Waste management is one of the problems which have to be solved to be able to reach our overall goal towards a sustainable society. The amount of waste from industry, forestry, agriculture and household is continuously increasing, causing problems to environment and human health. However, the right treatment of different waste materials can make them as raw materials for producing some value-added products. In the connection of this very important actual development area this PhD thesis deals with the utilization of one of the most difficult waste streams, keratin rich materials, to produce alternative energy in the form of biohydrogen. This study aims to solve two problems at the same time by decreasing waste streams to land filling areas and also decreasing our dependence on fossil fuels.

The work covers two main areas, the development and scale up of two-stage fermentation system for the utilization of different protein-rich substrates for biohydrogen production followed by a molecular biotechnological study on construction of a protein overexpression system aiming to create a recombinant *E. coli* with keratinase activity. The thesis has a well-proportioned, extensive and perspicuous layout including 16 figures. The language is correct making the text of the thesis clear and distinct.

The *Introduction* part briefly summarizes the background and sets the goal of this work. The first main capital of the thesis gives an *Overview of the literature*, covering approximately half of the study. In this capital Balázs Bálint gives a professional review on his knowledge within four main areas connected to his studies. He starts with the characteristics and decomposition of protein-rich waste materials and then discusses possible ways for hydrogen production. I specially liked the presentation in the following two sub capitols dealing with hydrogenase enzymes, which play an important role in hydrogen producing metabolisms, and with different hyperthermophilic archea species responsible for hydrogen production.

The literature references are presented correctly, although I have a comment to Figure 1 on page 11, which shows the three-dimentional structure of [NiFe] hydrogenase.
purified from *Desulfovibrio gigas*. The connected reference which this figure was adapted from is missing. Similarly, the connected reference could not be found either to Figure 4 on page 18, showing hydrogenases and the hydrogen metabolism of *P. furiosus*.

The following two capitals Aims of the study and Material and methods both are accurate and correct.

The next capital, the Results, gives a very demonstrative picture of the overall work. This study is based on extensive laboratory work, using techniques from basic microbiological methods through process optimization and scale up in high performance bioreactors to modern techniques used in molecular biology, all leading to well-documented results. The well-designed experimental setups clearly give the answers for the investigated problems.

However, I have some comments. In sub capital 4.1 the decomposition of chicken feathers was investigated using *Bacillus licheniformis* KK1. Before performing the microbiological digestion the feather was pretreated by heating up the sample to 140 °C in for 20 minutes. Then the pretreated sample was mixed with the cultivation media and the mixture was transferred to the fermenter followed by sterilization at 125 °C in 20 minutes. This is actually an additional heat treatment for the feathers. Could only one of these two treatments result in the expected effect, leading in that case to a decrease in the overall energy demand of the process?

The efficiency of the protein degradation was followed by two different ways, measuring the solubilized protein concentration in the broth and detection of the protein pattern of the samples taken from the broth by polyacrylamide gel electrophoresis. Figure 6, page 32 presents the results from both measurement techniques. In the text there are statements explaining the one on Figure 6B, page 31: “After 138 h of treatment with *B. licheniformis* KK1, about 75% of the initially insoluble keratin was solubilized and could detected in the fermentation broth”; and then the other on Figure 6C, page 33: “Nearly complete degradation of chicken feather meal was observed in the fermenter within 138 hours of incubation”. These are two different conclusions regarding to the efficiency of the protein degradation in same samples taken from the same cultivation. In my opinion degradation rate of 75% is still not a nearly complete degradation.

Sub capital 4.2.4 deals with investigations on relative performance of feather meal hydrolysate vs standard substrates. On page 36 it is reported that “In this experiment, same amounts of feather hydrolysate or Bacto Peptone were used to feed *T. litoralis*” also the same confusing statement is found in figure text at Figure 9A, page 37: “Hydrogen
production of \textit{T. litoralis} on 42 and 84 mg of feather hydrolysate compared to cultures grown on the same amounts of Bacto Peptone. " Obviously, the Candidate means feather hydrolysate containing same amount of protein as the equivalent amount of Bacto Peptone. However, this clearly explanation I could only find in the published article \textit{(Appl Microbiol Biotechnol, 2005, 69:404-410)} in which this part of the study is reported.

The volume of the measured produced hydrogen depends on the actual temperature and pressure during each measurement. These actually measured gas volume data therefore usually are used for the calculation of the gas content at normal conditions and then reported as \( \text{NmL} \) produced gas in the headspace. I could not find any indication on at which conditions the accumulated hydrogen production is reported in the study. Moreover, the hydrogen production normalized on the amount of nutrients consumed can also be called as the hydrogen production yield and the hydrogen production normalized on the cell concentration is often called as the specific hydrogen production. Finally, I only would like to point out an erratum I have found on page 37. The hydrogen production normalized on the amount of nutrients is shown on Figure 9C and not on Figure 9B, as it is stated in the text.

The Discussion is a well-constructed capital taking several aspects and arguments into account, which all prove that Balázs Bálint is an independent researcher, who is able to summarize, critically analyze and conclude his results. That fact is also proved in his thesis statements there he briefly summarizes in 9 points the most important results of his study.

As a final point, I can only congratulate to this high quality research work which is additionally strengthened by the attached publications, six of them published in international journals. These articles are reviewed and accepted by international research authorities and they speak for themselves therefore, do not need any additional review. I set a high value on the two patents connected to this work too.

Balázs Bálint has demonstrated in his thesis that there is a possibility for biological hydrogen production based on waste materials as resources. However, as he also mentioned in the end of the thesis, so far, the economy of the investigated waste treatment system is hard to estimate. Nevertheless, I have one question I would like the Candidate to answer during the defense: Which kind of advantages and disadvantages, if any, biohydrogen has compared with other alternative renewable energy sources produced by biological methods, like biogas and bioethanol, especially taking the actual conditions in Hungary into account?
On the whole, I can conclude that Balázs Bálint presents here a very accurate PhD thesis based on extensive experimental work resulting in highly reliable data of excellent quality. I warmly recommend therefore, this thesis to be taken for public defense and the PhD degree to be obtained for the Candidate, Balázs Bálint, after he successfully completed the defense.

Borás, 2009.02.16.

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Review report on the PhD thesis of Balázs Bálint

Titled:
Utilization of protein-rich animal waste materials to produce biohydrogen

The thesis is complete and the structure of the thesis is well-proportioned and logical. It contains: a short summary of the premises, an overview of the literature, aims, methods, results, a discussion and conclusions of the work. There are no scientific nor terminological errors in the Abstract. The use of the English language is excellent and the thesis is written quite clearly throughout. Finally, the thesis possesses a very nice aesthetic quality.

The topic is currently of high interest in a twofold manner:
(i) it explores a potential solution to get rid of a risky slaughterhouse waste in a sustainable way as an attractive alternative to the conventional expensive and environmental unfriendly methods,
(ii) it uses the protein rich waste as a renewable source for producing hydrogen as an alternative to fossil fuels

In addition, the work described in this thesis builds on the contemporary status of the scientific knowledge of the field and brings it a step forward through:

- Developing and optimizing a hydrolysate production process using chicken feathers as substrate and a dedicated strain of Bacillus licheniformis for which a patent has been filed
- Selection of an appropriate microbial candidate to ferment the hydrolysate to hydrogen
- Testing the obtained best candidate on hydrolysates of other keratin sources
- Adaptation of the fermentation process to another substrate, i.e. meat meal, with a scaling-up step included
- An attempt to enhance keratin degradation through a concentrated keratin solution obtained via genetic engineering instead of whole-cell cultures

The literature was well studied concerning the various topics that were applied in the research, although some introduction on genetic engineering on Thermococcales would have been useful.

The introduction contained information regarding the structure and breakdown of keratins, manners of biological hydrogen production including the hydrogenases involved, and carbohydrate, peptide and hydrogen metabolism in the order Thermococcales, which is essential to perform the research. The Introduction builds on reliable background knowledge published in recently as well as in the past.

The study makes use of both classical and modern microbiological methods. The classical methods are especially applied to the way the microorganisms were cultivated.
The modern techniques were used in the genetic engineering of the keratinase gene, i.e. the cloning and heterologous expression techniques. Many steps were carried out with sophisticated preparation kits and use of PCR. The important fermentations were performed in controlled bioreactors, which are essential for this kind of microbial research.

The novel scientific results can be summarized as follows:

a) a new minimal medium has been developed for several species of the Thermococcales group
b) a methodology has been developed for evaluation of numerous organic materials as nutrient sources for hyperthermophilic hydrogen producing microorganisms
c) improvement of the degradation of chicken feathers by Bacillus licheniformis strain KK1 in an aerobic process
d) selection of an appropriate hydrogen-producing thermophile that can use keratin hydrolysate as a well-suited substrate
e) other appropriate proteinaceous animal wastes can be combined with the chicken feathers to form an adequate substrate for biohydrogen production
f) determination of the sequence of the keratinase gene in B. licheniformis KK1
g) overproducing this gene in E. coli

The candidate disseminated his work at various levels, i.e. as publications in well-known international peer-reviewed journals, as patents and as proceedings to symposia. In addition, the candidate has been involved at the same time in related research that resulted in more peer-reviewed publications.

In conclusion, the thesis is indeed suitable as a subject of defense and considering the work accomplished, published and described, the candidate deserves to obtain a PhD degree.

However, there are several questions for which I like to have an answer from the candidate:

1) Concerning the media: Did you ever question yourself whether T. litoralis and P. furiosus really need these high Mg\(^{2+}\) concentrations in the medium. You have taken over this quantity of 10.6 g MgCl\(_2\)·6H\(_2\)O in your own minimal medium. Was there never a problem with precipitations? From the high NaCl concentration I gather both archaea are marine microorganisms?

2) The hydrolysis of the chicken feathers in the fermenter was performed under aerobic conditions, because you state an air flow of 0.5 L/min (page 23). However, there is never a mentioning of monitoring the oxygen concentration in the culture. How do you know that your culture was well aerated?

3) Page 37, Hydrogen production from chicken feathers (F) or Bacto Peptone (P). There are some differences in hydrogen production with F or P as a substrate. Especially at 84 mg F you obtained less H\(_2\)/OD compared to 84 mg P (Fig. 9c). This strongly suggests that other by-products are formed. Did you check this with
HPLC-analysis? There is no deep discussion on this either in the Discussion section (page 47). Please, motivate your answer.

4) Page 38, Figure 10. Less than twice the amount of hydrogen was produced on 84 mg P. Were you aware of \textit{P. furiosus} being sensitive to hydrogen? Did you check for the by-products (e.g. alanine production)?

5) Page 47. I would be interested to see a more detailed calculation on the theoretical energy yield on the conversion of 30,000 tons of feather waste to hydrogen. For instance, did you include any energy that you have to put into the process to release the hydrogen?

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