzymatically hydrolysed. The results demonstrated that after adaptation of the genus *Clostridium* bacteria to the new substrate, the growth kinetics and sugar consumption of these bacteria were similar to the ones obtained in traditional culture media.

Key words: *biofuel, biobutanol, agricultural waste, hydrolysis.*

INTRODUCTION

The decrease in global fossil fuels and increasing cost of energy has motivated developments in the production of energy from biomass. During the past decades, the production of ethanol from high energy crops, starch, lignocellulose and agricultural waste, and the subsequent introduction of ethanol into gasoline has become a common practice [1]. Further developments have shown that biobutanol is more advantageous as a biofuel than bioethanol, as it has a higher energy content, higher blending rate with gasoline and can be transported via pipelines. Biobutanol is traditionally obtained via acetone–butanol–ethanol (ABE) fermentation of genus *Clostridia* bacteria, where sugars (glucose, xylose, arabinose, cellobiose) are used as carbon sources [2].

Current research has shown that effective biobutanol production is possible from agricultural waste and biomass not suitable for food [3, 4]. However, before fermentation a pre-treatment and subsequent hydrolysis steps are still necessary to make sugars accessible for bacteria. Cellulose or hemicelluloses are broken down during the hydrolysis. This can be performed either chemically by sulphuric acid or other acids, or enzymatically by cellulolytic enzymes [5]. Lately the latter approach has been used more often, since it is regarded as environmentally friendly and enzymes can be recovered and used again. However, since cellulose and hemicelluloses are covered with lignin structures in the biomass, pre-treatment prior hydrolysis is necessary. Mechanical (e.g. milling, grinding), chemical (e.g. alkali, dilute acid), thermal (e.g. steam treatment) or enzymatic (e.g. wood degrading fungi) pre-treatment techniques have been developed and used to destroy lignin structures [5, 6], but most of

them are still regarded as expensive and time consuming. Mostly these techniques require highly trained personnel and are not reliable on small scale, at local biofuel pilot plants. Here in this paper we demonstrate the development of a simple approach to obtain fermentable sugars from lignocellulosic biomass not used in food production, to subsequently produce biobutanol.

MATERIALS AND METHODS

Preparation of cellulolytic enzymes

Laboratory scale preparation of cellulolytic enzymes was performed from white rot fungi *Irpex lacteus* IBB 104, grown on agar plates (0.8 g/L KH_2PO_4; 0.2 g/L K_2HPO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 2 g/L NH_4NO_3; 3 g/L yeast extract; 5 g/L glucose; 18 g/L agar; pH = 6.0). The fungi were placed into 250 mL flasks containing 100 mL of liquid medium (10.0 g/L glucose; 2.0 g/L NH_4NO_3; 0.8 g/L KH_2PO_4; 0.4 g/L K_2HPO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 2.0 g/L yeast extract; pH = 5.5–5.8) and incubated on an orbital shaker (150 rpm) at 27 °C. After 5–7 days the fungal pellets were homogenized with glass beads. Then 10 mL of homogenized fungal cultures where re-inoculated in the same media with the exception that glucose was substituted with agricultural substrate (hay, raw and dried giant hogweed or Avicel (Sigma)). Enzyme activity assays were performed regularly. After 2 weeks the biomass was removed and $(NH_4)_2SO_4$ (0.5 kg/L) was added to the supernatant, diluted and incubated at 4 °C for 24–48 hours. After incubation the enzyme was sedimented by centrifugation (4000 rpm; 10 min) and stored in 0.05 *M* sodium citrate buffer at 4 °C for further use.

Enzyme activities were measured according to IUPAC recommendations [7, 8] and included the measurements of carboxymethylcellulase (CMCase), filter paper and xylanase assays.

Substrate pre-treatment and hydrolysis

After harvesting, hay or giant hogweed was ground (to 2–5 mm) and either directly diluted in 0.05 M sodium citrate buffer (3% w/v) and hydrolysed, or boiled for 5 minutes, or sterilized at 121°C for 15 minutes and then hydrolysed. For process optimization, filtration of the substrate prior to hydrolysis was tested.

For hydrolysis, the enzyme (0.2 FPU/mL, 20 FPU/g) was added to the pre-sterilized or non-sterilized substrates diluted in sodium citrate buffer and incubated on an orbital shaker for 24 hours at 27 °C. For process optimization, aerobic or anaerobic hydrolysis was tested. Then the mixture was centrifuged at 4000 rpm for 10 minutes to separate solid particles, and subsequently filtered through 1.2, 0.45 and 0.22 μm pore size filters.

Measurements of reducing sugars

Reducing sugar concentration was measured by dinitrosalicylic acid (DNS) method [7]. In brief, to 0.1 mL of 0.05 *M* sodium citrate buffer in glass tubes 0.6 mL of DNS and 0.1 mL of centrifuged (10 min) sample supernatant was added. For blank control, distilled water was used instead of the sample. Then all samples were boiled for 5 min and transferred to cold water. Then 4 mL of distilled water was added. Absorption was measured with spectrophotometer at

540 nm. To obtain absolute concentrations, a standard curve against glucose was constructed.

Bacterial strains and culture medium

Clostridium acetobutylicum ATCC®824, *Clostridium beijerinckii* DSM 6422 and *Clostridium tetanomorphum* ATCC®49273 were inoculated into a nutrient rich medium (5 g/L peptone, 5 g/L Lab-Lemco, 10 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.5 g/L $K_2HPO_4 \cdot 2H_2O$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 2H_2O$, 0.01 g/L NaCl, 0.001 g/L *p*-aminobenzoic acid; 0.00001 g/L biotin; 30 g/L glucose or hydrolysed substrate containing sugars) and grown anaerobically (AnaeroGen, Oxoid) at 37 °C for 24 hours. The inoculum was further used for batch and fermentation studies. For cell adaptation to lignocellulosic hydrolysates, all cultures were inoculated on a regular basis into media containing higher concentration (0%, 5%, 10% etc.) of substrate.

For substrate inhibition studies, overnight cultures of *C. beijerinckii* (final concentration $\sim 1 \cdot 10^6$ cells/mL) were inoculated into decimal dilution tubes containing either hay hydrolysate only, or hay hydrolysate mixed with growth media (above).

Fermentation studies

Prior to fermentation, the cultures were double-washed with sterile phosphate buffer (pH = 7.2), and a known concentration (final concentration $\sim 10^5$ cells/mL) was inoculated into LAMBDA MINIFOR bench-top laboratory fermenter (Czech Republic) with temperature (37 °C), pH, pO_2 (below 0.1 mg/mL) and air flow rate (0.1 L/min) control, or in batch tubes. Fermentation media contained up to 50% of hydrolysate.

For analyses of cell concentration (total bacterial counts), sugar utilization and product formation, samples were collected directly either from the fermenter or tubes.

RESULTS AND DISCUSSION

Pre-treatment and hydrolysis strategies

Lignocellulose substrate grinding alone as a physical treatment technique is usually not regarded effective enough, and subsequent combination with other techniques has been suggested [5]. Our studies have shown that during the initial phase of hydrolysis, sugar formation occurred with the same trend only for the ground and ground-autoclaved (treated with pressurized air at elevated temperature) substrates (Fig. 1). However, the sugar concentration started to decrease after three hours. Besides suggestions on enzyme adsorptive loss through inhibition by lignin degradation products [9], microscopic analyses showed increasing microbial activity, which effectively consumed any sugars formed during the hydrolysis. Thus, substrate sterilization was introduced. To simplify the treatment and reduce energy requirements, simple substrate boiling was tested, and it was demonstrated that boiling for 5 minutes was sufficient to obtain the same sugar concentration (higher than 0.3 g per gram of substrate) as obtained by heat and pressure treatment (Fig. 1). The lignocellulosic biomass typically contains 50–80% of carbohydrates (dry mass fraction) [5], however, analyses on giant hogweed (data not shown) indicated that the total concentration of celluloses and hemicelluloses did not exceed 40%. Thus,

mechanical grinding with subsequent boiling was used as the pre-treatment technique for future studies.

The enzyme concentration for hydrolysis (20 FPU/g) was chosen according to recommendations [9]. Decrease in enzyme concentration below 5 FPU/g with the purpose of cost reduction gave lower sugar formation rates, and was not used further. Batch enzyme recovery tests showed that on average 40–80% of the enzyme could be recovered and used again.

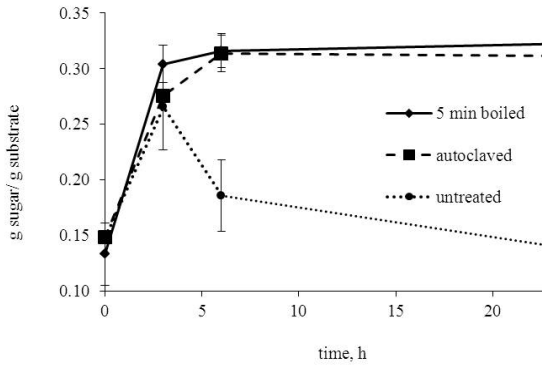

Fig. 1. The effect of ground substrate (dried giant hogweed) heat pre-treatment on reduced sugar yield during hydrolysis. Standard deviation represents the data dispersion from 3 separate studies.

Due to the observation that after effective pre-treatment the liquid already contained cellulose and hemicellulose degradation products [10], substrate filtration prior to hydrolysis was assessed. Our studies showed that the solid fraction contained much higher amounts of hydrolysable material than the liquid fraction (Fig. 2). Separate hydrolysis of liquid [10] and solid fractions is regarded as more labour intensive and complicated, thus eliminated from further studies. Additionally, no difference was observed upon performing hydrolysis in aerobic or anaerobic conditions (Fig. 2). The simpler and cheaper aerobic approach was chosen for future work. Enzyme recovery studies showed that higher recoveries were obtained from samples containing only the liquid fraction (78%), whereas solid particles prevented the extraction of enzyme and only around 45% recovery was possible on laboratory scale. This can be connected to enzyme adsorption to lignin structures [9], and requires further work with respect to separation technologies.

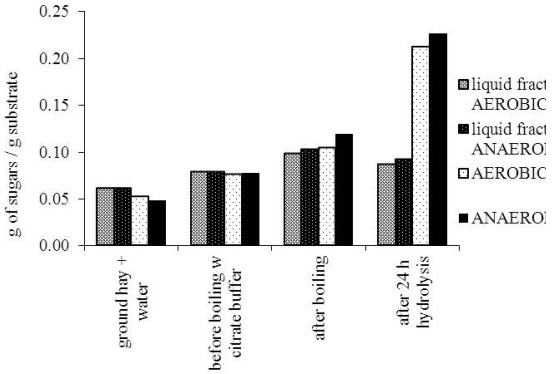

Fig. 2. Comparison of sugar release by aerobic and anaerobic hydrolysis of solid-separated and mixed substrates.

Apart from the traditional three step (pre-treatment, hydrolysis and fermentation) cellulosic alcohol production, simultaneous saccharification and fermentation has been suggested [4]. The approach was tested with pre-treated hay substrates, where enzyme and pure culture of *C. beijerinckii* were added simultaneously and incubated anaerobically for 72 hours. The results indicated that no significant growth of *C. beijerinckii* occurred (below 1 log after 48 hours), and subsequently no formation of ABE was observed after 72 hours of incubation.

Fermentation studies

To analyse the ability of *Clostridia* to ferment sugars produced from lignocellulosic biomass, a series of growth studies were performed. Initially all cultures showed a strong inhibition by the prepared hydrolysates (Fig. 3), and almost no growth and no ABE production was observed for samples containing 100% hay hydrolysate. Samples containing culture media along with 1% and 0.1% of the hydrolysate showed similar growth rate to the positive control, and no inhibition was observed for these samples. Inhibitory properties of the hydrolysate were observed in the samples of 10% hydrolysate. Since no chemical pre-treatment was performed, the presence of inhibitory compounds like furfurals or hydroxymethylfurfurals was not expected [11]. A mere inhibition by the substrate itself was assumed. Further, the cultures were adapted to an increasing concentration of hydrolysate and a decreasing amount of culture media.

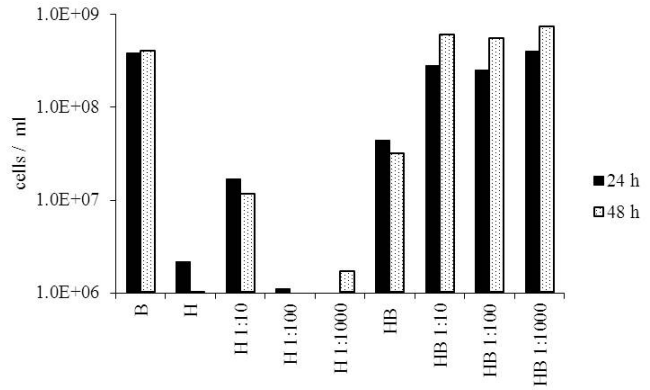

Fig. 3. Inhibition of *Clostridium beijerinckii* growth by hay hydrolysate.
B – represents growth media; H – hydrolysate; numbers indicate dilution rate of the hydrolysate.

Following an adaptation period, fermentation with all three ABE producing *Clostridia* species was performed. Results showed that the adapted *Clostridia* were able to grow in 50% hay hydrolysate and had similar sugar consumption rate as in culture media (Fig. 4 *A,B*).

In both types of media, all cultures had similar growth rates. Only *C. acetobutylicum* showed a rapid decrease in the growth rate after 24 hours in media supplemented with hay. In the culture media, the substrate degradation coefficients for C. *acetobutylicum* and *C. tetanomorphum* were 0.008 h^{-1} and 0.009 h^{-1} (Pearson criteria, P = –0.84 and –0.98, respectively), whereas for *C. beijerinckii* it was higher (0.046 h^{-1}, P = –0.83) (Fig. 4*B*). A slight reduction in substrate degradation was observed for *C. beijerinckii* in the supplemented media (0.023 h^{-1}, P = –0.96), which allowed a more prolonged stationary

phase – the substrate was utilized more slowly, and a longer stationary phase was observed (after 72 hours the cells were still found in a stationary growth phase), during which the low molecular weight products are usually formed along with initiation of sporulation [12]. The onset of stationary phase and shift from acetogenic to solventogenic phase was also demonstrated first by a rapid decrease of system pH (from ~ 6 to 4), then stabilization and subsequent increase. For *C. beijerinckii* at early stationary phase the pH value often decreased below 4.

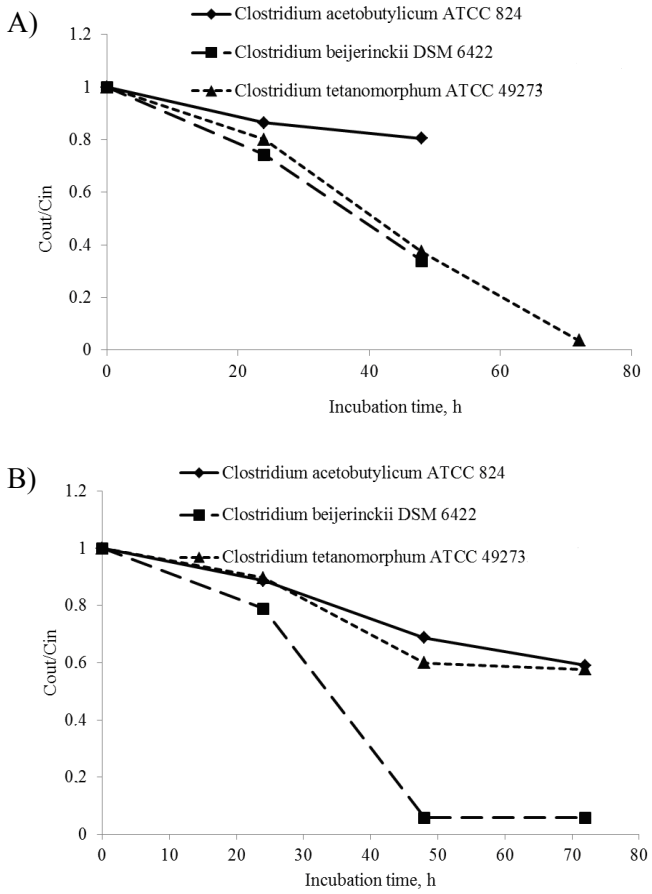

Fig. 4. Growth and subsequent sugar consumption of *C. acetobutylicum*, *C. beijerinckii* and *C. tetanomorphum* in media containing 50% hay (*A*), or in pure culture media (*B*). Values are expressed as the ratio between sugar concentration in the samples after and before cell addition (C/C_{in}), versus incubation time.

Production of acetone, butanol and ethanol (ABE) from the fermentation of *Clostridia* is a common technology [1]. However, its industrial application has been limited due to low product yield, which results from the inhibition of fermenting bacteria during the increase of alcohol concentration in the process broth [2]. The observed total product (ABE) concentration reached 0.66% with classic medium, but decreased when waste hydrolysate was added. A similar trend was observed for adapted cultures, too. Subsequently, the low concentration of butanol in the fermentation broth caused high product recovery costs. To increase the ABE production, it was necessary to keep the ABE con-

centration in the fermenter below the toxic level, *e.g.*, using the gas-stripping process. Consequently, the yield can be higher by up to 13 times [13]. A number of recent studies are based on complicated commercial technologies and large plant construction, whereas the focus of this research was to develop a simple system for waste processing on small scale, *e.g.*, at individual farms. In this case, the boiling pre-treatment of hydrolysate, *Clostridium* cell adaptation and inhibitor removal by dilution of hydrolysate are optimal and cost effective methods.

CONCLUSIONS

Agricultural waste (giant hogweed, hay) can be converted to fermentable sugars in a simple and environmentally friendly (no use of chemicals) manner, and used for biobutanol production on small scale.

Adaptation of *Clostridia* to hydrolysates allowed decreasing of substrate inhibition on cell growth, and the observed sugar consumption rate was similar for pure culture media and for a substrate containing 50% of hydrolysate.

Acknowledgement

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BIOBUTANOLA IEGŪŠANA NO LAUKSAIMNIECĪBAS ATKRITUMIEM: VIENKĀRŠOTA PARAUGU PRIEKŠAPSTRĀDES UN HIDROLĪZES METODIKA

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K O P S A V I L K U M S

Samazinoties pasaules naftas rezervēm, arvien lielāka uzmanība tiek pievērsta alternatīvās enerģijas ieguves veidiem. Viena no alternatīvām degvielām var būt biobutanols, ko fermentācijas laikā dabiski veido klostrīdiju ģints baktērijas. Līdz šim par vienu no kavējošiem aspektiem biobutanola plašākai lietošanai tika uzskatītas salīdzinoši dārgās ražošanas un substrāta izmaksas. Pētījumā parādīts, kā iespējams iegūt biobutanolu, izmantojot pārtikai nepiemēroto biomasu (siens, latvāņi). Procesa vienkāršošanai un izmaksu samazināšanai tradicionālās ķīmiskās un augstspiediena priekšapstrādes un hidrolīzes tehnoloģijas tika aizvietotas ar vienkāršu substrāta vārīšanu un enzimātisko hidrolīzi. Konstatēts, ka pēc nelielas klostrīdiju adaptēšanas jaunajos vides apstākļos klasiskajā augšanas barotnē un vidē ar pievienotu hidrolizātu būtiski neatšķīrās šūnu augšana un fermentējamo cukuru patēriņš. Tādējādi secināts, ka optimizācijas procesā iespējams samazināt biobutanola iegūšanas izmaksas un vienkāršot tehnoloģiskos procesu.