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Biorefinery with Open Mixed Cultures for Biofuels and Chemicals Production from Organic Waste: Biodegradation of Unpretreated Cellulose

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This work fits in the general research area of organic waste conversion to chemicals and fuels using anaerobic digestion. In particular this study investigates the ability of undefined mixed microbial cultures to ferment cellulose to ethanol and organic acids without any chemical or physical pretreatment of the feed. The anaerobic conversion of microcrystalline cellulose was investigated in four batch experiments, carried out at 25 ˚C without any pretreatment of the cellulose. The mixed culture effectively fermented the substrates, however cellulose degradation only occurred after the microorganisms had been acclimated to cellulose in continuous runs, while cellulose was not degraded by unacclimated microorganisms. Acetic acid was the main metabolic product while ethanol, butyric acid and propionic acid were also present in low concentrations. Within 100 days from the start of the batch tests, the cellulose removal was in the range 40-50 %. The maximum concentration of acetic acid observed was 8.8 g/L while the maximum ethanol concentration was 0.6 g/L.
The experimental results demonstrates the capability of open mixed microbial culture to ferment cellulose under mild conditions. Even though the rates observed in this study are still too low for industrial exploitation, they indicate the potential of mixed cultures to biodegrade cellulose even in the absence of any pretreatments. The next step of the study will be aimed at finding the conditions that increase the cellulose biodegradation rate.

1. Introduction

Anaerobic digestion of organic waste is usually aimed at the production of methane. Although methane is an important source of renewable energy, there is increasing interest in the literature to drive anaerobic digestion to the production of more valuable chemicals, e.g. alcohols, or organic acids (Kleerezebem et al., 2007; Dionisi et al., 2015; Kleerezebem et al., 2015). Organic wastes that could be potentially used for conversion to chemicals and fuels are, among the others, agricultural waste, wood waste and forestry residues. One of the main problems in the use of these wastes in anaerobic digestion is their lignocellulosic nature, which makes their microbial degradation difficult. Currently, most of the research in this area uses chemical or physical pretreatments to breakdown lignin and cellulose, making the sugars available for microbial fermentation (Wyman et al., 2011). However, chemical-physical pretreatments typically require high temperature and pressure and the external addition of chemicals and they are therefore very expensive and not environmentally friendly. Therefore, the use of anaerobic digestion for the valorisation of lignocellulosic waste is still very limited.
As an alternative to current pretreatment technologies, this study investigates the feasibility of using open mixed microbial cultures for the biodegradation of cellulosic materials without any prior chemical or physical pretreatment. The aim is to investigate the ability of open mixed cultures to hydrolyse unpretreated cellulose and to convert it to valuable chemicals. Biodegradation of unpretreated cellulose has so far mainly been investigated using pure cultures of selected cellulolytic strains (e.g. Pavlostathis et al., 1988) but little research has been carried out on the direct biodegradation of unpretreated cellulose using open mixed cultures.

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This study reports our experimental study on the biodegradation of microcrystalline cellulose using open mixed cultures. The study was carried out using batch tests during which the concentration of volatile suspended solids (VSS), total carbohydrates, and liquid phase products (volatile organic acids and ethanol) was measured.

2. Materials and Methods

2.1 Media composition

Crystalline cellulose of 20 µm particles (Sigmacell type 20, Sigma-Aldrich, Dorset, UK, product number S-3504) was used for the cellulose degradation experiments as the sole carbon source. In addition to cellulose, the media comprised of 69.6 g/L K_2HPO_4, 48 g/L NaH_2PO_4, 2 g/L NH_4Cl, 0.125 g/L $MgCl_2.6H_2O$ and 0.09 g/L $CaCl_2.6H_2O$. Tap water was used to prepare the media. The phosphate salts acted as pH buffer, since all experiments were run without external pH control.

2.2 Acclimation to cellulose before batch tests

The microorganisms used in the batch tests had been acclimated with cellulose in continuous runs which were inoculated with: 1) soil that was obtained from Craibstone, Aberdeen, UK; 2) anaerobic digester sludge collected from Gask anaerobic digester, Turriff, Aberdeenshire, UK. The soil was homogenized with a 150 µm mesh prior to use and the large solids in the sludge were removed by filtration through a Buchner funnel prior to use.
The continuous runs were carried out in 200 ml glass reactor with a feed comprised of 20 g/l cellulose, as only carbon source, and the mineral media reported in section 2.1. The reactors were purged with N_2 gas for 5 minutes, prior to inoculation, to attain anaerobic conditions. The continuous runs were carried out at 25 °C with a hydraulic residence time (HRT) of 2 days.

2.3 Experimental set up for batch tests

Four 250 ml Duran vessels were used for the batch experiments with a working volume of 200 ml. Batch 1 was filled with 200 ml culture from a continuous reactor inoculated with 1 % (w/v) soil, Batch 2 was filled with 200 ml culture from a continuous reactor inoculated with 5 % (w/v) sludge, Batch 3 was filled with 200 ml culture from a continuous reactor inoculated with 5 % (w/v) soil. Batch 1 was filled with the culture after 2 months of continuous run, Batch 2 and Batch 3 were filled after 1 month of continuous run. A fourth reactor (Batch 4) was filled with unacclimated soil, i.e. without the soil being ever exposed to cellulose before the start of the batch tests. All the reactors were incubated at 25 ^{0}C with shaking at 200 RPM. Each experiment was performed once.

2.4 Analytical methods

To monitor the fermentation process, 7ml was withdrawn from the culture broth periodically using a syringe. The VSS were measured according to the Standard Methods (APHA, 1999). The total carbohydrate and the soluble sugars were estimated using the Anthrone reagent method (Koehler, 1952). The product spectrum was determined by gas chromatography (GC), using a capillary column (30m x 0.25mm, TG-WaxMS A), from Thermoscientific, coupled to a Flame ionization detector (FID). The initial temperature of the column was 80 °C for 2 min followed with a ramp of 20 °C/min and a final temperature of 200 °C for 1 min; the injector and detector temperatures were 200 °C and 250 °C respectively. Hydrogen was used as the carrier gas at a flowrate of 35 ml/min. The samples were acidified with H_3PO_4 as described by Raposo et al. (2015).

3. Results and discussion

3.1 Batch experiment with cellulose

Figure 1 shows the cellulose conversion profile during the experiments. As measured by the removal of total carbohydrates, 48%, 37% and 51% cellulose conversion was observed in Batch 1, Batch 2 and Batch 3 respectively. On the other hand, when microorganisms were not previously adapted to cellulose (Batch 4), no cellulose degradation took place over the whole length of the test. It can be observed that in Batch 1, 2 and 3 the initial concentration, measured both as VSS and total carbohydrates, at the beginning of the test was higher than in the feed to the continuous reactors. This was because mixing in the continuous reactor was not perfect leading to the accumulation of solids inside the reactors. The difference in the initial substrate concentration can influence the cellulose fermentation in these experiments. Further research is required to determine the effect of the initial substrate concentration on the process. The cellulose degradation rate in Batch 1, 2, and 3, based on total carbohydrates, was similar and in the range 0.2 - 0.3 g L^{-1} day^{-1}. Soluble carbohydrates were also measured during the tests, but their concentration was always found to be negligible. This indicates that, once cellulose is hydrolysed, the produced glucose is immediately consumed and therefore cellulose hydrolysis is

the rate limiting step for cellulose degradation, as also reported in other literature studies (e.g. Liang et al., 2014). Figure 2 shows the profiles of the fermentation products during the batch tests.

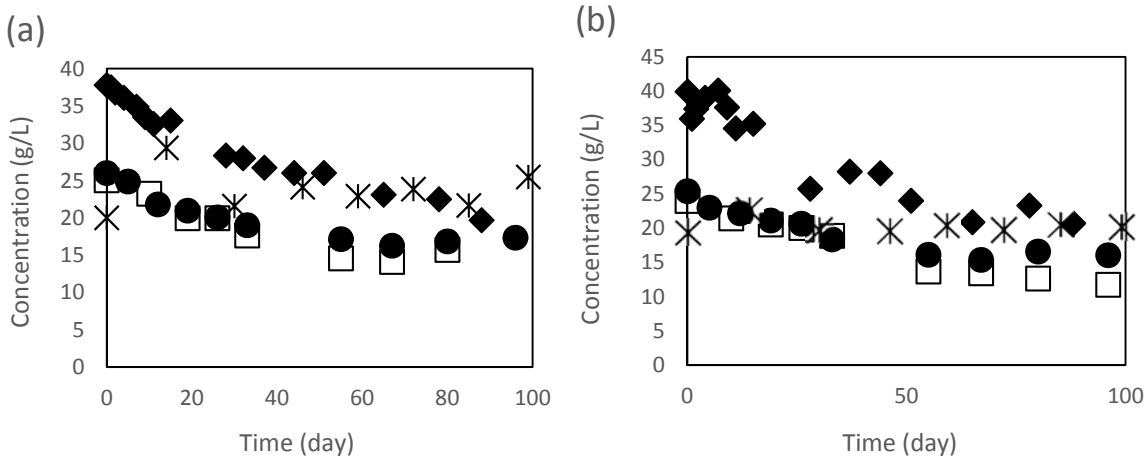

Figure 1: Comparison of microcrystalline cellulose fermentation by mixed culture (a) VSS (b) Total carbohydrates. (◆) Batch 1, (●) Batch 2, (□) Batch 3, (✳) Batch 4

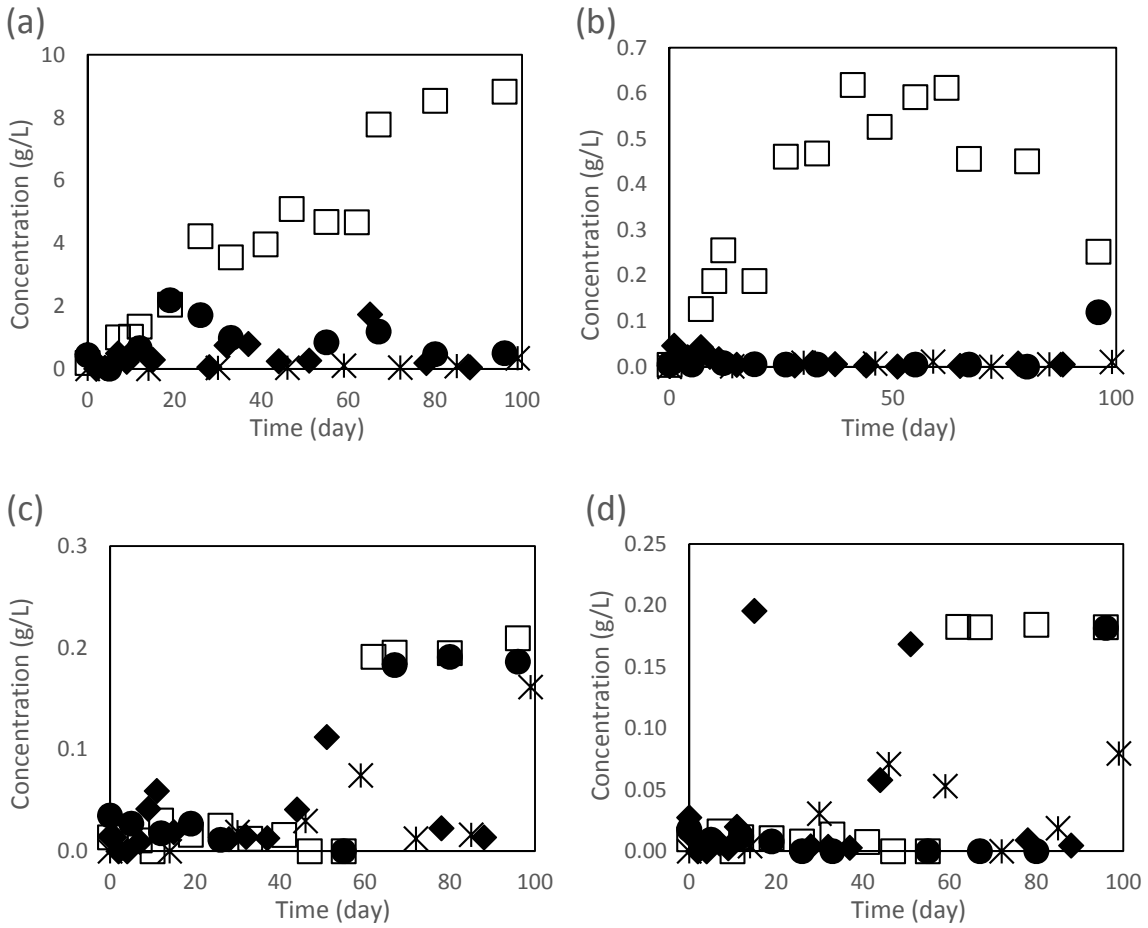

Figure 2: Main products of cellulose fermentation (a) Acetic acid (b) Ethanol (◆) (c) Propionic acid (d) Butyric acid (◆) Batch 1, (●) Batch 2, (□) Batch 3, (✳) Batch 4

Even though each experiment was performed once, the results clearly indicate that acclimation to cellulose is an important factor in the rate of biodegradation, since a significant cellulose biodegradation rate was observed in all the tests with previous acclimation of the biomass (Batches 1,2 and 3), while no cellulose degradation was observed in the test without previous acclimation (Batch 4).
The only test where products were detected in high concentrations was Batch 3. In that case, acetate was the main product, reaching a concentration of 8.8 g/L, and ethanol was also observed at a concentration of up to 0.6 g/L. Propionic and butyric acids were also observed but at very low concentrations. The fact that the product concentration was much higher in Batch 3, compared to Batch 1 and 2, in spite of the similar cellulose removal, probably indicates a higher activity of methanogenic microorganisms in Batch 1 and 2 than in Batch 3. This is probably due, in turn, to the different conditions used for the acclimation of the microorganisms to cellulose prior to the batch experiments.
Figure 3 shows the evolution of pH during Batch 3. The pH slowly decreased, due to the accumulation of acetate, but always remained in the range 6.4 - 7.0. No significant pH change was observed in the other batch tests, where the pH remained constant at approximately 6.9 throughout the length of the tests (data not shown). This is consistent with the observation that very little acids accumulation took place in the other batch tests.

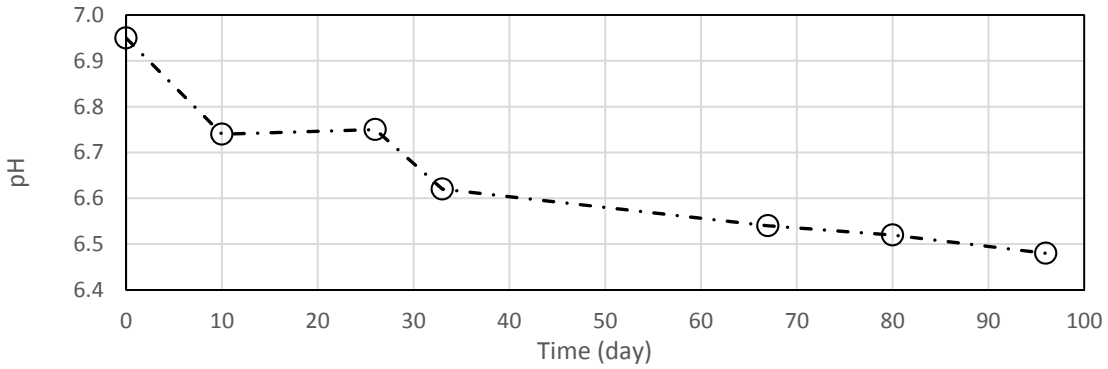

Figure 3: Change in the pH of the Batch 3 culture medium during the process

The rate of cellulose degradation obtained in this study is compared with other literature studies which used the same type of cellulose (Sigmacell Type 20) in Table 1. Little evidence is available in the literature about Sigmacell 20 degradation by anaerobic mixed cultures, therefore the only meaningful comparison, reported in Table 1, is with pure culture studies. It is evident that with the selected cellulolytic bacteria *Ruminococcus flavefaciens* and *Fibrobacter succinogens* higher cellulose degradation rates have been obtained than in our study. However, at least part of the difference can be attributed to the different temperature used, which was 39 ˚C with the pure culture studies and 25 ˚C in our mixed culture study.

Table 1: Comparison of cellulose (Sigmacell 20) degradation rate (D=dilution rate)

Rate of degradation ($gL^{-1}day^{-1}$)	**Conditions**	**Reference**
1.42	D = 0.019 h^{-1}, pH 7.19, 39 °C *Ruminococcus flavefaciens*	Shi and Weimer (1992)
6.44	D = 0.101 h^{-1}, pH 6.56, 39 °C *Ruminococcus flavefaciens*	Shi and Weimer (1992)
1.29	D = 0.0136 h^{-1}, pH 6.19, 39 °C *Fibrobacter succinogenes*	Weimer (1993)
1.28	D = 0.016 h-1, pH 6.19, 39 °C *Fibrobacter succinogenes, Ruminococcus flavefaciens and Ruminococcus albus*	Chen and Weimer (2001)
0.2 – 0.3	Batch, pH 7, 25 °C mixed culture	This study

It is also important to observe that the cellulose degradation rates in mixed cultures can be further enhanced by enrichment of the culture with the substrate, and therefore the rates obtained in this study are not be considered the maximum possible rates obtainable by mixed cultures. Increase in substrate degradation rate by sequential enrichment of mixed cultures has already been reported in many areas of biotechnology, e.g. for polyhydrxyalkanoates production (Majone et al., 2006), and will be carried out with cellulose as next step of our experimental study. Apart of the degradation rates, it is important to observe that mixed cultures give several potential advantages over pure culture processes, e.g. the possibility of direct single-stage ethanol production from cellulose and the absence of sterilisation costs.

3.2 Mass Balances

The COD in anaerobic systems is conserved due to the absence of oxygen. The contributions of the various products to the COD balance for Batch 3 is reported in Table 2. Overall, over 80% of the COD of the removed cellulose was recovered as the measured products in the liquid phase. Most of the COD was recovered as acetate, which accounted for 72% of the removed COD. Other contributions to the COD balance which were not measured probably included mainly microorganisms and hydrogen. For Batch 1 and 2, on the other end, the COD of the recovered products accounted for just a very little fraction of the COD of the removed cellulose (data not shown), and this is a strong indication of the occurrence of methanogenesis in these tests.

Table 2: Products and COD mass balance on Batch 3

Parameters	Concentration g-COD/L	COD recovery[a]
Initial Cellulose[b]	25.63	
Cellulose removed	13.09	
Acetic acid	9.44	0.72
Ethanol	0.53	0.04
Propionic acid	0.32	0.02
Butyric acid	0.33	0.03
Total COD recovery (%)		**81.07**

[a]COD balance = COD VFA/ COD cellulose removed
[b]Measured as total carbohydrates

It is interesting to compare the measured COD recovery as acetate (from Table 2) with the stoichiometric equation for glucose conversion to acetic acid, Eq(1) below. According to Eq(1), glucose can be converted to acetate with a 67% yield on a COD basis, and, taking the experimental error into account, this is in good agreement with the 72% yield reported in Table 2. This again indicates that Eq(1) describes the product distribution of cellulose degradation during Batch 3 reasonably well.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (1)$$

4. Conclusions

This study represents a contribution towards the development of a pretreatment-free process for the biological conversion of organic waste into chemicals using open mixed cultures. Anaerobic mixed microbial fermentation of cellulose was investigated in four batch experiments, at 25 °C, which gave a cellulose conversion of up to 50% in approximately 100 days. Acetate was the main fermentation product, reaching a concentration of up to 8.8 g/l. Cellulose degradation activity was only observed after the mixed cultures had been acclimated to cellulose as only carbon source, while unacclimated microorganisms were not able to degrade cellulose. The data indicates that cellulose can be effectively degraded by open mixed culture without chemical pretreatment, however the rates obtained are still too low for industrial applications. Therefore, further study, e.g. enrichment tests, is needed to identify the conditions which give higher cellulose degradation rates.

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