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Biological treatment of chicken feather waste for improved biogas production

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Abstract

A two-stage system was developed which combines the biological degradation of keratin-rich waste with the production of biogas. Chicken feather waste was treated biologically with a recombinant *Bacillus megaterium* strain showing keratinase activity prior to biogas production. Chopped, autoclaved chicken feathers (4%, *W*/*V*) were completely degraded, resulting in a yellowish fermentation broth with a level of 0.51 mg/mL soluble proteins after 8 days of cultivation of the recombinant strain. During the subsequent anaerobic batch digestion experiments, methane production of 0.35 Nm^3/kg dry feathers (i.e., 0.4 Nm^3/kg volatile solids of feathers), corresponding to 80% of the theoretical value on proteins, was achieved from the feather hydrolyzates, independently of the pre-hydrolysis time period of 1, 2 or 8 days. Cultivation with a native keratinase producing strain, *Bacillus licheniformis* resulted in only 0.25 mg/mL soluble proteins in the feather hydrolyzate, which then was digested achieving a maximum accumulated methane production of 0.31 Nm^3/kg dry feathers. Feather hydrolyzates treated with the wild type *B. megaterium* produced 0.21 Nm^3 CH_4/kg dry feathers as maximum yield.

Key words: anaerobic digestion; feather waste; keratinase, *Bacillus licheniformis*; *Bacillus megaterium*; gene expression

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Introduction

The consumption of chickens generates a large amount of feather waste per year (Onifade et al., 1998; Sangali and Brandelli, 2000). Feather meal is generally used as animal feed because it's high protein content. The utilization of waste for animal feed is becoming tighter (Commission of the European Communities, 2000) and moreover the high treatment costs make the process economically not feasible.

Anaerobic digestion is an environmentally and economically promising process to recover feather waste for renewable energy production. Besides of the production of valuable products as methane and digested residue, which can be used as fertilizer, it also destroys pathogens presented in the feather waste (Salminen and Rintala 2002a, 2002b).

Although, the theoretical methane yield is 0.496 Nm^3/kg VS (volatile solids) from proteins (Davidsson, 2007), just around 0.21 Nm^3/kg VS (Salminen et al., 2003) can be obtained from untreated feather wastes, because of the compact structure of keratins making up the feathers. In bird feathers the protein is presented as β-keratin, which contains disulfide bonds, hydrogen bondings and hydrophobic interactions, resulting in a very resistable protein against the attack of most proteolytic enzymes (Ramnani and Gupta, 2007; Coward-Kelly et al., 2006).

Several pretreatment methods, including chemical, enzymatic or biological treatments, have been therefore investigated in the past few years to improve the digestibility of feathers (Coward-Kelly et al., 2006; Wang and Parsons, 1997; Latshaw et al., 1994; Papadopoulos, 1989). As a result of a chemical treatment, using calcium hydroxide at 150°C, 80% up to 95% of the feather keratin could be dissolved (Coward-Kelly et al., 2006). However, the high temperature and the addition of chemicals make this method economically and environmentally less attractive. Ramnani and Gupta (2007) have found that conventional proteases as pepsin and trypsin cannot degrade feather, while savinase and subtilisin are able to solubilize feathers.

In nature keratin can be an appealing supply for keratin-degrading microorganisms such as *Bacillus licheniformis* (Lin et al., 1992), and for various dermatophyta fungi. These organisms have made it possible to develop several environmentally friendly methods utilizing keratinolytic enzymes for handling the keratin-containing wastes (Sangali and Brandelli, 2000). Moreover, to improve the performance of the degradation process, the attention is

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turned to the development of recombinant strains with increased keratinase production (Rao et al., 1998). *Bacillus megaterium* has previously been reported as a productive host cell for the production of extracellular enzymes such as amylases and penicillin amidase (Malten et al., 2005). Using this system resulted in an efficient recombinant protein production due to its high level of protein secretion capacity and low level of protease activity.

The aim of this study was to develop a two-stage system which combines the biological degradation of keratin-rich waste with the production of biogas. Keratinase gene from *B. licheniformis* ATCC® 53757 was cloned into an expression vector, pHIS1525.SP_{lipA} followed by the expression of the recombinant keratinase in *Bacillus megaterium* ATCC® 14945. The recombinant strain was then used for the degradation of chicken feather waste as a pretreatment step prior to biogas production. Different feather hydrolyzates obtained after different long pretreatment times were utilized in the second step for biogas production.

1 Materials and methods

1.1 Bacterial strains, plasmid, and medium

Bacterial strains, vectors and primers used in this study are given in Table 1. *B. licheniformis* ATCC® 53757, with high keratinolytic activity, was used as a source for the keratinase gene, and the strain *B. megaterium* ATCC® 14945 was used for transformation and cloning. Both bacterial strains were obtained from the American Type Culture Collection (ATCC, USA), maintained on LB agar (1.5%, *W*/*V*) and subsequently grown in LB broth (pH 7.5).

Protoplasts of *B. megaterium* strain used for the transformation were prepared and maintained in RHAF medium as described by McCool and Canoon (2001) with modifications in lysozyme concentration and incubation time.

To find recombinants with high proteolytic activity, the recombinants were plated on milk agar plates (5 g/L peptone, 3 g/L yeast extract, 100 mL/L sterile ultra-heat treated (UHT) non-fat milk, and 12 g/L agar) (Riffel and Brandelli, 2006) containing tetracycline (10 μg/mL) and xylose (0.5%, *W*/*V*).

1.2 DNA manipulations

Chromosomal DNA of *B. licheniformis* ATCC® 53757 was isolated according to molecular biology protocols as described by Ausubel et al. (1996) and was used as a template for PCR amplification of the *ker* gene. The primers BglII-B.M-kerA-Foward and SphI-B.M-kerA-Reverse (Invitrogen, USA) were designed using the software Vector NTI Advance.v11.0 (Table 1). The vector used was pHIS1525.SP_{lipA} (MoBitech GmbH, Germany), which is a *B. megaterium* expression vector, carrying a secretion signal peptide sequence, a 6xHis-tag, and a xylose inducible promoter (Table 1). PCR products were obtained using the following amplification conditions: 96°C for 2 min; 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min (35 cycles); and 72°C for 10 min (final extension). The concentrations of reagents were as described by the supplier of DNA polymerase (Invitrogen, USA) and the reactions were performed in PCR system (TRIO-Thermoblock, Biometra GmbH, Germany). The PCR product was purified from an agarose gel by using EASYTRAP Gel Extraction Kit Ver. 2 (TaKaRa Biomedicals, Japan) and subsequently cloned into *Bgl*II and *Sph*I sites of *B. megaterium* expression vector, pHIS1525.SP_{lipA}. The enzymes used in cloning and restriction analysis were obtained from MoBitech GmbH.

1.3 Expression of *ker* gene in *B. megaterium*

The PCR products of *ker* gene were cleaved by *Bgl*II and *Sph*I and then inserted between the *Bgl*II and *Sph*I sites of the vector, pHIS1525.SP_{lipA} resulting in a recombinant plasmid, pKERHIS1525.SP_{lipA}. Subsequently, the strain *B. megaterium* ATCC® 14945 was transformed using protoplast transformation as described by McCool and Canoon (2001).

1.4 Affinity chromatography and enzyme activity

Purification of ker-6xHis protein was performed under native conditions as described by Janknecht et al. (1991), using Ni-NTA affinity column chromatography QIAexpress system (Qiagen, Hilden, Germany). The protein fractions were further determined by SDS-PAGE (12%, *V*/*V*) according to Ausubel et al. (1996). The molecular mass was determined by comparing with the mobility of 10–250 kDa rainbow molecular weight markers (Prestained SDS-PAGE Standards, Broad Range, Biorad, USA).

The protease activity of the samples was measured according to the method described by Secades and Guijarro (1999), using azocasein (Sigma) as substrate. The absorbance of the samples was measured at wavelength

Table 1 Strains, oligonucleotide primers and plasmid used in this study

Strains/Primers/Plasmid		Reference/source
Strain		
B. licheniformis ATCC53757	Wild type (37°C)	ATCC, USA
B. megaterium ATCC®14945	Wild type (37°C)	ATCC, USA
Primer	Sequence (5′-3′)*	
BglII-B.M-kerA-Foward	GCGGCGAGATCTATGATGAGGAAAAAGAGTTTTTGG	This study
SphI-B.M-kerA-Reverse	ATTACTGCATGCTGAGCGGCAGCTTCGA	This study
Plasmid		
pHIS1525.SPlipA	Tetr, Ampr, 1136 bp ker ORF cloned at *Bgl*II and *Sph*I sites of pHIS1525 under PxlyA	MoBitech GmbH, Germany

* Restriction sites are underlined.

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of 420 nm (A_{420}) using a spectrophotometer (UV-160A Schimadzu, Japan). One unit of protease activity was set to be equal to the amount of enzyme required for an increase in absorbance (A_{420}) by 0.01 after incubation of the samples at 37°C for 30 min.

1.5 Degradation of keratin-rich wastes

Feather degradation was carried out as described by Bálint (2008). Chicken feathers including barbs, calamuses, and rachises were initially chopped into 5 mm segments. 200 mL of 0.5 mmol/L phosphate buffer (pH 8.0) and 8 g of intact air-dried chicken feathers were mixed in 1-L Erlenmeyer flasks, and the suspension was heated up at 135°C for 30 min. After cooling, 1% (V/V) LB medium was added, and subsequently the keratinaceous media were inoculated (inoculation ratio 1:100, V/V) with overnight cultures of wild type of *B. megaterium* ATCC® 14945 or *B. licheniformis* ATCC® 53757 or the recombinant *B. megaterium* strain developed in this study. In the case of the recombinant strain the medium was also supplied with 10 μg/mL of tetracycline and 0.5% (W/V) xylose. In each case three cultivations were started up in parallel and the samples were then incubated at 37°C with continuous shaking at 120 r/min. One of the three parallel cultivations was stopped after 1 day, the second after 2 days, while the third was stopped after 8 days of cultivation. Samples were taken to measure the keratin degradation level. The remainder of the cultivation broths was then centrifuged at 4°C and 20,000 ×g for 15 min and the cell-free broths (henceforth referred to as feather hydrolyzates), were stored at –20°C and used for further determination of methane potential in anaerobic batch digestion systems.

1.6 Determination of soluble protein concentration

Samples taken for monitoring the keratin degradation were centrifuged at 20°C and 12,000 r/min for 10 min prior to determining the protein concentration in the supernatant by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as reference. The optical absorbance was measured at 595 nm using a spectrophotometer (SP-870 Turner®, USA).

1.7 Batch digestion experiments

The batch experiments were carried out at thermophilic conditions (55°C) according to the method described by Hansen et al. (2004). The inoculum (TS: 1.9%; VS: 1.2%; VFA: 2214 mg/L; NH_4^+-N: 2206 mg/L; pH 7.78) was obtained from a large-scale municipal solid waste digester (Borås Energi and Miljö AB, Sweden) operating at thermophilic conditions. The inoculum was obtained from a large-scale municipal solid waste digester (Borås Energi and Miljö AB, Sweden). Serum glass bottles with a total volume of 118 mL, closed with butyl rubber seals and aluminum caps, were used as reactors. For each flask containing 33 mL inoculum, biologically treated or untreated feathers with 0.25 g volatile solids (VS) were added. Blanks containing phosphate buffer instead of the substrate were also performed to determine the methane production of only the inoculum. All experimental setups were performed in triplicates. After putting together the reaction mixture, the reactors were sealed and flushed with a gas mixture of 80% N_2 and 20% CO_2 to obtain anaerobic conditions and then incubated at 55°C. During the 50-day incubation period, the bottles were shaken and moved around in the incubator once a day. Gas samples of 0.25 mL were regularly taken from the head space of the reactors, using a pressure-tight syringe, and measured directly by gas chromatograph (GC). Excess gas was released through a hospital needle after each analysis, to avoid overpressure higher than 2 bars built up in the head space of the flasks. Gas samples from the head space of each reactor were taken again after the release of overpressure and measured by GC. Gas analyses of the batch series were carried out two to three times a week during the first two weeks of the experiment, and once a week in the following weeks.

1.8 Gas analysis and calculations

Methane was analyzed by using a gas chromatograph (Auto System, Perkin Elmer, USA) equipped with a packed column (Column 8000 PKD, Perkin Elmer, USA) and a thermal conductivity detector (Perkin Elmer, USA) with inject temperature of 150°C. The carrier gas was nitrogen operated with a flow rate of 20 mL/min at 60°C. A 250 μL pressure-tight gas syringe (VICI, Precision Sampling Inc., USA) was used for the gas sampling. Assuming ideal gas mixtures and using the ideal gas law, the methane content in the reactor head space can be calculated by using the data from the GC measurements without measuring the actual pressure in the bottle (Hansen et al., 2004). A gas with known composition was used as standard on each measuring occasion. All methane volumes are presented at standard conditions (0°C and 101.3 kPa).

2 Results

2.1 Production and purification of recombinant keratinase

Recombinants were selected upon the upcoming clearing zone when tested for proteolytic activity on milk agar plates (Fig. 1). Colonies achieving the largest clearing zone were chosen for further investigations. The overnight culture induced with 0.5% (W/V) xylose addition yielded a protease activity of 3.4 U/mL after 9 hr of growth in LB tetracycline medium, which was seven times higher than that of the wild type strain (Table 2).

Purification of the recombinant 6xHis-tagged protein was carried out using the QIAexpress system by Ni-NAT affinity column chromatography. To see the effect of the length of incubation, purification was performed after incubations for both 9 and 18 hr, respectively. Maximum protease activity of 29.5 U/mL was obtained in the elution fraction after 18 hr (Table 2).

The purified keratinase from *B. megaterium* was further investigated by SDS-PAGE, and the recombinant protease was found as a band equivalent to approximately 33 kDa molecular weight, as shown in Fig. 2.

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Table 2 Determination of proteolytic activity of the recombinant *B. megaterium* compared with that of the wild type strain

Sample	Optical density (A_{420})	Proteolytic activity (U/mL)
In the broth after 9 hr cultivation of the wild-type strain	0.005	0.5
In the broth after 9 hr cultivation of the recombinant strain	0.034	3.4
Purified after 9 hr cultivation of the recombinant strain	0.126	12.6
Purified after 18 hr cultivation of the recombinant strain	0.295	29.5

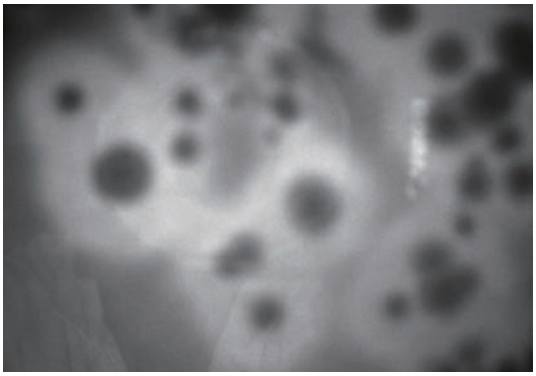

Fig. 1 Extracellular keratinase produced by the recombinant *B. megaterium* strain after incubation on milk agar plates for 24 hr. The clearing zones indicate the extracellular keratinase production.

2.2 Degradation of chicken feather waste by the recombinant strain

To verify the ability of the developed recombinant *B. megaterium* strain to degrade feather keratin, the strain was cultivated on a medium containing chicken feather waste. Additionally, wild type of *B. megaterium* strain and *B. licheniformis* strain with natural keratinase activity were also cultivated under similar conditions as control. The degradation of feather particles started after 24 hr of incubation by the action of the recombinant strain, while no degradation could be observed using the wild type strain. The digestion of feather residues increased considerably upon prolonged incubation time, resulting in a color change from a roughly colorless medium with particles to a yellowish fermentation broth after 8 days of incubation.

The hydrolysis of chicken feathers by the recombinant *B. megaterium* strain generated a higher amount of soluble proteins than the hydrolysis by the other two strains (Fig. 3). The protein concentration sharply increased to 0.19 mg/mL during the first two days of the incubation time, and finally 0.51 mg/mL soluble proteins were achieved after 8 days. As shown in Fig. 3, the soluble protein concentrations were lower in samples hydrolyzed by *B. licheniformis*, i.e., 0.08 mg/mL after 2 days of incubation and 0.25 mg/mL after total 8 days, respectively. Moreover, very low amounts of soluble proteins were measured from samples which were hydrolyzed by the wild type *B. megaterium* strain. After 8 days of incubation, more than twice the amount of soluble proteins could be obtained from feather hydrolyzates treated by the recombinant *B. megaterium* strain, compared with feather hydrolyzates treated by *B. licheniformis*, the strain which producing keratinase naturally (Fig. 3).

2.3 Biogas production from feather hydrolyzates

Figure 4 shows the accumulated methane production after the 50-day long anaerobic digestion of feather hydrolyzates compared with the accumulated methane production from untreated feathers. The methane yield from untreated feathers was 0.18 Nm^3/kg VS, which was improved to more than a double amount of about 0.4 Nm^3 CH_4/kg VS feathers as a result of the pre-hydrolysis by the recombinant *B. megaterium* strain. The methane yield of the feather hydrolyzates performed during different, i.e., 1-, 2- or 8-day, cultivation periods, respectively, did

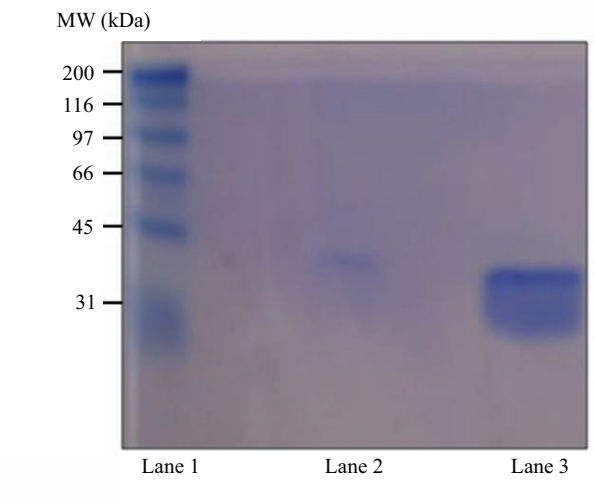

Fig. 2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of extracellular keratinase produced after 9 hr cultivation by the recombinant *B. megaterium* (Lane 3) compared with that of the wild type strain (Lane 2). Lane 1: 10–250 kDa rainbow molecular weight markers.

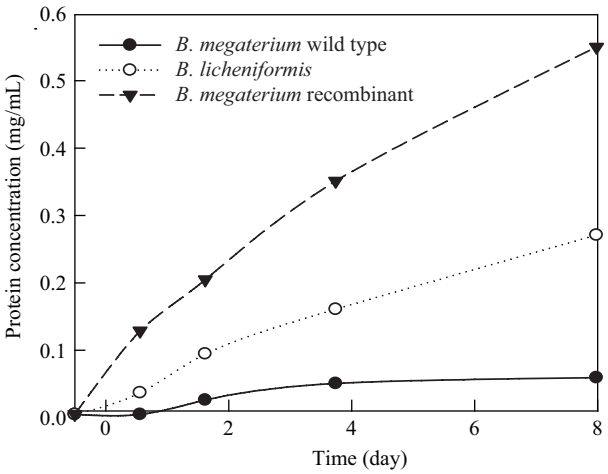

Fig. 3 Soluble protein concentration determined in the broth during the feather degradation step using the recombinant *B. megaterium* strain, compared with that of the wild type *B. megaterium* strain, and the native keratinase producing *B. licheniformis* strain.

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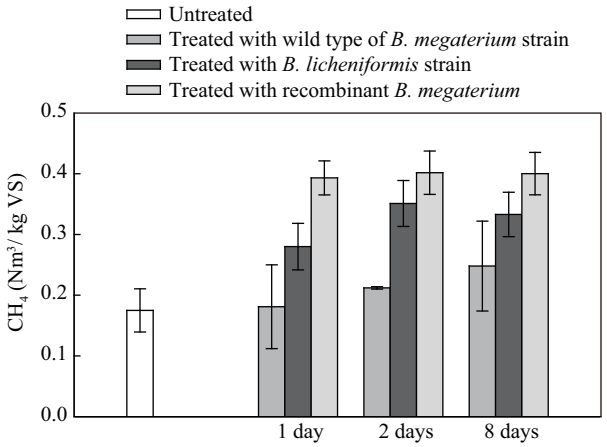

Fig. 4 Accumulated methane production obtained after 50 days of anaerobic digestion of the feather hydrolyzates compared with that from untreated feather waste. The feather hydrolyzates were performed by cultivation of the recombinant *B. megaterium*, wild type *B. megaterium* or the native keratinase producing *B. licheniformis* strain, respectively, in feather containing media for 1, 2 or 8 days.

not show any significant differences. Methane production around 0.4 Nm^3/kg VS (i.e., 0.39, 0.40 and 0.41 Nm^3/kg VS after 1, 2 and 8 days of degradation, respectively), corresponding to 80% of the theoretical maximum of 0.496 Nm^3/kg VS proteins (Davidsson 2007), was achieved independently of the treatment time (Fig. 4). In contrast, pre-hydrolyzed feathers by the wild type *B. megaterium* showed 0.18, 0.21 and 0.24 Nm^3/kg VS methane yields after 1, 2 and 8 days of degradation, respectively, which are not significantly higher than the methane yield from untreated feathers (0.18 Nm^3/kg VS). Treatment with the naturally feather degrading strain, *B. licheniformis* resulted in methane yields of 0.28, 0.35, 0.33 Nm^3/kg VS, counting up to 66% of the theoretical value from proteins (Davidsson, 2007), after 1, 2 and 8 days of pretreatment times, respectively (Fig. 4).

3 Discussion

A two-step system was developed for the utilization of keratin-rich chicken feather waste for biogas production. A recombinant *B. megaterium* strain carrying keratinase gene from *B. licheniformis* was developed for the feather degradation step. Previously, Bálint et al. (2005) have found that the fermentation broth obtained after microbial degradation of protein-rich feather wastes using a native *B. licheniformis* strain is an ideal source for the next biohydrogen production step. In addition, several studies have reported that keratinase gene from *B. licheniformis* could be successfully expressed under a xylose inducible promoter in *B. megaterium* (Radha and Gunasekaran, 2008, 2009). The recombinant *B. megaterium* strain developed in this study could effectively degrade feathers prior to biogas production, which resulted in up to 80% of the theoretical methane yield from proteins. In contrast, up to 66% of the theoretical methane yield could be achieved after similar treatment by the native keratinase producing strain, *B. licheniformis*. The recombinant strain showed a maximum proteolytic activity of 29.5 U/mL after 18 hr incubation in LB medium (Table 2). This is comparable with results reported by Ouled Haddar et al. (2009) when developing recombinant *B. subtilis* strains for feather degradation, where protease activities of 13 U/mL were reported for the three different strains after 24 hr of incubation. Park and Son (2009) reported a keratinolytic activity of 58 U/mL after 5 days of cultivation of *B. megaterium* F7-1, a feather-degrading mesophilic bacterium.

The extracellular enzyme produced by the recombinant strain in this study was purified using Ni-NTA affinity chromatography and the product was determined and controlled using SDF-PAGE. The molecular mass of the purified keratinase was determined to be 33 kDa (Fig. 2) which corresponds to that of purified keratinase of *B. licheniformis* PWD reported by Lin et al. (1992).

To crack the stabilizing disulfide bonds in keratin, the feathers were first treated at 135°C for 30 min. With some margin, this treatment also fulfilled the requirement of EU legislation of handling of slaughterhouse waste, which must undergo a thermal treatment prior to anaerobic digestion to avoid contamination by pathogenic microorganisms (EC byproduct regulation, 2002). Subsequently, the pretreated material was exposed to further degradation by the three investigated strains. The biodegradation was then followed up by determining the amount of soluble proteins in the broth (Fig. 3). After 8 days of cultivation on an initial feather concentration of 4% (*W*/*V*), 0.51 mg/mL soluble proteins could be obtained using the recombinant *B. megaterium* strain, which is two times higher than that of using the native feather degrading strain, *B. licheniformis*. In comparison, Ouled Haddar et al. (2009) measured around 0.17 mg/mL soluble proteins in feather hydrolyzate containing initially 1% (*W*/*V*) feathers, after 4 days of incubation by a recombinant *B. subtilis* strain.

To determine whether the incubation period affects the methane yield during the following biogas production, the strains were cultivated for 1, 2 or 8 days on feathers prior to the anaerobic digestion. The best methane yields could be achieved after treatment by the recombinant *B. megaterium* strain, with an increase by 122% compared to the methane yield obtained from untreated feathers. On the other hand, biological treatment by the wild type *B. megaterium* had no significant effect on the methane yield from feathers (Fig. 4). Furthermore, treatment by *B. licheniformis* for 1, 2 or 8 days, resulted in 55%, 94% or 83%, improvement, respectively, in the accumulated methane production (Fig. 4). Moreover, there were no significant differences between the methane potentials of the hydrolyzates obtained after the different long treatment periods (Fig. 4), when the recombinant strain was used for the feather degradation. The keratinase production was controlled by a strong xylose inducible promoter in the developed recombinant strain which resulted in an immediate and effective production of the keratin degrading enzyme without showing any lag phase in the enzyme production. In comparison, in the case of the native keratinase producing strain, *B. licheniformis*, the longer the cultivation period on feathers was, the higher methane yield could be achieved under the subsequent anaerobic digestion. However, the best

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methane yield could be obtained after 2 days of cultivation, which was any way lower (0.35 Nm^3/kg VS) than that of after a 1-day treatment with the recombinant strain (0.40 Nm^3/kg VS).

Salminen et al. (2003) reported previously a methane potential of untreated feathers of 0.21 Nm^3/kg VS, which was improved by 37% to 51% after combined thermal (120°C, 5 min) and enzymatic treatment using commercial alkaline endopeptidases for a 24-hr treatment period. Beside the better performance in methane production, an additional advantage of the developed two-stage system for feather utilization in this study is that it is economically more feasible by saving the costs for the production and purification of enzymes.

4 Conclusions

The developed two-stage system for utilizing keratin-rich feather waste for biogas production, using a biological degradation step prior to the anaerobic digestion step, is an economically feasible and environmentally friendly alternative. One day long biological pretreatment by the recombinant *B. megaterium* strain resulted in a methane production of 0.35 Nm^3/kg dry feathers (corresponding to 0.4 Nm^3 CH_4/kg VS), which is 222% of the methane yield of untreated feathers. As a control a similar two stage system using the native keratinase producing *B. licheniformis* strain for feather degradation was also investigated. One day treatment with this strain resulted in 155% of methane production of untreated feathers during the followed anaerobic digestion step.

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