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(71) Applicant: **DSM IP ASSETS B.V.** [NL/NL]; Het Overloon 1, 6411 TE HEERLEN (NL).

(72) Inventors: **SMITS, Johannes Petrus**; P.O. Box 4, 6100 AA ECHT (NL). **BOULAND, Frank Maarten**; P.O. Box 4, 6100 AA ECHT (NL).

(74) Agent: **DSM NUTRITIONAL PRODUCTS LTD** et al.; SCHWANDER, Kuno, Wurmisweg 576, 4303 Kaiseraugst (CH).

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(54) Title: PROCESS FOR THE PRODUCTION OF BIOGAS

(57) Abstract: The invention relates to a process for the production of biogas.



PROCESS FOR THE PRODUCTION OF BIOGAS

Field

5 The application relates to a process for the production of biogas.

Background

Animal agriculture is a significant source of ammonia (NH₃) emitted into the atmosphere. Ammonia is a toxic gas that emitted from for example manure contributes to odor and is a direct
10 and indirect pollutant, contaminating water, soil, and air. Moreover, atmospheric ammonia leads to the formation of fine particulates contributing to respiratory and cardiovascular diseases. Atmospheric ammonia can also uncontrolled deposit on land and surface water affecting flora and fauna, leading to reduction in biodiversity

Manure management is one of the greatest challenges for farmers. Storage is a required
15 step within the farm animal manure management, necessary to safely store manure until climate and/or crop are in the right conditions for soil application. As well known, gaseous emissions generally occur during field spreading activities, but also the storage phase is responsible for a large amount of uncontrolled nitrogen and carbon compound losses.

One of the possible solutions to reduce nitrogen and carbon compound emissions is to
20 provide manure treatments. Existing treatments applied are for example solid–liquid separation (SLS), which reduces the formation of ammonia from urea originated from urine and/or uric acid, or anaerobic digestion (AD), which allows organic material to be converted into biogas. The SLS solution is only effective on fresh manure and requires investment in SLS equipment and buildings. Anaerobic digestion produces renewable energy in the form of biogas. Biogas can be captured and
25 combusted to generate electricity, directly burned for heating applications, or further upgraded for use in higher value applications such as vehicle fuel or base chemicals. However, manure processing at biogas plants also incurs in non-desired emissions of ammonia and methane, especially during storage of manure.

One of the solutions to decrease ammonia emissions during storage of manure is covering
30 manure storage reservoirs and capture atmospheric ammonia from the off-gas of the storage facility by air-scrubbing technologies. However, the costs are high and the effectiveness is depending on construction of the storage facility, covering material quality and the effectiveness of the scrubbing technology.

For environmental reasons, it is therefore desirable to find new and innovative process
35 features aimed at capturing ammonia and consequently reducing ammonia emissions during manure storage.

Summary

An object of the application is to provide an improved process for the production of biogas. The process is improved in that ammonia emission during the production process are reduced. In particular, the ammonia emission during storage of the organic material to be digested is reduced.

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Detailed description

Throughout the present specification and the accompanying claims, the words "comprise" and "include" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows. The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.* to one or at least one) of the grammatical object of the article. By way of example, "an element" may mean one element or more than one element.

Described herein is a process for the production of biogas from organic material, which process comprises the steps of (a) contacting biomass with enzymes and/or a culture to obtain a first composition, (b) fermenting the first composition to obtain a second composition comprising organic acids, (c) contacting the second composition comprising organic acids with the organic material to obtain a third composition, and (d) digesting the third composition to produce biogas. In an embodiment the biomass already comprises sufficient intrinsic culture and only enzymes are contacted with the biomass. In a preferred embodiment the biomass is contacted with enzymes and a culture.

Described herein is also a process for the production of biogas from organic material, which process comprises the steps of (a) contacting biomass comprising organic acids with organic material to obtain a composition, and (b) digesting the composition to produce biogas.

The term biogas as used herein means the product produced by the anaerobic digestion or fermentation of organic materials. Biogas comprises primarily methane and carbon dioxide and may have small amounts of hydrogen sulphide, ammonia, nitrogen and moisture. The composition of biogas depends among others on the organic material that is digested or fermented, the equipment, additives like minerals and nutrients, and process conditions during the digestion.

In step (a) biomass, enzymes and a culture are contacted to form a first composition. The term "a culture" as used herein encompasses a single culture, *i.e.* a culture comprising a single strain, but also a mixed culture, *i.e.* a culture comprising several strains, *e.g.* two strains, three strains, four strains, five strains, six strains, seven strain or even eight or more strains. The strains can be of the same type of microorganism, *e.g.* two or more bacterial strains. The strains can also be of different types of microorganisms, *e.g.* one or more bacterial strains, one or more yeast strains and/or one or more fungal strains.

In an embodiment the culture should be capable of producing one or more organic acids. In an embodiment the culture is capable of producing one or more organic acids from sugars. In an embodiment the culture is capable of producing one or more organic acids selected from, but not

limited to, the group consisting of lactic acid, acetic acid, propionic acid, formic acid, butyric acid, succinic acid, citric acid and itaconic acid. In a preferred embodiment the culture is capable of producing acetic acid.

The culture may comprise one or more bacterial strains, one or more yeast strains, one or more fungal strains or a combination thereof. Fungal strains include, but are not limited to, strains of *Rhizopus arrhizus*, *Rhizopus oryzae*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus wentii*, *Trichoderma reesei*, *Talaromyces emersonii*. Yeast strains include, but are not limited to, strains of *Candida lignohabitans*, *Candida catenula*, *Candida guilliermondii*, *Yarrowia lipolytica*, *Candida tropicalis*, *Kluyveromyces*, *Saccharomyces*. Bacterial strains include, but are not limited to, strains of *Bacillus*, *Pseudomonas*, *Acetobacter*, *Gluconobacter*, *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Aerococcus* spp, *Leuconostoc* spp, *Enterococcus* and *Propionibacterium*.

In a preferred embodiment the culture is a bacterial culture. The bacterial culture can be a culture comprising a single bacterial strain, but may also be a mixed culture comprising two or more different bacterial strains. The term "a bacterial culture" as used herein encompasses a culture comprising at least a bacterial strain. The bacterial culture may also comprise one or more non-bacterial strains, e.g. a yeast strain and/or fungal strain.

In an embodiment the bacterial culture comprises lactic acid bacteria. Suitable lactic acid bacteria include, but are not limited to, *Lactococcus lactis* ssp. *lactis* ATCC 19435, *Lactococcus lactis* ssp./*lactis* AS211, *Lactobacillus delbrueckii* ssp. *delbrueckii* ATCC 9649, *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081, *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus helveticus* (thermophilic), *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Pediococcus acidilactici*, *Streptococcus thermophilus*, *Streptococcus* spp., *Enterococcus* spp, *Pediococcus* spp, *Aerococcus* spp, *Leuconostoc* spp, *Lactobacillus salivarius*, *Lactobacillus brevis* such as *Lactobacillus brevis* (DSM 23231), *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactobacillus hilgardii*, *Lactobacillus coryniformis*, *Lactobacillus curvatus*, *Lactobacillus plantarum* such as *Lactobacillus plantarum* (DSM 19457), *Lactobacillus casei*, *Lactobacillus curvatus*, *Leuconostoc mesenteroides* sub-sp. *cremoris*, *Lactococcus lactis*, *Enterococcus faecium*, *Lactobacillus kefir* such as *Lactobacillus kefir* (DSM 19455), *Lactobacillus buchneri*, *Lactobacillus paracasei*, *Lactobacillus diovilorans*, *Pediococcus pentosaceus*, *Lactobacillus rhamnosus*, *Pediococcus parvulus* and *Propionibacterium acidipropionici*. In an embodiment the bacterial culture comprises the commercially available product called BioStabil® Biogas.

In an embodiment the bacterial culture is heterofermentative. The term "heterofermentative" as used herein means that the bacterial culture is capable of fermenting glucose into a variety of organic acids, like lactic acid, acetic acid, formic acid, citric acid, succinic acid and carbon dioxide. Heterofermentative bacteria include, but are not limited to, *Leuconostoc* spp, *Lactobacillus salivarius*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactobacillus hilgardii*, *Lactobacillus coryniformis*, *Lactobacillus curvatus*, *Lactobacillus plantarum*,

Lactobacillus casei, *Lactobacillus curvatus*, *Oenococcus*, *Weissella* or any of the other bacterial cultures as described herein that are "heterofermentative".

In an embodiment the bacterial culture is capable of fermenting hexose sugars and pentose sugars. Hexose sugars include, but are not limited to, glucose, galactose and mannose. Penstose
5 sugars include, but are not limited to, arabinose and xylose.

Biomass as used herein includes any polymeric sugar containing material. Preferably, biomass as used herein includes cellulosic material and/or lignocellulosic material and/or hemicellulosic material and/or starch material and/or pectin material and/or lipid material. Biomass suitable for use in the processes as described herein includes virgin biomass and/or non-virgin
10 biomass such as agricultural biomass, processed agricultural biomass, commercial organics, construction and demolition debris, municipal solid waste, household waste, waste paper and yard waste, waste of the dairy industry (comprising for example lactose), waste from the beet and cane sugar industry (comprising for example sucrose (saccharose)) and by-products of oil seeds (for example rape seed meal, sunflower meal and soy husks). Common forms of biomass include trees,
15 shrubs and grasses, wheat, rye, oat, wheat straw, sugar cane, cane straw, sugar cane bagasse, switch grass, miscanthus, energy cane, cassava, molasse, barley, corn, corn stover, corn fiber, corn husks, corn cobs, canola stems, soybean stems, sweet sorghum, corn kernel including fiber from kernels, distillers dried grains (DDGS), brewery spent grains, silage such as corn silage, grass silage and beet silage, products and by-products from milling of grains such as corn, wheat and
20 barley (including wet milling and dry milling) often called "bran or fibre" as well as municipal solid waste, waste paper and yard waste. The biomass can also be, but is not limited to, herbaceous material, agricultural residues, forestry residues, municipal solid wastes, waste woods (type A, B and/or C), waste paper, and pulp and paper mill residues. "Agricultural biomass" includes branches, bushes, canes, corn and corn husks, energy crops, forests, fruits, flowers, grains, grasses,
25 herbaceous crops, leaves, bark, needles, logs, roots, saplings, short rotation woody crops, shrubs, switch grasses, trees, vegetables, fruit peels, vines, sugar beet, sugar beet pulp, wheat midlings, oat hulls, and hard and soft woods (not including woods with deleterious materials). In addition, agricultural biomass includes organic waste materials generated from agricultural processes including farming and forestry activities, specifically including forestry wood waste. Agricultural
30 biomass may be any of the afore-mentioned singularly or in any combination or mixture thereof. In a preferred embodiment the biomass is silage.

The biomass can be pretreated before it is contacted with the enzymes and the culture. Pretreatment methods are known in the art and include, but are not limited to, heat, mechanical, chemical modification, biological modification and any combination thereof. The biomass may also
35 be washed before it is contacted with the enzymes and the culture.

The biomass may be added batch-wise, fed-batch wise or continuously to a reactor. The enzymes may be added batch-wise, fed-batch wise or continuously to a reactor. The culture may be added batch-wise, fed-batch wise or continuously to a reactor.

In an embodiment the enzymes are in the form of an enzyme composition. The enzyme composition may be an aqueous composition (including for example a slurry) or a solid composition.

In an embodiment the enzymes are fungal enzymes. In an embodiment the enzymes are selected from the group consisting of amylases, cellulases, hemicellulases, pectinases and lipases.

5 In an embodiment the enzymes are in an enzyme composition. In an embodiment the enzyme composition is from a fungus, preferably a filamentous fungus. In an embodiment the enzyme composition is produced by a fungus, preferably a filamentous fungus. In an embodiment the enzymes in the enzyme composition are derived from a fungus, preferably a filamentous fungus. In an embodiment the enzyme composition comprises a fungal enzyme, preferably a filamentous
10 fungal enzyme. "Filamentous fungi" include all filamentous forms of the subdivision *Eumycota* and *Oomycota* (as defined by Hawksworth *et al.*, In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK). Filamentous fungi include, but are not limited to *Acremonium*, *Agaricus*, *Aspergillus*, *Aureobasidium*, *Beauveria*, *Cephalosporium*, *Ceriporiopsis*, *Chaetomium*, *paecilomyces*, *Chrysosporium*, *Claviceps*, *Cochiobolus*, *Coprinus*,
15 *Cryptococcus*, *Cyathus*, *Emericella*, *Endothia*, *Endothia mucor*, *Filibasidium*, *Fusarium*, *Geosmithia*, *Gilocladium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Myrothecium*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Panerochaete*, *Pleurotus*, *Podospora*, *Pyricularia*, *Rasamsonia*, *Rhizomucor*, *Rhizopus*, *Scylatidium*, *Schizophyllum*, *Stagonospora*, *Talaromyces*, *Thermoascus*, *Thermomyces*, *Thielavia*, *Tolypocladium*, *Trametes*
20 *pleurotus*, *Trichoderma* and *Trichophyton*. Several strains of filamentous fungi are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

25 The enzymes may be prepared by fermentation of a suitable substrate with a suitable microorganism, e.g. a fungus, wherein the enzymes are produced by the microorganism. The microorganism may be altered to improve or to make the enzymes. For example, the microorganism may be mutated by classical strain improvement procedures or by recombinant DNA techniques. Therefore, the microorganisms mentioned herein can be used as such to produce
30 the enzymes or may be altered to increase the production or to produce altered enzymes which might include heterologous enzymes, e.g. amylases, cellulases, hemicellulases, pectinases, thus enzymes that are not originally produced by that microorganism. Preferably, a fungus, more preferably a filamentous fungus is used to produce the enzymes.

The enzymes are used to hydrolyse the biomass (release sugars from biomass that
35 comprises polysaccharides). Biomass as used herein comprises polysaccharides, oligosaccharides, monosaccharides, pectin, proteins and/or lipids. The polysaccharides can be starch, celluloses (glucans) and/or hemicelluloses (xylans, heteroxylans and xyloglucans). Starch may be present as amylose or amylopectin. In addition, hemicellulose may be present as

glucomannans, for example in wood-derived biomass. Pectin consists of an associated group of polysaccharides. The enzymatic hydrolysis of these polysaccharides to soluble sugars, including both monomers and multimers, for example glucose, cellobiose, xylose, arabinose, galactose, fructose, mannose, rhamnose, ribose, galacturonic acid, glucuronic acid and other hexoses and pentoses occurs under the action of different enzymes acting in concert (e.g. amylases, cellulases, hemicellulases and pectinases). A sugar product comprises soluble sugars, including both monomers and multimers such as polymers. In an embodiment the sugar product comprises for example glucose, galactose and arabinose. Examples of other sugars are cellobiose, xylose, arabinose, galactose, fructose, mannose, rhamnose, ribose, galacturonic acid, lactose, saccharose, sucrose, glucuronic acid and other hexoses and pentoses. In addition, the biomass may comprise pectins and other pectic substances such as arabinans, which may make up considerably proportion of the dry mass of typically cell walls from non-woody plant tissues (about a quarter to half of dry mass may be pectins). Furthermore, the biomass may comprise lignin.

Enzymes that may be used in the processes as described herein are described in more detail below.

Enzymes such as lytic polysaccharide monooxygenases, endoglucanases (EG) and exo-cellobiohydrolases (CBH) catalyze the hydrolysis of insoluble cellulose to products such as celooligosaccharides (cellobiose as a main product), while β -glucosidases (BG) convert the oligosaccharides, mainly cellobiose and cellotriose, to glucose.

Xylanases such as for example α -L- arabinofuranosidases, feruloyl and acetylxylan esterases, glucuronidases, and β -xylosidases catalyze the hydrolysis of hemicellulose.

An enzyme composition for use in the processes as described herein may comprise at least two activities, although typically a composition will comprise more than two activities, for example, three, four, five, six, seven, eight, nine or even more activities. Typically, an enzyme composition for use in the processes as described herein comprises at least two cellulases. The at least two cellulases may contain the same or different activities. The enzyme composition for use in the processes as described herein may also comprises at least one enzyme other than a cellulase, e.g. a hemicellulase, a pectinase, an amylase or a combination thereof.

In an embodiment an enzyme composition for use in the processes as described herein at least comprises an endoglucanase (EG), a cellobiohydrolase (CBH), an endoxylanase (EX), a beta-xylosidase (BX) and a beta-glucosidase (BG). An enzyme composition for use in the processes as described herein may comprise an endoglucanase (EG), a cellobiohydrolase I (CBHI), a cellobiohydrolase II (CBHII), a beta-glucosidase (BG), an endoxylanase (EX) and a beta-xylosidase (BX). An enzyme composition as described herein may also comprise a lytic polysaccharide monooxygenase (LPMO). An enzyme composition may comprise more than one enzyme activity per activity class. For example, a composition may comprise two endoglucanases, for example an endoglucanase having endo-1,3(1,4)- β glucanase activity and an endoglucanase having endo- β -1,4-glucanase activity.

The enzyme composition for use in the processes as described herein may comprise a cellulase and/or a hemicellulase and/or a pectinase and/or an amylase and/or a lipase and/or a protease.

As used herein, a cellulase is any polypeptide which is capable of degrading or modifying cellulose. A polypeptide which is capable of degrading cellulose is one which is capable of catalyzing the process of breaking down cellulose into smaller units, either partially, for example into cellobextrins, or completely into glucose monomers. A cellulase as described herein may give rise to a mixed population of cellobextrins and glucose monomers. Such degradation will typically take place by way of a hydrolysis reaction.

As used herein, a hemicellulase is any polypeptide which is capable of degrading or modifying hemicellulose. That is to say, a hemicellulase may be capable of degrading or modifying one or more of xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan. A polypeptide which is capable of degrading a hemicellulose is one which is capable of catalyzing the process of breaking down the hemicellulose into smaller polysaccharides, either partially, for example into oligosaccharides, or completely into sugar monomers, for example hexose or pentose sugar monomers. A hemicellulase as described herein may give rise to a mixed population of oligosaccharides and sugar monomers. Such degradation will typically take place by way of a hydrolysis reaction.

As used herein, a pectinase is any polypeptide which is capable of degrading or modifying pectin. A polypeptide which is capable of degrading pectin is one which is capable of catalyzing the process of breaking down pectin into smaller units, either partially, for example into oligosaccharides, or completely into sugar monomers. A pectinase as described herein may give rise to a mixed population of oligosaccharides and sugar monomers. Such degradation will typically take place by way of a hydrolysis reaction.

The term "amylase" as used herein means enzymes that hydrolyze alpha-1,4-glucosidic linkages in starch, both in amylose and amylopectin, such as alpha-amylase (EC 3.2.1.1), beta-amylase (EC 3.2.1.2), glucan 1,4-alpha-glucosidase (EC 3.2.1.3), glucan 1,4-alpha-maltotetraohydrolase (EC 3.2.1.60), glucan 1,4-alpha-maltohexaosidase (EC 3.2.1.98), glucan 1,4-alpha-maltotriohydrolase (EC 3.2.1.116) and glucan 1,4-alpha-maltohydrolase (EC 3.2.1.133), and enzymes that hydrolyze alpha-1,6-glucosidic linkages, being the branch-points in amylopectin, such as pullulanase (EC 3.2.1.41) and limit dextrinase (EC 3.2.1.142).

As used herein, a lactase (EC 3.2.1.21) is an enzyme that hydrolyses terminal, non-reducing beta-D-glucosyl residues with release of beta-D-glucose.

As used herein, a sucrase (EC 3.2.1.26 (invertase)) catalyzes the hydrolysis of sucrose into fructose and glucose.

As used herein, a lipase is an enzyme that catalyzes the hydrolysis of fats (lipids). Examples include, but are not limited to triacylglycerol lipases, phospholipases (such as A₁, A₂, B, C and D), cutinases and galactolipases.

As used herein, a protease is a protein hydrolyzing or modifying proteins. Examples include, but are not limited to, endo-acting proteases (serine proteases, metalloproteases, aspartyl proteases, thiol proteases), exo-acting peptidases that cleave off one amino acid, or dipeptide, tripeptide etceteras from the N-terminal (aminopeptidases) or C-terminal (carboxypeptidases) ends of the polypeptide chain.

Accordingly, an enzyme composition for use in the processes as described herein may comprise one or more of the following enzymes, a lytic polysaccharide monooxygenase (e.g. GH61), a cellobiohydrolase, an endo- β -1,4-glucanase, a beta-glucosidase, and a β -(1,3)(1,4)-glucanase. A composition for use in the processes as described herein may also comprise one or more hemicellulases, for example, an endoxylanase, a β -xylosidase, a α -L-arabinofuranosidase, an α -D-glucuronidase, an acetyl xylan esterase, a feruloyl esterase, a coumaroyl esterase, an α -galactosidase, a β -galactosidase, a β -mannanase and/or a β -mannosidase. A composition for use in the processes as described herein may also comprise one or more pectinases, for example, an endo-polygalacturonase, a pectin-methyl esterase, an endo-galactanase, a beta-galactosidase, a pectin-acetyl esterase, an endo-pectin lyase, pectate lyase, alpha-rhamnosidase, an exo-galacturonase, an expolygalacturonate lyase, a rhamnogalacturonan hydrolase, a rhamnogalacturonan lyase, a rhamnogalacturonan acetyl esterase, a rhamnogalacturonan galacturonohydrolase, and/or a xylogalacturonase. In addition, one or more of the following enzymes, an amylase, a protease, a lipase, a ligninase, a hexosyltransferase, a glucuronidase, an expansin, a cellulose induced protein or a cellulose integrating protein or like protein, a lactase, a sucrase, a catalase may be present in a composition for use in the processes as described herein.

As used herein, lytic polysaccharide monooxygenases are enzymes that have recently been classified by CAZy in family AA9 (Auxiliary Activity Family 9) or family AA10 (Auxiliary Activity Family 10). *Ergo*, there exist AA9 lytic polysaccharide monooxygenases and AA10 lytic polysaccharide monooxygenases. Lytic polysaccharide monooxygenases are able to open a crystalline glucan structure and enhance the action of cellulases on lignocellulose substrates. They are enzymes having cellulolytic enhancing activity. Lytic polysaccharide monooxygenases may also affect cello-oligosaccharides. According to the latest literature, (see Isaksen et al., Journal of Biological Chemistry, vol. 289, no. 5, p. 2632-2642), proteins named GH61 (glycoside hydrolase family 61 or sometimes referred to EGIV) are lytic polysaccharide monooxygenases. GH61 was originally classified as endoglucanase based on measurement of very weak endo-1,4- β -d-glucanase activity in one family member but have recently been reclassified by CAZy in family AA9. CBM33 (family 33 carbohydrate-binding module) is also a lytic polysaccharide monooxygenase (see Isaksen et al, Journal of Biological Chemistry, vol. 289, no. 5, pp. 2632-2642). CAZy has recently reclassified CBM33 in the AA10 family.

In an embodiment the lytic polysaccharide monooxygenase comprises an AA9 lytic polysaccharide monooxygenase. This means that at least one of the lytic polysaccharide monooxygenases in the enzyme composition is an AA9 lytic polysaccharide monooxygenase. In

an embodiment, all lytic polysaccharide monooxygenases in the enzyme composition are AA9 lytic polysaccharide monooxygenase.

In an embodiment the enzyme composition comprises a lytic polysaccharide monooxygenase from *Thermoascus*, such as *Thermoascus aurantiacus*, such as the one described in WO 2005/074656 as SEQ ID NO:2 and SEQ ID NO:1 in WO2014/130812 and in WO 2010/065830; or from *Thielavia*, such as *Thielavia terrestris*, such as the one described in WO 2005/074647 as SEQ ID NO: 8 or SEQ ID NO:4 in WO2014/130812 and in WO 2008/148131, and WO 2011/035027; or from *Aspergillus*, such as *Aspergillus fumigatus*, such as the one described in WO 2010/138754 as SEQ ID NO:2 or SEQ ID NO: 3 in WO2014/130812; or from *Penicillium*, such as *Penicillium emersonii*, such as the one disclosed as SEQ ID NO:2 in WO 2011/041397 or SEQ ID NO:2 in WO2014/130812. Other suitable lytic polysaccharide monooxygenases include, but are not limited to, *Trichoderma reesei* (see WO 2007/089290), *Myceliophthora thermophila* (see WO 2009/085935, WO 2009/085859, WO 2009/085864, WO 2009/085868), *Penicillium pinophilum* (see WO 2011/005867), *Thermoascus sp.* (see WO 2011/039319), and *Thermoascus crustaceus* (see WO 2011/041504). Other cellulolytic enzymes that may be comprised in the enzyme composition are described in WO 98/13465, WO 98/015619, WO 98/015633, WO 99/06574, WO 99/10481, WO 99/025847, WO 99/031255, WO 2002/101078, WO 2003/027306, WO 2003/052054, WO 2003/052055, WO 2003/052056, WO 2003/052057, WO 2003/052118, WO 2004/016760, WO 2004/043980, WO 2004/048592, WO 2005/001065, WO 2005/028636, WO 2005/093050, WO 2005/093073, WO 2006/074005, WO 2006/117432, WO 2007/071818, WO 2007/071820, WO 2008/008070, WO 2008/008793, US 5,457,046, US 5,648,263, and US 5,686,593, to name just a few. In an embodiment the lytic polysaccharide monooxygenase is from *Rasamsonia*, e.g. *Rasamsonia emersonii* (see WO 2012/000892).

As used herein, endoglucanases are enzymes which are capable of catalyzing the endohydrolysis of 1,4- β -D-glucosidic linkages in cellulose, lichenin or cereal β -D-glucans. They belong to EC 3.2.1.4 and may also be capable of hydrolyzing 1,4-linkages in β -D-glucans also containing 1,3-linkages. Endoglucanases may also be referred to as cellulases, avicelases, β -1,4-endoglucan hydrolases, β -1,4-glucanases, carboxymethyl cellulases, celludextrinases, endo-1,4- β -D-glucanases, endo-1,4- β -D-glucanohydrolases or endo-1,4- β -glucanases.

In an embodiment the endoglucanase comprises a GH5 endoglucanase and/or a GH7 endoglucanase. This means that at least one of the endoglucanases in the enzyme composition is a GH5 endoglucanase or a GH7 endoglucanase. In case there are more endoglucanases in the enzyme composition, these endoglucanases can be GH5 endoglucanases, GH7 endoglucanases or a combination of GH5 endoglucanases and GH7 endoglucanases. In a preferred embodiment the endoglucanase comprises a GH5 endoglucanase.

In an embodiment the enzyme composition comprises an endoglucanase from *Trichoderma*, such as *Trichoderma reesei* or *Trichoderma longibrachiatum*; from *Humicola*, such as a strain of *Humicola insolens*; from *Aspergillus*, such as *Aspergillus aculeatus* or *Aspergillus*

kawachii; from *Erwinia*, such as *Erwinia carotovora*; from *Fusarium*, such as *Fusarium oxysporum*; from *Thielavia*, such as *Thielavia terrestris*; from *Humicola*, such as *Humicola grisea* var. *thermoidea* or *Humicola insolens*; from *Melanocarpus*, such as *Melanocarpus albomyces*; from *Neurospora*, such as *Neurospora crassa*; from *Myceliophthora*, such as *Myceliophthora thermophila*; from *Cladorrhinum*, such as *Cladorrhinum foecundissimum*; and/or from *Chrysosporium*, such as a strain of *Chrysosporium lucknowense*. In an embodiment the endoglucanase is from *Rasamsonia*, such as a strain of *Rasamsonia emersonii* (see WO 01/70998). In an embodiment even a bacterial endoglucanase can be used including, but are not limited to, *Acidothrmus cellulolyticus* endoglucanase (see WO 91/05039; WO 93/15186; US 5,275,944; WO 96/02551; US 5,536,655, WO 00/70031, WO 05/093050); *Thermobifida fusca* endoglucanase III (see WO 05/093050); and *Thermobifida fusca* endoglucanase V (see WO 05/093050). In an embodiment the enzyme composition comprises the commercially available endoglucanase called GoldFerm®.

As used herein, beta-xylosidases (EC 3.2.1.37) are polypeptides which are capable of catalysing the hydrolysis of 1,4- β -D-xylans, to remove successive D-xylose residues from the non-reducing termini. Beta-xylosidases may also hydrolyze xylobiose. Beta-xylosidase may also be referred to as xylan 1,4- β -xylosidase, 1,4- β -D-xylan xylohydrolase, exo-1,4- β -xylosidase or xylobiase.

In an embodiment the beta-xylosidase comprises a GH3 beta-xylosidase. This means that at least one of the beta-xylosidases in the enzyme composition is a GH3 beta-xylosidase. In an embodiment all beta-xylosidases in the enzyme composition are GH3 beta-xylosidases.

In an embodiment the enzyme composition comprises a beta-xylosidase from *Neurospora crassa*, *Aspergillus fumigatus*, *Trichoderma longibrachiatum* or *Trichoderma reesei*. In an embodiment the enzyme composition comprises a beta-xylosidase from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 2014/118360).

As used herein, an endoxylanase (EC 3.2.1.8) is any polypeptide which is capable of catalysing the endohydrolysis of 1,4- β -D-xylosidic linkages in xylans. This enzyme may also be referred to as endo-1,4- β -xylanase or 1,4- β -D-xylan xylanohydrolase. An alternative is EC 3.2.1.136, a glucuronoarabinoxylan endoxylanase, an enzyme that is able to hydrolyze 1,4 xylosidic linkages in glucuronoarabinoxylans.

In an embodiment the endoxylanase comprises a GH10 xylanase. This means that at least one of the endoxylanases in the enzyme composition is a GH10 xylanase. In an embodiment all endoxylanases in the enzyme composition are GH10 xylanases.

In an embodiment the enzyme composition comprises an endoxylanase from *Aspergillus aculeatus* (see WO 94/21785), *Aspergillus fumigatus* (see WO 2006/078256), *Penicillium pinophilum* (see WO 2011/041405), *Penicillium sp.* (see WO 2010/126772), *Thielavia terrestris* NRRL 8126 (see WO 2009/079210), *Talaromyces leycettanus*, *Thermobifida fusca*, or *Trichophaea saccata* GH10 (see WO 2011/057083). In an embodiment the enzyme composition comprises an

endoxylanase from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 02/24926).

As used herein, a beta-glucosidase (EC 3.2.1.21) is any polypeptide which is capable of catalysing the hydrolysis of terminal, non-reducing β -D-glucose residues with release of β -D-glucose. Such a polypeptide may have a wide specificity for β -D-glucosides and may also hydrolyze one or more of the following: a β -D-galactoside, an α -L-arabinoside, a β -D-xyloside or a β -D-fucose. This enzyme may also be referred to as amygdalase, β -D-glucoside glucohydrolase, cellobiase or gentobiase.

In an embodiment the enzyme composition comprises a beta-glucosidase from *Aspergillus*, such as *Aspergillus oryzae*, such as the one disclosed in WO 02/095014 or the fusion protein having beta-glucosidase activity disclosed in WO 2008/057637, or *Aspergillus fumigatus*, such as the one disclosed as SEQ ID NO:2 in WO 2005/047499 or SEQ ID NO:5 in WO 2014/130812 or an *Aspergillus fumigatus* beta-glucosidase variant, such as one disclosed in WO 2012/044915, such as one with the following substitutions: F100D, S283G, N456E, F512Y (using SEQ ID NO: 5 in WO 2014/130812 for numbering), or *Aspergillus aculeatus*, *Aspergillus niger* or *Aspergillus kawachi*. In another embodiment the beta-glucosidase is derived from *Penicillium*, such as *Penicillium brasilianum* disclosed as SEQ ID NO:2 in WO 2007/019442, or from *Trichoderma*, such as *Trichoderma reesei*, such as ones described in US 6,022,725, US 6,982,159, US 7,045,332, US 7,005,289, US 2006/0258554 US 2004/0102619. In an embodiment, even a bacterial beta-glucosidase can be used. In another embodiment the beta-glucosidase is derived from *Thielavia terrestris* (WO 2011/035029) or *Trichophaea saccata* (WO 2007/019442). In an embodiment the enzyme composition comprises a beta-glucosidase from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 2012/000886).

As used herein, a cellobiohydrolase (EC 3.2.1.91) is any polypeptide which is capable of catalyzing the hydrolysis of 1,4- β -D-glucosidic linkages in cellulose or cellotetraose, releasing cellobiose from the ends of the chains. This enzyme may also be referred to as cellulase 1,4- β -cellobiosidase, 1,4- β -cellobiohydrolase, 1,4- β -D-glucan cellobiohydrolase, avicelase, exo-1,4- β -D-glucanase, exocellobiohydrolase or exoglucanase.

In an embodiment the enzyme composition comprises a cellobiohydrolase I from *Aspergillus*, such as *Aspergillus fumigatus*, such as the Cel7A CBH I disclosed in SEQ ID NO:6 in WO 2011/057140 or SEQ ID NO:6 in WO 2014/130812; from *Trichoderma*, such as *Trichoderma longibrachiatum* or *Trichoderma reesei*; from *Chaetomium*, such as *Chaetomium thermophilum*; from *Talaromyces*, such as *Talaromyces leycettanus* or from *Penicillium*, such as *Penicillium emersonii*. In an embodiment the enzyme composition comprises a cellobiohydrolase I from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 2010/122141).

In an embodiment the enzyme composition comprises a cellobiohydrolase II from *Aspergillus*, such as *Aspergillus fumigatus*, such as the one in SEQ ID NO:7 in WO 2014/130812 or from *Trichoderma*, such as *Trichoderma longibrachiatum* or *Trichoderma reesei*, or from *Talaromyces*, such as *Talaromyces leycettanus*, or from *Thielavia*, such as *Thielavia terrestris*,

such as cellobiohydrolase II CEL6A from *Thielavia terrestris*. In an embodiment the enzyme composition comprises a cellobiohydrolase II from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 2011/098580).

In an embodiment the enzyme composition also comprises one or more of the below mentioned enzymes.

As used herein, a β -(1,3)(1,4)-glucanase (EC 3.2.1.73) is any polypeptide which is capable of catalysing the hydrolysis of 1,4- β -D-glucosidic linkages in β -D-glucans containing 1,3- and 1,4-bonds. Such a polypeptide may act on lichenin and cereal β -D-glucans, but not on β -D-glucans containing only 1,3- or 1,4-bonds. This enzyme may also be referred to as licheninase, 1,3-1,4- β -D-glucan 4-glucanohydrolase, β -glucanase, endo- β -1,3-1,4 glucanase, lichenase or mixed linkage β -glucanase. An alternative for this type of enzyme is EC 3.2.1.6, which is described as endo-1,3(4)-beta-glucanase. This type of enzyme hydrolyses 1,3- or 1,4-linkages in beta-D-glucanase when the glucose residue whose reducing group is involved in the linkage to be hydrolysed is itself substituted at C-3. Alternative names include endo-1,3-beta-glucanase, laminarinase, 1,3-(1,3;1,4)-beta-D-glucan 3 (4) glucanohydrolase. Substrates include laminarin, lichenin and cereal beta-D-glucans.

As used herein, an α -L-arabinofuranosidase (EC 3.2.1.55) is any polypeptide which is capable of acting on α -L-arabinofuranosides, α -L-arabinans containing (1,2) and/or (1,3)- and/or (1,5)-linkages, arabinoxylans and arabinogalactans. This enzyme may also be referred to as α -N-arabinofuranosidase, arabinofuranosidase or arabinosidase. Examples of arabinofuranosidases that may be comprised in the enzyme composition include, but are not limited to, arabinofuranosidases from *Aspergillus niger*, *Humicola insolens* DSM 1800 (see WO 2006/114094 and WO 2009/073383) and *M. giganteus* (see WO 2006/114094).

As used herein, an α -D-glucuronidase (EC 3.2.1.139) is any polypeptide which is capable of catalysing a reaction of the following form: α -D-glucuronoside + H₂O = an alcohol + D-glucuronate. This enzyme may also be referred to as α -glucuronidase or α -glucosiduronase. These enzymes may also hydrolyse 4-O-methylated glucuronic acid, which can also be present as a substituent in xylans. An alternative is EC 3.2.1.131: xylan α -1,2-glucuronosidase, which catalyses the hydrolysis of α -1,2-(4-O-methyl)glucuronosyl links. Examples of α -glucuronidases that may be comprised in the enzyme composition include, but are not limited to, α -glucuronidases from *Aspergillus clavatus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Humicola insolens* (see WO 2010/014706), *Penicillium aurantiogriseum* (see WO 2009/068565) and *Trichoderma reesei*.

As used herein, an acetyl xylan esterase (EC 3.1.1.72) is any polypeptide which is capable of catalysing the deacetylation of xylans and xylo-oligosaccharides. Such a polypeptide may catalyze the hydrolysis of acetyl groups from polymeric xylan, acetylated xylose, acetylated glucose, α -naphthyl acetate or p-nitrophenyl acetate but, typically, not from triacetyl glycerol. Such a polypeptide typically does not act on acetylated mannan or pectin. Examples of acetyl xylan

esterases that may be comprised in the enzyme composition include, but are not limited to, acetylxylin esterases from *Aspergillus aculeatus* (see WO 2010/108918), *Chaetomium globosum*, *Chaetomium gracile*, *Humicola insolens* DSM 1800 (see WO 2009/073709), *Hypocrea jecorina* (see WO 2005/001036), *Myceliophthora thermophila* (see WO 2010/014880), *Neurospora crassa*,
 5 *Phaeosphaeria nodorum* and *Thielavia terrestris* NRRL 8126 (see WO 2009/042846). In an embodiment the enzyme composition comprises an acetyl xylin esterase from *Rasamsonia*, such as *Rasamsonia emersonii* (see WO 2010/000888)

As used herein, a feruloyl esterase (EC 3.1.1.73) is any polypeptide which is capable of catalysing a reaction of the form: feruloyl-saccharide + H₂O = ferulate + saccharide. The saccharide
 10 may be, for example, an oligosaccharide or a polysaccharide. It may typically catalyse the hydrolysis of the 4-hydroxy-3-methoxycinnamoyl (feruloyl) group from an esterified sugar, which is usually arabinose in 'natural' substrates. p-nitrophenol acetate and methyl ferulate are typically poorer substrates. This enzyme may also be referred to as cinnamoyl ester hydrolase, ferulic acid esterase or hydroxycinnamoyl esterase. It may also be referred to as a hemicellulase accessory
 15 enzyme, since it may help xylanases and pectinases to break down plant cell wall hemicellulose and pectin. Examples of feruloyl esterases (ferulic acid esterases) that may be comprised in the enzyme composition include, but are not limited to, feruloyl esterases from *Humicola insolens* DSM 1800 (see WO 2009/076122), *Neosartorya fischeri*, *Neurospora crassa*, *Penicillium aurantiogriseum* (see WO 2009/127729), and *Thielavia terrestris* (see WO 2010/053838 and WO
 20 2010/065448).

As used herein, a coumaroyl esterase (EC 3.1.1.73) is any polypeptide which is capable of catalysing a reaction of the form: coumaroyl-saccharide + H₂O = coumarate + saccharide. The
 saccharide may be, for example, an oligosaccharide or a polysaccharide. This enzyme may also be referred to as trans-4-coumaroyl esterase, trans-p-coumaroyl esterase, p-coumaroyl esterase
 25 or p-coumaric acid esterase. This enzyme also falls within EC 3.1.1.73 so may also be referred to as a feruloyl esterase.

As used herein, an α-galactosidase (EC 3.2.1.22) is any polypeptide which is capable of catalysing the hydrolysis of terminal, non-reducing α-D-galactose residues in α-D-galactosides,
 including galactose oligosaccharides, galactomannans, galactans and arabinogalactans. Such a
 30 polypeptide may also be capable of hydrolyzing α-D-fucosides. This enzyme may also be referred to as melibiase.

As used herein, a β-galactosidase (EC 3.2.1.23) is any polypeptide which is capable of catalysing the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides.
 Such a polypeptide may also be capable of hydrolyzing α-L-arabinosides. This enzyme may also
 35 be referred to as exo-(1→4)-β-D-galactanase or lactase.

As used herein, a β-mannanase (EC 3.2.1.78) is any polypeptide which is capable of catalysing the random hydrolysis of 1,4-β-D-mannosidic linkages in mannans, galactomannans and
 glucomannans. This enzyme may also be referred to as mannan endo-1,4-β-mannosidase or endo-

1,4-mannanase.

As used herein, a β -mannosidase (EC 3.2.1.25) is any polypeptide which is capable of catalysing the hydrolysis of terminal, non-reducing β -D-mannose residues in β -D-mannosides. This enzyme may also be referred to as mannanase or mannase.

As used herein, an endo-polygalacturonase (EC 3.2.1.15) is any polypeptide which is capable of catalysing the random hydrolysis of 1,4- α -D-galactosiduronic linkages in pectate and other galacturonans. This enzyme may also be referred to as polygalacturonase pectin depolymerase, pectinase, endopolygalacturonase, pectolase, pectin hydrolase, pectin polygalacturonase, poly- α -1,4-galacturonide glycanohydrolase, endogalacturonase; endo-D-galacturonase or poly(1,4- α -D-galacturonide) glycanohydrolase.

As used herein, a pectin methyl esterase (EC 3.1.1.11) is any enzyme which is capable of catalysing the reaction: pectin + n H₂O = n methanol + pectate. The enzyme may also be known as pectinesterase, pectin demethoxylase, pectin methoxylase, pectin methylesterase, pectase, pectinoesterase or pectin pectylhydrolase.

As used herein, an endo-galactanase (EC 3.2.1.89) is any enzyme capable of catalysing the endohydrolysis of 1,4- β -D-galactosidic linkages in arabinogalactans. The enzyme may also be known as arabinogalactan endo-1,4- β -galactosidase, endo-1,4- β -galactanase, galactanase, arabinogalactanase or arabinogalactan 4- β -D-galactanohydrolase.

As used herein, a pectin acetyl esterase is defined herein as any enzyme which has an acetyl esterase activity which catalyses the deacetylation of the acetyl groups at the hydroxyl groups of GalUA residues of pectin.

As used herein, an endo-pectin lyase (EC 4.2.2.10) is any enzyme capable of catalysing the eliminative cleavage of (1 $\rightarrow$ 4)- α -D-galacturonan methyl ester to give oligosaccharides with 4-deoxy-6-O-methyl- α -D-galact-4-enuronosyl groups at their non-reducing ends. The enzyme may also be known as pectin lyase, pectin *trans*-eliminase; endo-pectin lyase, polymethylgalacturonic transeliminase, pectin methyltranseliminase, pectolyase, PL, PNL or PMGL or (1 $\rightarrow$ 4)-6-O-methyl- α -D-galacturonan lyase.

As used herein, a pectate lyase (EC 4.2.2.2) is any enzyme capable of catalysing the eliminative cleavage of (1 $\rightarrow$ 4)- α -D-galacturonan to give oligosaccharides with 4-deoxy- α -D-galact-4-enuronosyl groups at their non-reducing ends. The enzyme may also be known polygalacturonic transeliminase, pectic acid transeliminase, polygalacturonate lyase, endopectin methyltranseliminase, pectate transeliminase, endogalacturonate transeliminase, pectic acid lyase, pectic lyase, α -1,4-D-endopolygalacturonic acid lyase, PGA lyase, PPase-N, endo- α -1,4-polygalacturonic acid lyase, polygalacturonic acid lyase, pectin *trans*-eliminase, polygalacturonic acid *trans*-eliminase or (1 $\rightarrow$ 4)- α -D-galacturonan lyase.

As used herein, an alpha rhamnosidase (EC 3.2.1.40) is any polypeptide which is capable of catalysing the hydrolysis of terminal non-reducing α -L-rhamnose residues in α -L-rhamnosides or alternatively in rhamnogalacturonan. This enzyme may also be known as α -L-rhamnosidase T, α -

L-rhamnosidase N or α -L-rhamnoside rhamnohydrolase.

As used herein, exo-galacturonase (EC 3.2.1.82) is any polypeptide capable of hydrolysis of pectic acid from the non-reducing end, releasing digalacturonate. The enzyme may also be known as exo-poly- α -galacturonosidase, exopolygalacturonosidase or exopolygalacturanosidase.

As used herein, exo-galacturonase (EC 3.2.1.67) is any polypeptide capable of catalysing: $(1,4\text{-}\alpha\text{-D-galacturonide})_n + \text{H}_2\text{O} = (1,4\text{-}\alpha\text{-D-galacturonide})_{n-1} + \text{D-galacturonate}$. The enzyme may also be known as galacturan 1,4- α -galacturonidase, exopolygalacturonase, poly(galacturonate) hydrolase, exo-D-galacturonase, exo-D-galacturonanase, exopoly-D-galacturonase or poly(1,4- α -D-galacturonide) galacturonohydrolase.

As used herein, exopolygalacturonate lyase (EC 4.2.2.9) is any polypeptide capable of catalysing eliminative cleavage of 4-(4-deoxy- α -D-galact-4-enuronosyl)-D-galacturonate from the reducing end of pectate, i.e. de-esterified pectin. This enzyme may be known as pectate disaccharide-lyase, pectate exo-lyase, exopectic acid transeliminase, exopectate lyase, exopolygalacturonic acid-*trans*-eliminase, PATE, exo-PATE, exo-PGL or (1 $\rightarrow$ 4)- α -D-galacturonan reducing-end-disaccharide-lyase.

As used herein, rhamnogalacturonan hydrolase is any polypeptide which is capable of hydrolyzing the linkage between galactosyluronic acid and rhamnopyranosyl in an endo-fashion in strictly alternating rhamnogalacturonan structures, consisting of the disaccharide [(1,2- α -L-rhamnoyl-(1,4)- α -galactosyluronic acid)].

As used herein, rhamnogalacturonan lyase is any polypeptide which is any polypeptide which is capable of cleaving α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalpA linkages in an endo-fashion in rhamnogalacturonan by beta-elimination.

As used herein, rhamnogalacturonan acetyl esterase is any polypeptide which catalyzes the deacetylation of the backbone of alternating rhamnose and galacturonic acid residues in rhamnogalacturonan.

As used herein, rhamnogalacturonan galacturonohydrolase is any polypeptide which is capable of hydrolyzing galacturonic acid from the non-reducing end of strictly alternating rhamnogalacturonan structures in an exo-fashion.

As used herein, xylogalacturonase is any polypeptide which acts on xylogalacturonan by cleaving the β -xylose substituted galacturonic acid backbone in an *endo*-manner. This enzyme may also be known as xylogalacturonan hydrolase.

As used herein, an α -L-arabinofuranosidase (EC 3.2.1.55) is any polypeptide which is capable of acting on α -L-arabinofuranosides, α -L-arabinans containing (1,2) and/or (1,3)- and/or (1,5)-linkages, arabinoxylans and arabinogalactans. This enzyme may also be referred to as α -N-arabinofuranosidase, arabinofuranosidase or arabinosidase.

As used herein, endo-arabinanase (EC 3.2.1.99) is any polypeptide which is capable of catalysing endohydrolysis of 1,5- α -arabinofuranosidic linkages in 1,5-arabinans. The enzyme may also be known as endo-arabinase, arabinan endo-1,5- α -L-arabinosidase, endo-1,5- α -L-

arabinanase, endo- α -1,5-arabanase; endo-arabanase or 1,5- α -L-arabinan 1,5- α -L-arabinanohydrolase.

"Protease" includes enzymes that hydrolyze peptide bonds (peptidases), as well as enzymes that hydrolyze bonds between peptides and other moieties, such as sugars (glycopeptidases). Many proteases are characterized under EC 3.4 and are suitable for use in the processes as described herein. Some specific types of proteases include, cysteine proteases including pepsin, papain and serine proteases including chymotrypsins, carboxypeptidases and metalloendopeptidases.

"Lipase" includes enzymes that hydrolyze lipids, fatty acids, and acylglycerides, including phosphoglycerides, lipoproteins, diacylglycerols, cutinases and the like. In plants, lipids are used as structural components to limit water loss and pathogen infection. These lipids include waxes derived from fatty acids, as well as cutin and suberin.

"Ligninase" includes enzymes that can hydrolyze or break down the structure of lignin polymers. Enzymes that can break down lignin include lignin peroxidases, manganese peroxidases, laccases and feruloyl esterases, and other enzymes described in the art known to depolymerize or otherwise break lignin polymers. Also included are enzymes capable of hydrolyzing bonds formed between hemicellulosic sugars (notably arabinose) and lignin. Ligninases include but are not limited to the following group of enzymes: lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13), laccases (EC 1.10.3.2) and feruloyl esterases (EC 3.1.1.73).

"Hexosyltransferase" (2.4.1-) includes enzymes which are capable of catalysing a transferase reaction, but which can also catalyze a hydrolysis reaction, for example of cellulose and/or cellulose degradation products. An example of a hexosyltransferase which may be used is a β -glucanosyltransferase. Such an enzyme may be able to catalyze degradation of (1,3)(1,4)glucan and/or cellulose and/or a cellulose degradation product.

"Glucuronidase" includes enzymes that catalyze the hydrolysis of a glucuronoside, for example β -glucuronoside to yield an alcohol. Many glucuronidases have been characterized and may be suitable for use, for example β -glucuronidase (EC 3.2.1.31), hyaluronoglucuronidase (EC 3.2.1.36), glucuronosyl-disulfoglucosamine glucuronidase (3.2.1.56), glycyrrhizinate β -glucuronidase (3.2.1.128) or α -D-glucuronidase (EC 3.2.1.139).

Expansins are implicated in loosening of the cell wall structure during plant cell growth. Expansins have been proposed to disrupt hydrogen bonding between cellulose and other cell wall polysaccharides without having hydrolytic activity. In this way, they are thought to allow the sliding of cellulose fibers and enlargement of the cell wall. Swollenin, an expansin-like protein contains an N-terminal Carbohydrate Binding Module Family 1 domain (CBD) and a C-terminal expansin-like domain. As described herein, an expansin-like protein or swollenin-like protein may comprise one or both of such domains and/or may disrupt the structure of cell walls (such as disrupting cellulose structure), optionally without producing detectable amounts of reducing sugars.

A cellulose induced protein, for example the polypeptide product of the *cip1* or *cip2* gene or similar genes (see Foreman *et al.*, J. Biol. Chem. 278(34), 31988-31997, 2003), a cellulose/cellulosome integrating protein, for example the polypeptide product of the *cipA* or *cipC* gene, or a scaffoldin or a scaffoldin-like protein. Scaffoldins and cellulose integrating proteins are multi-functional integrating subunits which may organize cellulolytic subunits into a multi-enzyme complex. This is accomplished by the interaction of two complementary classes of domain, *i.e.* a cohesion domain on scaffoldin and a dockerin domain on each enzymatic unit. The scaffoldin subunit also bears a cellulose-binding module (CBM) that mediates attachment of the cellulosome to its substrate. A scaffoldin or cellulose integrating protein may comprise one or both of such domains.

A catalase; the term "catalase" means a hydrogen-peroxide: hydrogen-peroxide oxidoreductase (EC 1.11.1.6 or EC 1.11.1.21) that catalyzes the conversion of two hydrogen peroxides to oxygen and two waters. Catalase activity can be determined by monitoring the degradation of hydrogen peroxide at 240 nm based on the following reaction: $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$. The reaction is conducted in 50 mM phosphate pH 7.0 at 25°C with 10.3 mM substrate (H_2O_2) and approximately 100 units of enzyme per ml. Absorbance is monitored spectrophotometrically within 16-24 seconds, which should correspond to an absorbance reduction from 0.45 to 0.4. One catalase activity unit can be expressed as one micromole of H_2O_2 degraded per minute at pH 7.0 and 25°C.

A phytase (myo-inositol hexakisphosphate phosphohydrolase); the term "phytase" means any type of phosphatase enzyme that catalyzes the hydrolysis of phytic acid (myo-inositol hexakisphosphate) which is an indigestible, organic form of phosphorus that is found in grains and oil seeds, and releases a usable form of inorganic phosphorus.

An enzyme composition for use in the processes as described herein may be composed of enzymes from (1) commercial suppliers; (2) cloned genes expressing enzymes; (3) broth (such as that resulting from growth of a microbial strain in media, wherein the strains secrete proteins and enzymes into the media; (4) cell lysates of strains grown as in (3); and/or (5) plant material expressing enzymes. Different enzymes in a composition of the invention may be obtained from different sources. The enzyme composition may also be a solid composition comprising one or more microbial strains excreting enzymes during growth on solids present in the solid composition.

The enzymes can be produced either exogenously in microorganisms, yeasts, fungi, bacteria or plants, then isolated and added, for example, to biomass. In the uses and processes described herein, the components of the compositions described above may be provided concomitantly (*i.e.* as a single composition *per se*) or separately or sequentially.

In an embodiment the enzyme composition is selected from the group consisting of MethaPlus® L100/L120, Axiase™ 100, eBREAK™ 1000F, Roxazyme® G2, Ronozyme® WX, Ronozyme® A, Ronozyme® VP, Ronozyme® Multigrain, Cellic® CTec, Cellic® Htec, Accelerase® 1000/15000/Trio, Ronozyme® Rumistar, Ronozyme® HiStarch, Avantec®, Liquozyme®,

Termamyl®.

In an embodiment the enzyme composition comprises a whole fermentation broth of a fungus, preferably a whole fermentation broth of a filamentous fungus. The whole fermentation broth can be prepared from fermentation of non-recombinant and/or recombinant filamentous fungi.

5 In an embodiment the filamentous fungus is a recombinant filamentous fungus comprising one or more genes which can be homologous or heterologous to the filamentous fungus. In an embodiment, the filamentous fungus is a recombinant filamentous fungus comprising one or more genes which can be homologous or heterologous to the filamentous fungus wherein the one or more genes encode enzymes that can degrade a cellulosic substrate. The whole fermentation broth
10 may comprise any of the polypeptides described above or any combination thereof.

Preferably, the enzyme composition is a whole fermentation broth wherein the cells are killed. The whole fermentation broth may contain organic acid(s) (used for killing the cells), killed cells and/or cell debris, and culture medium.

Generally, filamentous fungi are cultivated in a cell culture medium suitable for production
15 of enzymes capable of hydrolyzing a cellulosic substrate. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable culture media, temperature ranges and other conditions suitable for growth and cellulase and/or hemicellulase and/or pectinase production are known in the art. The whole fermentation broth can be prepared by growing the filamentous fungi to stationary phase and
20 maintaining the filamentous fungi under limiting carbon conditions for a period of time sufficient to express the one or more cellulases and/or hemicellulases and/or pectinases. Once enzymes, such as cellulases and/or hemicellulases and/or pectinases and/or amylases, are secreted by the filamentous fungi into the fermentation medium, the whole fermentation broth can be used. The whole fermentation broth of the present invention may comprise filamentous fungi. In some
25 embodiments, the whole fermentation broth comprises the unfractionated contents of the fermentation materials derived at the end of the fermentation. Typically, the whole fermentation broth comprises the spent culture medium and cell debris present after the filamentous fungi is grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis (particularly, expression of cellulases and/or hemicellulases and/or pectinases and/or amylases).

30 In some embodiments, the whole fermentation broth comprises the spent cell culture medium, extracellular enzymes and filamentous fungi. In some embodiments, the filamentous fungi present in whole fermentation broth can be lysed, permeabilized, or killed using methods known in the art to produce a cell-killed whole fermentation broth. In an embodiment, the whole fermentation broth is a cell-killed whole fermentation broth, wherein the whole fermentation broth containing the
35 filamentous fungi cells are lysed or killed. In some embodiments, the cells are killed by lysing the filamentous fungi by chemical and/or pH treatment to generate the cell-killed whole broth of a fermentation of the filamentous fungi. In some embodiments, the cells are killed by lysing the filamentous fungi by chemical and/or pH treatment and adjusting the pH of the cell-killed

fermentation mix to a suitable pH. In an embodiment, the whole fermentation broth comprises a first organic acid component comprising at least one 1-5 carbon organic acid and/or a salt thereof and a second organic acid component comprising at least 6 or more carbon organic acid and/or a salt thereof. In an embodiment, the first organic acid component is acetic acid, formic acid, propionic acid, a salt thereof, or any combination thereof and the second organic acid component is benzoic acid, cyclohexanecarboxylic acid, 4-methylvaleric acid, phenylacetic acid, a salt thereof, or any combination thereof.

The term "whole fermentation broth" as used herein refers to a preparation produced by cellular fermentation that undergoes no or minimal recovery and/or purification. For example, whole fermentation broths are produced when microbial cultures are grown to saturation, incubated under carbon-limiting conditions to allow protein synthesis (e.g., expression of enzymes by host cells) and secretion into cell culture medium. Typically, the whole fermentation broth is unfractionated and comprises spent cell culture medium, extracellular enzymes, and microbial, preferably non-viable, cells.

If needed, the whole fermentation broth can be fractionated and the one or more of the fractionated contents can be used. For instance, the killed cells and/or cell debris can be removed from a whole fermentation broth to provide a composition that is free of these components.

The whole fermentation broth may further comprise a preservative and/or anti-microbial agent. Such preservatives and/or agents are known in the art.

The whole fermentation broth as described herein is typically a liquid, but may contain insoluble components, such as killed cells, cell debris, culture media components, and/or insoluble enzyme(s). In some embodiments, insoluble components may be removed to provide a clarified whole fermentation broth.

In an embodiment, the whole fermentation broth may be supplemented with one or more enzyme activities that are not expressed endogenously or expressed at relatively low level by the filamentous fungi, to improve the degradation of the cellulosic substrate, for example, to fermentable sugars such as glucose or xylose. The supplemental enzyme(s) can be added as a supplement to the whole fermentation broth and the enzymes may be a component of a separate whole fermentation broth, or may be purified, or minimally recovered and/or purified.

In an embodiment, the whole fermentation broth comprises a whole fermentation broth of a fermentation of a recombinant filamentous fungus overexpressing one or more enzymes to improve the degradation of the biomass. Alternatively, the whole fermentation broth can comprise a mixture of a whole fermentation broth of a fermentation of a non-recombinant filamentous fungus and a recombinant filamentous fungus overexpressing one or more enzymes to improve the degradation of the biomass.

In an embodiment enzymes are added by supplementing fermented organic solids. These solids may have been fermented with fungal strains, yeast strains, bacterial strains or a combination of these strains which are capable of secreting enzymes belonging to the group of enzymes to

improve the degradation of the biomass. In an embodiment, the fermented organic solids are obtained from the first composition of a previous batch.

In an embodiment step (a) and step (b) of the processes for producing biogas as described herein are done in different reactors, preferably they are however done in the same reactor. In an embodiment steps (a) and/or (b) are done in a single reactor, but steps (a) and/or (b) may also be done in multiple reactors. In an embodiment steps (a) and/or (b) are done in a reactor having a volume of 0.1 – 50 m³, preferably 0.2 - 20 m³, more preferably of 0.5 - 5 m³. In case multiple reactors are used in steps (a) and/or (b), they may have the same volume, but also may have a different volume. In an embodiment the reactor(s) used in steps (a) and/or (b) have a ratio height to diameter of to 1:1 to 10:1.

In an embodiment the fermentation step (i.e. step (b)) is a simultaneous hydrolysis and fermentation. In an embodiment the pH is not controlled before and/or during the fermenting step. Preferably, the pH changes to 4.5, preferably to 4.2, more preferably to 4.0 and most preferably to 3.8 during fermentation.

In an embodiment the enzymes are used in the fermentation step (i.e. step (b)) in an amount of 100-1000 ppm on dry matter weight of biomass.

In an embodiment the dry matter content in the fermentation step (i.e. step (b)) is from 5% to 30% (w/w), preferably 5% to 20% (w/w). In an embodiment the fermentation step (i.e. step (b)) is conducted at a temperature of 10°C – 65°C, preferably 35°C – 40°C.

In an embodiment the culture is used in the fermentation step (i.e. step (b)) in an amount of 10⁵ to 10⁸ CFU/g dry matter, preferably 10⁶ CFU/g dry matter. The third composition may comprise culture. Consequently, the third composition may be used to inoculate the second composition.

As described above in step (b) of the processes of the invention is fermenting the first composition to obtain a second composition comprising organic acids. In an embodiment the second composition comprises at least 5% organic acids (w/w on dry matter).

As described above in step (c) of the processes of the invention is contacting the second composition comprising organic acids with the organic material to obtain a third composition.

In an embodiment the organic material is organic material comprising ammonia, urea and/or uric acid. In an embodiment the ammonia is dissolved in the organic material and is the result of urea and/or uric acid degradation. The organic material can be selected from the group consisting of manure, dung, waste, silage, sludge, biomass from purification, fermentation or digestion processes or any combination thereof. In a preferred embodiment the organic material comprises manure, from cows, pigs, goats, poultry, sheeps or horses. The manure may consist of combined faeces and urine.

In an embodiment the organic material has an organic matter content of 5 – 50% dry matter. By organic matter content of the organic material is meant the dry matter content of the organic

material minus ash. BOD (Biological Oxygen Demand) test is commonly used to indirectly measure the amount of organic matter content of the organic material, see for example ISO 5815 (1989).

In an embodiment the fermentation (step (b)) is done in a first reactor. In an embodiment the contacting of the second composition comprising organic acids with the organic material to
5 obtain a third composition is done in a second reactor. In other words, the fermentation step can be done in a reactor different from the reactor that is used to contact the second composition comprising organic acids with the organic material to obtain a third composition.

In a preferred embodiment the organic material is added to the second composition comprising organic acids. In other words, the organic material is added to a reactor already
10 comprising the second composition comprising organic acids.

In an embodiment the process further comprises the step of aerating the organic material before digestion. The organic material can be aerated before, during and/or after it is contacted with the second composition comprising organic acids. When the organic material is aerated after it is contacted with the second composition comprising organic acids, this means that the third
15 composition is aerated (the organic material and the second composition comprising organic acids together form the third composition). The organic material can also be aerated by aerating the second composition comprising organic acids and contacting the aerated second composition comprising organic acids with the organic material. Aeration can be done by blowing an oxygen-containing gas, such as air, into the organic material and/or second composition comprising organic
20 acids and/or third composition. Blowing an oxygen-containing gas into the organic material and/or second composition comprising organic acids and/or third composition can be done by means of a sparger and/or nozzles and will result in mixing the second composition comprising organic acids with the livestock manure reducing the methane production during the storage of the obtained third composition. Aeration can be done continuously or discontinuously. The mixing of the third
25 composition reduces sedimentation of the solids in the third composition, thus keeping the third composition homogeneous for further transfer, transport and processing of the third composition to the anaerobic digester.

In an embodiment the third composition can be stored from 1 hour to 2000 hours before digestion. In an embodiment the third composition can be stored from 1 hour to 1500 hours before
30 digestion. In an embodiment the third composition can be stored from 1 hour to 1000 hours before digestion. In an embodiment the third composition can be stored from 1 hour to 900 hours before digestion. In an embodiment the third composition can be stored from 1 hour to 800 hours before digestion. In an embodiment the third composition can be stored from 1 hour to 700 hours before digestion. In an embodiment the third composition can be stored from 1 hour to 600 hours before
35 digestion. In an embodiment the third composition can be stored from 1 hour to 360 hours before digestion. Storage can be done in the reactor wherein the second composition comprising organic acids is contacted with the organic material to obtain a third composition. Storage of the third

composition can however also be done in a separate storage reactor in which aeration is maintained. In an embodiment storage time is determined by the pH.

In an embodiment, the reactor for storage of the third composition is continuously or discontinuously emptied to the anaerobic digester and refilled afterwards (batch mode). In another embodiment, the reactor for storage of the third composition is continuously or discontinuously partially emptied to the anaerobic digester and meantime supplied with freshly prepared third composition during emptying (continuous mode).

In an embodiment biomass is added to the third composition prior to and/or during the digestion. In an embodiment the third composition is contacted with biomass prior to and/or during the digestion. In an embodiment the third composition is mixed with biomass prior to and/or during the digestion. When biomass and manure are digested together this is called co-digestion. Suitable biomasses for co-digestion have been described above.

In an embodiment the digestion of the organic material (e.g. the third composition) is anaerobic. Examples of reactors that can be used for the digestion (i.e. the biogas production) are Covered Anaerobic Lagoon Digester, Plug-flow Digester, Continuous Mixing Digester, upflow reactor, Fixed Film Digester, Sequencing Batch Reactor, High Solids Anaerobic Digester, to name just a few.

The pH during the biogas production will in general be between pH of 6 and 8, preferably between 7 and 7.5. Generally no measures have to be taken to control the pH, the system is capable to maintain this pH itself. In case the substrate of the biogas production is outside this pH range or outside the preferred pH range, so for example at pH of 6 or lower, or at pH of 8 and higher, the pH of this substrate can be neutralized to for example between 6 and 8.

A general problem associated in the production of biogas is the production of carbon dioxide, which is an unwanted side-product and a greenhouse gas, and a loss of carbon. One way to reduce the amount of carbon dioxide is to feed external hydrogen to the anaerobic digester or produce hydrogen inside the anaerobic digester. Details of the biogas production step e.g. size of digester, which bacteria are used, which enzymes are used etc can be found in Anukam et al., Processes, 7, 504, pages 1-19 (2019).

EXAMPLES

Example 1

Addition of organic acids to reduce pH of manure

Freshly dropped cow feces was collected from a local farm, mixed by stirring and transferred in portions of 750 g into six glass bottles of 1 liter within 3 hours upon collection. The weight of manure added to each bottle was measured.

The content of three bottles, further on referred to as “acidified manure”, were adjusted to pH 5.5 with 90% DL-lactic acid while stirring. The amount of lactic acid used is expressed as mmol lactic acid per kg manure.

The pH of the three remaining bottles, further on referred to as “reference”, were not adjusted. The bottles were loosely closed with a cap to avoid overpressure and placed in a water bath of 24 °C.

The pH of the six bottles was measured regularly during 24 days. The content of the bottles was mixed prior to pH measurement. The results are shown in Table 1.

The lactic acid added reduced the pH of manure to 5.5 and the pH of “acidified manure” remained below that of the “reference” for at least 24 days. The amount of lactic acid required to obtain pH 5.5 was the ratio between the concentration of ammonia as gas (NH₃) and the concentration of ammonium in solution (NH₄⁺) which is pH dependent:

$$\begin{aligned} [NH_3] \cdot [H^+] / [NH_4^+] &= K_a \\ \rightarrow \\ [NH_3] / [NH_4^+] &= K_a / [H^+] \end{aligned}$$

wherein K_a is the acid dissociation constant which is constant at constant temperature.

Based on this equation, R% the ratio [NH₃]/[NH₄⁺] is calculated as the amount of gaseous ammonia (NH₃) as percentage of the amount of aqueous ammonium (NH₄⁺) using the pH value of each sample ($[H^+] = 10^{-pH} \text{ mol/L}$) and $K_a = 5.6 \times 10^{-10} \text{ mol/L}$.

The result of the calculations shows the effect of lactic acid added to the manure: the amount of gaseous ammonia (NH₃) relative to the amount of ammonium (NH₄⁺) is 0.2% or less, if the manure is acidified with lactic acid.

Example 2

Acidification of silage biomass

In this example the additional acidification of silage during incubation with enzymes and cultures is shown.

Corn silage, containing 41% dry matter, was obtained from a local farm. The corn silage was used as such. Corn silage (97 g) was added to water (903 g) in a closed stirred vessel (MiniFors, Infors AG, Basel, Switzerland) equipped with a heating jacket, a probe for online pH measurement, a PT100 probe for temperature control and a condenser at the gas-outlet to limit water evaporation. Nitrogen was supplied through a gas inlet into the headspace to obtain anaerobic conditions as to mimic oxygen limitations which commonly occur in poor-mixable slurry fermentations on large scale. The stirred vessel content was heated and maintained at temperature-setpoint value throughout the experiment.

The incubations were executed in fivefold. Enzymes were added to the silage mixture once the temperature reached its setpoint value.

Vessel 1 (reference) was used without any addition. In vessels 2 and 4, a composition of cellulolytic and proteolytic feed enzymes (Ronozymes® VP, Ronozymes® ProAct and Ronozymes® WX, DSM Nutritional Products, Basel, Switzerland) was added to stimulate acidification. A composition of two cellulolytic biogas enzymes (Methaplus L100 and Axiase 100 from DSM) was added to vessel 3 and 5. Vessels 4 was supplemented with a heterofermentative lactobacilli culture (DelvoCheese® CT151, DSM Food Specialties, Delft, Netherlands). The culture used has its optimum growth temperature at 25°C. Vessel 5 was supplemented with a protease producing lactobacilli culture (DelvoAdd® 200-H, DSM Food Specialties, Delft, Netherlands) which has its optimum growth temperature at 38°C. The setpoint temperature of the five vessels was 25, 25, 25, 25 and 38°C, respectively.

The incubations lasted 3 days after which the vessels were cooled to room temperature and caustic (4N sodium hydroxide solution) was added until a pH 5.5 was reached. The net amount of caustic used per kg silage dry matter to bring the pH to 5.5 was determined and compared to the amount used in vessel 6 containing silage without incubation period and without addition of enzymes and cultures.

The results (see Table 2) show that, although corn silage is acidified during the silage process, additional acidification occurs during the incubation with enzymes and enzymes and cultures. During the incubation of silage with feed enzymes or biogas enzymes; with or without lactobacilli cultures added, up to 37% additional acidification occurs, producing up to 3.5 times the amount of acid needed to bring manure to pH 5.5 as done in Example 1. The addition of cultures resulted in additional acidification.

Fermented biomass obtained from vessels 1 and 6 as well as acidified biomass obtained after fermentation from vessels 2-5 (being examples of the second composition comprising organic acids as defined herein) are further contacted with organic material (manure) to obtain a third composition and said third composition is further digested to produce biogas. The biogas process utilizing acidified biomass (vessel 2-5) is improved over the regular biomass (vessel 1 and 6) in that for instance the biogas yield is increased and/or more methane is produced. The relative increase in biogas yield and/or methane production is at least 1%, 2%, 5%, 10% or more,

As such the addition of enzymes and/or cultures to the initial biomass surprisingly showed a positive effect on the biogas production during anaerobic digestion.

Example 3

Acidification of lignocellulosic biomass

Wheat straw was obtained from a local farm and contained 91% dry matter. The straw was cut in pieces of 1 – 3 cm before application. Cut straw (28 g) was added to water (914 g) in the closed stirred vessel described in Example 2. The stirred vessel content was heated and maintained at $50 \pm 1^\circ\text{C}$ throughout the experiment. Enzyme was added to the straw mixture once the temperature reached $50 \pm 1^\circ\text{C}$.

The experiment was executed in twofold; one with cellulolytic enzymes for biogas applications (Axiase® 100, DSM Nutritional Products, Basel, Switzerland) which has a pH optimum between pH 6 and 8 and temperature optimum is around 50°C, and the other without enzymes added.

5 The incubations lasted 2 days after which the vessels were cooled to room temperature and the pH was brought to 5.5 with caustic (4N sodium hydroxide solution). The amount of caustic used determines the acidification obtained with the straw.

10 The results (see Table 3) show that 48 hours incubation of wheat straw at 50°C result in acidification which, expressed as mmol NaOH per kg wheat straw, reaches the amount of acid needed to bring 1 kg manure to the pH of 5.5. When enzymes are added, the production of acids exceeds the amount needed to acidify manure and results in 14% additional acidification, despite the suboptimal pH conditions for this biogas enzyme.

15 Fermented biomass obtained from vessels 1 as well as acidified biomass obtained after fermentation from vessels 2 (being an example of the second composition comprising organic acids as defined herein) are further contacted with organic material (manure) to obtain a third composition and said third composition is further digested to produce biogas. The biogas process utilizing acidified biomass (vessel 2) is improved over the regular biomass (vessel 1) in that for instance the biogas yield is increased and/or more methane is produced. The relative increase in biogas yield and/or methane production is at least 1%, 2%, 5%, 10% or more,

20 As such the addition of enzymes to the initial biomass surprisingly showed a positive effect on the biogas production during anaerobic digestion.

Example 4

Acidification of protein-rich biomass

25 A feedstock mix of 1:1:1 rapeseed meal, sunflower meal and soy husks was prepared containing on as-is bases 23% cellulose, 11% hemicellulose, 7% starch, 25% crude protein and 11% moisture.

30 The feedstock mix (105 g) was added to water (942 g) in the closed stirred vessel described in Example 2. The stirred vessel content was heated and maintained at $38 \pm 1^\circ\text{C}$ throughout the experiment. Enzymes were added to the feedstock mix slurry once the temperature reached $38 \pm 1^\circ\text{C}$.

35 The experiment was executed in twofold. To vessel 1, no enzymes were added (reference). To vessel 2, 500 ppm cellulolytic enzyme Ronozyme® VP was added. The dosage used was in accordance with the dosage prescribed by the enzyme supplier (DSM Nutritional Products, Basel, Switzerland) for feed applications and expressed as ppm on feedstock dry matter.

The incubations lasted 2 days after which the vessels were cooled to room temperature and the pH was brought to 5.5 with caustic (4N sodium hydroxide solution). The amount of caustic used determines the acidification obtained with the feedstock.

The concentration of total organic acids (lactic acid, acetic acid, formic acid) was measured in the liquid fraction of the slurry after centrifugation, using HPLC equipped with a Aminex HPX-87H analytical column (Biorad, Hercules, CA).

The results (see Table 4) show that protein-rich feedstock acidify, even without additional enzymes or cultures. The addition of cellulolytic enzymes however resulted in an increase in acidification of 41%. The acidification 1 kg feedstock mix exceeds the amount of acid needed to acidify 1 kg manure from Example 1 to pH by 2.5 – 4 times.

Fermented biomass obtained from vessels 1 as well as acidified biomass obtained after fermentation from vessels 2 (being an example of the second composition comprising organic acids as defined herein) are further contacted with organic material (manure) to obtain a third composition and said third composition is further digested to produce biogas. The biogas process utilizing acidified biomass (vessel 2) is improved over the regular biomass (vessel 1) in that for instance the biogas yield is increased and/or more methane is produced. The relative increase in biogas yield and/or methane production is at least 1%, 2%, 5%, 10% or more,

As such the addition of enzymes to the initial biomass surprisingly showed a positive effect on the biogas production during anaerobic digestion.

Table 1: Addition of organic acids to reduce pH of manure

	Reference						Acidified manure					
	Bottle 1		Bottle 2		Bottle 3		Bottle 4		Bottle 5		Bottle 6	
Weight of manure (g)	750.0		750.1		750.0		750.1		750.0		750.1	
pH before adjustment	8.56		8.50		8.53		8.58		8.50		8.47	
Lactic acid added (mmol/kg manure)	0		0		0		201		210		185	
Incubation time (days)	pH	R%	pH	R%	pH	R%	pH	R%	pH	R%	pH	R%
0	8.56	20,3	8.50	17,7	8.53	19,0	5.47	0,0	5.35	0,0	5.70	0,0
2	7.25	1,0	7.31	1,1	7.31	1,1	5.36	0,0	5.36	0,0	5.54	0,0
7	6.87	0,4	6.84	0,4	6.85	0,4	5.34	0,0	5.31	0,0	5.82	0,0
10	6.83	0,4	6.78	0,3	6.78	0,3	6.37	0,1	5.82	0,0	6.50	0,2
14	6.74	0,3	6.71	0,3	6.72	0,3	6.47	0,2	6.44	0,2	6.48	0,2
17	6.67	0,3	6.64	0,2	6.66	0,3	6.55	0,2	6.49	0,2	6.48	0,2
21	6.61	0,2	6.58	0,2	6.60	0,2	6.48	0,2	6.44	0,2	6.50	0,2
24	6.58	0,2	6.57	0,2	6.52	0,2	6.38	0,1	6.40	0,1	6.49	0,2

Table 2: Acidification of silage biomass

	Vessel 1	Vessel 2	Vessel 3	Vessel 4	Vessel 5	Vessel 6
Water (g)	903	903	903	903	903	901
Silage (g)	97	98	97	98	98	94
Enzyme added	-	Feed enzymes	Biogas enzymes	Feed enzyme	Biogas enzymes	-
Enzyme dosage (ppm on dry matter)	-	1094	503	1102	1094	-
Culture added	-	-	-	DelvoCheese® CT151	DelvoAdd® 200-H	-
Temperature setpoint (°C)	25	25	25	25	38	25
Culture dosage (g/kg silage slurry)				1	1	
Incubation time (h)	pH	pH	pH	pH	pH	pH
1	3,85	3,85	3,86	3,83	3,66	3,85
12	3,87	3,85	3,86	3,82	3,81	NA
24	3,85	3,84	3,86	3,81	3,85	NA
36	3,84	3,84	3,85	3,8	3,83	NA
48	3,82	3,82	3,84	3,79	3,89	NA
60	3,8	3,81	3,83	3,77	3,89	NA
70	3,79	3,8	3,82	3,77	3,89	NA
Caustic usage (mmol NaOH/kg silage DM)	629	636	653	684	693	526
Additional acidification	24%	25%	27%	34%	37%	0%

Table 3: Acidification of lignocellulosic biomass

	Vessel 1	Vessel 2
Water (g)	913	914

Wheat straw (g)	31	30
Enzyme added	-	Axiase® 100
Enzyme dosage (ppm on dry matter)	-	750
Temperature setpoint (°C)	50	50
Incubation time (h)	pH	pH
1	6,96	6,88
12	6,74	6,82
24	4,47	4,49
36	4,41	4,45
48	4,41	4,43
Caustic usage (mmol NaOH/kg wheat straw DM)	207	236
Acidification	100%	114%

Table 4: Acidification of protein-rich biomass

	Vessel 1	Vessel 2
Water (g)	942	942
Feedstock mix	105	106
Enzyme added	-	Ronozyme® VP
Enzyme dosage (ppm on dry matter)	-	750
Temperature setpoint (°C)	38	38
Incubation time (h)	pH	pH
1	5.90	5.96
12	4.74	4.52
24	3.64	4.01
36	3.65	3.93
48	3.65	3.89
Caustic usage (mmol NaOH/kg wheat straw DM)	585	827
Acidification (%)	100%	141%
Total organic acid (g/L) at 48 h	7.5	10.5
Increase in total organic acids (%)	0%	41%

CLAIMS

1. A process for the production of biogas from organic material, which process comprises the steps of:
 - 5 a) contacting biomass with enzymes and/or a culture to obtain a first composition,
 - b) fermenting the first composition to obtain a second composition comprising organic acids,
 - c) contacting the second composition comprising organic acids with the organic material to obtain a third composition, and
 - d) digesting the third composition to produce biogas.
- 10 2. A process according to claim 1, wherein the organic material is manure.
3. A process according to claim 1 or 2, wherein the culture is a bacterial culture.
- 15 4. A process according to any of the claims 1 to 3, wherein the bacterial culture comprises lactic acid bacteria.
5. A process according to claim 3 or 4, wherein the bacterial culture is heterofermentative.
- 20 6. A process according to any of the claims 3 to 5, wherein the bacterial culture is capable of fermenting hexose sugars and pentose sugars.
7. A process according to any of the claims 1 to 6, wherein the enzymes comprise cellulases, hemicellulases, pectinases and/or lipases.
- 25 8. A process according to any of the claims 1 to 7, wherein the fermenting step is a simultaneous hydrolysis and fermentation.
9. A process according to any of the claims 1 to 8, further comprising aerating the organic material and/or third composition before digestion.
- 30 10. A process according to any of the claims 1 to 9, wherein the digestion of the third composition is anaerobic.
- 35 11. A process according to any of the claims 1 to 10, wherein the fermentation is done in a first reactor.

12. A process according to any of the claims 1 to 11, wherein contacting the second composition comprising organic acids with the organic material to obtain a third composition is done in a second reactor.
- 5 13. A process according to any of the claims 1 to 12, wherein the organic material is added to the second composition comprising organic acids.
14. A process according to any of the claims 1 to 13, wherein the third composition is stored from 1 hour to 2000 hours before digestion.
- 10 15. A process according to any of the claims 1 to 13, wherein the third composition is contacted with biomass prior to and/or during the digestion

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069327

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P5/02 C12P39/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	abstract; claims 1-14; figures 1-3 paragraphs [0056] - [0080] -----	9
X	US 9 074 179 B2 (ZITOMER DANIEL [US]; UNIV MARQUETTE [US]) 7 July 2015 (2015-07-07) abstract; claims 1-20; example 1 -----	1,3,5, 8-10,13, 15
X	US 2013/186823 A1 (HAZEWINKEL JACOB HENDRIK OBBO [NL]) 25 July 2013 (2013-07-25) abstract; claims 1-9 paragraphs [0005], [0016], [0018] ----- -/-	1-3,5,8, 10-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

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Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Schröder, Gunnar

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2021/069327

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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International application No

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