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Evaluation Of Biological Degraded Keratin for Biogas Production Using Dry Anaerobic Digestion System

[a]Sinta Setyaningrum[*], [b]Regina J. Patinvoh, [c]Ronny Purwadi,
[d]Mohammad Taherzadeh

[a]Chemical Engineering Department, Politeknik Negeri Bandung, Bandung 40559, Indonesia
[b]Chemical and Polymer Engineering, Lagos State University, Ojo 102101, Nigeria
[c]Chemical Engineering Department, Faculty of Industrial and Technology, Bandung Institute of Technology, Bandung 40132, Indonesia
[d]Swedish Centre for Resource Recovery, University of Boras, Borås 503 32, Sweden

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ABSTRACT

To produce biogas, an anaerobic digestion utilized various types of organic waste as substrate for the reaction process. Keratin waste is a protein complex of organic waste that mainly produced from the poultry and farming industry. Biological pretreatment was selected because it more energy saver and generating diverse types of amino acid monomers. Three types of keratins used in this research were feathers, wool, and hair. Culture of Bacillus sp. C4 were inoculated into keratins and incubated for 24 hours, 48 hours, and 72 hours. The chicken feather and sheep wool produced the highest soluble proteins as 45.99 mg/ml and 38.75 mg/ml respectively with 72h incubation time. Meanwhile, keratin hair cannot be degraded by Bacillus sp. C4. Dry anaerobic digestion system prepared using oxygen free capped vials then incubated at 37°C for 50 days. Free ammonia formed by hydrolysis of proteins is suspected to be an inhibitor in the methanogenesis process, as total methane produced from degraded keratin only 256,6 ml C4/gr VS in 36 days retention time.

KEYWORDS

Keratin
Hydrolysis
Bacillus sp. C4
Dry anaerobic digestion

INTRODUCTION

A growing population followed by economic growth is a major factor in increasing energy needs. Until now, the fulfillment of global energy needs is still based on petroleum fuels, coal, and natural gas. The use of fossil fuels has a detrimental effect on the environment and its availability is not sustainable. Therefore, meeting energy needs to be balanced with a sustainable supply of renewable energy. Biogas is renewable energy in the form of methane gas. The excess of biogas energy is environmentally friendly, easy to produce, and inexpensive. (Deublein & Steinhauser, 2010)

Biogas is produced from the anaerobic fermentation of methane. The anaerobic reaction process is complex because it consists of 4 reaction phases: hydrolysis, acetogenic, acidogenic,

*Corresponding Author: sinta.setyaningrum@polban.ac.id

and methanogenesis. Various types of organic materials can be used as substrates for biogas production. Types of waste commonly used for biogas production include forest and plantation waste, urban organic waste, and livestock waste.

One of the most common organic materials is keratin. Keratin is a protein-rich material and has a complex physiochemical structure. Keratin has two types, namely α-keratin and β-keratin. α-keratin has an α-helical structure and it is elastic. Examples types of α-keratin include wool, hair, and horns. Meanwhile β-keratin has a pleated sheet structure and is not elastic (Hill et al., 2010). Several periods of keratin waste from poultry feathers are used as a source of nitrogen for animal feed. This utilization option is further constrained by regulations related to the outbreak of the avian influenza virus (Mezes & Tamas, 2015). Another benefit is the industrial production of the enzyme keratinase. The industrial enzyme production process requires high costs in the purification process. Another option that can be done is to use keratin waste as a substrate for biogas production. (Brandelli et al., 2010)

Keratin is not easy to decompose. To reuse keratin, pretreatment is often done. The function of pretreatment is to break down the complex structure of keratin into amino monomers. Some pretreatment methods are mostly done by thermal and pressure, chemical, enzymatic, and biological. Enzymatic and biological pretreatment is known to require a long time but does not require a large amount of energy. Biological hydrolysis results as desired because enzymes work in specific processes (Forgacs et al., 2013). To be able to degrade keratin, microorganisms that have proteolytic keratinase enzymatic activity are needed. Various literature reports the many types of microorganisms: bacteria, yeast, and fungi with keratinase activity. In this study, a bacterium with a high potency of the keratinase enzyme, *Bacillus sp*. strain C4. A strain of *Bacillus sp*. C4 is able to degrade keratin poultry feathers and sheep wool completely. (Fellahi, 2014)

Anaerobic digestion operating system is a system used to produce biogas. Dry anaerobic digester (DAD) is an operating condition with a total solid substrate character of more than 10%. The advantages of dry digestion systems include organic loading rates of high solid substrates, low energy consumption, smaller reactor volumes, and the production of less liquid leachate (De Baere, 2000)(Rocamora et al., 2020)(Pavan et al., 2000). The purpose of this study was to evaluate the production of biogas by the dry anaerobic digestion process using a substrate resulting from the degradation of keratin complex proteins.

MATERIALS AND METHODS

Materials

Three types of keratin were used as the primary substrate in this study, including poultry feathers, sheep's wool, and human hair. Each keratins was washed and chopped before biological pretreatment experiments.

This experiment used two different types of bacterial inoculums, namely bacterial inoculums with strains of *Bacillus sp*. C4 and anaerobic bacterial flocculant granules inoculum. *Bacillus sp*. C4 is a bacteria that has enzymatic keratinase activity and has been registered under the University of Gothenburg culture collection as CCUG 66887. *Bacillus sp*. C4 is used as an

inoculum culture in keratin pretreatment experiments. Anaerobic flocculant bacterial inoculums were used in the dry digestion experiments. Anaerobic flocculant granules inoculum were obtained from the UASB wastewater treatment reactor in Hammarby Sjostad, Sweden.

Three types of media were used in this study as bacterial growth media and enrichment media at each stage of the experiment. Luria-Bertani (LB) is used as a growing medium for *Bacillus sp*. C4 Modified Basal Medium II was used in the keratin pretreatment experiment as an enrichment medium. MBM II composition is 0.5 g/L NH_4Cl; 0.5 g/L NaCl; 0.5 g/L K_2HPO_4; 0.4 g/L KH_2PO_4; 0.1 g/L $MgCl_2.6H_2O$; 1.5 g/L yeast extract; and 1 g/L peptone. In the dry digestion experiment for biogas production, Basal Medium is used as an enrichment medium for flocculant anaerobic culture. The composition of Basal Medium is divided into 5 stock solutions. Stock A of solution consists of 100 g/L NH_4Cl; 10 g/L NaCl; 10 g/L $MgCl_2.6H_2O$; and 5 g/L $CaCl_2.2H_2O$. Stock of solution B consists of 200 g/L $K_2HPO_4.3H_2O$. Stock of C solution is a 0.5 g/L resazurin solution. Stock D solution is a trace metal solution consisting of 2 g/L $FeCl_2.4H_2O$; 0.05 g/L H_3BO_3; 0.05 g/L $ZnCl_2$; 0.038 g/L $CuCl_2.2H_2O$; 0.05 $MnCl_2.4H_2O$; 0.05 $(NH_4)6Mo_7O_{24}.4H_2O$; 0.05 $AlCl_3$; 0.05 $CoCl_2.6H_2O$; 0.092 $NiCl_2.6H_2O$; 0.5 ethylenediaminitetraacetate; 1 ml of concentrated HCl, 0.1 g/L $Na_2SeO_3.5H_2O$. Stock of solution E is a vitamin solution, consisting of 2 mg/L biotin; 2 mg/L folic acid; 10 mg/L pyridoxine acid; 5 mg/L riboflavin; 5 mg/L thiaminehydrochloride, 0.1 mg/L cyanocobalamine; 5 mg/L nicotinic acid, 5 mg/L P-aminobenzoic acid, 5 mg/L lipoic acid; and DL-pantothenic acid. To make 1 L Basal Media, 10 ml stock A solution, 2 ml stock B solution, 1 ml stock C solution, 1 ml stock D solution, and 1 ml stock E solution are added to 975 ml distilled water.

Biological Pretreatment Keratin

In this study, the keratin pretreatment process used a biological method with the help of bacterial culture with the enzymatic activity of keratinase, *Bacillus sp*. C4. The total work volume in the pretreatment stage was 20 ml in a 250 ml Erlenmeyer flask. As much as 20% $^{w}/_{v}$ of each type of keratin (4g dry weight keratin) was added to Modified Basal Medium II and sterilized using an autoclave. Inoculum of bacteria *Bacillus sp*. C4 was then inoculated into a mixture of sterilized media and keratin. The incubation process of the keratin pretreatment stage was carried out in a 160 rpm water bath shaker at 37°C. Two variations in the amount of inoculum used, namely 5% $^{v}/_{v}$ and 10% $^{v}/_{v}$. The variation of incubation time for pretreatment is divided into three, namely 24 hours, 48 hours, and 72 hours.

Dry Anaerobic Digestion System

Dry anaerobic digestion experiments were carried out using selected degraded keratin substrates. The basis for selecting keratin substrate samples was the average pretreatment operating conditions that have enzymatic activity of keratinase and high dissolved protein concentrations. Variations of the experiment were made by the ratio of the keratin substrate and the flocculant anaerobic inoculum. There were two kinds of substrate dry weight: inoculum ratio used, which was 1: 1 and 1: 2 ratio. Dry anaerobic digestion is carried out using a vacuum vial as a reactor with a total volume of 118 ml, while the working volume of the reaction is no

more than two-thirds of the total volume. Anaerobic conditions are created by exchanging gases in the reactor using a mixture of 80% nitrogen gas and 20% CO_2 gas. All variations of the experiment were incubated at 37°C for 50 days.

Analysis

The results of the keratin pretreatment experiment were analyzed into three general methods, namely biological, physical, and chemical analysis. Biological analysis for samples of keratin pretreatment results is the calculation of the number of viable bacteria in the total broth of the experiment. The number of viable bacteria is determined using the Total Plate Count method. Physical and chemical analysis of the keratin pretreatment experimental sample was carried out by separating solid and liquid material from the total broth using vacuum. The solid sample obtained was then analyzed to obtain the weight loss of keratin treated with pretreatment. The liquid sample is then analyzed to obtain the final pH value of the pretreatment experiment, the concentration of dissolved protein, and the amount of enzymatic activity of keratinase. Analysis of dissolved protein concentrations was carried out using the Lowry analysis method.

Biogas production activities in the form of methane and carbon dioxide are carried out periodically during the incubation process of dry anaerobic digestion. The biogas production analysis was carried out using Gas Chromatography (Perkin-Elmer, USA) equipped with columns (6 'x 1.8" OD, 80/100, Mesh, Perkin Elmer, USA) and thermal conductivity detectors (Perkin Elmer, USA), and with a 150°C temperature injector (Teghammar et al., 2013).

THE RESULTS AND DISCUSSION

Biological Pretreatment Keratin

Keratin pretreatment is done so that complex proteins can be broken down into amino acid monomers and more easily digested by microorganisms in the anaerobic fermentation process. Keratin biological pretreatment was carried out with the help of enzymatic activities of keratinase enzymatic. In previous experiments reported by Patinvoh, *Bacillus sp*. C4 successfully degrades keratin from poultry feathers into dissolved protein by up to 70% after incubation for 5 days with the provision of 5% $^{w}/_{v}$ keratin against total broth (Patinvoh et al., 2016). The use of three types of keratins, two concentrations of inoculum, and keratin with a high load were carried out in this study with the hope of getting optimum dissolved protein yields.

From Figure 1, Graph A can be seen that for the keratin substrate of poultry feathers and sheep's wool, *Bacillus sp*. C4 can grow up to an incubation time of 72 hours, but *Bacillus sp*. C4 was no longer viable after 24 hours of incubation on human hair substrates. Inoculum culture cells *Bacillus sp*. C4, which can grow in the keratin substrate, correlates with the production of the enzyme keratinase. The keratinase enzyme is alkaline, so the mixture of substrate and media that has been regulated at a neutral acidity level of pH 7 will turn into alkaline when keratinase activity increases. This result was similar according to experiments by Suntornsuk (Suntornsuk & Suntornsuk, 2003). Increasing the acidity is an indication of the presence of the enzyme keratinase. The highest value of the enzymatic activity was produced through variations

that used 5% inoculum and after 48 hours incubation time for pretreatment of sheep's wool, which was 49 U/ml.

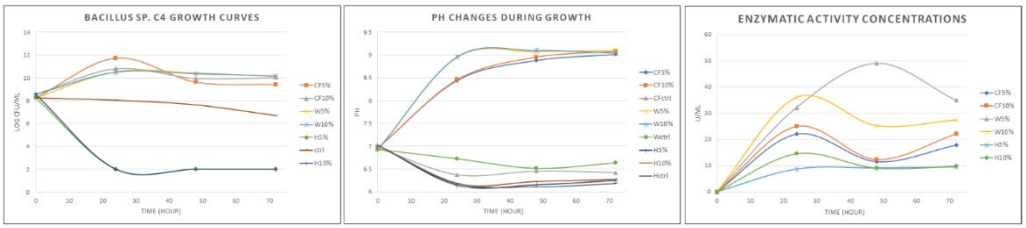

Figure 1. Keratin biological pretreatment results graph. A) Bacillus sp. Growth. C4; B) concentration of enzymatic activity; C) changes in the degree of acidity

Another analysis to see the success of the degradation test is through the calculation of the weight loss of keratin and the concentration of dissolved protein. Figure 2 is a graph of weight percent loss of keratin and dissolved protein concentrations from each treatment of keratin degradation test. The most lost keratin mass is poultry feather keratin. With 72 hours incubation time treatment, the mass of poultry feather keratin with inoculum variations of 5% and 10% can disappear by 27.7% and 26.3%, while sheep's keratin wool disappears by an average of 14.9%. No amount of keratin hair mass was lost in this experiment. The most keratin that is converted to dissolved protein after biodegradation testing is poultry feathers. Laba also reported that hair keratin and pig bristle were hardly to degraded by keratinase microorganisms (Laba & Rodziewicz, 2014). In the incubation range of 24 hours and 48 hours, a number of degraded keratin feathers produce a high dissolved protein with a difference of 25.1 mg/ml (5% inoculum) and 25.2 mg/ml (10% inoculum).

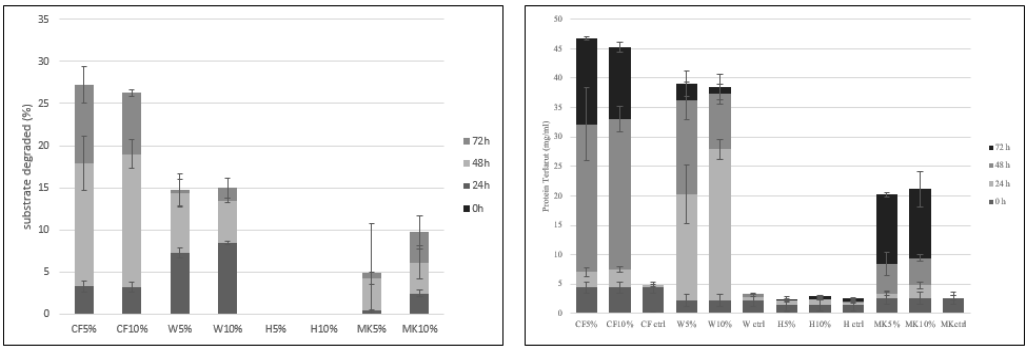

Figure 2. Keratin biological pretreatment results graph. A) percent lost by keratin; B) dissolved protein concentration (CF: feather, W: wool, H: hair, and MK: mixed keratin)

Dry Anaerobic Digestion

Figure 3 is a graph of cumulative methane gas production per day with hydrolyzed keratin substrate. Six variations of experimental treatment were performed for each use of the keratin substrate. The keratin substrate used is keratin which has been hydrolyzed for 48 hours. The

timing of this keratin pretreatment is due to the fact that 48 hours have been able to degrade keratin in an optimum amount.

The cumulation graph of methane gas production with hydrolyzed poultry feather substrate can be observed in Figure 3A. There are 2 graphs that have the highest value, namely the cumulative methane gas production graph variant CFctrl (1: 2) and CF5% (1: 2) reaching the amount of 169.25 ml CH_4/gr VS after 6 days incubation and 152.03 ml CH_4/gr VS after incubation for 18 days in a row. Based on the graph, it can be seen that the production of methane gas for all variants began to be invisible after the 6th day, while the control methane gas production graph continued to increase until the retention time of the 24th day. It can be assumed that there is an inhibition of the process of methanogenesis, because of the low C/N ratio in the biogas substrate composition (Kovács et al., 2013).

Figure 3B is a graph of the accumulation of methane gas production with sheep's wool substrate from several variations. Based on Figure 3B it can be seen that the highest methane gas production rate occurs at 6 days retention time with a value of 146.7 ml CH_4/gr VS for variants W5% (1: 2) and W10% (1: 2), and 126.1 ml CH_4 / gr VS for the Wctrl variant (1: 2). Methane gas production continues to increase after the retention time of the 6th day to the 50th day, although it is not high. In the anaerobic digester process with hydrolyzed wool substrate, there is inhibition, just like the anaerobic digester process with degraded feather poultry substrate. The graph of cumulative methane gas production in the DAD system with keratin hair substrate is shown in Figure 3C. The methane gas production activity of the DAD system for hair substrate is very different from the methane gas production activity of the DAD system for poultry feathers and sheep wool substrates. In the DAD system the hair substrate does not inhibit the process of methanogenesis, so that the methane gas can continue to be produced at a stable rate. This can be attributed to the results of biological hydrolysis test on hair, i.e. the hair detected cannot be hydrolyzed by microorganisms with keratinase *Bacillus sp*. C4 It can be assumed that the inhibitor of the process of methanogenesis originates from the hydrolysis of poultry feathers and sheep wool (Lange et al., 2016).

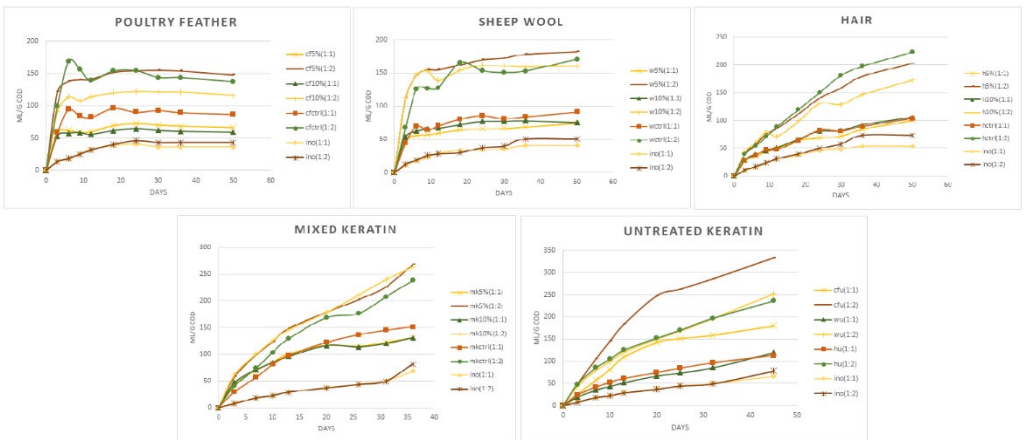

Figure 3. Graph of total methane gas production through the dry anaerobic digestion system. A) poultry feather keratin substrate; B) sheep's wool; C) hair; D) mixed keratin; E) untreated keratin

3D image is a graph of cumulative methane gas production from the DAD system with a mixture of wool and hair keratin (Mixed Keratin = MK). Similar to the results of methane gas production in other keratin substrate DAD systems, this graph can also be seen that the DAD system by adding 1: 2 gr of substrate and inoculum gives the highest methane gas yield reaching an average of 256.6 ml CH_4/gr VS. The activity and rate of methane gas production were monitored to be stable and there was no apparent inhibition of the anaerobic digestion process. It can be assumed that the presence of inhibiting components of the methane gas production process is far below the limit so that it does not affect the DAD MK substrate system (Korniłłowicz-Kowalska & Bohacz, 2011).

A DAD system using a keratin substrate without pretreatment was carried out to compare the results of methane gas production across all treatment variations. Through this treatment it was observed that the yield of methane gas from substrates without pretreatment produced was much higher than the yield of methane gas produced from hydrolyzed keratin substrates. Even though methane gas yield from keratin substrate without pretreatment is higher, the rate of methane gas production with hydrolyzed keratin substrate is faster.

The use of substrates and inoculums with a ratio of 1: 2 gr VS can always produce biogas which is higher than the ratio of 1: 1 gr VS. This is consistent with the theory that states that the use of the substrate: inoculum ratio that provides the best yield is in the range of 1: 2 to 1: 4 (Kayhanian, 1999). The production of methane gas from the keratin substrate of poultry feathers and hydrolyzed sheep wool occurs rapidly, which is only about 6-9 days incubation, then the production of methane gas appears to be inhibited. Keratin substrate without pretreatment (Figure 3E) will produce biogas with a high amount of accumulation, but requires a long retention time, around 40 days.

CONCLUSION

Based on the analysis results in each stage of the experiment, it can be concluded that the use of 200 mg/ml substrate and *Bacillus sp*. C4 as a degrading bacterium with an initial cell density of 1.8 x 10^8 and 3.6 x 10^8 cfu/ml provide insignificant degradation results to the poultry feathers and sheep wool. The incubation time of 48 hours is the best incubation time compared to 24 hours or 72 hours, which was able to degrade keratin poultry feathers and sheep wool with an average of 35 mg/ml of soluble protein, but hair keratin cannot be degraded by *Bacillus sp*. C4. The biogas production process with a dry anaerobic digestion system utilizing degraded keratin waste cannot operate optimally, assuming that the low ratio of C/N in the substrate and the inhibition mechanism by free ammonia in the reactor system.

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REFERENCES

Brandelli, A., Daroit, D. J., & Riffel, A. (2010). Biochemical features of microbial keratinases and their production and applications. *Applied Microbiology and Biotechnology*, *85*(6), 1735–1750. https://doi.org/10.1007/s00253-009-2398-5

De Baere, L. (2000). Anaerobic digestion of solid waste: state-of-the-art. *Water Science and Technology : A Journal of the International Association on Water Pollution Research*, *41*(3), 283–290.

Deublein, D., & Steinhauser, A. (2010). Biogas from Waste and Renewable Resources: An Introduction, Second Edition. In *Biogas from Waste and Renewable Resources: An Introduction, Second Edition*. https://doi.org/10.1002/9783527632794

Fellahi, S. (2014). A Bacillus Strain Able to Hydrolyze Alpha- and Beta-Keratin. *Journal of Bioprocessing & Biotechniques*, *04*(07). https://doi.org/10.4172/2155-9821.1000181

Forg??cs, G., Lundin, M., Taherzadeh, M. J., & Horv??th, I. S. (2013). Pretreatment of chicken feather waste for improved biogas production. *Applied Biochemistry and Biotechnology*, *169*(7), 2016–2028. https://doi.org/10.1007/s12010-013-0116-3

Hill, P., Brantley, H., & Van Dyke, M. (2010). Some properties of keratin biomaterials: Kerateines. *Biomaterials*, *31*(4), 585–593. https://doi.org/10.1016/j.biomaterials.2009.09.076

Kayhanian, M. (1999). Ammonia Inhibition in High-Solids Biogasification: An Overview and Practical Solutions. *Environmental Technology*, *20*(4), 355–365. https://doi.org/10.1080/09593332008616828

Kornilłowicz-Kowalska, T., & Bohacz, J. (2011). Biodegradation of keratin waste: Theory and practical aspects. *Waste Management*, *31*(8), 1689–1701. https://doi.org/10.1016/j.wasman.2011.03.024

Kovács, E., Wirth, R., Maróti, G., Bagi, Z., & Rákhely, G. (2013). Biogas Production from Protein-Rich Biomass: Fed-Batch Anaerobic Fermentation of Casein and of Pig Blood and Associated Changes in Microbial Community Composition) Biogas Production from Protein-Rich Biomass: Fed-Batch Anaerobic Fermentation of Casein and. *PLoS ONE*, *8*(10), 77265. https://doi.org/10.1371/journal.pone.0077265

Laba, W., & Rodziewicz, A. (2014). Biodegradation of hard keratins by two Bacillus strains. *Jundishapur Journal of Microbiology*, *7*(2), 1–7. https://doi.org/10.5812/jjm.8896

Lange, L., Huang, Y., & Busk, P. K. (2016). Microbial decomposition of keratin in nature—a new hypothesis of industrial relevance. *Applied Microbiology and Biotechnology*, *100*(5), 2083–2096. https://doi.org/10.1007/s00253-015-7262-1

M??zes, L., & Tam??s, J. (2015). Feather Waste Recycling for Biogas Production. *Waste and Biomass Valorization*, *6*(5), 899–911. https://doi.org/10.1007/s12649-015-9427-7

Patinvoh, R. J., Feuk-Lagerstedt, E., Lundin, M., Sárvári Horváth, I., & Taherzadeh, M. J. (2016). Biological Pretreatment of Chicken Feather and Biogas Production from Total Broth. *Applied Biochemistry and Biotechnology*, *August*. https://doi.org/10.1007/s12010-016-2175-8

Pavan, P., Battistoni, P., Mata-Alvarez, J., & Cecchi, F. (2000). Performance of thermophilic semi-dry anaerobic digestion process changing the feed biodegradability. *Water Science and Technology : A Journal of the International Association on Water Pollution Research*, *41*(3), 75–81.

Rocamora, I., Wagland, S. T., Villa, R., Simpson, E. W., Fernández, O., & Bajón-Fernández, Y. (2020). Dry anaerobic digestion of organic waste: A review of operational parameters and their impact on process performance. *Bioresource Technology*, *299*, 122681. https://doi.org/10.1016/J.BIORTECH.2019.122681

Suntornsuk, W., & Suntornsuk, L. (2003). Feather degradation by Bacillus sp. FK 46 in submerged cultivation. *Bioresource Technology*, *86*(3), 239–243. https://doi.org/10.1016/S0960-8524(02)00177-3

Teghammar, A., Castillo, M. D. P., Ascue, J., Niklasson, C., & Sárvári Horváth, I. (2013). Improved anaerobic digestion by the addition of paper tube residuals: Pretreatment, stabilizing, and synergetic effects. *Energy and Fuels*, *27*(1), 277–284. https://doi.org/10.1021/ef301633x