ETHANOL AND HYDROGEN PRODUCTION FROM AGRICULTURAL RESIDUES BY HYPERTHERMOPHILES

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Abstract

Agricultural residues are abundant non-food valued materials that can be used for production of renewable energy sources. The major obstacle for bioconversion of the residues is the utilization of insoluble hemi/cellulose by microorganisms. Various methods for the pretreatment of the materials have been developed, but problems appear because of toxicity and pollution among many others. It is highly desirable to use minimal pretreatment of the raw materials and achieve maximum bioconversion to renewable energy sources, such as ethanol and hydrogen. Hyperthermophiles are a group of microorganisms that can grow at temperatures at 90°C and above. They possess highly thermostable hydrolytic enzymes, such as xylanase, cellulase, amylase and more, which have great potential in the application of bioconversion of agricultural residues. Several hyperthermophilic strains, such as *Thermotoga* sp. *Pyrococcus* sp. and *Thermococcus* sp. were used to investigate their capability of producing ethanol and hydrogen. Results obtained indicate that they are ethanol and hydrogen producers and show advantage of bioconversion at high temperatures. Key enzymes functioning in the bioconversion process were also characterized and they may be useful for future development of novel biotechnology.

Introduction

Consuming fossil energy sources, such as oil and coal, imposes negative impact on our environments. Renewable fuels that can be derived from biological materials including all the agricultural, forestry and municipal solid wastes are the alternative of our future energy source (1). Ethanol and hydrogen are considered among the major and most important biofuels, which can be obtained by the fermentation of biomass using microorganisms (2,3). In principal, biotechnological aspects of biomass-to-ethanol conversion are developed by using yeasts, *Zymomonas mobilis*, recombinant *Escherichia coli* and *Clostridia*. However, no single microorganism is suitable to fulfill all of the requirements, which are high productivity, selectivity and broad substrate utilization to deal with different nature of the various types of biomass. Furthermore, none of them is capable of growing at high temperatures. Therefore, enhanced thermotolerance of fermenting organisms and the use of thermostable enzymes are among the highly desirable traits. In addition, biological hydrogen production has been investigated using various microorganisms, which have showed great potential of high efficiency of biomass-to-hydrogen conversion. Agricultural residues are a major type of non-food valued biomass that can be used for producing biofuels. However, their major components hemi/cellulose are not readily accessible to most of the microorganisms, and various methods of pretreatment are required, which may generate toxic byproducts and increase operation cost. It is possible that the bioconversion rate of this type of biomass may be better because of higher solubility and better enzymatic activities are available at high temperatures.

Hyperthermophilic microorganisms grow optimally at temperatures of 80°C and above, which include both *Archaea* and *Bacteria* (4). They possess the most thermostable enzymes known. Those related to the hydrolysis of biomass are α-amylases, α-glucosidases, pullulanases, xylanases, cellobiohydrolase, β-xylosidases, β-glucosidases and proteinases (5, 6). Their sugar metabolism involves both Entner-Doudoroff and Embden-Meyerhof pathways in which pyruvate is a metabolic intermediate (7). Furthermore, some of them have novel thermostable pyruvate decarboxylase and alcohol dehydrogenase, which are key enzymes involved in ethanol production (8, 9). They also possess thermostable hydrogenases that catalyze the production of hydrogen from the utilization of different biomass (10). Therefore, they are in principal able to convert various types of biomass into ethanol and hydrogen. However, there is no information available for the direct application of any of these organisms in any industrial bioprocess. Our goal is to develop a continuous fermentation and an on-line recovery system for the production of ethanol and hydrogen from biomass including agricultural residues.

Results

1. Ethanol and hydrogen were produced by hyperthermophilic microorganisms (Table 1)
2. Agricultural residues and biopolymers were used for the production of ethanol and hydrogen by *Thermotoga hypogea* (Fig. 1), *Thermotoga maritima* (Fig. 2) and *Thermotoga neapolitana* (Fig. 3).
3. High cell density of *T. neapolitana* promoted higher production of hydrogen up to 30% (Fig. 4).
4. The use of buffer in the growth media resulted in the highest production of hydrogen near 40% (Fig. 5)
5. Thermostable xylanase was isolated from *T. hypogea* (Table 2).

Table 1. Production of Ethanol and Hydrogen by Hyperthermophilic Microorganisms

Microorganisms	Growth Temp. (T_{opt}, °C)	Substrates	Production	
			Ethanol (mM)	Hydrogen (%)
Pyrococcus furiosus	100	Maltose, starch, cellobiose	0.1 - 0.8	24 - 33
Thermococcus guaymasensis	98	Glucose, starch, maltose	0.2 - 0.5	19 - 30
T. maritima	80	Glucose, starch, maltose, xylan	0.1 - 0.5	14 - 15
T. neapolitana	80	Glucose, starch, cellobiose, cellulose, xylose, xylan, wheat straw, barley straw, corn husk	1 - 2	4 - 40
T. hypogea	70	Glucose, fructose, sucrose, maltose, starch, cellobiose, cellulose, xylose, xylan, wheat straw, barley straw, corn husk, corn cob, soybean straw	0.1 - 0.4	3 - 16

Results

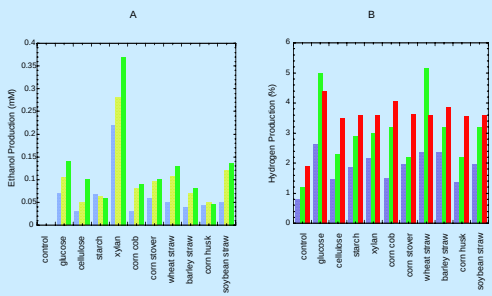

Figure 1. Ethanol (A) and hydrogen (B) production from different substrates by *T. hypogea*. A: blue bar, 14 hours; yellow bar, 48 hours; green bar 90 hours. B: blue bar, 24 hours; green bar, 48 hours; red bar, 90 hours.

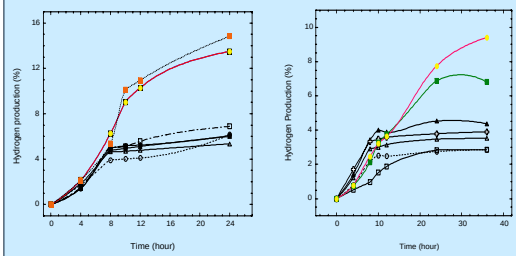

Figure 2. Hydrogen production by *T. maritima* using various substrates. Open circle, control; orange square, glucose; filled yellow circle, xylan; filled triangle, barley straw; filled black circle, wheat straw; open square, corn stover; filled black square, corn husk; open triangle, soybean straw.

Figure 3. Hydrogen production by *T. neapolitana* using various substrates. Open circle, control; filled yellow circle, xylan; filled green circle, corn husk; filled triangle, barley straw; open cross, wheat straw; open square, corn stover; open triangle, soybean straw.

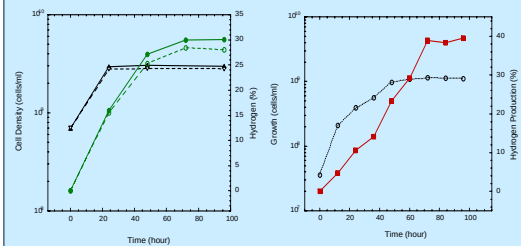

Figure 4. Growth and H_2 production by *T. neapolitana* at high cell density. Filled triangle, growth without air; open triangle, growth in the presence of 2% (v/v) air; filled circle, hydrogen production from the growth without air; open circle, hydrogen production from the growth in the presence of 2% (v/v) air.

Figure 5. Growth and H_2 production by *T. neapolitana* with buffer present in the media. 50 mM Tris. Open triangle, growth (cell density); filled square, hydrogen production.

Table 2. Properties of xylanase of *T. hypogea*

	Characteristics
Location	Xylanase activity was found to be cell-associated but not membrane-bound (3-8 U/mg); virtually no activity in the growth media supernatant
Purified enzyme	Molecular weight: 39 kDa Optimal pH: 5.5 Optimal temperature: 90°C Apparent K_m for xylan: 0.07% (w/v) Apparent V_{max}: 2500 U/mg $t_{1/2}$ at 90°C: 110 min Products of hydrolysis of xylan: xylobiose and oligosaccharides $[X]_n$ ($n \geq 3$)

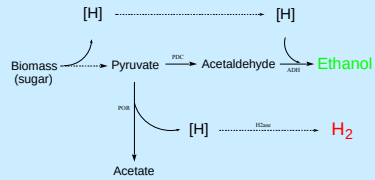

Figure 6. Fermentation of ethanol and hydrogen by hyperthermophilic microorganisms - a tentative model. [H], reducing equivalents; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; POR, pyruvate ferredoxin oxidoreductase; H2ase, hydrogenase

Discussion and Summary

It is clearly showed that all the microorganisms tested have the ability to produce ethanol and hydrogen, which indicates that all enzymes involved in the metabolic pathways are present. Alcohol dehydrogenase and hydrogenase activities were detected (unpublished results of our laboratory). Pyruvate decarboxylase was also detected previously (8). However, no CoA-dependent aldehyde dehydrogenase has been found in hyperthermophiles yet. It is likely that only two enzymes are involved in the production of ethanol from pyruvate (Fig. 6). Hydrogenases can use either ferredoxin or NAD(P)H as electron donors for the production of hydrogen (11). Small amount of oxygen had almost no effect on both growth and hydrogen production by *T. neapolitana* (Fig. 4), which may be advantageous to large-scale fermentation. This result is consistent with previously published results but no stimulation of hydrogen production was observed in the presence of oxygen (12).

Compared to ethanol production, hydrogen was produced in a much higher rate. Based on sugar metabolic pathways that are operating in these microorganisms (7), hydrogen production favors a metabolism for more efficient energy conservation, by which growth rate is higher. It is possible to decrease the hydrogen production for shifting the metabolism to ethanol production (Fig. 6). *Thermotoga* sp. may serve as a model system to study regulation of the metabolism of ethanol and hydrogen in hyperthermophiles. *T. neapolitana* can produce a very high level of hydrogen (~40%; Fig. 5), which will have great potential in application of biological hydrogen production. Higher production of hydrogen may still be achieved. Therefore, the very near term goal is to optimize the production of hydrogen using these microorganisms.

The hyperthermophiles used for this study have thermostable hydrolases (Table 2), however, they grew with a limited rate using agricultural residues (data not showed). This is not surprising because the residues are insoluble materials. Their accessibility at higher temperatures was expected to be higher but not high enough. Further investigation of both pretreatment methods and growth conditions will be required to maximize the efficiency of bioconversion of agricultural residues. New data obtained will provide further insight into the application of hyperthermophiles in producing renewable energy sources ethanol and hydrogen.

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