Effect of xylose and nutrients concentration on ethanol production by a newly isolated extreme thermophilic bacterium

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ABSTRACT

An extreme thermophilic ethanol-producing strain was isolated from an ethanol high-yielding mixed culture, originally isolated from a hydrogen producing reactor operated at 70 °C. Ethanol yields were assessed with increasing concentrations of xylose, up to 20 g/l. The ability of the strain to grow without nutrient addition (yeast extract, peptone and vitamins) was also assessed. The maximum ethanol yield achieved was 1.28 mol\textsubscript{ethanol}/mol\textsubscript{xylose consumed} (77% of the theoretical yield), at 2 g/l of initial xylose concentration. The isolate was able to grow and produce ethanol as the main fermentation product under most of the conditions tested, including in media lacking vitamins, peptone and yeast extract. The results indicate that this new organism is a promising candidate for the development of a second generation bio-ethanol production process.

Key words | anaerobic, bioethanol, extreme thermophilic

INTRODUCTION

Renewed interest in alternative energy sources has been rising in the last decade, together with the awareness that the use of fossil fuels has been causing great damage to the environment. The depletion of the planet’s natural resources has led to increased efforts of establishing processes for alternative fuel production that comply with the definitions of sustainability and environmental-friendly.

The production of bio-ethanol from sugar or starch containing biomass referred to as ‘first-generation’ bio-ethanol is now an established and mature process. In 2008, of a total of 65 billion litres of ethanol produced worldwide, 89% were produced in the USA and Brazil (Renewable Fuels Association 2010). However, the main feedstock for ethanol production in the USA has been the processed starch fraction of yellow corn (maize), while in Brazil sucrose from sugar cane is used (Taylor et al. 2009). The use of land resources to produce biofuels instead of food or food grade resources has raised criticism (Tenenbaum 2008) and therefore alternative feedstocks need to be used, so that bio-ethanol production becomes truly sustainable.

Agricultural residues such as straw, corn stover, bagasse, and waste such as household waste, also contain fermentable carbohydrates and can also be used for ethanol production in the so called ‘second-generation’ processes. However, these processes face several challenges. One of them is that often the agricultural organic residues are of lignocellulosic structure, and therefore, the carbohydrates are not readily available to the fermenting microorganisms. Moreover, lignocellulosic biomass contains more than 20% of pentose sugars, such as xylose and arabinose (Talebnia et al. 2010), which cannot be used by the common ethanol commercial strains, such as Saccharomyces cerevisiae, the most used organism in first-generation bio-ethanol production (Talebnia et al. 2010).

In the light of these facts, much of the ongoing research has been focused on either improving the existing strains by metabolic engineering (Jeffries 2006), or in finding new organisms with attractive features towards ethanol production from pentose sugars. One possible and interesting approach is the use of thermophilic ethanologenic bacteria, as reviewed by Taylor et al. (2009). These organisms can have several advantages over mesophilic organisms in a second-generation bio-ethanol production process, such as the ability to ferment, in addition to hexoses and pentoses, hydrolysate materials and even more complex carbohydrates like cellulose (Karakashev et al. 2007). Furthermore, other advantages, though less important...
when using high-temperature fermentation, are easier product recovery due to ethanol evaporation and reduced risk of contamination of the process with the typical microorganisms present in the feedstock, since most of these cannot grow in the thermophilic temperature range.

Promising thermophilic candidates to produce bio-ethanol from second-generation feedstocks include engineered strains of *Thermoanaerobacter marthani* (Georgieva & Ahring 2007), *Geoactillus thermoglucosidasius* (Cripps et al. 2009) and *Thermoanaerobacterium saccharolyticum* (Shaw et al. 2008). However, none of these organisms has yet achieved the high yields and high ethanol tolerance characteristic of the commonly used mesophilic species such as *S. cerevisae* or *Zymomonas mobilis*. The latter can typically achieve active growth in up to 12–14% v/v ethanol and achieve yields higher than 90% of the theoretical yield.

In this work, we used a mixed enriched thermophilic culture as a starting point for isolation of interesting candidates for bio-ethanol production. This culture had previously achieved 95% of the theoretical ethanol yield from xylose under specific cultivation conditions (Zhao et al. 2010). These included the supply of high concentrations of nutritional supplements and nitrogen sources such as yeast extract (YE) and peptone, which would be undesirable in a large-scale process. Dominant organisms in this enrichment culture were phylogenetically affiliated to some representatives of the genus *Thermoanaerobacter*. Although mixed cultures are also able to produce high yields of bioethanol, ultimately a single strain is desirable, as relatively small changes of the environmental conditions could give advantage to other species in the mixed, with different fermentation product profile as result (Zhao et al. 2010).

Thus, the objectives of this study were (i) to isolate the organism(s) present in the starting mixed culture capable of achieving high yields of ethanol from xylose and (ii) to assess for the influence of xylose and other nutrients in ethanologenic yields and determine whether these can be omitted from the process.

**MATERIALS AND METHODS**

**Inoculum and media**

The inoculum was a high-yielding ethanol and hydrogen mixed culture (Zhao et al. 2009) enriched from a bio-hydrogen producing reactor operation at 70 °C using xylose as the main carbon source (Kongjan et al. 2009) The initial inoculum was obtained from a laboratory scale bio-hydrogen producing continuously stirred tank reactor (CSTR), fed with household solid waste. This reactor was operated at 70 °C and had a hydraulic retention time of 3 days (Liu et al. 2008).

The cultivation media were based on the basic anaerobic (BA) medium described in Angelidaki & Sanders 2004, but cysteine hydrochloride was excluded. Anaerobic cultivation vials containing BA medium were autoclaved at 121 °C during 20 min; pH 7 was achieved by sparging the bottles with 80/20% N2/CO2 gas mixture, while pH 5.5 by omitting sodium bicarbonate and using CO2 as sparging gas. When used, xylose, YE, peptone, vitamins and Na2S were added to each vial just before inoculation, from anaerobic, sterile-filtered stock solutions.

**Isolation and batch experiments**

Isolation was carried out anaerobically at 70 °C and pH 7 using the roll-tube technique (Hungate 1969). The medium was obtained by mixing pre-sterilized double strength BA medium (as described above) with a hot solution of 22 g/l Gelrite and 4 g/l MgCl2 in equal parts. 2 g/l xylose, 1 g/l YE, vitamin solution and Na2S were added directly to the 25 ml tubes, to which 5 ml of medium was added. The tubes were then flushed with 80/20% N2/CO2 gas mixture, inoculated (OD600 = 0.1), capped, rolled under a stream of cold water, and finally incubated. Colonies were picked with sterile Pasteur pipettes, diluted in liquid BA medium with 2 g/l xylose and pH 7 and re-incubated. When growth was observed (by detecting hydrogen production and measuring OD600), new roll tubes were inoculated with the growing culture. This procedure was repeated until only one colony type was present in the roll tubes.

All batch experiments to evaluate the influence of xylose concentration and other nutrients and to assess ethanol yields were performed in triplicate. Controls without addition of xylose were included. 250 ml serum vials containing 100 ml of BA medium with 10% (v/v) inoculum were used for all experiments. The concentrations tested were 0, 2, 5, 10 and 20 g/l xylose; 0, 0.5, 1, 1.5 and 2 g/l YE; 0, 0.5, 1, 1.5 and 5 g/l peptone. Furthermore, experiments where vitamin and/or YE were omitted from the medium and trials with pH 5.5 were also performed.

**PCR-DGGE**

To confirm the purity of the isolated strain and for preliminary identification, PCR-DGGE technique was used, as
described in Zhao et al. (2009). Genomic DNA was extracted from a sample of liquid culture obtained after the final isolation step and purified using a QIAmp DNA Stool Mini Kit (QIAGEN, 15504). Universal primer 1492-r and bacteria-specific primer 27-f were used for the first PCR. Primers 518-r and 357-f were used to amplify the V3 region in the second PCR.

The bands corresponding to the products of the second PCR were excised from the DGGE bands and sent for sequencing (MilleGen, Labège, France). The V3 region of the genome was deposited in the GenBank database under the accession number GU176611.

Analytical methods

Hydrogen concentration in the headspace was measured in a gas chromatography equipment (MicroLab, Aarhus, Denmark) with a thermal conductivity detector (TCD) and a s-m stainless column packed with Porapak Q (50/80 mesh). Nitrogen was used as the carrier gas.

Xylose and lactic acid were determined by high-performance liquid chromatography (HPLC) (Agilent) using a refractive index detector and a Bio-Rad Aminex HPX-87H column operated at 65.5 °C; 4 mM H2SO4 was used as an eluent at a flow rate of 0.6 ml/min. Detection limits for xylose and lactic acid were 0.001% (w/v) and 0.0035% (w/v), respectively.

Volatile fatty acids (VFAs) and alcohols (ethanol, butanol) were analyzed using a gas chromatograph (HP 5890 series II) equipped with a flame ionization detector (FID) and an HP FFAP column. The GC-TCD and GC-FID conditions were set according to (Liu et al. 2008). Detection limit for VFAs (acetic acid, butyric acid, valeric acid, propionic acid and hexanoic acid) was 0.002% (w/v). Detection limit for ethanol and butanol was 0.0094% (w/v).

RESULT AND DISCUSSION

After the third repetition of the isolation procedure, 1–2 mm diameter, cream-colored, round colonies were the only type that could be found in the roll tubes. This isolate was preliminarily called strain DTU01, and was used in the subsequent experiments.

V3 region sequencing results placed the new strain DTU01 within the order Clostridiales. Further work regarding morphological, physiological and genetic characterization of strain DTU01 revealed that it falls within the newly described genus Caldicoprobacter (Yokoyama et al. 2010).

Effect of initial xylose concentration

Ethanol production and xylose removal were investigated in batch cultivations at different initial xylose concentrations: 2, 5, 10 and 20 g/l (Figure 1).

For concentrations 2–5 g/l, ethanol production started within the first 24 h of cultivation and occurred at very close rates. Stationary phase was reached within 71 h. The xylose consumption profiles for these concentrations also show a similar trend, although at 10 g/l xylose was not totally degraded within the same time frame.

The highest ethanol yield was obtained for an initial concentration of xylose of 2 g/l and was 1.28 mole ethanol/mole xylose consumed, which is 77% of the theoretical value (1.67 mole ethanol/mole xylose) (Figure 2). To date, this is the maximum value reported in the literature for thermophilic ethanol production from xylose with a non-engineered pure culture.

Ethanol yields decreased with the increasing initial xylose concentration and at 20 g/l, no significant ethanol production was observed. At concentrations above 5 g/l, there was a significant amount of xylose remaining in the

Figure 1 | Time course of ethanol production and xylose consumption for different initial concentrations of xylose.
bottles (see also Table 1). This could indicate substrate inhibition, which has previously been reported for other thermophilic organisms (van Niel et al. 2003).

Another reason for the decreasing ethanol yields could be product inhibition, by accumulation either of fermentation products or of chemical xylose conversion products resulting from caramelization of xylose (Buera et al. 1987) or Maillard reaction (Hill & Patton 1947). In fact, change of the colour to brown with cultivation time was observed in the serum flasks. The darkness of the brown colour increased over time for all the flasks and the intensity of browning was higher in the vials with higher initial xylose concentration. This assumption was also supported by the incomplete COD balance for the 10 and 20 g/l initial xylose cases (see Table 1). After 150 h of cultivation, xylose fermentation products in amounts corresponding to its consumption, were not detected, indicating that xylose had been converted to other compounds, undetectable by the analytical methods used.

Nevertheless, it is worth to remember that the inoculum had been cultivated at 2 g/l of xylose during all the isolation process. Adaptation to higher substrate concentrations has been reported for thermophilic cultures (Kongjan et al. 2009) and repeated batch cultivation in higher values could lead to higher yields. Obtaining higher ethanol yields at high substrate concentrations is important, since typical sugar concentrations in, for example, pre-treated lignocellulosic substrates can reach values as high as 51.3 g/l (Almeida et al. 2009).

The maximum ethanol concentration obtained in the flasks was 1.6 g/l, for 5 g/l initial xylose concentration. Although this value is far from the desirable range of concentrations for efficient and economic downstream processing (Vane & Alvarez 2008), it falls within concentrations reported for other ethanologenic thermophilic bacteria (Sveinsdottir et al. 2009). Furthermore, given the volatile properties of ethanol, downstream processing of ethanol produced at high temperatures will not require such high concentrations. Once the ethanol is in the gas phase, its removal can be done by mild vacuum application, or stripping, as opposed to conventional distillation (Vane & Alvarez 2008).

**Effect of peptone and yeast extract concentration**

In the enriched culture used as initial inoculum for this isolation process, the highest ethanol yields were achieved in medium supplemented with 2 g/l YE and 5 g/l peptone. These values are higher than the typical amounts of nutrients and supplements added to culture media. Therefore, the effect of peptone and YE concentration in ethanol production by this isolate was evaluated. The initial concentration of xylose used in these tests was 5 g/l.

Ethanol to acetate and lactate ratios in Figure 3, show that in almost all the cases, ethanol was the predominant fermentation product. Generally lower yields were achieved in the series of batches were YE was tested, probably due to the high peptone concentration used in these (5 g/l). Lower concentrations of peptone (0–1.5 g/l) did not seem to have an influence on ethanol yield.

Xylose consumption seems to increase until a certain point with both peptone and YE. It has been reported in several studies that supplementing the growth medium with YE generally has beneficial effects to the fermentation process, such as increased hydrogen yields (Xu et al. 2008; Kongjan et al. 2009) and improved sugar consumption and increased ethanol yields (He et al. 2009). This is due to the highly rich

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**Table 1** | Major components of the cultivation medium after 150 h of cultivation, for each initial xylose concentration. All values are in mmol except where indicated

<table>
<thead>
<tr>
<th>Initial xylose</th>
<th>Remaining xylose (%)</th>
<th>Ethanol</th>
<th>Acetate</th>
<th>Lactate</th>
<th>Other VFA</th>
<th>Hydrogen</th>
<th>COD balance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.56 (2 g/l)</td>
<td>1.81</td>
<td>1.89</td>
<td>0.54</td>
<td>0.13</td>
<td>0.03</td>
<td>0.91</td>
<td>79</td>
</tr>
<tr>
<td>3.49 (5 g/l)</td>
<td>6.79</td>
<td>3.25</td>
<td>0.58</td>
<td>0.63</td>
<td>0.02</td>
<td>1.01</td>
<td>69</td>
</tr>
<tr>
<td>19.52 (10 g/l)</td>
<td>40.33</td>
<td>2.26</td>
<td>0.28</td>
<td>0.10</td>
<td>0.02</td>
<td>1.01</td>
<td>47</td>
</tr>
<tr>
<td>30.02 (20 g/l)</td>
<td>70.99</td>
<td>0.22</td>
<td>0.28</td>
<td>0.10</td>
<td>0.02</td>
<td>0.03</td>
<td>10</td>
</tr>
</tbody>
</table>
composition of YE, which includes peptides, amino acids, vitamins and carbohydrates (Eurasyp 2010). A similar effect can be expected from the addition of peptone, which contains mostly amino acids.

However, in this case, the highest concentration of peptone (5 g/l) yielded less ethanol and also inverted the growing xylose consumption trend. This can once again be explained by the Maillard reaction, which is a reaction of reducing sugars with amino acids (Hill & Patton 1947), given that in these particular serum flasks. In the lower range of peptone added (0–1.5 g/l), the concentration did not seem to have a significant effect in the ethanol yield. In the case of YE, a trend was not observed, but the highest yield was achieved for 1.5 g/l YE added to the medium. Neither YE nor peptone seemed to have an influence in hydrogen production, which remained constant in all cases (results not shown).

The fact that the isolate was not significantly affected by the different concentrations of supplements reinforces its value as a promising bio-ethanol producer. The addition of high quantities of nutritional supplements in an industrial-scale process using second-generation feedstocks as a raw material for ethanol production would be expensive and eradicate the sustainable character of the process.

**Other nutrient requirements and effect of lowering the pH**

According to Zhao et al. (2010), 5.5 was the optimal pH value for ethanol production in the enriched culture. This was the value of pH used throughout the experiments. However, typical pH values reported for optimal growth of other anaerobic and thermophilic organisms are usually in the range of 6–8 (Larsen et al. 1997).

The ability of the new isolate to growth without the addition of vitamins and YE was also tested. The concentration of xylose used in these tests was 5 g/l. Figure 4 compares ethanol accumulation with xylose consumption over fermentation time. Ethanol production...
occurred faster and reached higher values at pH 7. This corresponded to the fastest xylose consumption rate and almost full conversion. The decrease in ethanol concentration observed after 60 h of fermentation corresponded to a simultaneous increase both in lactate and acetate concentrations (data not shown). This suggests a shift in the metabolic pathways occurring at lower concentrations of xylose and/or higher concentrations of ethanol.

For the batches at pH 5.5 and where vitamins and YE were omitted from the medium, neither ethanol production rates nor xylose consumption rates varied significantly. The main difference was noticed when the media contained YE but not vitamins: more xylose was consumed when compared to the medium lacking both supplements. This confirms the observation in the previous section that addition of YE to the medium seems to benefit the fermentation towards total sugar utilization.

Figure 5 shows that the highest ethanol yield was obtained for pH 7 and was 1.12 mol\(_{\text{ethanol}}\)/mol\(_{\text{xylose consumed}}\), which accounts for 69% of the theoretical yield. This value is higher than the 0.8 mol\(_{\text{ethanol}}\)/mol\(_{\text{xylose consumed}}\) achieved when the isolate was first cultured with 5 g/l initial xylose concentration (Figure 1). Accordingly, xylose consumption was 97%, which is also slightly higher than in the beginning for the same conditions –92% (Figure 1). This indicates that the isolate has the capacity of adaptation, which is a valuable asset to any potential candidate microorganism to be used in an industrial process.

Although the yields achieved were lower when vitamins and YE were omitted from the culture medium, the isolate was still able to grow and utilize more than 60% of the xylose added to the medium. In all the situations ethanol was the main fermentation product. Interestingly, the highest ethanol to acetate and lactate ratio was achieved in the non-supplemented medium, with a value of 2.1.

CONCLUSIONS

An extreme thermophilic organism was successfully isolated from a high ethanol and hydrogen yielding thermophilic enrichment culture. This isolate yielded ethanol as the main fermentation product, followed by acetate and lactate. Ethanol yields as high as 77 and 69% of the theoretical yield were achieved, respectively, 2 and 5 g/l of initial xylose concentration in the medium. Up to this date, 1.28 mol\(_{\text{ethanol}}\)/mol\(_{\text{xylose consumed}}\) is the highest yield reported in the literature for an extreme thermophilic, non-engineered, pure strain producing ethanol from pure xylose.

Ethanol was produced in xylose concentrations up to 10 g/l, and although substrate inhibition was detected at 20 g/l of xylose, the isolate showed potential to adapt to higher concentrations of xylose, when the yield at 5 g/l increased after several cultivations.

The isolate was also able to grow and produce significant yields of ethanol in media lacking nutrient sources such as vitamins, peptone or YE. This is a valuable asset for a candidate microorganism for second-generation bioethanol production, meaning this can be achieved without costly addition of supplements to the lignocellulosic biomass.

The isolated organism is therefore a new, extremely interesting and very promising organism for the establishment of a sustainable ethanol production process.

REFERENCES


Renewable Fuels Association


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