Utilization of protein-rich animal waste materials to produce biohydrogen

Ph.D. Thesis

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Introduction

Today, the generation of electricity, heating and transportation highly depend on fossil fuels. Combustion of natural gas, oil and coal releases enormous quantities of carbon dioxide, which is considered as the major cause of global warming and climate change. In addition, the formation of fossil fuels is much slower than their consumption rate and their depletion is expected within a couple of decades. Concerns about the limited availability of fossil fuels and their negative effect on the environment urge the scientific community to seek for clean energy carriers that can be produced from renewable sources. Hydrogen is among the best candidates since it burns to clean water vapor with zero carbon dioxide emission. It can be generated from many renewable energy sources including solar energy, hydropower, wind power and biomass.

A great number of microorganisms are known to produce hydrogen and the possible use of microbes for hydrogen production is extensively studied. Photosynthetic cyanobacteria, green algae and purple bacteria are potential candidates for solar energy driven biohydrogen production while fermentative bacteria and archaea are well suited for fermentative hydrogen evolution using cheap organic substrates.

Agriculture and related industries produce large quantities of by-products, which are rich in carbohydrates or peptides. Currently, these wastes are mainly disposed or decomposed through expensive procedures. In principle, these organic waste materials could be used to cover the biomass requirement of fermentative hydrogen-producing facilities. The combination of biological waste decomposition and fermentative biohydrogen production is economically very promising strategy as it can solve two problems at the same time. It offers an environmentally sound, cheap alternative for the treatment of agricultural wastes and additionally it produces environmentally energy carriers replacing fossil fuels.

Aims of the study

The aim of the present study was the development of a novel biological waste utilization system that combines microbial degradation of protein-rich animal waste materials (feathers, pig hair, meat meal) with the production of a useful product, biohydrogen.

1. **Construction of a two-stage fermentation system to utilize keratinaceous wastes for biohydrogen production**.
    - Evaluation of chicken feathers, digested by *B. licheniformis* KK1, as a carbon and energy source for dark hydrogen fermentation. Testing the ability of potential known hydrogen producer strains to utilize keratin hydrolysate for hydrogen evolution.
    - Optimization of the keratin degradation step to produce a fermentation broth that is ideal for the next hydrogen production step.
    - Optimization and scaling-up of the hydrogen-producing fermentation step.
    - Testing the two-stage system with additional keratin wastes (i.e. goose feathers, pig hair).
    - Determination of overall conversion yields for different keratinaceous wastes.

2. **Adaptation of the established two-stage system for meat meal.**
    - Evaluation of meat meal as a nutrient for dark hydrogen fermentation.
    - Scaling-up of the hydrogen production step on meat meal hydrolysate.
    - Determination of overall conversion yield for meat meal.

3. **Enhancing the waste degradation step using molecular biology techniques.**
    - Cloning and sequencing the keratinase gene from *B. licheniformis* KK1.
    - Heterologous expression of the keratinase in *E. coli.*

Methods

Hydrolysis of chicken feathers was carried out with *B. licheniformis* KK1 both in Erlenmeyer flasks and in 1 L glass vessels of a Braun Biostat DCU 3 fermenter. Feather degradation was followed by monitoring the changes in protein concentration and protein pattern of the fermentation broth over time. Hydrolysates obtained from the bacterial decomposition of feather waste were added to nutrient-stripped minimal media and tested in hydrogen producing fermentations with potential hydrogen evolving microorganisms (*Caldicellulosiruptor saccharolyticus*, *Escherichia coli* K12, *Thermococcus litoralis* and *Pyrococcus furiosus*). Microbial hydrogen production was followed by gas chromatography. In addition to chicken feather hydrolysate, fermentation broths prepared from goose feathers, pig hair and meat meal were also evaluated as possible nutrient sources for microbial hydrogen production. Scale-up studies on the hydrogen-producing fermentations with *T. litoralis* were performed in a 6.9 L batch fermenter.

The keratinase gene of *B. licheniformis* KK1 was amplified via high fidelity PCR and its nucleotide sequence was determined. DNA manipulations and analyses were performed according to standard techniques and the manufacturer's recommendations. For heterologous expression of the *kerA* in *E. coli* a gene expression cassette was constructed based on the pBAD/gIII system. Keratinase activity was assayed spectrofotometrically using the chromogenic peptide substrate N-succinyl-Ala-Ala-Pro-Phe-pNA.

Results

My results are summarized in the following points:

1. I have developed a minimal medium (CMSY) and methodology for the evaluation of numerous organic materials as nutrient sources for hyperthermophilic hydrogen-producing microorganisms.

2. Decomposition of chicken feather was performed in shaken Erlenmeyer-flasks using the *Bacillus licheniformis* KK1 strain, and I have proven that near complete degradation of feather occurs within 84 hours of incubation, which was accompanied by accumulation of small-sized peptides in the fermentation broth. I have determined the degradation time optimal for the concomitant hydrogen-producing fermentation.

3. I have evaluated several potential hydrogen-producing microorganisms (*Escherichia coli, Caldicellulosiruptor saccharolyticus T. litoralis* and *Pyrococcus furiosus*) and demonstrated *Thermococcus litoralis* to be the best candidate for the hydrogen-producing fermentation on keratin hydrolysate.

4. I have disclosed that milling is required for chicken feather fermentation carried out with stirring. I have achieved a 3.5-times scale-up of the keratin fermentation under well-controlled conditions. Monitoring the keratin degradation process in fermenter I have shown that the feather meal disappeared within 138 hours of incubation and, in paralel, peptides were accumulated in the fermentation broth.

5. I have proven that feather hydrolysate is a well-suited nutrient for *T. litoralis* comparable to the expensive commercial peptidic substrate, Bacto Peptone.

6. I have carried out a 125 times scale up of the hydrogen production step on feather hydrolysate using a high temperature fermenter. I have shown that the highest hydrogen concentration and the best overall conversion yield on feather hydrolysate can be achieved when the hydrogen fermentation step is carried out in fermenter.

7. I have adapted the two-stage fermentation system for the utilization of additional substrates including goose feather, pig hair and meat meal. I have thus proven that it is possible to combine the decomposition of numerous animal waste materials with the production of biohydrogen.

8. I have isolated the *kerA* gene coding for the keratinase in *B. licheniformis* KK1 and determined its nucleotide sequence.

9. I have created a protein overexpression construct for the production of the keratinase in *E. coli*. I have detected the presence of active keratinase in the induced periplasmic fraction of *E. coli* cells transformed with pBLK-BAD.

Publications related to the thesis

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