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Using the second law of thermodynamics for enrichment and isolation of microorganisms to produce fuel alcohols or hydrocarbons

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HIGHLIGHTS

- Thermodynamics determines net metabolite flow in fermentation processes.
- Gibbs energy change (ΔG) must be negative for reactions supporting microbial growth.
- ΔG was calculated for different fermentation conditions and products.
- Under certain conditions, ΔG was negative for making alcohols or alkanes.
- Under these conditions, organisms were isolated for biofuel production.

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ABSTRACT

Fermentation of crops, waste biomass, or gases has been proposed as a means to produce desired chemicals and renewable fuels. The second law of thermodynamics has been shown to determine the net direction of metabolite flow in fermentation processes. In this article, we describe a process to isolate and direct the evolution of microorganisms that convert cellulosic biomass or gaseous CO_2 and H_2 to biofuels such as ethanol, 1-butanol, butane, or hexane (among others). Mathematical models of fermentation elucidated sets of conditions that thermodynamically favor synthesis of desired products. When these conditions were applied to mixed cultures from the rumen of a cow, bacteria that produced alcohols or alkanes were isolated. The examples demonstrate the first use of thermodynamic analysis to isolate bacteria and control fermentation processes for biofuel production among other uses.

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1. Introduction

Fossil fuel extraction is becoming more difficult and expensive, and burning this fuel accelerates climate change. Therefore, there has been an intense interest in developing microorganisms that can ferment energy crops or waste biomass to renewable fuels (Ragauskas et al., 2006). Understanding what conditions allow organisms to thrive while producing desired products like alcohols or hydrocarbons may enable the use of microorganisms to produce these products.

Physical chemists have understood that all chemical reactions are controlled by kinetic or thermodynamic mechanisms (Chang, 1981). With kinetic control, the rates of reactions depend on substrate concentrations or enzyme activities, and these enzyme activities in turn may depend on microbial growth or enzyme synthesis. The profile of products formed depends on relative rates of different competing reactions. With thermodynamic control, the feasibility of reactions and the availability of pathway branches depend on the second law of thermodynamics. This law governs whether or not a reaction can proceed spontaneously in the forward direction based on the concentrations of reactants and products.

Biologists have focused on controlling kinetic elements of fermentation such as enzyme function, microbial activity, gene expression, or provision of substrates. However, the present analysis is based on the theory that fermentation is often controlled by thermodynamics. For example, in a mixed-culture anaerobic bioreactor (e.g. cow's rumen, and anaerobic digester) as soon as a glucose molecule is released by digestion of cellulose there are several microbes that can transport it into their cells and metabolize it to any number of products. The amount of energy that any particular organism can obtain depends on the concentration of all products of the reaction relative to all reactants. Since the free glucose concentration is very low due to competition among

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microorganisms in the fermentation, and the products of fermentation are removed slowly, only very efficient microbes can use the low concentration of glucose at all. And they can only use it when concentrations of the products which they produce are low. Therefore, when their products start to build up, they can no longer obtain Gibbs energy by converting the reactant to a product, and they leave the glucose behind for another microorganism that produces a different product. As the different products build up, the free glucose concentration increases enabling higher concentrations of the original products. In this way, a predictable ratio of products is produced.

In chemistry, whether or not a reaction can proceed spontaneously in the forward direction is represented by the change in Gibbs energy (ΔG), which can be calculated based on the ratio of products and reactants in the system (Chang, 1981). Using this calculation, a strongly negative ΔG indicates that the reaction could proceed strongly in the forward direction without the addition of energy to the system. A strongly positive value of ΔG indicates the reaction cannot proceed in the forward direction without addition of energy to the system, and it may even run in the reverse direction.

Previous research demonstrated by three different methods that the profile of products within some fermentation systems (e.g. cow's rumen) is explained as near-equilibrium in accordance with the second law of thermodynamics. First, the values of ΔG are near zero for many reactions in the rumen (Kohn and Boston, 2000; Ungerfeld and Kohn, 2006), or a manure or a sewage digester (Hoh and Cord-Ruwisch, 1997; Jackson and McInerney, 2002) including methane or acetic acid production from CO_2 and H_2 and interconversion of acids. Furthermore, the rates of interconversion of one acid to another are similar in each direction as measured using isotopic labels (Ungerfeld and Kohn, 2006). Finally, perturbation studies in which a certain acid is added to the rumen result in a decrease in production of the added acid and an increase in conversion to other acids (Seal and Parker, 1994). All of these results support the theory that the profile of acids and gases in these fermentation systems is near thermodynamic equilibrium.

The current studies use the theory describing the direction of metabolite flow in fermentation as a function of thermodynamics to define conditions for production of desired biofuels and for enrichment and isolation of bacteria that make those fuels. Since many fermentation systems are near equilibrium, when these systems are perturbed, the rates of reactions adjust to restore the equilibrium. In the process, microbes that carry out the favored reactions thrive and grow. Under these conditions, microbes can be enriched and isolated to produce desired products.

2. Materials and methods

Q2 Methods and compositions described are protected by U.S. Patent numbers 8,178,329 (Kohn and Kim, 2012a, 2012b) and Q3 8,535,921 (Kohn and Kim, 2013). Other U.S. and European patents are pending. All rights are reserved, and permission is required before using or making the patented inventions.

2.1. Digestion and fermentation.

In vitro digestion was orchestrated according to Goering and Van Soest (1970). Rumen fluid samples were taken from a non-lactating fistulated Holstein cow in accordance with the Institutional Animal Care and Use Committee guidelines. Contents from a cow's rumen, or other source when indicated, were prepared by blending for 1 min and straining through four layers of cheese cloth followed by glass wool. Buffers, macrominerals, microminerals, tryptic digest of casein, ammonia, sulfide, and cysteine reducing agents, and resazurin were used (Goering and Van Soest, 1970). Bicarbonate salts were replaced with equi-molar phosphate salts when not using carbon dioxide as the headspace gas. Starting pH of medium was 6.8.

These digestions were typically carried out in flasks or test tubes under anaerobic conditions at 39 °C. Media were boiled under 100% CO_2 or N_2 (for H_2 preparations). Carbon dioxide or nitrogen, when used as the headspace gases, was run through a copper column to remove residual O_2 before perfusing over rumen contents and into containers to maintain anaerobic conditions. The same media formulae were used for initial enrichment, agar roll tubes, broths, slant tubes, and agar plates. However, roll tubes, slant tubes and agar plates also contained 2% agar (BactoAgar). Media used for pure cultures (everything but the initial enrichments) were combined with 20% clarified rumen fluid (centrifuged at 12,000*g* for 30 min) to provide potential unknown growth factors.

2.2. Enrichment

Media usually contained 2% timothy hay ground through a 1-mm screen of a Wiley mill. In other cases, the source of energy was VFA, and in other cases only synthesis gases (e.g. CO_2 or CO and H_2) provided energy to select for organisms that grow on synthesis gases. The rationale for the different sources of carbohydrate or other source of energy was to enrich for microorganisms that could digest and utilize the type of carbohydrate for energy.

Media (45 ml) were transferred to each 140-ml Wheaton bottle under perfusion of gas, and rumen inocula (5 ml) were added to each flask. Flasks were incubated in a shaking water bath for at least 48 h. New flasks were prepared with 45 ml of media with additional energy, and 5 ml of subculture was added from the previous fermentation. These flasks were again incubated for 48 h or longer and again sub-sampled. Each enrichment process typically included at least three cycles of sub-culturing and growth. For each enrichment and roll tube, the temperature of incubation typically was 39 °C. To prevent wash out of some slower growing microorganisms, some enrichments were carried out for greater than 48 h. For example, enrichments in high alcohol medium or using CO_2 and H_2 for energy were continued for up to 10 days and ended when signs of active fermentation were evident.

The enrichments were conducted while maintaining conditions to favor enrichment of the microorganisms of interest. Such conditions are defined for specific examples that follow. In general, provision of undesired products (e.g. VFA, gases) in the fermentation shifts metabolism against further production of those products because the fermentations are near equilibrium. Therefore, the desired products were maintained in low concentration relative to the other products to increase further production of only the desired products.

2.3. Isolation

Isolation of microorganisms was carried out under conditions to select for organisms that are more likely to produce an alcohol, or produce an alkane greater than one-carbon in length. Specific modifications to isolate organisms that produce each type of product are provided as examples. In general, conditions were established to be thermodynamically favorable for desired end products and to be unfavorable for end products that were not desired.

Sterile roll tubes were used to isolate colonies from sources of microorganisms with or without enrichment (Hungate, 1950). The media were transferred to sterile tubes with agar media while still hot from autoclaving. Tubes were cooled to 55 °C and inoculated under H_2 and CO_2 pressures. The microbial inocula were diluted serially in media to obtain cultures from 1 to 10^{-14} viable cells per 0.5 ml inocula. Roll tubes were prepared for each level of dilution,

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