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Butanol fermentation of the brown seaweed *Laminaria digitata* by *Clostridium beijerinckii* DSM-6422

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**Highlights**

- High butanol yield of 0.42 g/g achieved by fermentation of *L. digitata* hydrolysate.
- Final butanol concentration of 7.16 g/L achieved by batch fermentation.
- High molar ratio of butanol to acetone-butanol-ethanol (0.85) achieved.
- Consumption of lactic acid, mannuronic acid and guluronic acid observed.

**Abstract**

Seaweed represents an abundant, renewable, and fast-growing biomass resource for 3rd generation biofuel production. This study reports an efficient butanol fermentation process carried out by *Clostridium beijerinckii* DSM-6422 using enzymatic hydrolysate of the sugar-rich brown seaweed *Laminaria digitata* harvested from the coast of the Danish North Sea as substrate. The highest butanol yield (0.42 g/g-consumed-substrates) compared to literature was achieved, with a significantly higher butanol: acetone-butanol-ethanol (ABE) molar ratio (0.85) than typical (0.6). This demonstrates the possibility of using the seaweed *L. digitata* as a potential biomass for butanol production. For the first time, consumption of alginate components was observed by *C. beijerinckii* DSM-6422. The efficient utilization of sugars and lactic acid further highlighted the potential of using this strain for future development of large-scale cost-effective butanol production based on (ensiled) seaweed.

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1. Introduction

Researchers worldwide work on developing alternative sustainable energy production processes to reduce the greenhouse gas emissions from fossil fuels. While ethanol fermentation is the most mature biochemical process for liquid biofuels production, butanol fermentation receives increasing attention due to its advantages such as better gasoline-blending properties and higher energy density (Swana et al., 2011).

Seaweed represents a huge un-exploited marine bioresource consisting of thousands of species, of which the growth does not compete for arable land, fertilizer or fresh water resources with land plants. Seaweed biomass is composed of varieties of sugar polymers while containing low or no lignin. Thus, there is no need of harsh pretreatment which generally complicates the utilization of lignocellulosic biomasses (van Hal et al., 2014). The above merits show the promising potential of utilizing seaweed biomass as feedstock for production of 3rd generation liquid biofuels. The reported extremely high glucose content (56.7%) in a wild-growing brown seaweed species (*Laminaria digitata*, harvested in summer 2012 from Denmark) highlighted the suitability of using this seaweed biomass as a potential fuel feedstock (Hou et al., 2015). Moreover, as a native species in the northern Atlantic, substantial efforts for cultivation are on-going, with some documentation of high biomass yields (Burton et al., 2009). In the present paper, the potential of using *L. digitata*, harvested from the coast of Danish North Sea in the summer of 2014, as feedstock for biobutanol production was studied.

The main structural components of *L. digitata* are alginate and cellulose, while the main energy storage compounds are laminarin and mannitol. Alginate is composed of mannuronic acid and...
guluronic acid being covalently linked together in sequence. Cellulose consists of a β-(1,4)-linked glucose units. Laminarin consists of a β-(1,3) glucan chain as backbone with side chains of β-(1,6). Due to the high β-glucan content in this seaweed species, enzymes e.g. cellulases mixture with different β-glucanases and β-glucosidases were effective on liberation of glucose (Hou et al., 2015). The liberated glucose can be fermented to biobutanol by bacteria such as Clostridium spp.

Biobutanol production by Clostridium spp. takes place through the acetone-butanol-ethanol (ABE) fermentation pathway. It is a complex two-phase hetero-fermentative process, where a wide spectrum of substrates, including monomers (e.g. glucose, mannitol) and polymers (e.g. gums), can be converted to various intermediates and end products, of which butanol is normally found in the highest fraction. The conversion process can be divided into acidogenesis, where organic acids such as lactic acid, acetic acid, and butyric acid are mainly formed, followed by solventogenesis where the organic acids are re-fermented for the production of the solvents acetone, butanol and ethanol. Thus, the organic acids can be regarded as both byproducts and substrates of ABE fermentation (Jones and Woods, 1986).

Acetone, as one of the final products of ABE fermentation, is not favorable due to its recovery cost and incompatibility to rubber engine parts. Hence, reducing acetone production relative to butanol is preferred for the economic feasibility of butanol fermentation. However, production of butanol without co-production of acetone and acids using solvent-producing Clostridia is difficult due to the requirement for cellular redox balance (Jones and Woods, 1986).

The fermentation strain used in this study, Clostridium beijerinckii DSM-6422 (also known as C. butylicum NNRL B-592 and VPI 13436), is in general well described as a butanol-producing organism. This strain is phenotypically closely related to the ABE fermentation model strain C. acetobutylicum ATCC 824, but genetically quite distinct (Johnson et al., 1997). Up to date, only few seaweed species have been tested as substrate for butanol fermentation by bacterial strains from class of Clostridia, and the produced butanol yields are in the range of 0.1–0.4 g/L (Potts et al., 2012; Huesemann et al., 2012; van der Wal et al., 2013; Bikker et al., 2016). This study for the first time tested the brown seaweed L. digitata as substrate for butanol fermentation by the strain of Clostridium beijerinckii DSM-6422.

2. Material and methods

2.1. Material

Wild grown sugar-rich L. digitata was collected in August 2014 from the coast of Danish North Sea, and was stored in freezer (−20 °C) after harvesting until use. After thawing, the material was prepared by oven drying at 50 °C and milling according to the procedures as described by Hou et al. (2015). This batch of wild harvested L. digitata contained 47.8% DM glucose (the equivalent from gums), 4.1% DM mannitol, 5.6% DM crude protein and 13.3% DM ash, as determined by the published composition analysis methods (Hou et al., 2015).

2.2. Enzymatic hydrolysis

The prepared L. digitata was re-dried in plastic bags in a ventilated oven at 70 °C overnight. 30 kg of the re-dried material was added to 600 L water in pre-disinfected 800 L steel reactor with continuous stirring. pH was adjusted to 5.04 by 20% H₂PO₄ and temperature was regulated to 45 °C. Cellulases mixture (NS81016; Novozymes A/S), which contained mixture of different β-glucanases and β-glucosidases, was added corresponding to an enzyme loading of 10 % (v/w) of seaweed dry weight. The hydrolysis process was stopped after 24 h, due to the detection of contamination signs e.g. consumption of glucose (data not shown, paper in preparation). 96.5 L liquid fraction (hydrolysate) was collected by pumping from the top of the reactor, after 2 h sedimentation. The collected liquid i.e. seaweed hydrolysate was stored at −18 °C, until further investigations (Section 2.4).

2.3. Inoculum preparation

Freeze dried culture of Clostridium beijerinckii DSM-6422 (DSMZ, Germany) was propagated in 50 ml anaerobic vials with 28 ml N₂ flushed cultivation medium (glucose 10 g/L, peptone 10 g/L, beef extract 10 g/L, yeast extract 3 g/L, NaCl 5 g/L, sodium acetate 3 g/L, resazurin (0.025 % w/v) 4 ml/L, L-cysteine HCl 0.5 g/L). The vials were incubated at 36.5 °C in an incubator (Grant Bio, ES-20) with horizontal shaking speed at 120 rpm. The cell growth was monitored by optical density at 600 nm (OD₆₀₀) by spectrophotometer (V-1200, VWR). When OD₆₀₀ reached 1.5–2, 4 ml of the cultivated cells were transferred into 60 mL fresh medium in 100 ml anaerobic vials. These vials were incubated under the same conditions (36.5 °C, 120 rpm) until OD₆₀₀ reached to 1.5–2 before being inoculated to the prepared seaweed hydrolysate for butanol fermentation.

2.4. Butanol fermentation

The butanol fermentations were performed as duplicates in 100 ml anaerobic vials with 60 ml working volume of the thawed seaweed hydrolysate stored in −18 °C (Section 2.2) and the control medium (nutrient-rich medium: glucose 12.5 g/L, mannitol 4.0 g/L, peptone 10 g/L, beef extract 10 g/L, yeast extract 3 g/L, NaCl 5 g/L, sodium acetate 3 g/L, resazurin (0.025 % w/v) 4 ml/L, L-cysteine HCl 0.5 g/L). Sodium acetate was added in the control medium to improve and stabilize solvent production by C. beijerinckii (Chen and Blaschek, 1999). All the vials were flushed with N₂ for 50 min and pH of both the seaweed hydrolysate and the control medium were adjusted to 6.8–7, before being autoclaved at 121 °C for 15 min. After cooling the vials to room temperature, 4 ml prepared inoculum culture (OD₆₀₀ = 1.5–2) was inoculated into 60 ml medium under anaerobic condition. The vials were incubated at 36.5 °C in the orbital shaker-incubator with rotation speed of 120 rpm. The fermentation was followed for 213 h by taking samples under sterilized condition at 0, 11, 26.5, 50.5, 101, and 213 h. pH of the samples was recorded and the samples were stored at −18 °C until analysis.

2.5. Chemical analysis

Glucose, mannitol and lactate content were quantified by an HPLC system equipped with an Aminex HPX-87H column (Bio-Rad Laboratories Ltd., USA), running at 63 °C with 4 mM H₂SO₄ as eluent at a flow rate of 0.6 ml/min. Acetate, butyrate, acetone, ethanol and butanol content were analyzed by gas chromatography (GC; Hewlett Packard, HPS890 series II) equipped with a flame ionization detector (FID) and a HP FFAP column (30 m × 0.53 mm × 1.0 µm). Content of glucose (for soluble glu- can), guluronic acid and manuronic acid was analyzed both directly and after weak acid hydrolysis (in 1 M H₂SO₄ at 100 °C for 2 h) in the samples before and after fermentation. In case of acid post-hydrolysis, samples were first neutralized with BaCO₃ using bromophenol blue as indicator (final pH > 4.6, max 8.2). Analysis was performed using HPAEC-PAD (ICS3000, Dionex, Sunnyvale, CA) equipped with a CarboPac PA1 column, a guard column and post-column addition of NaOH. After sample injection while using 15 mM NaOH as mobile phase, pure water was used first.
as eluent for sugar alcohols and reducing sugars and, subsequently, a gradient of NaOH and Na acetate was used as eluent for uronic acids. Standards of guluronic acid and mannuronic acid were purchased as sodium salt from CarboSynth Limited (UK).

2.6. Calculations

The butanol yields in this study are based on: 1) gram of produced butanol divided by gram of total fermentable substrates; 2) gram of produced butanol divided by total consumed substrates.

Butanol:ABE ratios are based on both weights and moles.

Carbon recovery was calculated as carbon yield of all the organic products after fermentation (C-mol). Acetate and lactate can be both products (from acidogenic phase) and substrates (from solventogenic phase), of which the reactions are reversible (Jones and Woods, 1986). Acetate/lactate was thus considered as product or substrate for the final carbon recovery calculation, depending on its final net accumulation or consumption after fermentation. Overall, the following formula was used:

\[
\text{% Carbon recovery} = \frac{\text{Produced [2-}M(\text{ethanol} + \text{acetate}) + 3-M(\text{acetone} + \text{lactate}) + 4-M\text{butyrate} + 4-M\text{butanol}]}{\text{Consumed [6-M(\text{glucose} + \text{mannitol} + \text{mannuronic acid} + \text{guluronic acid})] + 3-M(\text{glycerol} + \text{lactate}) + 2-M\text{acetate}}} \]

3. Results and discussion

3.1. Butanol fermentation in nutrient-rich control medium

First, a control experiment was performed to compare the fermentation performance and ensure the accuracy of the analysis. The results showed that the fermentation process was fast. After 11 h, 50% glucose was consumed, along with the production of ABE (Fig. 1A). Sequential utilization glucose and mannitol was observed i.e. mannitol consumption initiated after ~50 h when 93% glucose was consumed (Fig. 1A). This is due to the glucose repression on the PTS (phosphotransferase system) by which mannitol is transported into cells of C. beijerinckii (Mitchell et al., 1995). Further, it may take time for the cells to activate the production of enzymes associated with mannitol utilization (e.g. mannitol-1-phosphate dehydrogenase), which may not be required by the inoculum culture grown in the medium without mannitol. Moreover, mannitol may be more favored for butyrate production rather than for butanol production, due to its more reduced form than glucose (Crabbendam et al., 1985; Huesemann et al., 2012). In the control medium, C. beijerinckii DSM 6422 showed almost full consumption of glucose and mannitol (Fig. 1A, Table 1). The final butanol:ABE molar ratio of 0.66 (Table 1) corresponds well with the typical theoretical ratio of 0.6 (Jones and Woods, 1986). The butanol yield of 0.26 g/g-converted total substrate (Table 1) is within the normally experienced range (Jones and Woods, 1986). It is worth mentioning that the exponential growth phase had already passed at the second sampling (t = 11 h) by measurement of OD600 (Fig. 1A), and no obvious cell growth was observed after 25 h. Total carbon recovery of the fermentation was calculated to be 85.3%, due to the non-measured CO2 production and carbons accumulated in the produced bacterial cell biomass (Jones and Woods, 1986). Altogether, the control experiment mapped the fermentation characteristics of C. beijerinckii DSM 6422 and verified the accuracy of the experiments and analysis.

3.2. Butanol fermentation in seaweed hydrolysate

The composition of the seaweed hydrolysate is listed in Table 1. In addition to the expected components such as glucose and mannitol, components of lactic acid, glycerol, mannuronic acid and guluronic acids were also detected. The minor amount of alginate compounds (guluronic and mannuronic acid monomers and oligomers) could result from the partial hydrolysis by organic acids of a small amount of released alginate from the cellulose matrix broken by cellulases. Lactic acid and glycerol in the hydrolysate could have been produced by e.g. the natural seaweed-associated bacteria (Singh and Reddy, 2014) responsible for the observed contamination during the pilot scale enzymatic hydrolysis.

Fig. 1B shows the fermentation performance in the seaweed hydrolysate. As discussed in the Section 3.1, a similar pattern of sequential utilization of glucose-mannitol was observed i.e. mannitol utilization initiated when 90% of glucose was consumed. This was also reported by López-Contreras et al. (2014) for ABE fermentation of brown seaweed. In addition, 46% of the soluble glucan in the hydrolysate was utilized by C. beijerinckii DSM 6422 (Table 1), which could be due to the strain’s ability of production and excretion of saccharolytic enzymes (Huesemann et al., 2012). pH (initially 6.8) dropped rapidly to a constant level (5.4) after ~11 h, which was in accordance with the stop of acetate and butyrate production, indicating a fast transfer of acidogenesis to solventogenesis (Fig. 1B). The consumption of substrate i.e. glucose, glucan and mannitol and the production of solvents i.e. acetone, butanol and ethanol stopped after ~100 h (Fig. 1B), indicating cell inactivation. Such inactivation could be caused by e.g. accumulation of produced butanol (usually occurring at higher butanol concentrations of 10–20 g/L, Moon et al., 2016) or cell sporation (usually occurring concomitantly with active solventogenesis, as observed in this study, Zheng et al., 2009).

As mentioned above, the seaweed hydrolysate contained small amounts of alginate compounds i.e. monomers and oligomers of mannuronic and guluronic acid. Interestingly, some consumption of both guluronic acid monomer and oligomer, as well as mannuronic acid monomer and soluble oligomer was observed (Table 1). Since alginate can account up to 40% dry weight of brown seaweed biomass (Jung et al., 2013), it seems meaningful to identify the ionic acid metabolism (i.e. to which metabolites alginate compounds are converted) in C. beijerinckii.

In addition, it was observed that lactic acid in the seaweed hydrolysate (1.33 g/L) was completely consumed by C. beijerinckii DSM 6422. Previous studies reported that fermentation by Clostridium strains of combined sugars and lactic acid at suitable ratios resulted in the conversion of lactic acid to butanol, due to the compensation of redox power from sugar metabolism (Yoshida et al., 2012). Nowadays, the interest in utilizing seaweeds for bioenergy production is substantially increasing. However, finding a cost- and energy-efficient preservation method for seaweed biomass with typically high water content (e.g. 80–90%) is still a challenge. Ensilage is considered a promising preservation method with comparatively low energy input (Herrmann et al., 2015). The ensiled seaweed biomass contains lactic acid and C5 and C6 sugars. The capacity of converting lactic acid in combination with C5 and C6 sugars to ABE by Clostridium indicates the potential of using ensiled seaweed biomass for liquid biofuel production, based on which a cost-effective seaweed-butanol production process could be developed.

Reviewing literature, only a few seaweed species i.e. brown seaweed Saccharina spp. (Huesemann et al., 2012; López-Contreras et al., 2014) and green seaweed Ulva spp. (Potts et al.,
Fig. 1. Substrate consumption and product formation by *C. beijerinckii* DSM 6422 during the fermentation on (A) control medium and (B) enzymatic hydrolysate of seaweed *L. digitata*. Data presented are average of two independent experiments and error bars indicate the ranges of measurements. ×: Glucose, +: Mannitol, ▲: Acetate, ■: Butyrate, ▲: Acetone, ●: Butanol, ■: Ethanol, ◇: OD600, △: pH.
referred study (0.02 g/L/h; Huesemann et al., 2012), an average experiment (0.05 g/L/h) and also significantly higher than in the control (0.12 g/L/h during the first 50.5 h) was higher than in the control that although the volumetric productivity in seaweed hydrolysate by upscaled fermentation with e.g. continuous mode. All the interesting findings presented are the average of duplicates.

Table 1
<table>
<thead>
<tr>
<th>Substrate (g/L)</th>
<th>Seaweed hydrolysate</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before fermentation</td>
<td>After fermentation</td>
</tr>
<tr>
<td>Glucose monomer</td>
<td>9.02</td>
<td>1.38</td>
</tr>
<tr>
<td>Glucose from soluble glucan</td>
<td>11.11</td>
<td>6.02</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.31</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>4.58</td>
<td>1.82</td>
</tr>
<tr>
<td>Mannuronic acid monomer</td>
<td>0.034</td>
<td>0.027</td>
</tr>
<tr>
<td>Mannuronic acid from soluble oligomer</td>
<td>0.027</td>
<td>0.018</td>
</tr>
<tr>
<td>Guluronic acid monomer</td>
<td>0.016</td>
<td>0.004</td>
</tr>
<tr>
<td>Guluronic acid from soluble oligomer</td>
<td>0.068</td>
<td>0.059</td>
</tr>
<tr>
<td>Product (g/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butanol</td>
<td>-</td>
<td>7.16</td>
</tr>
<tr>
<td>Acetate</td>
<td>-</td>
<td>0.85</td>
</tr>
<tr>
<td>Ethanol</td>
<td>-</td>
<td>0.12</td>
</tr>
<tr>
<td>Butyrate</td>
<td>-</td>
<td>1.01</td>
</tr>
<tr>
<td>Substrate/product (g/L)</td>
<td>Acetic acid</td>
<td>0.32</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>1.33</td>
<td>-</td>
</tr>
<tr>
<td>Butanol Yield g/g-total-substrate</td>
<td>0.27</td>
<td>-</td>
</tr>
<tr>
<td>g/g-consumed-substrate</td>
<td>0.42</td>
<td>-</td>
</tr>
<tr>
<td>Butanol:ABE molar:molar</td>
<td>0.85</td>
<td>-</td>
</tr>
<tr>
<td>g/g</td>
<td>0.88</td>
<td>-</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>89.3</td>
<td>-</td>
</tr>
</tbody>
</table>

- Under detection limit. NA: Not applicable.
- Acetic acid and lactic acid can be both product (from acidogenic phase) and substrate (for solventogenic phase). For the calculation of final carbon yield, if acetate or lactate was finally accumulated in the medium, it was considered as product; if acetate or lactate is consumed after fermentation, it was considered as substrate. Data presented are the average of duplicates.

4. Conclusions

This study showed that the enzymatic hydrolysate of brown seaweed *L. digitata* favored the metabolic carbon flux in *Clostridium beijerinckii* DSM-6422 towards butanol production, resulting in a high butanol:ABE molar ratio and a high butanol yield. The efficient utilization of sugars, lactic acid, and for the first time reported, alginic components by this strain highlighted its potential for being used in future development of cost-effective (ensiling-based) seaweed-butanol industrial process.

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