Utilization of protein-rich animal waste materials to produce biohydrogen

Ph.D. Thesis

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Abbreviations

ABC transporter	ATP-binding cassette transporter
ACS	acyl-coenzyme A synthetase
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
Amp	ampicillin
AOR	aldehyde ferredoxin oxidoreductase
ATP	adenosine triphosphate
ATPase	adenosine triphosphate hydrolase
CM	complex medium for hyperthemophiles
CMSY	minimal medium for hyperthermophiles
CTAB	hexadecyltrimethylammonium bromide
DIG	dioxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DSMZ	German Collection of Microorganisms and Cell Cultures
Ech	energy converting hydrogenase
EDTA	ethylenediaminetetraacetic acid
EM	Embden-Meyerhof pathway
EtBr	ethidium bromide
EU	European Union
Fd	ferredoxin
FH	feather hydrolysate
FHL	formate hydrogenlyase
FNOR	ferredoxin NADP oxidoreductase
FTIR	Fourier transform infrared spectroscopy
GAPOR	glyceraldehyde-3-phosphate ferredoxin oxidoreductase
GC	gas chromatograph
GDH	glutamate dehydrogenase
GTPase	guanosine triphosphate hydrolase
H_2ase	hydrogenase
IOR	indolepyruvate ferredoxin oxidoreductase
KGOR	2-ketoglutarate oxidoreductase
Mbh	membrane-bound hydrogenase
MM	meat meal
MMH	meat meal hydrolysate
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PHH	pig hair hydrolysate
pNA	para-nitroaniline
POR	pyruvate ferredoxin oxidoreductase
PSII	photosystem II
TAE	tris-acetate-EDTA
TCD	thermal conductivity detector
Tris	tris(hydroxymethyl)aminomethane
TSE	Transmittable Spongiform Encephalopathy
VOR	2-ketoisovalerate ferredoxin oxidoreductase
X-phosphate	5-bromo-4-chloro-3-indolyl phosphate

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 air pollutants such as carbon dioxide, sulfur oxides and nitrogen oxides. The latter two compounds are responsible for acid rains while elevated levels of carbon dioxide in the atmosphere are considered the major cause of global warming and climate change. In addition, the formation of fossil fuels is much slower than their consumption rate and therefore 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 hydrogen fermentation 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 (Claassen et al. 1999). The combination of biological waste decomposition and fermentative biohydrogen production is promising 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 might produce energy carriers that can reduce our dependence on fossil fuels.

1. Overview of the literature

1.1. Proteinaceous animal wastes - processes for decomposition

1.1.1. Meat meal

Animal carcasses, by-products from slaughterhouses, seized raw meat and meat products unsuitable for human consumption are collected and processed in dedicated industrial facilities. Closed disinfectors are used to sterilize and cook the collected materials applying high pressure and temperature. The resulting pulp is subsequently dried *in situ* followed by physical (e.g. extrusion) or chemical fat removal. Dried materials with reduced fat content are finally milled to yield meat meal (Szél and Gál 1980).

For a long time, meat meal has been applied as feedstuff for farmed animals. However, current EU regulations (1774/2002/EU) introduced a ban on such use and tightly restricted the use of animal by-products. Consequently, nowadays in Hungary this material is mainly incinerated.

1.1.2. Keratinaceous wastes

1.1.2.1. Structure of keratins

Keratins are insoluble fibrous structural proteins that can be found in feathers, wool and animal hair in large quantities. Their unique matrix-filament texture (Jones and Pope 1985), strengthened by a large number of disulfide bonds (Lynch et al. 1986), makes keratin highly resistant against physical, chemical and biological agents, resulting in an ideal protective layer for animals. Based on their secondary structure, keratins are divided into α and β keratins. In mammals,α keratins predominate (Fraser et al. 1986) while β keratins are more characteristic to reptiles and birds (Fraser and Parry 2008). X-ray diffraction studies confirm the presence of tightly packed helical filaments that consist of repeated units both in animal hair and in feathers. Filaments found in avian feathers and reptilian scales consist of a pair of twisted β-sheet domains, each composed by a 32-residue domain (Fraser and Parry 2008). The other part of the same protein makes up the matrix of the keratinaceous structures. Mammalian keratins contain filaments that are built up from heterodimeric rod-like particles (Steinert 1990). These building blocks are formed by spooling a type I (acidic) and a type II (neutral/basal) α keratin resulting in a coiled coil structure. Non-helical N terminal and C terminal domains support the multimerisation of the rods yielding long filaments.

1.1.2.2. Keratin degradation techniques

Billions of tons of keratinaceous wastes are produced annually in the poultry, wool, fish, and meat industry (Williams et al. 1991; Frazer 2004). Pyrolysis (Kim and Agblevor 2007), chemical treatment of concentrated waste by reducing agents (Onifade et al. 1998) or hot NaOH (Kim and Patterson 2000) are commonly employed, although these technologies are energy demanding and environmentally harsh. As a protein polymer, keratin is an attractive feed source for those microbes that have the ability to degrade and utilize it. Several bacteria e.g. *Bacillus cereus* DCUW (Ghosh et al. 2008), *Bacillus subtilis* (Cai et al. 2008) *Streptomyces pactum* (Bockle et al. 1995), *Bacillus licheniformis* PWD-1 (Lin et al. 1992; Williams et al. 1990), *Chryseobacterium* sp. kr6 (Riffel et al. 2003), *Streptomyces fradiae* (Yu et al. 1969), *Bacillus halodurans* (Takami et al. 1992; Takami et al. 1999), the hyperthermophilic *Fervidobacterium* species (Friedrich and Antranikian 1996; Nam et al. 2002), several dermatophyta fungi (Kunert 1973; Yu et al. 1972) and recently a nonpathogenic *Trichoderma* strain (Cao et al. 2008) were reported to produce keratinolytic proteases capable of decomposing keratin. Many of these microorganisms are potential candidates for biotechnological keratin degradation applications.

1.1.2.3. Keratinases

Keratinases are proteases that are able to hydrolyze keratins. In general, proteases can be classified according to their cleavage habits. Exoproteases cleave peptide bonds at the amino termini (aminopeptidases) or at the carboxy termini (carboxypeptidases) of their substrates, while endopeptidases break non-terminal peptide bonds inside polypeptide chains (Rao et al. 1998). Based on the functional groups of their active sites, proteases can be further divided into four prominent groups: serine proteases, cysteine proteases, aspartic proteases and metalloproteases (Neurath 1989). The majority of known keratinases are endopeptidases belonging to the serine protease family.

Amino acid sequences of several *Bacillus* keratinases are known to show striking sequence homology to Carlsberg subtilisin (E.C. 3.4.21.62), a well-described member of the serine protease family. All three catalytic active sites (Asp32, His64, Ser221) characteristic to subtilisins can be identified in the primary sequence of keratinases.

Subtilisins and related extracellular proteases bear a triad of 'pre', 'pro' and 'mature' regions (Wells et al. 1983; Power et al. 1986). The N-terminal 'pre' part serves as a signal sequence directing the translocation of the newly synthesized precursor molecules through the cell membrane. The adjacent 'pro' region acts as an intramolecular chaperone that promotes

the correct fold of the protease domain and is a prerequisite for the protease maturation (Shinde et al. 1997). In the last step of maturation, the enzyme is activated via an autocatalytic removal of the 'pro' region (Power et al. 1986).

Kinetic parameters of *Bacillus licheniformis* KK1 keratinase and Carlsberg subtilisin were determined and compared using a set of para-nitroaniline (pNA) conjugated oligopeptides as substrates (Evans et al. 2000). Both enzymes showed similar kinetics with most of the oligopeptide substrates, preferentially cleaving next to hydrophobic and aromatic residues. The nearly identical protein sequence and the similar biochemical characteristics suggest a tight relationship between keratinases and subtilisins isolated from *Bacillus* strains.

1.2. Possible ways of hydrogen production

1.2.1. Physical and chemical hydrogen production

Annually, roughly 50 million tons of hydrogen is used in the world (NHA 2008). Most of it is generated via methane reforming or through partial oxidation of crude oil (Hancsók 2004). Currently, electrolysis of water cannot compete economically with the chemical production of hydrogen. However, this is expected to change since the price of fossil fuels tends to increase whilst electricity generated from renewable sources is expected to become cheaper over time.

1.2.2 Photobiological hydrogen production

Photobiological hydrogen production refers to biological production of hydrogen performed by phototropic microorganisms that can directly utilize solar energy. Photosynthetic microalgae and cyanobacteria split water into oxygen, protons and electrons using solar energy collected by their photosystem II (PS II). Theoretically, electrons can be redirected from PS II to a hydrogenase enzyme coupling the photolysis of water with the production of hydrogen gas. As a proof of the principle, Benemann and co-workers combined spinach chloroplast, a clostridial electron carrier molecule (ferredoxin) and a [FeFe] hydrogenase for the direct photoproduction of hydrogen (Benemann et al. 1973). Their system was shown to evolve hydrogen from water upon illumination, but the production of hydrogen stopped quickly since oxygen generated by PS II very soon inactivated the hydrogenase.

Indirect biophotolysis is an attempt to circumvent the inborn incompatibility between oxidative photosynthesis and hydrogen production. In this approach, the oxygen and hydrogen evolution stages are separated in time as it was demonstrated with the green alga

Chlamydomonas reinhardtii (Melis et al. 2000). In the first stage, microbial cultures are allowed to perform photosynthesis under aerobic conditions that leads to the accumulation of starch. Subsequently, the sulfur removal blocks PS II biosynthesis and cellular metabolism is switched towards anaerobiosis and hydrogen production. Under these circumstances cells consume carbohydrate storages and generate hydrogen. The drawback of the method is the rather low yield of hydrogen production.

Anoxygenic photosynthetic purple bacteria are also promising candidates for photosynthesis-driven hydrogen production. Their single photosystem cannot split water, but has a cyclic electron flow that does not release electrons. Therefore, external reduced compounds are required to feed the photosynthetic CO_2 fixation processes with electrons (Ehrenreich and Widdel 1994; Griffin et al. 2007). As their photosynthesis is anoxic, simultaneous operation of oxygen-sensitive proteins such as hydrogenases or nitrogenases is possible during photosynthetic growth. Recently, in the purple sulfur bacterium, *Thiocapsa roseopersicina*, a [NiFe] hydrogenase was shown to have a light dependent hydrogen evolving capacity (Rákhely et al. 2004).

1.2.3. Biohydrogen production via dark fermentation

Anaerobic heterotrophic microorganisms have various fermentative pathways for utilization of energy-rich organic materials. These microorganisms largely contribute to the decomposition process in nature and are able to metabolize numerous substrates such as sugars, starch, cellulose and proteins. Intracellular oxidation of sugars and amino acids yields reduced cofactors that need to be re-generated to ensure the continuous operation of the metabolic system. In the absence of electron acceptors, such as oxygen or nitrate, hydrogen production is one of the solutions for the disposal of the excess reducing power. Consequently, the dark hydrogen production of these microbes is tightly linked to the anaerobic fermentative pathways (Vignais and Colbeau 2004).

Pure cultures such as from *Clostridia* (Yokoi et al. 2001), *Caldicellulosiruptor* (Kádár et al. 2003) and *Enterobacterium* (Ito et al 2004) species as well as consortium of anaerobic bacteria (Massanet-Nicolau et al. 2008) are frequently employed for the lab-scale production of hydrogen. Laboratory studies are often carried out on pure carbohydrate substrates such as glucose, starch or cellulose although these purified substances are too expensive for large-scale applications. However, agriculture produces numerous energy-rich waste materials suitable for biohydrogen production such as cellulose-rich plant residues (Lo et al. 2008), starch (Yokoi et al. 2001) or fructans (Kyazze et al. 2008), animal manure (Zhu et al. 2007)

and sewage sludge (Massanet-Nicolau et al. 2008). Efficient hydrolysis of the biopolymers present in the biomass is essential for these processes. During dark hydrogen fermentation, carbon dioxide and organic acids are formed as end products in addition to biohydrogen. The produced organic acids might be suitable feedstock for photo-fermentation using photoorganothrophic bacteria. A promising combination of dark hydrogen fermentation and hydrogen photofermentation was recently described (Claassen and de Vrije 2006).

1.3. Hydrogenases

Nitrogenases and hydrogenases are metalloenzymes which participate in the hydrogen metabolism of a given cell. While nitrogenase produces hydrogen as by-product during nitrogen fixation, hydrogenases (H_2ases) are dedicated metalloenzymes that catalyze the reversible oxidation of hydrogen ($H_2 \leftrightarrow 2H^+ + 2e^-$) (Vignais and Colbeau 2004; Hederrich 2004). Usually, H_2ase activities in both reaction directions can be detected *in vitro* using artificial or natural electron carrier molecules. However, their *in vivo* function depends on the actual redox status of the cells. H_2ases are classified into three major classes based on the metal composition of their active sites (Vignais and Colbeau 2004). The [NiFe] H_2ases harbor a Ni and a Fe atom, whilst the [FeFe] H_2ases contain two Fe atoms in their active centers. The third class comprises archaeal enzymes with an iron-containing cofactor catalytic core (Lyon et al. 2004). Therefore, this family is termed "iron-sulfur-cluster-free" H_2ases. Computer assisted analysis of available sequences and three-dimensional structures disclosed that the three H_2ase classes are phylogenetically distinct (Vignais et al. 2001).

1.3.1. [NiFe] hydrogenases

[NiFe] hydrogenases were found in many bacteria and archaea. The core enzyme is a globular heterodimer formed by a large (α) and a small (β) subunit that have molecular weights of ~60 kDa and ~30 kDa, respectively. The crystal structures of various [NiFe] H_2ase dimers have greatly extended our knowledge on the generic folds and structural a properties of these enzymes (Volbeda et al. 1995) (Fig. 1). The bimetallic active center is located in the large subunit, and is coordinated by four conserved cysteine residues. X-ray and infrared spectroscopy have identified the presence of three inorganic ligands (2 CN^- and one CO) that are connected to the Fe atom in the active site (Volbeda et al. 1996; Happe et al. 1997). The small subunit is equipped with up to three [4Fe-4S] clusters that allow the electron transfer between the active site and the surface of the enzyme. These complex enzymes require

numerous maturation proteins catalyzing the folding and metallocenter assembly.

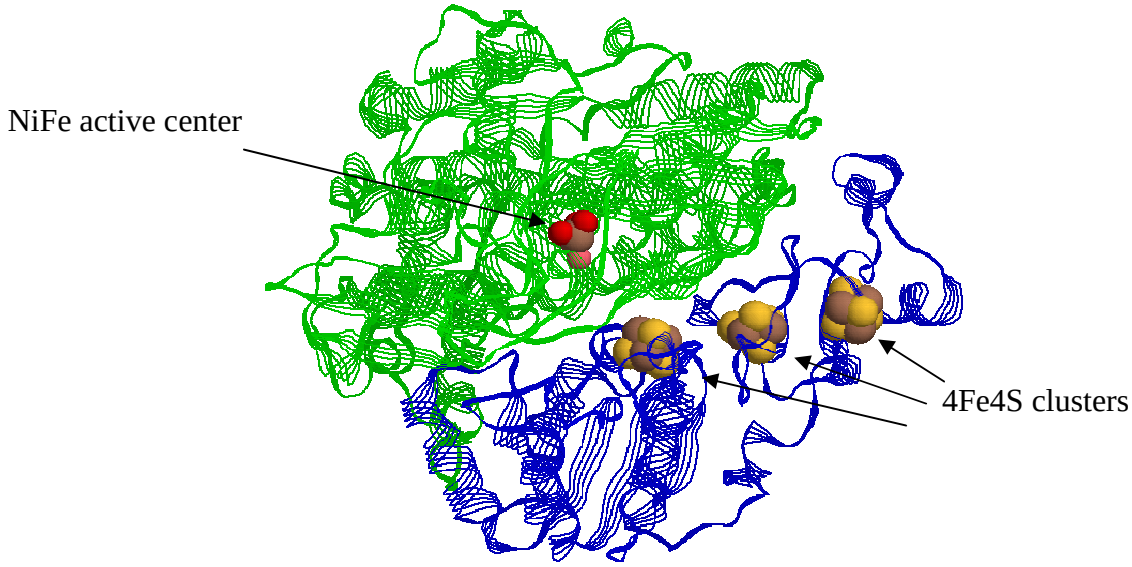


Figure 1 Three-dimensional structure of the [NiFe] hydrogenase purified from *Desulfovibrio gigas*. Green and blue ribbons represent large and small subunits respectively. The NiFe active center and 4Fe4S clusters are marked with arrows.

1.3.1.1. Classification of [NiFe] hydrogenases

[NiFe] H_2ases can be divided into four groups based on their sequence, structure and biochemical properties (Vignais et al. 2001; Vignais and Colbeau 2004).

Group 1: Uptake [NiFe] hydrogenases

Membrane-bound uptake H_2ases belonging to Group 1 generate reducing equivalents by supplying electrons to the anaerobic respiratory chains, methanogenesis, sulfate or metal reduction. Usually, a characteristic signal sequence can be found on the small subunit of the Group 1 hydrogenases that directs the transportation of the fully folded enzymes to the periplasm by means of the “tat” machinery (Weiner et al. 1998). Uptake H_2ases are linked to a cytochrome b type subunit that anchors the hydrogenase dimer to the cell membrane and transfers the electrons to the quinone pool (Vignais and Colbeau 2004). In this way, the oxidation of hydrogen can be coupled to the formation of a proton gradient that drives ATP synthesis.

Group 2: Cytoplasmic hydrogen sensors and cyanobacterial uptake [NiFe] hydrogenases

[NiFe] hydrogenases of Group 2 lack the N-terminal signal sequence. Some representatives, such as the *Rhodobacter capsulatus* HupUV H_2ase (Vignais et al. 2000) and the *Ralstonia eutropha* HoxBC H_2ase (Kleihues et al. 2000) do not participate in energy transduction. Instead, they act as a hydrogen sensor and regulate the expression of uptake hydrogenases depending on the presence or absence of H_2. Other members of the group include cyanobacterial uptake hydrogenases (HupSL) present in *Nostoc* strains (Axelsson and Lindblad 2002; Oxelfelt et al. 1998) and in *Anabaena variabilis* (Happe et al. 2000) that are induced under N_2 fixing conditions. Their main function is the reoxidation of hydrogen that is released by the nitrogenase as a by-product of nitrogen fixation.

Group 3: bidirectional heteromultimeric cytoplasmic [NiFe] hydrogenases

In addition to the standard dimeric core, bidirectional [NiFe] hydrogenases have further subunits that carry binding sites for different soluble cofactors such as cofactor 420, NAD^+ or $NADP^+$. These enzymes are able to oxidize or reduce their cofactor partners and are supposed to take part in maintaining the physiological redox balance of the cells. The first four-subunit NAD-depending [NiFe] hydrogenase was isolated from *R. eutropha* (Schneider and Schlegel 1976). Related enzymes were identified in cyanobacteria (Schmitz et al. 1995; Appel and Schulz 1996) and in the purple-sulfur bacterium *T. roseopersicina* (Rákhely et al. 2004). Bi-directional cytoplasmic H_2ases are commonly found also in Archaea (Bryant et al. 1989; Ma et al. 2000; Rákhely et al. 1999).

Group 4: hydrogen-evolving, energy-conserving, membrane-associated hydrogenases

H_2ases in this group form large, membrane-bound multisubunit complexes. A conserved core, which consists of two integral membrane proteins and four hydrophilic proteins, could be identified in all members of this group. These enzymes are distantly related to other [NiFe] H_2ases whereas their “extra” subunits show remarkable sequence homology to the subunits of the NADH:quinone oxidoreductase complex. Similarly to their homologous subunits in complex I, the integral membrane proteins of the membrane-bound H_2ase complex may act as proton pumps and may be involved in energy conservation (Hedderich 2004).

Heterohexameric membrane-bound hydrogenases (Ech) belonging to this group could be purified from *Methanosarcina barkeri* (Kurkin et al. 2002) and *Thermoanaerobacter tengcongensis* (Soboh et al. 2004). Recently, an operon presumably coding for an Ech

hydrogenase was identified in the genome of *Caldicellulosiruptor saccharolyticus* (van de Werken et al. 2008). Similarly, an operon coding for a 14-subunit H_2ase complex (Mbh) was found in *Pyrococcus furiosus* (Silva et al. 2000), and the corresponding protein complex was partially purified (Sapra et al. 2000). Based on partial DNA sequencing and biochemical data, a similar H_2ase complex is likely to be present in *Thermococcus litoralis* as well (Takács et al. 2008). These hydrogenases are proposed to have a primary role in the disposal of the excess reducing power formed in fermenting microorganisms. An operon coding for a protein complex similar to *E. coli* formate-hydrogen lyase (FHL) was also identified in the hyperthermophilic archaeon, *T. litoralis.* In *E. coli*, the FHL is connected to the carbohydrate metabolism via pyruvate/pyruvate-formate lyase. However, the expression pattern of the *T. litoralis* FHL suggested that this complex is instead linked to the peptide metabolism (Takács et al. 2008).

1.3.2. [FeFe] hydrogenases

The catalytic core of [FeFe] hydrogenases is termed as H-cluster and consists of a binuclear [FeFe] center bound to a [4Fe-4S] cluster via a bridging cysteine, and is attached to the protein chain by four cysteine residues (Adams 1990). FTIR spectroscopy identified CN^- and CO ligands associated to the Fe atoms in the active site, similarly to [NiFe] hydrogenases (Nicolet et al. 2001). [FeFe] H_2ases have generally larger activity towards hydrogen evolution as compared to [NiFe] H_2ases, therefore they are frequently found in anaerobic fermentative microorganisms. However, uptake hydrogenases also exist in this family, such as the periplasmic enzyme found in *D. gigas* (Pohorelic et al. 2002). [FeFe] hydrogenases are unique in a sense that they could also be found in eukaryotes such as the green algae *C. reinhardtii* (Happe and Naber 1993). Despite of their high hydrogen evolving activity, the biotechnological use of [FeFe] hydrogenases is difficult due to their extreme oxygen sensitivity.

1.4. Hyperthermophilic hydrogen-producing archaea

1.4.1. Archaea – the third domain of life

Less than 20 years passed since Woese and co-workers defined Archaea as the third major domain of life representing an ancient life form. On one hand, archaea have a prokaryotic cellular organization similar to bacteria. On the other hand, their DNA replication apparatus is rather eukaryotic like (Gribaldo and Brochier-Armanet 2006). Based on the

phylogenetic trees constructed with the analysis of ribosomal proteins, the domain Archaea was split into two major phyla: Euryarchaeota and Chenarchaeota (Woese et al. 1990).

1.4.2. The order Thermococcales

Thermococcales is the best-known order within the phylum Euryarchaeota encompassing three genera: *Thermococcus*, *Pyrococcus* and *Paleococcus*. Species that belong to this order are hyperthermophilic with optimal growth temperatures exceeding 80^{o}C. *Thermococcus* and *Pyrococcus* species are generally of great biotechnological interest since they are a gold mine of heat-stable enzymes. While most species of Thermococcales are unable to grow in the absence of sulfur, this element is not absolutely necessary for the growth of *T. litoralis* and *P. furiosus.* Nonetheless, sulfur does have a stimulatory effect on the cell yield of these archaea (Bertoldo and Antranikian 2006).

1.4.3. Metabolic properties of Thermococcales

Species belonging to Thermococcales order usually able to ferment peptides, proteins or sugars producing H_2, CO_2, organic acids, alcohols and alanine as end products (Bertoldo and Antranikian 2006). Several extracellular proteases (Voorhorst et al. 1996; Voorhorst et al. 1997; Pulido et al. 2006) as well as enzymes involved in degradation of complex carbohydrates have been characterized from *P. furiosus* and *T. litoralis* (Brown and Kelly 1993). In addition, ATP-binding cassette (ABC) transporters involved in maltose transport (Horlacher et al. 1998), and probably in the peptide uptake (Albers et al. 2004), have been described in *Thermococcus* and *Pyrococcus* species.

1.4.3.1. Carbohydrate metabolism

Carbohydrate metabolism of *P. furiosus* was extensively studied revealing a modified version of the Embden-Meyerhof pathway (summarized in Fig. 2). One of the fundamental changes is the presence of unique sugar kinase enzymes that use ADP instead of ATP for phosphorylation of glucose and fructose-6-phosphate (Kengen et al. 1994). Another difference is that the oxidative steps involve unusual tungsten-containing enzymes that use ferredoxin as a redox partner. (Ma et al. 1997; Mukund and Adams 1995). In the final step, acetyl-Coenzyme A (acetyl-CoA) produced from pyruvate is converted into acetate by two acetyl-CoA synthase isoenzymes. This reaction is accompanied by the synthesis of ATP from ADP via substrate level phosphorylation. Oxidation of one mole of glucose yields up to 4 moles of reduced ferredoxin.

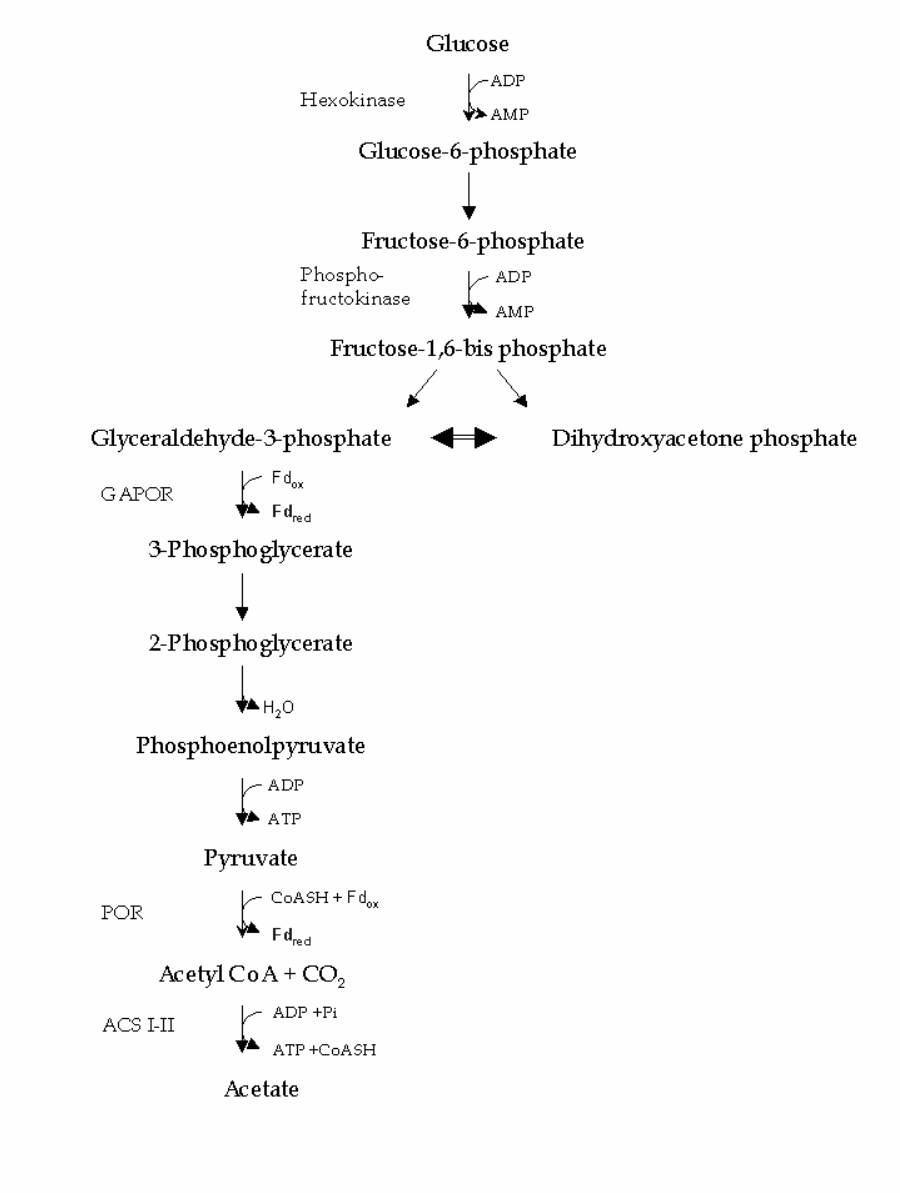


Figure 2 Carbohydrate metabolism in *P. furiosus* adapted from Kengen et al. 1994. ACS, acyl-Coenzyme A synthetase; GAPOR, glyceraldehyde-3-phosphate oxidoreductase; POR, pyruvate oxidoreductase;

1.4.3.2. Peptide metabolism

The peptide metabolism of the Thermococcales strains is less well known, although several key enzymes have been purified and characterized. In addition, whole genome DNA microarray analysis has revealed genes that might play important roles when *P. furiosus* is grown on peptides (Schut et al. 2003). The catabolism of amino acids is summarized in Fig. 3.

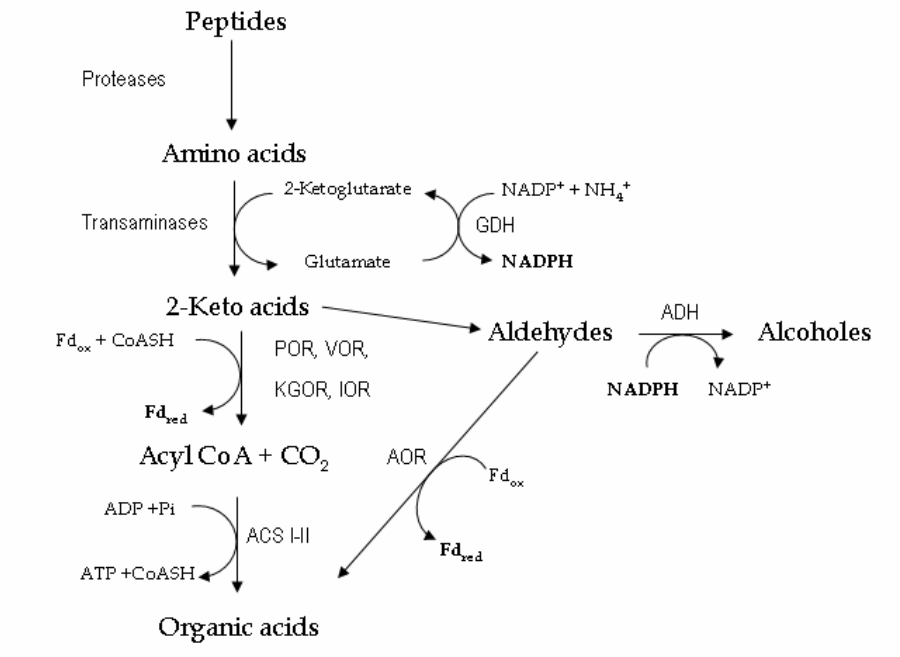


Figure 3 Peptide metabolism of *P. furiosus* adapted from Adams et al. 2001. Abbreviations: ACS, acyl-Coenzyme A synthetase; ADH, alcohol dehydrogenase; AOR, aldehyde ferredoxin oxidoreductase; GDH, glutamate dehydrogenase; IOR, indolepyruvate ferredoxin oxidoreductase; KGOR, 2-ketoglutarate oxidoreductase; POR, pyruvate oxidoreductase; VOR, 2-ketoisovalerate ferredoxin oxidoreductase

In the first step, amino acids are deaminated by transaminases. The produced 2-ketoacids are oxidized by four different 2-ketoacid ferredoxin oxidoreductases (POR, IOR, VOR, KGOR) to their corresponding CoA derivates, yielding reduced ferredoxin. Following the oxidation step, CoA derivates are converted into organic acids by two acetyl-CoA synthetase isoenzymes via substrate level phosphorylation coupled with the synthesis of ATP.

Besides the oxidation of 2-ketoacids, ketoacid-oxidoreductases also catalyze the decarboxylation of 2-ketoacids into aldehydes. The two reactions were shown to take place simultaneously *in vitro* and were supposed to be regulated by the availability of oxidized ferredoxin (Ma et al. 1997). Depending on the redox state of the cells, the aldehydes can be reduced into alcohols via ADH (Ma and Adams 1999) using electrons of NADPH, or they can be oxidized by AOR (Mukund and Adams 1991) yielding organic acids and reduced ferredoxin. In addition to reduced ferredoxin, NADPH can be also produced in the amino acid catabolism.

1.4.3.3. Hydrogen metabolism of Thermococcales

Several [NiFe] hydrogenases were described from members of Thermococcales including *P. furiosus*, one of the best-studied archaea. Hydrogen metabolism of *P. furiosus* is

tightly linked to the fermentation of carbohydrates and peptides since the reduction of protons is coupled to the regeneration of co-factors (ferredoxin, NADPH) produced in the oxidative steps (Fig. 4.). Two soluble heterotetrameric [NiFe] hydrogenases (Bryant and Adams 1989; Ma et al. 2000) and one membrane-bound [NiFe] multisubunit hydrogenase (Mbh) (Sapra et al. 2000; Silva et al. 2000) have been characterized in *P. furiosus* so far.

Mbh could be partially purified from the membrane and was shown to evolve hydrogen using reduced ferredoxin as an electron source. The enzyme has an extremely high hydrogen evolution:uptake ratio (250:1) (Silva et al. 2000) and belongs to the group of hydrogen-evolving hydrogenases (Group 4). The *mbh* operon consists of 14 ORFs with deduced sequences resembling the Ech hydrogenases of *M. barkeri* (Kurkin et al. 2002), *T. tengcongensis* (Soboh et al. 2004) and the CO-induced hydrogenase from *Rhodospirillum rubrum* (Fox et al. 1996). Based on the sequence similarity to the proton-translocating NADH:ubiquinone dehydrogenase complex (complex I), Mbh was proposed to act as a proton pump involved in energy conservation (Silva et al. 2000). Indeed, it was later demonstrated that the hydrogen evolution of Mbh was directly coupled to the proton gradient formation and ATP synthesis (Sapra et al. 2003).

The two cytoplasmic hydrogenases (Hyh1 and Hyh2) isolated from *P. furiosus* belong to the $NAD(P)^+$-reducing hydrogenase group (Group 3). Both enzymes catalyzed the hydrogen dependent reduction of S^0 to H_2S *in vitro* and were thus nominated as sulfhydrogenase I and sulfhydrogenase II, respectively. However, DNA microarray studies revealed that the sulfur reducing activity of the enzymes has little if any physiological relevance. There were only minor biochemical differences found between the two enzymes. Hydrogenase II was less active but was able to accept both NAD(H) and NADP(H) while hydrogenase I was active only with NADP(H) (Ma et al. 2000). The two soluble hydrogenases might be connected to the ferredoxin pool through the ferredoxin:NADPH oxidoreductase (FNOR, Sud) (Ma and Adams 2001). The two cytoplasmic enzymes are believed to maintain an intracellular redox balance of NAD(H) and NADP(H) (Silva et al. 2000; van Haaster et al. 2008).

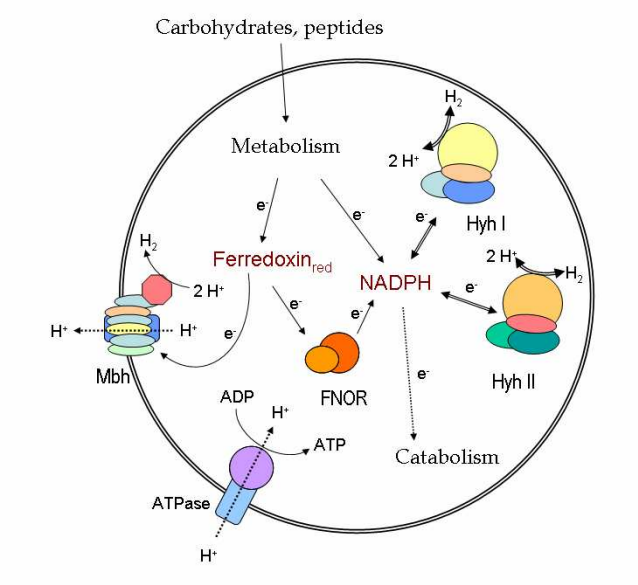


Figure 4 Hydrogenases and hydrogen metabolism of *P. furiosus.* FNOR, ferredoxin:NADPH oxidoreductase; Hyh I, soluble hydrogenase I; Hyh II, soluble hydrogenase II; Mbh, membrane-hydrogenase;

1.4.4. *Thermococcus litoralis* DSM 5473

T. litoralis strain DSM 5473 was isolated from narrow underwater solfataras near Naples in 1985 by Belkin and Jannash (Neuner et al 1990). Cells lack flagellation, have a spherical morphology with a varying width of 0.5-3 μm and are covered by a protein envelope. *T. litoralis* is neutrophilic, growing between pH=4.0 and pH=8.5 with a pH optimum around pH=6.0 and an optimal growth temperature of 85^{o}C.

The archaeon can be maintained on complex substrates such as yeast extract, peptone, tryptone, meat extract, and casein. No growth is observed solely on carbohydrates. Nevertheless, maltose has a stimulatory effect on cell yield in the presence of yeast extract, peptides or amino acids. Cells can reduce S^0 to H_2S, although the presence of sulfur is not absolutely necessary for growth. Several enzymes involved in the amino acid catabolism were isolated and characterized from *T. litoralis* (KGOR: Mai and Adams 1996; VOR: Heider et al. 1996; FOR: Dhawan et al. 2000). It is believed that both carbohydrate and peptide metabolism of *T. litoralis* resemble the pathways found in *P. furiosus*.

So far, only a cytoplasmic hydrogenase could be entirely purified and biochemically characterized from *T. litoralis* (Rákhely et al. 1999), thus our picture about its hydrogen metabolism is incomplete. The isolated heterotetrameric hydrogenase corresponds to the hydrogenase I of *P. furiosus*. Additionally, part of the gene of the Mbh large subunit has been

identified suggesting that a similar membrane bound hydrogenase complex might be present also in *T. litoralis* (Tóth unpublished). This was confirmed by indirect biochemical evidences (Takács et al. 2008). More recently, an octameric membrane-bound formate- hydrogen lyase complex without known counterpart in *P. furiosus* was described in the archaeon (Takács et al. 2008). The operon coding for the complex was shown to be up-regulated upon growth on peptides suggesting its involvement in peptide metabolism, although its exact position in the peptide metabolic pathway is not known. The metabolic background of *T. litoralis* makes it a promising candidate for biotechnological applications, such as biohydrogen production from various kinds of organic materials, including wastes.

2. Aims of the study

Agriculture and related meat industries are responsible for the mass production of various protein-rich by-products including feathers, animal hair, hoofs, horns and meat meal. Current methods for the decomposition of the waste stream are either expensive or environmentally harsh. 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. The specific goals were as follows:

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 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 a novel substrate, meat meal.

- Evaluation of meat meal as a nutrient for dark hydrogen fermentation.
- Scaling-up of the hydrogen production step.
- Determination of overall conversion yield for meat meal.

3. Enhancing the waste degradation step using molecular biology techniques.

- Cloning the keratinase gene from *B. licheniformis* KK1 and determining its nucleotide sequence.
- Heterologous expression of keratinase in *Escherichia coli.*

3. Materials and methods

3.1. Strains used

Strain	*Genotype*	*Reference*
Bacillus licheniformis KK1	wild type	Patent No.: P0004865
Escherichia coli XL-1 Blue MRF'	*recA1, gyrA96,* (F'*lacI*^{q} *Z*) Tc^{r}	Stratagene, Cat. No.: 200230
Escherichia coli TOP10	endA1, recA1, hsdR, deoR, mcrA, lacZΔM15, rpsL(Str^{R})	Invitrogen Cat. No.: V45001
Caldicellulosiruptor saccharolyticus (DSM 8903)	wild type	Rainey et al. 1994
Thermococcus litoralis (DSM 5473)	wild type	Neuner et al. 1990
Pyrococcus furiosus (DSM 3638)	wild type	Fiala and Stetter (1986)

3.2. Plasmids used

Plasmid name	*Genotype*	*Reference*
pBS+	ColE1 Amp^{r}	Stratagene Cat.No.: *discontinued*
pET15b	*lac*I, Amp^{r}, pBR322 ori, His-tag	Novagen Cat. No.: 69661-3
pBADgIIIA	rrnB, P_{BAD}, geneIII secr.signal, c-myc, pMB1 ori, HIS-tag, Amp^{r} , *ara*C	Invitrogen Cat. No.: V450-01

3.3. Media used

CM: Complex medium for *T. litoralis* and *P. furiosus* 1L contains: 24 g NaCl, 10.6 g $MgCl_2$•$6H_2O$, 4 g Na_2SO_4, 1.5 g $CaCl_2$•2 H_2O, 0.7 g KCl, 0.2 g $NaHCO_3$, 0.1 g KBr, 0.025 g SrCl, 0.03 g H_3BO_3, 0.2 mg resazurin, 195 mg filter-sterilized cysteine-HCl (added separately following heat sterilization of the medium), 1 g yeast extract and 5 g Bacto Peptone (Difco Laboratories, USA), pH was adjusted to 6.5.

CMM: Derivate of CM medium prepared with limited nutrient content. (same as CM except Bacto Peptone is omitted), pH adjusted to 6.5.

CMSY: Minimal medium for *T. litoralis* and *P. furiosus*. 1 L contains: 24 g NaCl, 10.6 g $MgCl_2$•$6H_2O$, 4 g Na_2SO_4, 1.5 g $CaCl_2$•2 H_2O, 0.7 g KCl, 0.2 g $NaHCO_3$, 0.1 g KBr, 0.025 g SrCl, 0.03 g H_3BO_3, 0.2 mg resazurin, 195 mg filter-sterilized cysteine-HCl (added separately following heat sterilization of the medium) and 0.05 g yeast extract, pH adjusted to 6.5.

LB: Complex medium for *Escherichia coli* and *Bacillus licheniformis* KK1. 1 L contains: 10 g tryptone, 5 g yeast extract, and 5 g NaCl. pH = 7.0.

M9: Minimal medium for *E. coli*. 1 L contains: 12.8 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NaCl, 2 ml of pre-sterilized 1 M $MgSO_4$ solution and 100 µl of pre-sterilized 1 M $CaCl_2$ solution added separately, following heat sterilization of the medium.

M640: Complex medium for *C. saccharolyticus* 1 L contains: 0.9 g NH_4Cl, 0.9 g NaCl, 0.4 g $MgCl_2$•$6H_2O$, 0.75 g KH_2PO_4, 1.5 g K_2HPO_4, 2 g peptone, 1 g yeast extract, 1 ml SL-10 trace element solution, 2.5 mg $FeCl_3$•$6H_2O$, 1 g cellobiose 0.75 g cysteine-HCl•H_2O, 0.5 mg resazurin.

SL-10: Trace element solution. 1 L contains: 10 ml HCl (25 %, 7.7 M), 1.5 g $FeCl_2$•$4H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2$•$4H_2O$, 6 mg H_3BO_3, 190 mg $CoCl_2$•$6H_2O$, 2 mg $CuCl_2$•$2H_2O$, 24 mg $NiCl_2$•$6H_2O$, 36 mg Na_2MoO_4•2 H_2O.

3.4. Chemical composition of meat meal

Meat meal was provided by ATEV JSC (Budapest, Hungary) with a chemical composition as follows:

Dry material content:	90 %
Protein:	54-57.9 %
Fat:	11-13.5 %
Ca:	5-7 %
P:	1.5-2.5 %

3.5. Microbiological and biotechnological procedures

3.5.1. Degradation of keratinaceous wastes in Erlenmeyer flasks

In 500 ml Erlenmeyer-flasks, 200 ml of 0.5 mM phosphate buffer (pH=8.0) was mixed with either 8 g of intact chicken feathers, or 8 g of intact pig hair, or 4 g of milled goose feathers. The suspensions were heat-treated at 140^{o}C for 20 minutes. After cooling, 1 % (v/v) LB medium was added and the keratinaceous media were inoculated with a *B. licheniformis* KK1 starter culture (inoculation ratio of 1:100). Cultures were incubated at 43^{o}C with continuous shaking at 240 rpm. Samples taken from the keratin degradation were centrifuged at 4^{o}C and 20000 × *g* for 15 minutes. Cell-free fermentation broths were stored at -20^{o}C prior to use.

3.5.2. Hydrolysis of chicken feathers in fermenter

Chicken feathers were chopped using a cutting mill, resulting in feather meal having an average particle size of roughly 5 mm. Twenty-eighth grams of feather meal suspended in 700 ml of 0.5 mM phosphate buffer (pH= 8.0) was heat treated for 20 minutes at 140^{o}C and subsequently supplemented with 1 % (v/v) LB medium. One drop (~ 10 µl) of anti-foam solution (Sigma-Aldrich, Cat. No.: A-6426) was also added to avoid foam formation. The resulting feather degradation medium was transferred to a one-liter glass vessel of a Biostat Q fermenter (B. Braun Biotech International) and was sterilized at 125^{o}C for 20 minutes. Liquid cultures of *B. licheniformis* KK1 were used to inoculate the fermenter with a 1:100 inoculation ratio. Fermentation was maintained using the following parameters:
Temperature: 43^{o}C; Stirring: 200 rpm; pH kept at 8.0; Air flow: 0.5 L/min

Samples were taken from the fermenter after 12, 18, 24, 36, 42, 48, 60, 91.5 and 137.5

hours of incubation. Cell-free fermentation broth was obtained after a centrifugation performed at 4^{o}C with 20 000 × *g* for 15 minutes. Fermentation broth samples were stored at -20^{o}C prior to usage.

3.5.3. Cultivation of *T. litoralis* and *P. furiosus*

Starter cultures of *T. litoralis* were grown overnight on CMM medium while *P. furiosus* starter was propagated on CM broth. Temperature of incubation was 85^{o}C for *T. litoralis* and 92^{o}C for *P. furiosus*. Both cultures were grown under N_2 atmosphere in gas-tight 60 ml hypovial bottles (Supelco) capped with rubber septum. Keratinaceous media with a final volume of 20 ml were prepared from CMSY supplemented with 5-20 % (v/v) keratin hydrolysate, which in turn contained 20-80 mg of digested feather protein. Inoculation was performed anaerobically in the workspace of a Bactron IV anaerobic chamber (Sheldon Manufacturing Inc) using 1:100 inoculation ratio.

3.5.4. Hydrogen production of *C. saccharolyticus* grown on keratin hydrolysate

Nutrient-stripped M640 medium was prepared and then supplemented with 10 % (v/v) of feather hydrolysate. In each gas tight 150 ml hypovial bottles (Supelco) 50 ml of the mixture was added. Inoculation was carried out with *C. saccharolyticus* using a ratio of 1:50. The hypovials were flushed with sterile N_2 for 10 minutes and the cultures were incubated at 70^{o}C. After 48 hours, the optical densities of the cultures as well as the hydrogen concentrations in the gas phases were determined.

3.5.5. Hydrogen production of *E. coli* grown on keratin hydrolysate

M9 minimal medium was completed with 10 % (v/v) of feather hydrolysate. In each gas-tight 60 ml hypovial bottles (Supelco) 20 ml of the mixture was poured and the keratinaceous medium was inoculated (ratio of 1:100) with *E. coli* K12 starter culture grown on LB liquid media. Next, the bottles were flushed with sterile N_2 for 10 minutes. Cells were propagated at 37^{o}C for 48 hours. Finally, the hydrogen content of the headspace and the optical density of the cultures were measured.

3.5.6. Large-scale cultivation of *T. litoralis*

A Biostat C CT5-2 fermenter (B. Braun Biotech International) with a total volume of 6.9 liter was used for the large-scale propagation of *T. litoralis*. For hydrogen production from waste materials, 2.5 liter of CMSY medium was completed either with 10 % (v/v) of keratin

hydrolysate or with 40 % (v/v) of pre-digested meat meal solution. One drop (~ 10 µl) of anti foam solution (Sigma-Aldrich, Cat. No.: A-6426) was added to avoid foaming. Media were heat sterilized *in situ* at 120°C for 20 minutes. The headspace of the fermenter was flushed with N_2 gas at 2.5 L/min for 15 minutes. Subsequently, the medium was supplemented with filter-sterilized cysteine-HCl solution yielding a final concentration of 0.0195 %. Fermentation parameters were kept as follows: temperature 85°C; pH=6.5; and stirring at 150 rpm. After the inoculation with *T. litoralis* (1:100) batch fermentation was performed in a closed system allowing hydrogen to accumulate.

3.5.7. Gas analysis

Hydrogen content in gas samples was determined using either a Hitachi 263-50 or an Agilent 6890N gas chromatograph equipped with 5Å molesieve column and a thermal conductivity detector (TCD).

3.6. DNA works

3.6.1. Purification of genomic DNA

Bacterial cultures (3-5 ml) were centrifuged in a microcentrifuge (Biofuge pico, Heraeus) at 15000 × *g* for 2 minutes. Cells resuspended in 567 µl of TE buffer (Tris/HCl 10 mM, EDTA 1 mM, pH= 7.5) were supplemented with 30 µl of 10 % SDS and subsequently treated with proteinase K (final concentration 100 µg / ml) for one hour at 37°C. Samples were incubated at 65°C for 10 minutes following the addition of 100 µl of 5M NaCl and 80 µl of CTAB in 0.7M NaCl. Cell components complexed to CTAB were removed via extraction with equal volume of chloroform/isoamyl alcohol followed by extraction with phenol-chloroform. Genomic DNA was precipitated with isopropanol, washed with 70 % ethanol and redissolved in ultra pure water.

3.6.2. Purification of plasmid DNA

Gene Elute™ plasmid miniprep kit (Sigma-Aldrich, Cat. No.: PLN70) was used for the purification of plasmid DNA from 3-4 ml of *E. coli* liquid cultures grown in LB medium supplemented with ampicillin with a final concentration of 100 µg / ml.

3.6.3. Restriction digestion and modification of DNA

DNA manipulations (restriction enzyme digestions, phosphorylation with polynucleotide kinase, Klenow-blunting, calf alkaline phosphatase treatment, ligation) were carried out according to the standard practice (Ausubel et al. 1996) or to the recommendation of the manufacturer. DNA restriction and modification enzymes were purchased from Fermentas.

3.6.4. Polymerase Chain Reaction

Polymerase chain reactions were performed in a PCR Express (Hybaid) thermocycler. The reaction mixture contained 1-3 µl of template DNA, 1 µM of primers, 200 µM of dNTP, Mg^{2+}, PCR buffer and DNA polymerase enzyme as advised by the manufacturers. For high fidelity PCR reactions Pfu DNA polymerase (Fermentas, Cat. No.: EPO501) was used, while routine PCR applications were performed with Dynazyme II (Finnzymes, Cat.No.: F-501L).

3.6.5. Agarose gel electrophoresis of DNA

Agarose gel electrophoresis of DNA was performed using gels containing 0.7-2 % of agarose and 0.5 µg/ml EtBr in TAE buffer (4 mM Tris-acetate, 1 mM EDTA) according to Current Protocols in Molecular Biology (Ausubel et al. 1996).

3.6.6. Isolation of DNA fragments from agarose gel

For isolation of DNA fragments from agarose gel, DNA Gel Extraction Kit (Fermentas, Cat. No.: #K0513) was used according to the manufacturer's recommendations, except that elution of DNA from the silica powder was performed at an elevated temperature of 65^{o}C for 5-10 minutes.

3.6.7. Southern hybridization

Genomic DNA samples of *B. licheniformis* KK1 digested with *Eco*RI, *Cla*I, *Eco*RV, *Sma*I, *Xba*I or *Xho*I (Fermentas) were separated by gel electrophoresis, and DNA was blotted to HybondN+ membrane (Amersham-Pharmacia) using capillary transfer technique (Ausubel et al. 1996). Membrane was washed and concomitantly dried at 80^{o}C under vacuum. Hybridization was performed at 68^{o}C using a 539 bp DIG-labeled DNA probe. For the detection of the probe, alkaline-phosphatase conjugated to anti-DIG antibody combined with NBT and X-phosphate was used as recommended by the manufacturer (DIG DNA Labeling

and Detection kit, Roche, Cat. No:11 093 657 910).

3.6.8. Plasmid constructions

3.6.8.1. Cloning the *kerA* gene

PCR primers BLK1N (5' ATGATGAGGAAAAAGAGT 3') and BLK1R (5' TTATTGAGCGGCAGCTTC 3') were used in high fidelity PCR reaction to obtain the full-length *kerA* gene in *B. licheniformis* KK1. The isolated PCR product was ligated in a pET15b vector (*Nco*I-*BamH*I digested, T4 polymerase-blunted) to yield pBLK.

3.6.8.2. Assembly of a protein expression construct based on pBAD/gIII system

BLK2N (5' CCATGGCTCAACCGGCGAAAAATGT 3') and BLK2R (5' TTGAGCGGCAGCTTCGACAT 3') primers were used in a high fidelity PCR reaction to amplify a truncated *kerA* gene, which lacks the “pre” region coding for a bacillus-type signal sequence (recognition sequence for *Nco*I is underlined for BLK2N). The isolated fragment was subsequently cloned into the *Sma*I site of pBS+ cloning vector and the resulting plasmid was cleaved with *Nco*I-*Kpn*I. A 1052 bp fragment was obtained and inserted into *Nco*I-*Kpn*I digested pBAD/gIIIA (Invitrogen, Cat. No.: V450-01) vector.

3.6.9. Transformation of *E. coli* with plasmid DNA

Preparation of chemical competent cells and transformation of *E. coli* with plasmids were carried out according to the simple and efficient method (SEM) (Inoue et al., 1990).

3.6.10. DNA sequencing

Sequencing of DNA was performed using a 3100 Genetic Analyzer (Applied Biosystems) operating in the DNA Sequencing Laboratory of the Biological Research Center (Hungarian Academy of Sciences). Trace files were base-called using the freeware program Chromas V1.3 (Technelysium Pty Ltd).

3.7. Protein works

3.7.1. Determination of protein concentration

Protein concentrations were estimated using the Micro Lowry method (Yeang et al. 1998) calibrated with serial dilutions of bovine serum albumin (BSA). Optical absorbances of samples were determined at 750 mm using a SmartSpec 3000 spectrophotometer (BioRad).

3.7.2. Polyacrylamide gel electrophoresis

Polyacrylamide gel casting, electrophoresis and silver staining were performed according to the Ausubel et al. 1996. Polyacrylamide gels containing a 15 % separating part and a 5 % stacking region were used.

3.7.3. Expression of *B licheniformis* KK1 keratinase in *E. coli*

E. coli TOP10 strain transformed with pBLK-BAD plasmid was cultivated at 37^{o}C in 100 ml of LB medium containing 100 μg / ml ampicillin. Arabinose was added to mid-log phase culture (OD_{600}=0.5) giving a final concentration of 0.4 %. Production of recombinant protein was allowed for 3-5 hours. Then, the cells were harvested and periplasmic protein fraction was prepared.

3.7.4. Preparation of *E. coli* periplasmic protein fraction

Induced *E. coli* cultures were centrifuged at 8000 × *g* for 15 min at 4^{o}C. Cells were resuspended in an appropriate amount of Tris-sucrose (20 % sucrose in 30 mM of Tris-HCl, pH=8.0) calculated with the formula:

$$buff = \frac{V}{1.5} \times OD_{600} \times 0.1 \quad \text{where}$$

buff : buffer needed for the resuspension of cells in ml

V: culture volume in ml

OD_{600}: optical density of the culture

For each ml of cell suspension, 2 μl of 0.5 M EDTA (pH=8.0) was added and the mixture was incubated at room temperature for 10 minutes with shaking. Subsequently, cells were centrifuged at 8000 × *g* for 10 min at 4^{o}C, and then resuspended in ice-cold 5 mM $MgSO_4$ solution. Following a 10-min incubation on ice, the periplasmic fraction was obtained as the supernatant after centrifugation at 8000 × *g* for 10 min at 4^{o}C.

3.7.5. Protease activity assay

For the protease activity measurements, N-succinyl-Ala-Ala-Pro-Phe-pNA (Sigma, Cat. No.: S7388), a substrate preferentially cleaved by keratinase (Evans et al. 2000), was used. 800 µl of reaction mixture for protease activity measurements (1.25 mM N-succinyl-Ala-Ala-Pro-Phe-pNA, 125 mM Tris-HCl, pH=8.0) was completed with 200 µl of periplasmic protein sample and then incubated at 50^{o}C for 1 hour. Optical density of the mixture was measured at 405 nm using a BioRad SmartSpec 3000 spectrophotometer.

4. Results

4.1. Decomposition of chicken feathers by *Bacillus licheniformis* KK1

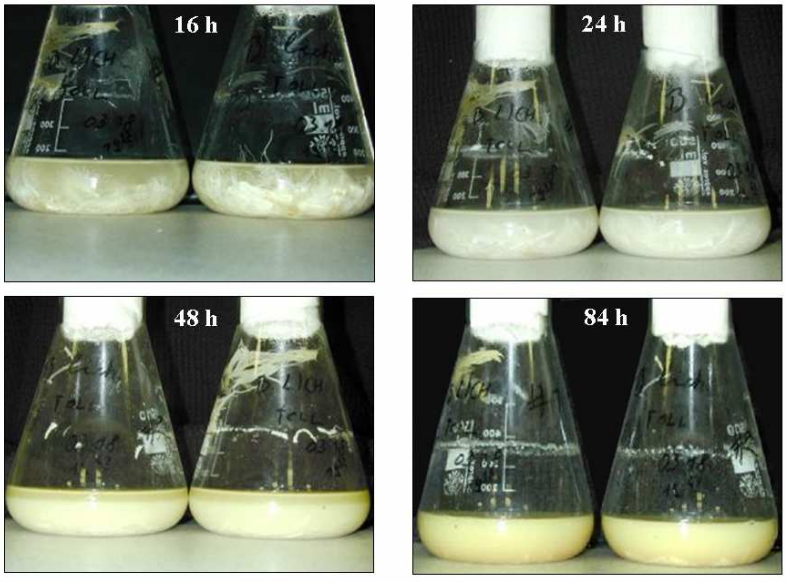
B. licheniformis KK1 previously isolated in our lab was shown to have a potential in the degradation of various proteinaceous materials including keratin (Patent No.: P0004865). Therefore, this strain was chosen for the keratin hydrolyzing step of our two stage system.

4.1.1. Degradation of whole chicken feathers in Erlenmeyer flasks

Preliminary keratin degradation experiments were carried out in 500 ml Erlenmeyer flasks with constant shaking, using a culture volume of 200 ml. Partial disintegration of feather barbs could already be seen after 16 hours of incubation with *B. licheniformis* KK1. In the course of time, the amount of macroscopic undigested feather residues decreased and the color of the fermentation broth turned yellowish. Complete decomposition of chicken feather was observed within 84 hours (Fig. 5A).

The soluble protein content of the fermentation broth was monitored and was found to increase continuously in time (Fig. 5B). Additionally, accumulation of small-sized peptide fragments was demonstrated by polyacrylamide gel electrophoresis (Fig. 5C). All these results indicate that *B. licheniformis* KK1 is able to liberate and digest proteins from chicken feathers thus producing a peptide-rich fermentation broth.

A



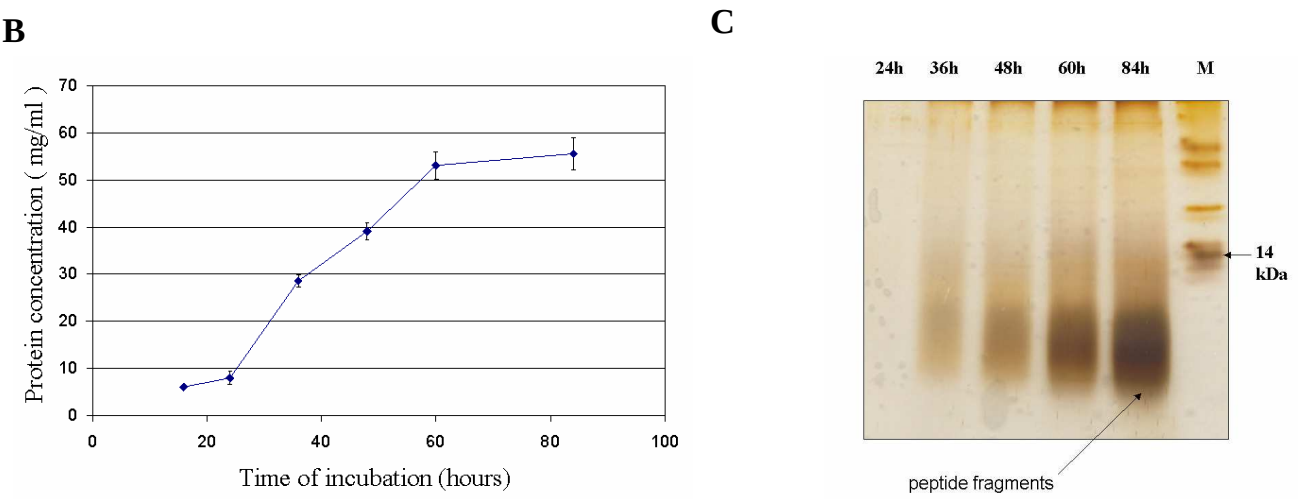


Figure 5 Degradation of whole chicken feathers by *B. licheniformis* KK1 in Erlenmeyer flasks. **A**: Photos of the feather degradation stages. **B**: The protein content of the cell-free medium at various time points. **C**: Protein pattern from the fermentation broth visualized on a silver-stained polyacrylamide gel. Lanes represent samples taken after 24, 36, 48, 60 and 84 hours of incubation with *B. licheniformis* KK1.

4.1.2. Degradation of milled chicken feathers in a pH-controlled fermenter

Hydrogen production experiments required large amounts of keratin hydrolysates, and therefore, scaling-up the keratin degradation step was necessary. Scale-up (3.5 times) studies and optimizations were performed in a 1000-ml glass vessel of a Biostat Q fermenter with a culture volume of 700 ml. Intact feathers tend to stick together when stirred in fermenter often causing physical damage to the stirrer or the engine. To overcome this difficulty, chicken feathers were crushed by a cutting mill producing a feather meal with a roughly 5 mm average particle size. Particles of this size did not influence the stirring allowing a better controlled, larger-scale bioprocessing of the waste material in the fermenter.

A remarkable change in the color of fermentation broth could be seen in parallel with the disappearance of the feather particles during the fermentation (Fig. 6A). Degradation of feathers was confirmed by the accumulation of soluble proteins in the fermentation broth in time (Fig. 6B). After 138 h of treatment with *B. licheniformis* KK1, about 75 % of the initially insoluble keratin was solubilized and could be detected in the fermentation broth.

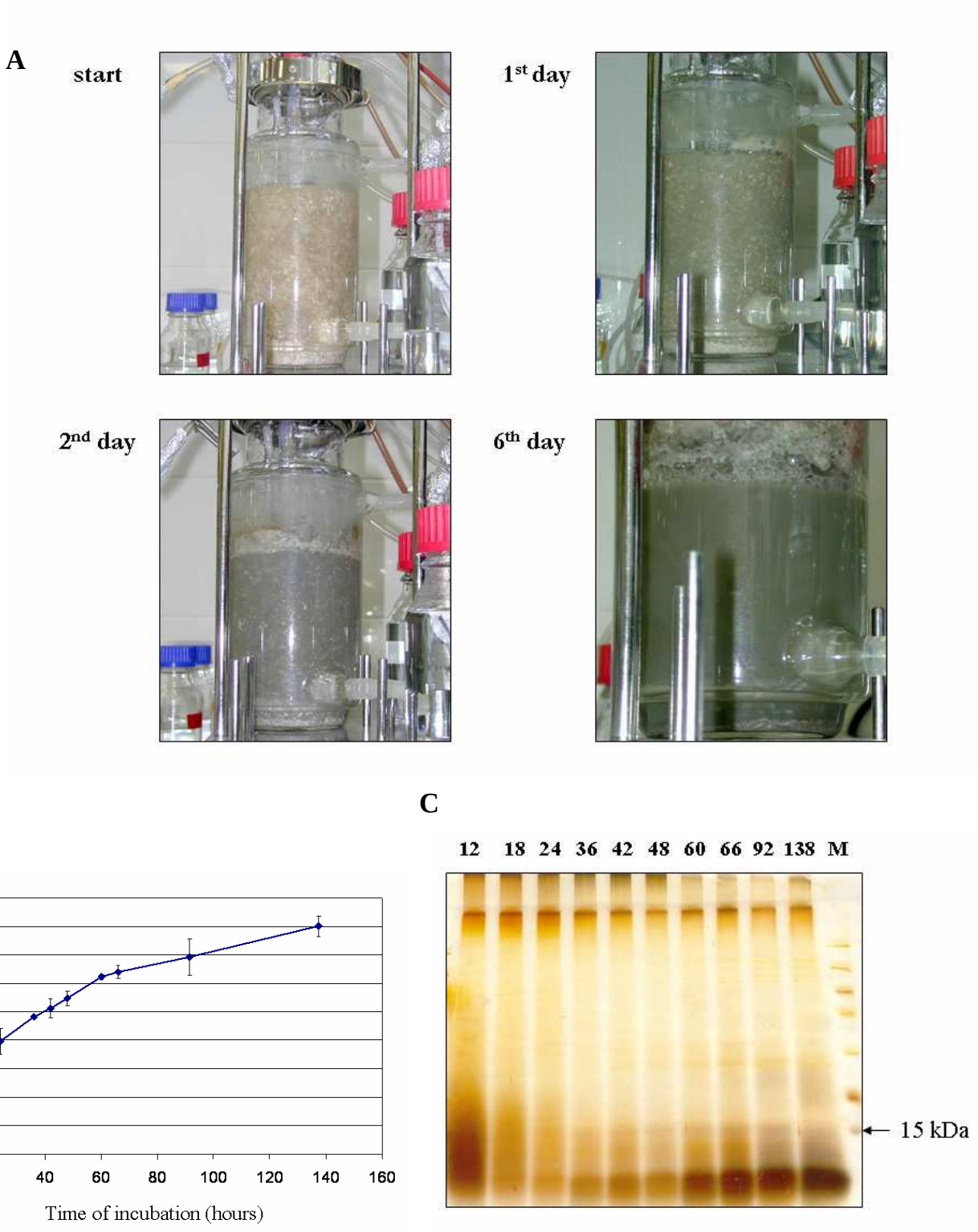


Figure 6 Degradation of chicken feather meal by *B. licheniformis* KK1 in fermenter. **A**: Photos of the feather degradation stages. **B**: Protein content of the fermentation broth measured at different stages of feather fermentation. **C**: Protein pattern of samples taken from the fermenter at various time intervals of hydrolysis (12-138 h), visualized on a silver-stained polyacrylamide gel.

A series of samples collected at different stages of fermentation were separated on a polyacrylamide gel revealing the enrichment in small peptides over time (Fig. 6C). Nearly complete degradation of chicken feather meal was observed in the fermenter within 138 hours of incubation. Additionally, it was demonstrated that chicken feathers can be stirred and processed in a fermenter following a physical disintegration performed in a cutting mill.

4.2. Hydrogen production using chicken feather hydrolysate

4.2.1. Optimization of CMSY minimal media

T. litoralis seems to have a complex hydrogen metabolism including a unique hydrogenase complex which is likely connected to the peptide metabolism. Therefore, this strain was tested for conversion of feather hydrolysate to biohydrogen.

To test if feather hydrolysate can be used as a feedstock for *T. litoralis*, a minimal medium had to be developed. The archaeon was inoculated on a series of culture media containing only the salts of the CM complex medium supplemented with various amounts of yeast extract. Medium completed with 0.05 g/L of yeast extract (nominated CMSY) was found to be ideal for the hydrogen production experiments, since cells grown solely on this medium showed barely detectable growth and produced a minimal amount of hydrogen. Conversely, cells displayed outstanding growth and hydrogen production on CMSY completed with an appropriate carbon and energy source such as Bacto Peptone (Table 1).

Medium used	*Hydrogen concentration % (V/V)*	OD_{600}
CM	8.12 ± 0.52	0.209 ± 0.023
CMM	3.47 ± 0.05	0.059 ± 0.004
CMSY	0.41 ± 0.00	0.037 ± 0.002
CMSY+ 0.5% P	7.72 ± 0.57	0.178 ± 0.007

Table 1 Hydrogen productions and optical densities of *T. litoralis* cultures after 25 hours of cultivation on media with varied nutrient contents. CM: *T. litoralis* complex medium; CMM: CM salts + 1g / L yeast extract; CMSY CM salts + 0.05 g/L yeast extract; P: Bacto Peptone

4.2.2. Effect of pH on the hydrogen production of *T. litoralis* grown on feather hydrolysate

CMSY minimal medium was supplemented with fermentation broth produced by *B. licheniformis* KK1 from chicken feather in Erlenmeyer flasks. Before mixing with the minimal media, pH of the feather hydrolysate was either adjusted to the optimal value of pH=6.5 or was left unaltered (pH=~8.0). The applied hydrolysate:CMSY volume ratio was 1:9 in all cases. Cumulative hydrogen production of *T. litoralis* cultures as well as changes in the pH of the culture media were monitored. CMSY medium turned out to have insufficient buffer capacity to maintain its original pH when the feather hydrolysate with unaltered pH (pH=~8) was added, i.e. the culture media became alkaline. Cells growing in this environment displayed smaller hydrogen production than those fed with pH-adjusted feather hydrolysate (data not shown). Thus, following this experiment, the pH of the fermentation broth was always altered to the optimal pH=6.5 value before the hydrogen production step.

4.2.3. Dependence of the hydrogen production on the feather degradation time

4.2.3.1 Degradation performed in Erlenmeyer flasks

Bacterial processing of feathers results in a fermentation broth with varying protein content and a continuously changing peptide composition. To identify the optimal feather degradation time for the subsequent hydrogen production stage, CMSY minimal medium was supplemented with 10 % (v/v) of pH-adjusted (pH=6.5) feather hydrolysates, which were collected at various time points of feather fermentation performed in Erlenmeyer flasks. Cumulative hydrogen production of *T. litoralis* cultures was monitored in time.

Increased hydrogen production could be seen for each culture growing on medium that was supplemented with feather hydrolysate compared to the control growing on CMSY medium without additives (Fig. 7). However, a significant variance in the hydrogen production ability of the archaeon that depended on the time of keratin degradation was observed. The early phase of feather fermentation (16 h and 24 h) yielded a hydrolysate with a moderate protein content that allowed hydrogen production to start without a lag phase, however the final hydrogen concentration in the headspace was rather small. This was probably due to the rapid depletion of the solubilized proteins. The mid phase of feather fermentation (48 h and 60 h) yielded a solution with a larger protein content that supported the highest final hydrogen concentration in the headspace (Fig. 7). Cells grown on samples derived from the late phase of fermentation (84 h) allowed moderate hydrogen production that

started only after a notable lag phase. To see if this lag phase is the result of an excessive protein content of the fermentation medium, a serial dilution of the 84-hour sample in CMSY minimal medium was prepared and inoculated with *T. litoralis*. Cultures grown on limited amounts of the 84-hour hydrolysate showed decreased hydrogen production compared to those cultures fed with CMSY supplemented with 10 % (v/v) of 84 hour sample (data not shown). This data suggests that, besides the changes in the protein concentration, time-dependent variations in the composition of the fermentation broth are also an important factor affecting the hydrogen production of *T. litoralis*.

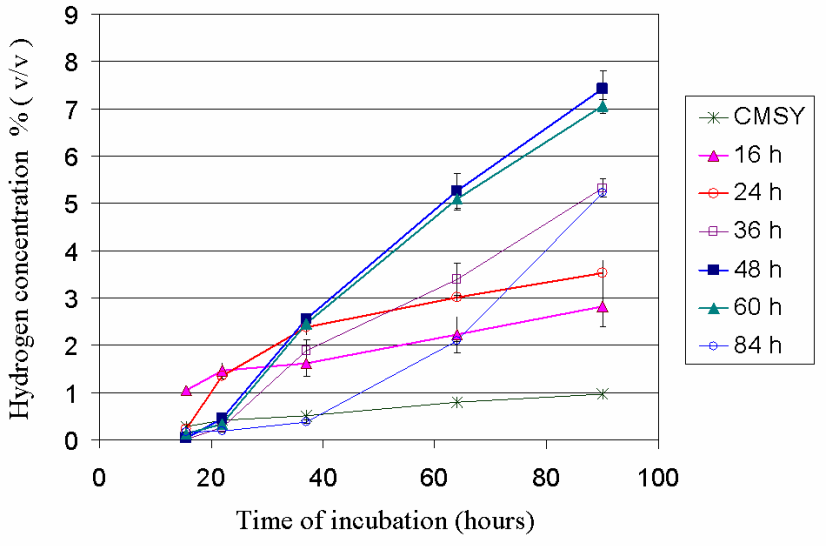


Figure 7 Cumulative hydrogen production of *T. litoralis* grown on feather hydrolysate samples collected at various time intervals. Degradation of feather was performed by *B. licheniformis* KK1 in Erlenmeyer flasks.

When hydrolysis is carried out in Erlenmeyer flasks, feather degradation performed by *B. licheniformis* KK1 for up to 60 hours was found to be optimal for the concomitant hydrogen production. Shorter degradation time yielded a fermentation broth with low protein content while longer fermentation time seems to render the keratin hydrolysate composition less suitable for hydrogen production with *T. litoralis*.

4.2.3.2. Degradation carried out in fermenter

To determine the optimal length of keratinolytic treatment carried out in the fermenter, *T. litoralis* cultures were grown on media supplemented with feather hydrolysate samples that represented different stages of feather fermentation.

Samples of the early fermentation stage (24 h) were shown to allow only moderate hydrogen production preceded by a notable lag phase (Fig. 8). Hydrolysis of chicken feathers

in the fermenter for 42 hours yielded a much more suitable feedstuff that supported intensive hydrogen generation. Best hydrogen production results were obtained with samples treated for 60 hours, while significantly longer proteolysis times (92 and 138 h) did not have significant effect on the hydrogen production capacity of the archaeon. Therefore, a keratin hydrolysis time of 60 h is recommended in the fermenter. Notably, cells growing on keratin hydrolysate prepared in fermenter displayed more intense hydrogen production than those fed with hydrolysates from Erlenmeyer flasks. Maximum hydrogen concentration in the headspace of *T. litoralis* cultures reached 6 % (v/v) already within 48 hours while a comparable hydrogen concentration was observed only after about 85 hours in the flask experiments. Along with the improved hydrogen production, an increased cell density could also be observed for the cultures grown on feather hydrolysates taken from the fermenter.

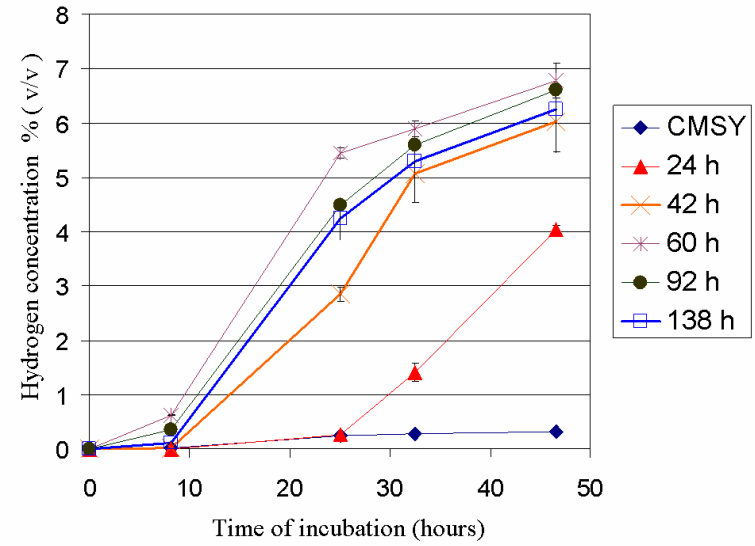


Figure 8: Cumulative hydrogen production of *T. litoralis* grown on feather hydrolysate samples collected at various time intervals. Degradation of feather was performed by *B. licheniformis* KKI in fermenter.

4.2.4. Relative performance of feather meal hydrolysate vs. standard substrates

The relative performance of feather hydrolysate as a media supplement was compared to Bacto Peptone, which is the standard peptidic growth substrate for *T. litoralis* in the DSM 623 medium. In this experiment, same amounts of feather hydrolysate or Bacto Peptone were used to feed *T. litoralis*.

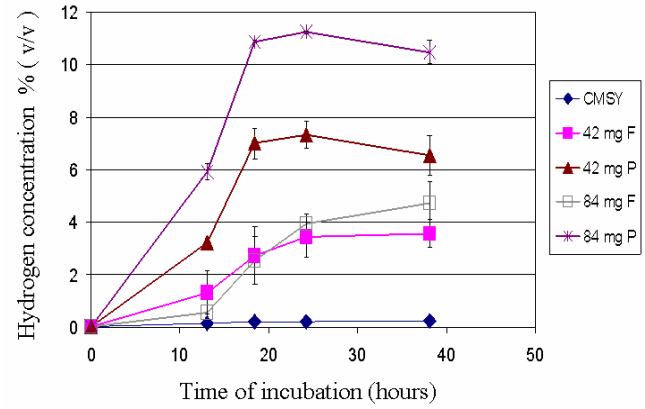
Cells grew better on Bacto Peptone and produced roughly two times more hydrogen than on feather hydrolysate (Fig. 9A). Hydrogen concentrations in the headspace after 48 hours of incubation were normalized to the optical densities of the cultures, allowing the calculation of a ratio between hydrogen production and cell growth for each substrate. Hydrogen productions normalized on the optical density of the cultures were found to be

nearly identical for both nutrients, revealing a direct connection between the hydrogen production capability and the culture's growth rate.

Both the optical density and the produced hydrogen doubled when the amount of peptone was increased twofold in the medium. In contrast, a higher substrate concentration did not result in a proportionally higher growth or increased hydrogen production in the case of feather hydrolysates indicating significant differences in the compositions of the two nutrients.

The hydrogen productions were normalized on amounts of nutrient the cells consumed. The value calculated for cultures grown on keratin hydrolysate was comparable to the one obtained for cultures growing on peptone (Fig. 9B). These data show that fermentation broth produced from keratin waste material via bacterial degradation is a suitable feedstuff for *T. litoralis* and is comparable with the gold standard Bacto Peptone.

A



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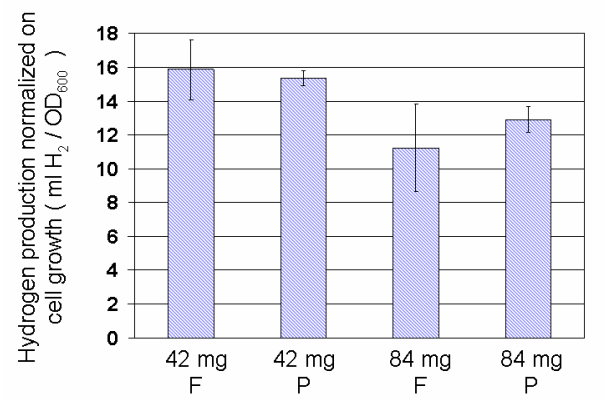

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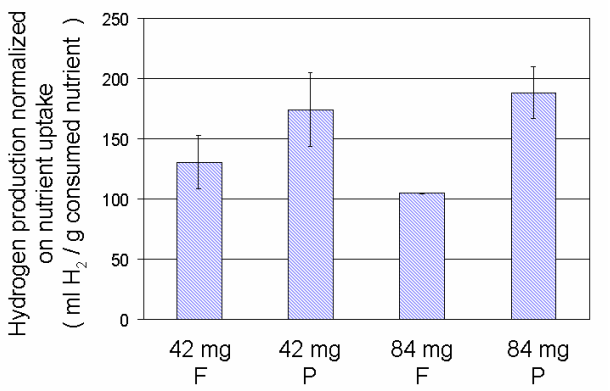


Figure 9 Relative performance of feather meal hydrolysate. **A:** Hydrogen production of *T. litoralis* on 42 and 84 mg of feather hydrolysate compared to cultures grown on the same amounts of Bacto Peptone. **B:** Hydrogen production of *T: litoralis* on feather hydrolysate (marked with F) or Bacto Peptone (marked with P) normalized on the cell growth after 38 hours of incubation. **C:** Ratio between produced hydrogen and consumed protein calculated for cultures grown on various substrates after 38 hours of incubation.

4.2.5. Hydrogen production of various microbes on feather hydrolysate

Besides the hyperthermophilic archaeon, *T. litoralis*, other potential hydrogen-producing microbes were tested for hydrogen production on feather hydrolysate. The Gram-negative mesophilic *Escherichia coli* K12, the Gram-positive thermophilic *Caldicellulosiruptor saccharolyticus* and the hyperthermophilic *Pyrococcus furiosus* were grown in their appropriate minimal media supplemented with 10 % (v/v) of fermentation broth. After 48 hours of incubation optical density of the cultures and hydrogen concentration in the headspace were determined. *C. saccharolyticus* and *E. coli* did not show significant growth on feather hydrolysate and displayed a negligible hydrogen production.

Out of the three microbes tested, only *P. furiosus* was able to utilize the keratinaceous nutrient source for hydrogen production. Cells grown on feather hydrolysate produced nearly the same amount of hydrogen that *T. litoralis* did in the previous experiments (Fig. 10). Remarkably, however, a twofold increase in added peptone did not result in doubled hydrogen production of *P. furiosus*. Along with this, cells displayed an inferior growth compared to *T. litoralis* when increased amount of Bacto Peptone was added.

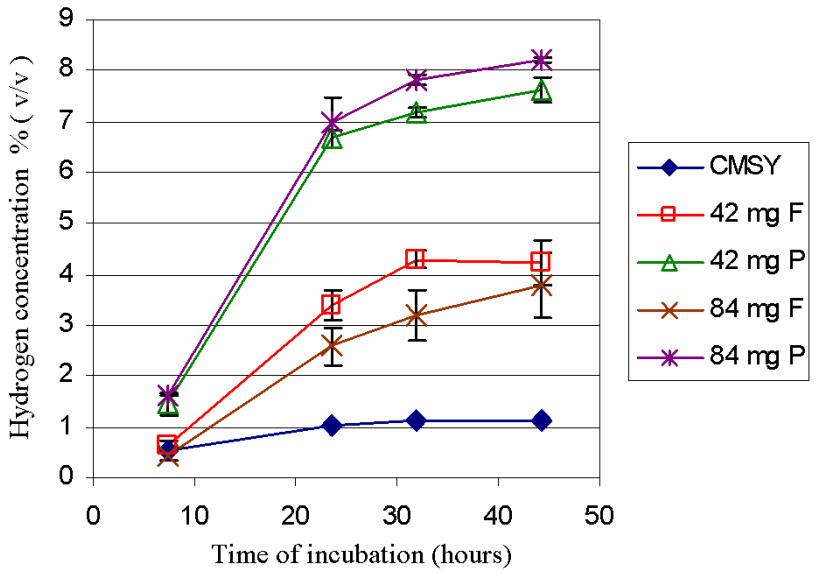


Figure 10 Hydrogen production of *P. furiosus* on identical amounts of feather hydrolysate (marked as F) and peptides (marked as P).

These findings suggest a limited growth / hydrogen production potential of *P. furiosus* on peptides. Based on the differences in hydrogen production capabilities on peptone and the optimal growth temperatures, *T. litoralis* was selected as a hydrogen producer for the scale-up hydrogen-producing fermentations.

4.2.6. Scale-up studies on the hydrogen production of *T. litoralis* on feather hydrolysate

Scale-up hydrogen-producing fermentations on feather hydrolysate were performed in a 6.9 L fermenter with a culture volume of 2.5 L containing 10 % (v/v) of chicken feather hydrolysate (derived from fermenter). Optical density and protein content of the fermentation broth as well as hydrogen concentration of the headspace were monitored in time.

Intensive cell growth (Fig. 11A) was observed during the first 24 h, and was accompanied by a significant consumption of proteins from the fermentation broth (Fig. 11B).

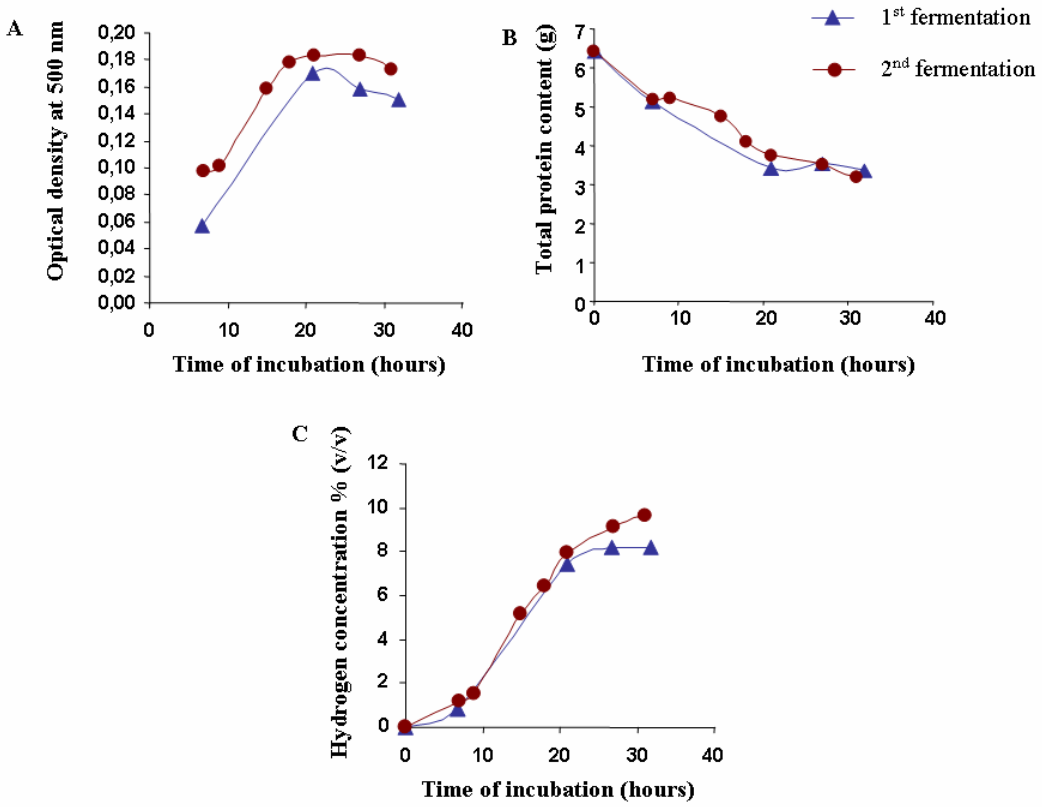


Figure11 Scale-up studies on the hydrogen-producing fermentation carried out with *T. litoralis* on feather hydrolysate. **A**: Time function of the optical density of *T. litoralis* culture grown on feather fermentation broth in fermenter. **B**: Consumption of nutrients from the fermentation broth by the archaeon during the fermentation. **C**: Time dependence of the hydrogen concentration in the headspace of the fermenter. The two curves represent two independent sets of experiments.

To maintain the pH of the culture, a substantial amount of alkali was required, indicating intense metabolic activity of the cells in the first 20 h. Rapid accumulation of hydrogen could be detected in the headspace. Within 48 h, the final concentration of hydrogen reached 10 % (v/v) (Fig. 11C), which is nearly 50 % more than was obtained for cultures growing in hypovial bottles on the same medium. In addition, the optical density of the

cultures cultivated in fermenter exceeded the values determined earlier for cultures grown in hypovials.

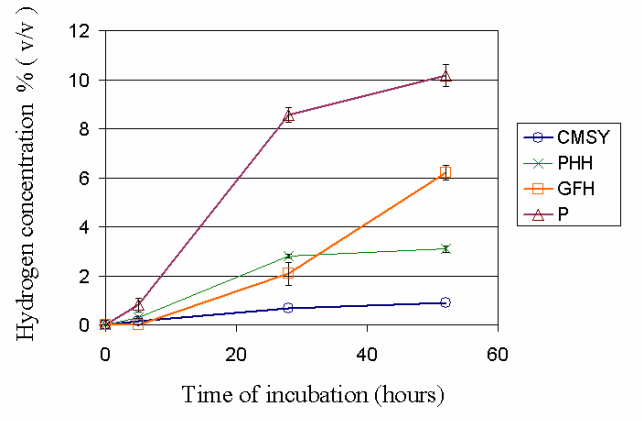
In conclusion, a 125 × scale-up of the hydrogen production step with an improved hydrogen production and growth rate could be achieved.

4.3. Adaptation of the two-stage hydrogen production system to other proteinaceous substrates

4.3.1. Hydrogen production on hydrolysates produced from pig hair and goose feathers

To test if the two-stage system can be operated with keratinaceous wastes other than chicken feathers, goose feather mail and intact pig hair were digested with *B. licheniformis* KK1 for 48 hours in Erlenmeyer flask and the resulting fermentation broths were evaluated as nutrient sources for *T. litoralis*. The archaeon displayed more intense hydrogen production on both keratin hydrolysates than did on CMSY minimal medium (Fig. 12A). The ratio between produced H_2 and consumed nutrients were calculated for both keratinaceous substrates (Fig. 12B) and were found to match the values calculated for chicken feather in previous experiments.

A



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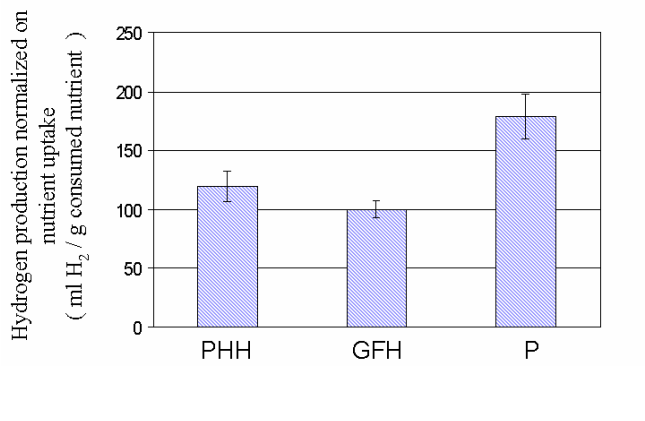

Figure 12 Hydrogen production of *T. litoralis* on various keratinaceous media. **A:** Hydrogen production of cells grown on pig hair fermentation broth (PHH) or goose feather hydrolysate (GFH) compared to cultures grown on identical amounts of Bacto Peptone (P). **B:** Ratio between produced hydrogen and consumed protein calculated for cultures grown on various keratinaceous substrates after 52 hours of incubation.

4.3.2. Hydrogen production of *T. litoralis* on raw meat powder

Due to the current EU regulations, meat meal cannot be further used for feeding farmed animals. However, industrial processing of proteinaceous animal waste materials still produces large amounts of meat meal that are mainly incinerated due to the lack of a more economical method.

Meat meal has nearly 40 % animal-digestible protein content and was considered as a potentially easy-to-utilize substrate for hydrogen production with *T. litoralis*. To test if the archaeon can digest meat meal with its own extracellular enzyme apparatus, cells were grown on CMSY minimal medium supplemented with various amount of raw meat meal. Surprisingly, *T. litoralis* failed to utilize meat meal for growth and hydrogen production. Moreover, a complete inhibition of hydrogen production was seen in the presence of 5 g/l meat material. (Table 2)

Culture medium	Hydrogen concentration (% v/v)	H_2 production compared to CMSY
CMSY	0.4 ± 0.02	
CMSY + 0.25 g/L MM	0.035 ± 0.07	no significant change
CMSY + 0.5 g/L MM	0,38 ± 0.03	no significant change
CMSY + 1 g/L MM	0.4 ± 0.003	no significant change
CMSY + 5 g/L MM	0 ± 0	complete inhibition

Table 2 Hydrogen production of *T. litoralis* on raw meat meal after 40 hours of incubation on CMSY medium supplemented with varied amount of raw meat meal (MM).

4.3.3 Effect of bacterial pretreatment of meat powder on the hydrogen production of *T. litoralis*

Pure microbial cultures of soil bacteria (*Bacillus licheniformis* KK1, *Bacillus megaterium*, *Pseudomonas koreensis*, *Rhodococcus erythropolis)* and fat / oil degrader isolates (L1, L2, L3, L4) as well as mixed consortia were used to treat meat meal. The fermentation outlets were tested for hydrogen production with *T. litoralis*. Meat meal hydrolysate obtained from the treatment of 3 % meat meal with *B. licheniformis* KK1 was found to give the best fermentation broth for the concomitant hydrogen production with *T. litoralis*.

Hyperthermophilic cultures grown on CMSY supplemented with 10-50 % (v/v) meat

meal hydrolysates produced 6-14 times more hydrogen than those grown on CMSY (Fig. 13). When the meat meal hydrolysate produced by *B. licheniformis* KK1 was used in the 10-40 % (v/v) range, the final hydrogen concentration in the headspace was proportional to the amount of substrate added.

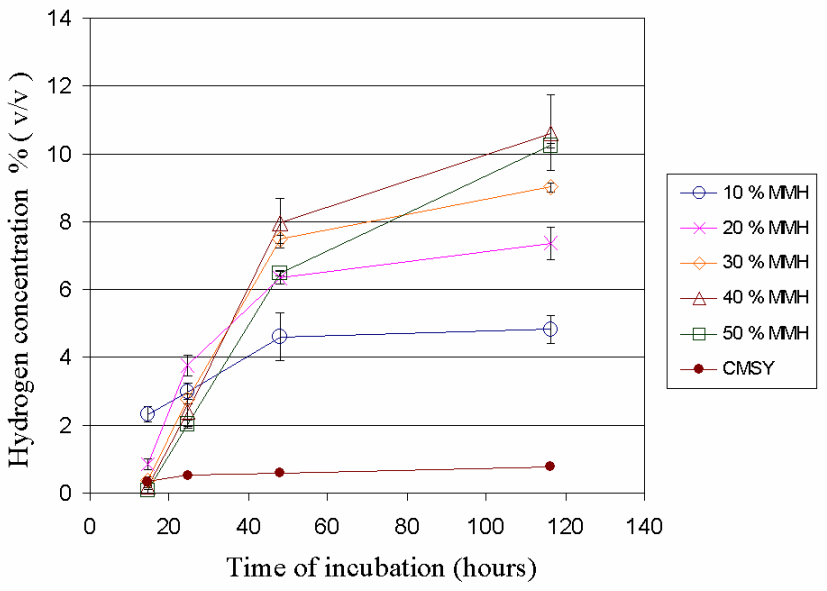

Figure 13 Hydrogen production of *T. litoralis* on pre-digested meat meal. Meat meal hydrolysate (MMH) was added in 10-50 % (v/v) to complement CMSY medium.

4.3.4. Scale-up studies on the hydrogen production of *T. litoralis* on meat meal hydrolysate

The meat meal hydrolysate giving the best hydrogen production results with *T. litoralis* in the small-scale experiments (40 % v/v) was selected for the scale-up studies in a 6.9 L fermenter with 2.5 L of culture volume.

Cells growing in the fermenter displayed outstanding hydrogen evolving capabilities with a final hydrogen concentration in the headspace reaching 25 % within 92 hours (Fig. 14A). Cultures grown on meat meal hydrolysate in the fermenter displayed a prolonged hydrogen production compared to cells grown on feather hydrolysate in previous experiments. Along with the accumulation of hydrogen in the headspace, a significant decrease in the protein content of the fermentation broth could be detected (Fig. 14B). *T. litoralis* was shown to consume roughly half of the nutrients added.

The two-stage system adapted to process meat meal yielded 37 ml hydrogen per g of waste material initially treated, which is very close to the value obtained for chicken feather waste.

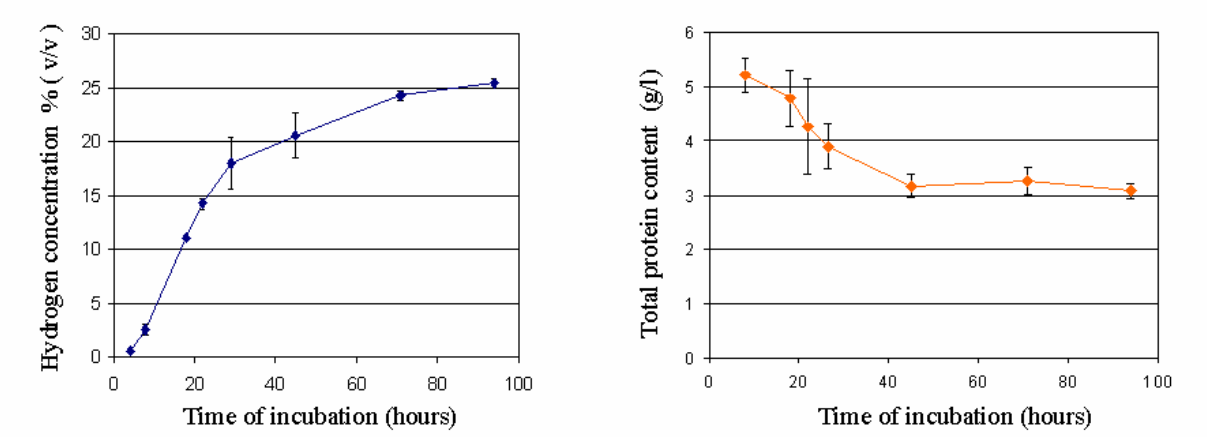

Figure 14 Scale-up studies on the hydrogen-producing fermentation carried out with *T. litoralis* on meat meal hydrolysate **A**: Cumulative hydrogen production of *T. litoralis*. **B**: Changes in the protein content of the fermentation broth during hydrogen fermentation. Curves represent the averages of two independent fermentations.

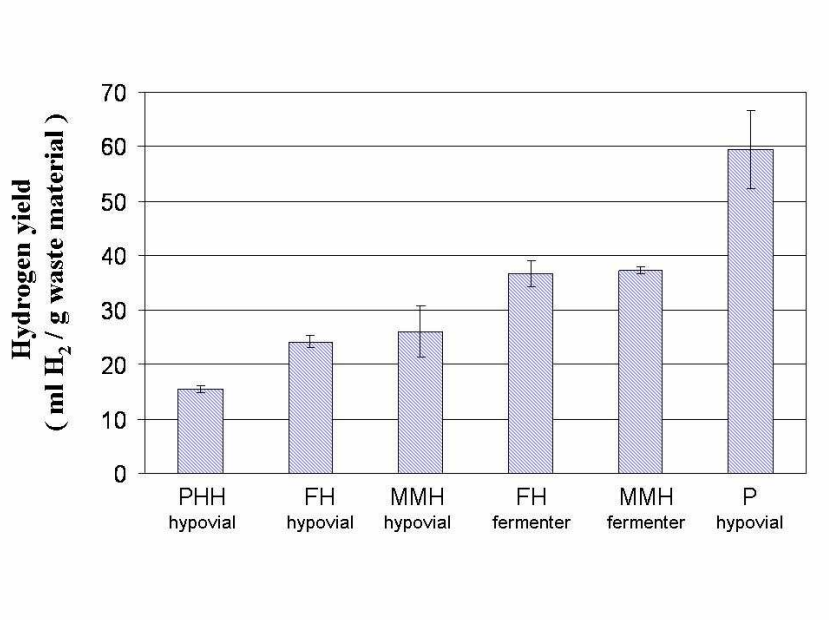

Figure 15 Overall hydrogen production yields of the two-stage fermentation system operated with various waste materials. The amount of hydrogen evolved in the hydrogen production stage was divided by the mass of waste materials treated in the degradation stage. PHH: pig hair hydrolysate; FH feather hydrolysate; MMH: meat meal hydrolysate; P: peptone; Hydrogen-producing fermentations were carried out either in hypo-vial bottles or in a high temperature fermenter.

4.3.5. Yields of the two-stage waste utilization system

Overall hydrogen production yields of the two-stage system were calculated for the different types of waste materials used. The volume of hydrogen produced in the second stage was normalized on the mass of waste material initially treated in the first step. Chicken

feather and meat meal gave the best results in the two-stage waste utilization system, while pig hair could be used only with moderated yields (Fig. 15). Yields on both substrates could be further increased when the hydrogen-producing fermentations were carried out in a high temperature fermenter.

4.4. Cloning and heterologous expression of the keratinase from *B. licheniformis* KK1

Use of concentrated enzyme solution instead of whole-cell in the feather/meat meal fermentation might improve the performance of the degradation process. For this, a heterologous/homologous system expressing the recombinant keratinase enzyme had to be developed.

4.4.1. Sequence analysis of the keratinase (*kerA*) gene

Primers designed on the consensus sequence of known *Bacillus* keratinase genes were used in high fidelity polymerase chain reaction to amplify an 1140 bp fragment containing the keratinase gene of *B. licheniformis* KK1.

Nucleotide sequence of the cloned fragment was determined, revealing one open reading frame with a corresponding protein sequence of 379 amino acids. BLASTP (Basic Local Alignment Tool for Proteins) analysis showed 98-99 % sequence identity towards known *Bacillus* keratinases over the entire length of the deduced protein sequence. All three characteristic polypeptide regions (pre-, pro- and mature) could be mapped exactly as it was previously found for the keratinase of *Bacillus licheniformis* PWD-1 strain (Lin et al. 1995). Southern hybridization experiments confirmed that the amplified gene is present in one copy in the *B. licheniformis* KK1 genome (data not shown).

4.4.2. Heterologous expression of KerA

For protein overexpression studies a truncated *kerA* gene was cloned into the pBAD/gIIIA vector, in which the expression of the gene of interest was under the control of the arabinose promoter. The original bacillus-type signal sequence (i.e. the pre region) was replaced with a plasmid-borne pIII signal sequence to redirect the expressed fusion protein into the periplasm of *E. coli*.

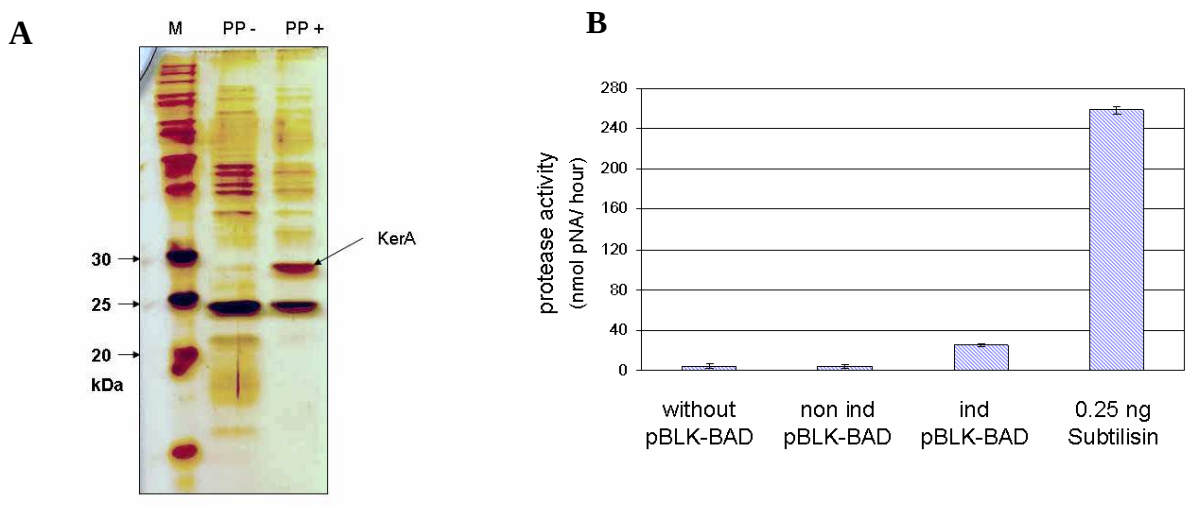

Figure 16 Heterologous expression of the *B. licheniformis* keratinase in *E. coli*. **A**: Periplasmic protein fractions of uninduced (PP-) and induced (PP+) *E. coli* transformed with pBLK-BAD analyzed on a silver-stained 12 % polyacrylamide gel. **B**: Protease activity of *E. coli* periplasmic samples against N-succinyl-Ala-Ala-Pro-Phe-pNA. Activities of induced (ind) and non induced (non ind) *E. coli* TOP10 strain transformed with pBLK-BAD plasmid were compared. Cells lacking the keratinase plasmid and 0.25 ng subtilisin were used as negative and positive control respectively. Proteinase activity is expressed as nanomoles of pNA released per hour.

An additional protein band with an estimated molecular weight of ~30 kDa could be detected in the periplasmic fraction after the induction of *E. coli* containing the expression cassette (Fig. 16A). It coincided with an increased periplasmic activity against N-succinyl-Ala-Ala-Pro-Phe-pNA, a chromogenic substrate that is a preferred substrate for keratinase (Evans et al. 2000) (Fig. 16B).

It has to be noted, that the enzyme activity against pNA-conjugated peptide substrate in the induced periplasmic sample is comparable with the activity of Carlsberg subtilisin within the pg/ml concentration range. This indicates that the amount of active keratinase is very low. Indeed, a substantial portion of the overexpressed keratinase was found aggregated into insoluble inclusion bodies and was detectable in the cell debris fraction resulting in a very limited keratinase yield.

5. Discussion

Agriculture and food industries produce enormous quantities of protein-rich by-products whose environmentally sound decomposition is a great challenge. Degradation of feathers and animal hair wastes are especially difficult to carry out at industrial scale, since they are resistant against a wide variety of physical, chemical and enzymatic decomposition agents due to the molecular structure of their main structural protein, keratin (Jones and Pope 1984; Lynch et al. 1986). Several physical and chemical techniques were developed for the breakdown of these materials, and potential use of the hydrolysates in the production of fertilizers, glues, and feed additives has been recognized (Onifade et al. 1998; Friedrich and Antranikian 1996). However, most of the methods tested are energy demanding and environmentally harsh. Microbial degradation of keratin was demonstrated with several bacteria possessing keratinase enzymes (Lin et al. 1992; Friedrich and Antranikian 1996; Nam et al. 2002; Onifade et al. 1998; Riffel et al. 2003). Biological fermentation systems based on innate keratin degradation abilities of microorganisms, being more cost efficient and environmentally friendly, have the potential to replace physical and chemical treatments.

We constructed a two-stage fermentation system, a processor of keratinaceous animal wastes combined with biohydrogen production. In the first step, keratin was degraded by *B. licheniformis* KK1, yielding a peptide-rich fermentation broth that could be utilized for hydrogen production in the second stage. The degradation of keratinaceous biowaste was demonstrated in detail for chicken feathers. The process was optimized to provide a fermentation broth that is best suited for the production of hydrogen in the next fermentation step. In addition to chicken feathers, degradation of pig hair and goose feathers were also carried out and the fermentation broths were used as nutrient source for the hydrogen-producing archaeon, T. litoralis. Hence, the two-stage system appears to work with all keratin-containing animal waste tested so far

The hydrogen evolution step was tested with the hyperthermophilic archaea *T. litoralis* (Neuner et al. 1990) and *P. furiosus* (Fiala and Stetter 1986), the Gram-positive bacterium, *C. saccharolyticus* (Rainey et al. 1994), and the *E. coli* K12 strains. Each strain is able to evolve hydrogen during fermentative growth (Bryant and Adams 1989; Joyner et al. 1977; Kádár et al. 2003; Rákhely et al. 1999). Substantial amounts of hydrogen from keratinolytic peptides could be obtained only with the archaea. Comparison of the two archaeal microbes revealed that their normalized hydrogen evolving capacities (hydrogen produced/substrate consumed) were similar, but *T. litoralis* performed better when the concentration of peptide

substrates was raised. Notably, *T. litoralis* was recently demonstrated to possess a membrane bound formate hydrogenlyase complex that is up-regulated in cells growing on peptidic media (Takács et al. 2008). This complex converts formate to hydrogen and carbon dioxide and is believed to be tightly coupled with the peptide fermentation pathway in *T. litoralis*. Lack of a similar complex in *P. furiosus* might be the factor limiting the hydrogen production when cells are grown in media containing peptides at higher concentrations.

T. litoralis was able to grow and produce significant amounts of hydrogen on keratin hydrolysates. Roughly half the quantity of hydrogen could be produced from the fermentation broth compared to identical amount of Bacto Peptone, a high-quality standard protein source generally used at lab scale as a nutrient for complex media. The results clearly demonstrated that keratinaceous waste stream was a good source of organic material for hydrogen production that can replace the expensive Bacto Peptone.

Both the keratin degradation and the biohydrogen production step were scaled up and performed in fermenters. Generally, for both steps, the performance was significantly improved. Parameters monitored through the hydrogen-producing fermentation step (optical density of the culture, protein content in the medium, hydrogen accumulation in the headspace, consumption of alkali for maintaining pH) confirmed intensive metabolism and superior hydrogen production of the archaeon in two independent experiments.

The yields of hydrogen produced from carbohydrates are generally compared to the theoretical maximum that is estimated based on the sugar fermentation pathways. However, for fermentation systems converting proteinaceous substrates to hydrogen such comparisons cannot be easily carried out given the complexity of pathways involved in the peptide metabolism. Thus, a simplified calculation was carried out dividing the volume of hydrogen produced by the amounts of waste material initially supplied to the degradation step. Best conversion of chicken feathers to hydrogen was obtained when both the degradation step and the hydrogen evolution were carried out in fermenter allowing the production of 36 ml of hydrogen per gram of chicken feathers. Based on the current performance of the two-stage system, utilization of all the nearly 30,000 tons of feather waste produced in Hungary in 2006 (source: Hungarian Central Statistical Office) could theoretically yield 12,6 GJ of energy, the equivalent to 371 000 m^3 of natural gas.

In addition to keratinaceous wastes, roughly 40,000 tons of meat meal unsuitable for human consumption is annually produced in Hungary (source: Mátra Power Plant JSC). This type of protein-rich material was widely used as animal feed stuff until 2002, when EU prohibited the utilization of animal wastes for feeding farmed animals. Since then, meat meal

is mainly combusted in cement kilns or thermal power plants. However, ignition of meat meal does not seem to be the optimal solution as it is associated with the release of air pollutants such as dioxin (Cheung et al. 2007), various nitrogen oxides and sulfur-dioxide (Cummins et al. 2006).

The two-stage fermentation system initially built for the utilization of keratinaceous materials was adapted to process meat meal, as an alternative substrate for hydrogen production. Many species of Thermococcales order are known to possess extracellular protease enzymes (Voorhorst et al. 1996; Voorhorst et al. 1997; Pulido et al. 2006) and elaborate peptide uptake mechanisms (Albers et al. 2004). Surprisingly, *T. litoralis* alone failed to produce hydrogen on raw meat meal. Instead, the material even had an inhibitory effect on the growth. However, proteolytic treatment of meat meal with *B. licheniformis* KK1 yielded a hydrolysate that was well-suited for hydrogen production step with *T. litoralis*. The archaeon fed with pre-treated meat meal displayed a hydrogen production performance similar to the previously grown on keratinaceous substrates. Best conversion of meat meal to hydrogen was achieved when the hydrogen production was performed in fermenter, when 1 g meat meal yielded 37 ml of H_2.

Yield calculations on the two-stage hydrogen-producing system operated with keratinaceous substrates revealed that the overall performance highly depends on the efficiency of the keratin degradation step. Use of a concentrated keratinase solution instead of whole cell culture can be beneficial by allowing shorter processing time, tighter control and might contribute to better hydrogen production yields. As a first step towards a stock of purified *B. licheniformis* KK1 keratinase, the *kerA* gene coding for the enzyme was cloned and its nucleotide sequence was determined. The deduced protein sequence was identified as a serine protease with high degree of identity (98-99 %) towards the known *Bacillus* subtilisins and keratinases, including the well-known enzyme of *B. licheniformis* PWD-1 keratin degrader strain. A heterologous expression system was constructed to overexpress the keratinase extracellularly. Presence of active keratinase enzyme could be detected in the periplasmic fraction of *E. coli* transformed with pBKL-BAD plasmid carrying the expression cassette. However, the amount of active keratinase enzyme in the periplasm was far below of our expectations since the majority of the overexpressed protein aggregated and formed insoluble inclusion bodies. The tested protein expression system could not yield enough active keratinase to support a cell-free application thus further optimization is therefore needed to obtain sufficient amounts of active keratinase.

The gas mixture produced from pre-digested waste materials with *T. litoralis* is

generally not pure enough for energy production in fuel cells. It contains up to 18 % (v/v) carbon dioxide which had to be removed prior to use. In tight co-operation with the Research Institute of Chemical and Process Engineering at University of Pannonia, a gas separation procedure was developed for the purification of hydrogen produced by *T. litoralis*. Using the combination of a porous polyethylene hollow-fiber membrane and a non-porous polyether-sulphone-polyimide composite membrane, hydrogen produced in our system could be concentrated three times (Bélafi-Bakó et al. 2006).

The economy of the waste treatment system is difficult to estimate at this point since the process needs further technological development. However, data presented here demonstrates the proof of the principle, that the decomposition of meat meal as well as a range of keratinaceous wastes can be linked to biohydrogen production.

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8. Összefoglalás (Summary in Hungarian)

Eredményeim a következő pontokban foglalhatóak össze:

1. Kifejlesztettem egy minimál tápoldatot (CMSY) és egy mérési módszert annak eldöntésére, hogy különféle szerves anyagok alkalmazhatóak-e hipertermofil hidrogéntermelő mikroorganizmusok tápanyagaként.

2. *Bacillus licheniformis* KK1 törzs felhasználásával, Erlenmeyer-lombikban 84 óra alatt sikerült egész csirketollat lebontatnom. Igazoltam, hogy hidrolízis során a tápoldatban kisméretű peptidek halmozódnak fel. Megállapítottam a hidrogéntermelés szempontjából legkedvezőbb keratin bontási időtartamot.

3. Több hidrogéntermelő mikroorganizmus (*Escherichia coli*, *Caldicellulosiruptor saccharolyticus*, *Thermococcus litoralis* és *Pyrococcus furiosus*) összehasonlítását követően megállapítottam, hogy a *T. litoralis* a legalkalmasabb jelölt a keratin hidrolizátumon történő hidrogéntermelésre.

4. Megállapítottam, hogy a csirketoll kevertetéses fermentációjához a szubszrátot előzetesen őrölni kell. Megvalósítottam a keratin bontó lépés 3,5-szeres léptéknövelését szabályozott, fermentoros körülmények között. Bizonyítottam, hogy a fermentáció 138. órájára a toll dara elbomlik, miközben kisméretű peptidekben gazdag tápoldat keletkezik.

5. Bizonyítottam, hogy a toll bontásából kapott tápoldat a költséges Bacto Peptone-hoz hasonlóan használható *T. litoralis* tápanyagforrásaként.

6. Egy magas hőmérsékletű fermentorban megvalósítottam a keratin hidrolizátumon végzett hidrogéntermelés 125-szörös léptéknövelését. A hidrogéntermelő fermentációt fermentorban elvégezve az addigi legmagasabb hidrogén koncentrációt és hidrogén kihozatalt tapasztaltam.

7. A kétlépéses fermentációs eljárást sertésszőr és húsliszt hasznosítására adaptáltam. Megmutattam, hogy a fermentációs eljárás révén számos állati eredetű hulladékból hidrogéngáz termeltethető.

8. Izoláltam a *B. licheniformis* KK1 keratinázát kódoló *kerA* gént, majd meghatároztam annak nukleotidsorrendjét.

9. A keratináz *E. coli*-ban történő túltermeltetéséhez létrehoztam egy fehérje túltermelő konstrukciót. A pBLK-BAD vektorral transzformált, indukált *E. coli* sejtek periplazmájában aktív keratináz jelenlétét mutattam ki.

9. Summary

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 Erlenmeyer flasks using the *Bacillus licheniformis* KK1 strain, and I have proven that near complete degradation of feather occurs within 84 hours of incubation accompanied by an 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 *T. 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 parallel, 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 pig hair and meat meal. I have 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.