Influence of substrate carbon on the metabolism of Clostridium thermohydrosulfuricum

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1. SUMMARY

The concentration of carbon sources has a significant influence on the growth, carbohydrate uptake and metabolite distribution in Clostridium thermohydrosulfuricum. The growing concentrations of glucose or starch increase the production of ethanol and lactate, the intracellular fructose-1,6-diphosphate (FDP) and the specific activity of lactate dehydrogenase (LDH), but decrease the ethanol/lactate ratio.

2. INTRODUCTION

C. thermohydrosulfuricum is able to ferment a wide variety of carbohydrates into ethanol, lactate, acetate, CO₂, and hydrogen. The 'associated' production of acetate and hydrogen is low and the direction of carbon units towards lactate biosynthesis decreases the ethanol yield. The nature and concentration of the carbon substrate influence the production of lactate, the specific activity of LDH and the intracellular level of FDP in Clostridium thermohydrosulfuricum [2]. We have studied the influence of glucose and starch on the growth, carbohydrate consumption, production of excretable metabolites, synthesis of LDH and the intracellular level of FDP in Clostridium thermohydrosulfuricum.

3. MATERIALS AND METHODS

3.1. Microorganism and culture conditions

Clostridium thermohydrosulfuricum 39E was isolated from Octopus Spring at Yellowstone National Park [3] and deposited at ATCC (ATCC 33223).

We used the fermentation medium proposed by Wiegel et al. [4] and modified by Mancuso [5], but did not add vitamins. The medium contained per litre (distilled water): Na₂HPO₄·12H₂O, 43.0 g; KH₂PO₄, 5.6 g; NH₄Cl, 0.5 g; mineral solution, 10.0 ml; resazurine, 2 mg; cysteine hydrochloride monohydrate, 0.6 g; yeast extract (YE) (Biomérieux); carbohydrate. The concentrations of YE and carbohydrate are indicated in the results.

The medium was prepared under anaerobic conditions and put into 250 ml culture bottles (Pyrex) under pressure of nitrogen. The pH was adjusted to 7.5 with 4 N NaOH. For the culture with constant pH, we used a 2 l fermentor (Bio-lafitte) under nitrogen pressure. It contained 1 l of medium with lower phosphate concentration (20
mM). The pH was maintained at 7.2 by addition of 2 N NaOH. The phosphate, the carbohydrates and the rest of the medium were separately sterilized at 121°C for 30 min. The culture bottles and the fermentor were inoculated with 2% (v/v) inoculum sampled from a preculture at exponential growth phase and incubated at 65°C. Agitation rate of the fermentation is 60 rpm. For the measurement of maximum substrate consumption, product formation and yields of fermentations, we took 10 ml samples at the beginning and the end of each fermentation process. Every hour a 3 ml volume was also taken during the course of fermentation.

3.2. Measurements of growth, carbon substrate and metabolites of the fermentation

The cell growth was followed by measuring the optical density at 660 nm in a Beckman DB-G spectrophotometer. One unit of optical density corresponds to 520 mg 1-1 of dry cell weight. The samples for the determinations of carbohydrates and products were centrifuged at 30,000 × g for 15 min at 3°C in a Beckman L5-50B ultracentrifuge. The supernatant solution was stored at 3°C. Glucose was determined by the hexokinase method (Glucouquant Test Combination – Boehringer) in a Technicon II autoanalyser. Starch determination was carried out by the phenol method using the Beckman DB-G spectrophotometer [6]. Ethanol and acetate were assayed by gas chromatography (chromatograph Intersmat IGC 121C). The 1-lactic acid assay was done by the enzymatic method using lactate dehydrogenase (Boehringer). The determination of FDP was described elsewhere [2].

3.3. Lactate dehydrogenase assay

The preparation method of the enzymatic extract was already described elsewhere [2]. The specific activity of the enzyme was determined by the method proposed by Vassault [7]. The reaction mixture of the assay (3.0 ml) contained: phosphate buffer pH 7.2, 100 mM; FDP, 0.2 mM; dithiothreitol, 2 mM, sodium pyruvate, 10 mM; NADH, 0.25 mM; enzymatic extract, 20 μl. The reaction which took place under anaerobic conditions at 30°C was followed at 340 nm. The enzymatic activity was expressed as enzyme units (U) which corresponds to the quantity of the enzyme necessary to transform 1 μmol of substrate into product(s) per min and its specific activity (Ac) was then expressed as U per mg of protein. The total protein determination was carried out according to the method of Lowry et al. [8].

4. RESULTS

4.1. Influence of glucose concentration on the growth and the production of metabolites

We studied the influence of different concentrations of glucose on the metabolism of Clostridium thermohydrosulfuricum growing on media containing 5 or 20 g l-1 of yeast extract (YE).

In the culture containing 5 g l-1 of YE as shown in Fig. 1a, an initial concentration of glucose less than 12 g l-1 was found to be totally consumed at the end of the fermentation. On the other hand, the glucose uptake, the biomass formation and the production of ethanol and lactate increased only a little in the fermentations using glucose concentrations higher than 12 g l-1. The ethanol/lactate ratio (Yeth/lac) fell at a constant rate as the glucose concentrations increased. As can be seen in Fig. 3, the Yeth/lac of 1.4 mol mol-1 was attained with weak glucose concentrations, as compared to that of 0.65 mol mol-1 given by high glucose.

In the culture containing 20 g l-1 of YE as shown in Fig. 1b, an initial concentration of glucose less than 30 g l-1 was found to be totally consumed at the end of the fermentation. At above 30 g l-1 of initial glucose, the sugar consumption and the biomass and metabolite formations did not increase. Yeth/lac remained constant (1.4 mol mol-1) (Fig. 3).

The maximum production of ethanol corresponding to the total consumption of glucose was 2.1 g l-1 on the medium containing 5 g l-1 of YE and 12 g l-1 of glucose, and 8.0 g l-1 on 20 g l-1 of YE and 30 g l-1 of glucose. This indicates a 3.8-fold increase in the production of ethanol.
whereas that of lactate was 2.4-fold greater under the same conditions.

At the end of pH controlled shake-flask fermentations with 20 g l\(^{-1}\) of YE, 40 g l\(^{-1}\) of glucose and 150 mM of phosphate at an initial pH of 7.5, the pH value fell to 5.3. With fermentations using the same YE and glucose concentrations but maintaining the pH at 7.2, the metabolite productions were not significantly different. Therefore, the limitations of growth and production were not due to changes in pH.

4.2. Influence of starch on growth and fermentation

As shown in Fig. 2, the evolution of growth, starch consumption and metabolic productions as functions of initial starch concentrations were found to be the same as those obtained with glucose media. Starch was not totally consumed even though the YE concentration was not limited. The consumption was found to be not more than 80% of the initial starch concentration less than 10

Fig. 1. Influence of initial glucose concentrations on growth, consumption of carbohydrate and the production of metabolites in *Clostridium thermohydrosulfuricum*. Fermentation media contain (a) 5 g l\(^{-1}\) or (b) 20 g l\(^{-1}\) YE, different concentrations of glucose and 150 mM phosphate buffer (initial pH 7.5).

Fig. 2. Influence of starch on the metabolism of *Clostridium thermohydrosulfuricum*. Fermentations in media containing different concentrations of starch and 5 g l\(^{-1}\) of YE were carried out under the same conditions as described in Fig. 1.
The highest values of ethanol and biomass were achieved with 15 g l\(^{-1}\) of starch. At higher starch concentrations YE became limiting and the lactate production was favored, but \(\alpha_{\text{eth/lac}}\) decreased. This decrease in the yield factor appears to be more prominent where there was a limitation in nitrogen source (Fig. 3).

### Table 1

<table>
<thead>
<tr>
<th>YE (g l(^{-1}))</th>
<th>Glucose (g l(^{-1}))</th>
<th>Starch (g l(^{-1}))</th>
<th>Ac LDH (U (mg prot)(^{-1}))</th>
<th>FDP ((\mu)mol gX(^{-1}))</th>
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\(\text{gX} = \text{gram of dry cell weight.}\)

### 4.3. Influence of substrate carbon on the activity of LDH and the intracellular formation of FDP

As shown in Table 1, the specific activity of LDH in the harvested cells growing at the exponential phase on glucose or starch medium with the same YE concentration as described in the former case, increased in parallel with the increment in the initial concentrations of these two carbon sources. For the same glucose concentration, the specific activity of this enzyme was found to decrease as the YE concentration increased.

The intracellular level of FDP varied in the same manner as the LDH activity. The former increased with the growing concentration of carbohydrate but decreased with that of YE. For the same ratio YE: glucose, the intracellular FDP concentrations and the specific activities of LDH appeared to be identical.

### 5. DISCUSSION

We have shown that carbon substrate concentration influences the production of ethanol and L-lactate and the relative yield of these metabolites in *Clostridium thermohydrosulfuricum*. The ratio ethanol:lactate decreases with the carbohydrate concentration when the culture is limited by YE, but only a little variation in the ratio is observed where there is no limitation in the nitrogen source.

The LDH activity and the intracellular FDP level increase together with the developing concentrations of carbon source.

It is possible that the diminution of the ethanol/lactate ratio is partially due to increased activity of LDH resulting from increased biosynthesis and activation by an elevated FDP. The latter's effect on the LDH activity was already reported elsewhere, but FDP is not necessary for the activity of this enzyme [2].

Ben-Bassat et al. [1] found significant differences in the level of intracellular FDP and the production of lactate according to the nature of the carbon substrate in *Thermoanaerobium brockii*. It is thus noticed that in the media containing glucose as the sole carbon source the intracellular FDP levels were 25 times higher than those in
media containing starch. The ethanol/lactate ratio was 4 times higher with starch than with glucose. We did not find any significant difference in the ethanol yields in relation to the nature of the carbon substrate.

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REFERENCES


