

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



W I P O | P C T



(10) International Publication Number
WO 2013/083801 A2

(43) International Publication Date
13 June 2013 (13.06.2013)

- (51) **International Patent Classification:** Not classified
- (21) **International Application Number:**
PCT/EP2012/074837
- (22) **International Filing Date:**
7 December 2012 (07.12.2012)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
11192849.5 9 December 2011 (09.12.2011) EP
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- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,

HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*



WO 2013/083801 A2

(54) **Title:** BIOGAS FROM SUBSTRATES COMPRISING ANIMAL MANURE AND ENZYMES

(57) **Abstract:** Present invention relates to a biogas production process comprising the steps of providing a substrate comprising manure, and: (a) adding one or more enzyme to the substrate, and then adding the substrate with the one or more enzyme to a biogas digester; or (b) adding the substrate to a digester tank and adding one or more enzyme to the tank.

TITLE: BIOGAS FROM SUBSTRATES COMPRISING ANIMAL MANURE AND ENZYMES**FIELD OF THE INVENTION**

5 The present invention relates to methods for producing biogas from a substrate comprising animal manure and at least one added enzyme.

BACKGROUND OF THE INVENTION

10 Most natural plant based material comprises a significant amount of lignocellulosic fibres that are undigestible or only slowly digestible in many biological systems. This has the consequence that for many biological processes converting plant based material a significant fraction of the treated material will not be digested or only digested in a low degree during the treatment.

15 For example in a usual biogas production plant manure is fermented under anaerobic conditions forming biogas and a waste material consisting to a large extent of lignocellulosic fibres that is hardly digested at all under to conditions of an anaerobic biogas process.

20 In areas with concentrated animal production, manure can often cause environmental problems. These include odor formation, pollution of waterways and the creation of eutrophicated land. As worldwide animal production continues to increase, so does the environmental impact. At the same time, manure is largely an unexploited renewable energy source, in particular the production of biogas such as methane.

25 The generation of biogas from manure is an old technology and today production facilities range from simple covered lagoons to sophisticated industrial plants with controlled process parameters. The industrial manure based plants of today have a low return on investment (ROI) due to the low energy intensity of raw manure (a combination of urine and feces) combined with the relatively large capital expenditure needed to erect a biogas plant. The use of this technology is typically limited unless the biogas or electricity production is subsidized (e. g. as in Germany). Due to low conversion of the lignocellulose present in the manure (currently achieving up to approximately 50% of theoretical methane production potential for dairy cow manure), high energy materials are commonly added to obtain additional biogas. Such materials include high energy crops or food processing waste. However, it is estimated that the limited availability and expense of high energy waste can limit the application of biogas extraction to only 5% of the available manure.

35 Anaerobic degradation of animal manure results in the reduction of waste and production of energy in form of biogas, which is composed mainly of methane and carbon dioxide. The methane potential of manure comes from the digestion of the organic components in the faeces and in the straw used as bedding material, which is mainly: carbohydrates, proteins and lipids. The theoretical methane productivity is higher in porcine (pig) manure (516

1/kgVS) than in dairy cattle manure (469 1/kgVS), while the practically obtainable methane yield in terms of VS is considerably higher in pig manure (356 1/kgVS) than in dairy cattle manure (148 1/kgVS). (*Methane productivity of manure, straw and solid fractions of manure*; Møller et al.; Biomass and Bioenergy 26 (2004) 485 - 495). VS is volatile solids.

5

It would be beneficial to provide a method for enhancing methane production from materials comprising lignocellulosic fibres, such as, porcine manure. This technology could facilitate a global change in manure management practices and turn an environmental problem into a profitable and environmentally beneficial solution.

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SUMMARY OF THE INVENTION

In a first aspect, the invention provides a biogas production process comprising the steps of providing a substrate comprising manure, and:

- (a) adding one or more enzyme to the substrate and then adding the substrate with one or more enzyme to a biogas digester or
- (b) adding the substrate to a digester tank and adding one or more enzyme to the tank.

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BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows production of biogas from the digester treated with enzymes and the reference digester from Example 1. Hollow diamond shapes represent reference (R2) and filled diamond shapes represent reactor with enzymes added (R1).

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Fig. 2 A-B show the concentrations of volatile fatty acids (VFA) in the two digesters of Example 1.

Fig. 3 shows the daily production of biogas from the digester treated with enzymes and the reference digester in Example 2.

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Fig. 4A-B show the concentrations of volatile fatty acids in the two digesters during the test in Example 2.

Fig. 5 shows the daily production of biogas from the digester treated with cellulase (R1) and the reference digester (R3) in Example 3.

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Fig. 6 shows the daily production of biogas from the digester treated with enzymes cocktail (R2) and the reference digester (R3) in Example 3.

Fig. 7. Solid diamond shapes shows the daily biogas production of R1 (reference reactor to R2) in example 4. Solid square shapes with dotted line shows the daily biogas production of reactor R2 in example 1. Solid triangle shapes shows the daily biogas production of R3 (reference reactor to R4) in example 4. Cross shapes with dotted line shows the daily biogas production of R4 in example 4.

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Fig. 8a Solid diamond shapes shows the daily biogas production (m^3/tons substrate) of the reference reactor in example 5. Hollow diamond shapes shows the daily biogas production (m^3/tons substrate) of the reactor added enzymes in example 5.

5 Fig. 8b Solid diamond shapes shows the difference in the daily biogas production as %, between the reactor added enzymes and the reference reactor in example 5. Hollow diamond shapes shows the difference in the daily biogas production as m^3 , between the reactor added enzymes and the reference reactor in example 5.

10 Fig. 9 Columns shows the difference in daily biogas production as % between R2/R3/R4/R5 compared to R1 (reference reactor). Each set (1-4) of columns represents different periods of the experiment. Set 1: the last week before adding enzymes; set 2-4; week one to three after adding enzymes to R2, R3 and R4. Black columns represent R2, gray columns represent R3, white columns represent R4 and striped columns represent R5.

DEFINITIONS

15 Biogas:

The term "biogas" is according to the invention intended to mean the gas obtained in a conventional anaerobic fermentor using manure. The main component of biogas is methane and the terms "biogas" and "methane" are in this application and claims used interchangeably.

20 The term "primary digester" is in this application and claims intended to mean the container wherein the first anaerobic fermentation takes place.

The term "secondary digester" is in this application and claims intended to mean the container wherein the second anaerobic fermentation takes place. Depending on the particular configuration of the biogas facility the primary digester may also serve as the secondary digester.

25

DETAILED DESCRIPTION OF THE INVENTION

The first aspect of the invention relates to a biogas production process comprising the steps of providing a substrate comprising manure, and:

- 30 (a) adding one or more enzyme to the substrate, and then adding the substrate with the one or more enzyme to a biogas digester; or
- (b) adding the substrate to a digester tank and adding one or more enzyme to the tank.

35 In a preferred embodiment of the first aspect, the content of porcine manure in the substrate is adjusted by continuous or stepwise addition of porcine manure during step (a) or (b). Preferably, the porcine manure constitutes above 15% wt-% DS, preferably above 20% wt-% DS, preferably above 25% wt-% DS, preferably above 30 wt-% DS, preferably above 35 wt-%

DS, , preferably above 40 wt-% DS, , preferably above 45 wt-% DS, , preferably above 50 wt-% DS, , preferably above 55 wt-% DS, , preferably above 60 wt-% DS, , preferably above 65 wt-% DS, more preferably above 70 wt-% DS of the substrate.

5 In a preferred embodiment of the first aspect, the substrate is degraded at a pH in the range from 7 to 10; preferably from 8 to 9; most preferably at around 8.5.

In a preferred embodiment of the first aspect, the substrate is degraded at a temperature in the range from 20-70°C, preferably 30-60°C, and more preferably 40-50°C.

A related aspect of present invention, the one or more enzyme of the first aspect is selected from the group consisting of an amylolytic enzyme, a lipolytic enzyme, a proteolytic
10 enzyme, a cellulolytic enzyme, an oxidoreductase and a plant cell-wall degrading enzyme.

In yet a related aspect, the one or more enzyme is selected from the group consisting of aminopeptidase, alpha-amylase, amyloglucosidase, arabinofuranosidase, arabinoxylanase, beta-glucanase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, ferulic acid esterase, deoxyribonuclease,
15 endo-cellulase, endo-glucanase, endo-xylanase, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannanase, mannosidase, oxidase, pectate lyase, pectin lyase, pectin trans-eliminase, pectin ethylesterase, pectin methylesterase, pectinolytic enzyme, peroxidase, protease, phytase, phenoloxidase, polygalacturonase, polyphenoloxidase,
20 proteolytic enzyme, rhamnogalacturonan lyase, rhamnogalucanase, rhamnogalacturonase, ribonuclease, SPS-ase, transferase, transglutaminase, xylanase and xyloglucanase.

In a related aspect, the one or more enzyme comprises 2 - 10 different enzymes.

In yet another related aspect, the invention relates to a process as described above, wherein the one or more enzyme is added in a dosage of 0.001 - 5.000 % (w/w; enzyme
25 product vs. dry organic mass substrate); preferably in in a dosage of 0.005 - 4.000 % (w/w; enzyme product vs. dry organic mass substrate); more preferably in a dosage of 0.010 - 3.000 % (w/w; enzyme product vs. dry organic mass substrate); even more preferably in a dosage of 0.050 - 2.000 % (w/w; enzyme product vs. dry organic mass substrate); and most preferably in a dosage of 0.100 - 1.500 % (w/w; enzyme product vs. dry organic mass substrate).

30 Alternatively, the one or more enzyme is added in a dosage of 0.001 - 5.000 mg enzyme protein per g dry organic mass substrate, VS; preferably in in a dosage of 0.005 - 4.000 mg enzyme protein per g dry organic mass substrate, VS; more preferably in a dosage of 0.010 - 3.000 mg enzyme protein per g dry organic mass substrate, VS; more preferably in a dosage of 0.020 - 2.000 mg enzyme protein per g dry organic mass substrate, VS; and most preferably in
35 a dosage of 0.040 - 1.800 mg enzyme protein per g dry organic mass substrate, VS.

The substrate used in present invention may be homogenized; preferably by milling, wet-

milling, grinding or wet-grinding prior to or during step (a) or prior to step (b) of the first aspect.

A base may be added to the substrate prior to or while it is being homogenized; preferably the base is NaOH, Na_2CO_3 , NaHCO_3 , $\text{Ca}(\text{OH})_2$, lime hydrate, ammonia and/or KOH.

5 The content of manure in the substrate may be adjusted by continuous or stepwise addition of manure during step (a) or (b) of the first aspect.

In a related aspect of present invention, the manure constitutes above 15% wt-% DS, preferably above 20% wt-% DS, preferably above 25% wt-% DS, preferably above 30 wt-% DS, preferably above 35 wt-% DS, , preferably above 40 wt-% DS, , preferably above 45 wt-% DS, , preferably above 50 wt-% DS, , preferably above 55 wt-% DS, , preferably above 60 wt-% DS, ,
10 preferably above 65 wt-% DS, more preferably above 70 wt-% DS of the substrate.

The substrate may be degraded at a pH in the range from 7 to 10; preferably from 8 to 9; most preferably at around 8.5 and at a temperature in the range from 20-70°C, preferably 30-60°C, and more preferably 40-50°C.

15 The substrate may be subjected to a microwave, chemical, mechanical, biological treatment and/or an ultrasonic irradiation treatment prior to step (a) or (b) of the first aspect.

The manure used in present invention may be porcine manure and/or cattle manure.

In yet a related aspect, the invention relates to a process wherein step (a) or (b) is followed by a fermentation step, which is optionally an anaerobic fermentation step.

20 Enzymes

Even if not specifically mentioned in context of a process or process of the invention, it is to be understood that the enzyme(s) as well as other compounds are used in an "effective amount". In a preferred embodiment of the first aspect of the invention, the one or more enzyme is selected from the group consisting of an amylolytic enzyme, a lipolytic enzyme, a proteolytic
25 enzyme, a cellulolytic enzyme, an oxidoreductase and a plant cell-wall degrading enzyme. Preferably, the one or more enzyme is selected from the group consisting of aminopeptidase, alpha-amylase, amyloglucosidase, arabinofuranosidase, arabinoxylanase, beta-glucanase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, ferulic acid esterase, deoxyribonuclease, endo-cellulase,
30 endo-glucanase, endo-xylanase, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannanase, mannosidase, oxidase, pectate lyase, pectin lyase, pectin trans-eliminase, pectin ethylesterase, pectin methylesterase, pectinolytic enzyme, peroxidase, protease, phytase, phenoloxidase, polygalacturonase, polyphenoloxidase, proteolytic enzyme,
35 rhamnogalacturonan lyase, rhamnogalacturonase, rhamnogalacturonase, ribonuclease, SPS-ase, transferase, transglutaminase, xylanase and xyloglucanase.

In a preferred embodiment, the one or more enzyme comprises 2 - 10 different

enzymes. Preferably, the one or more enzyme is added in a dosage of 0.001 - 5.000 % (w/w; enzyme product vs. dry organic mass substrate); preferably in a dosage of 0.005 - 4.000 % (w/w; enzyme product vs. dry organic mass substrate); more preferably in a dosage of 0.010 - 3.000 % (w/w; enzyme product vs. dry organic mass substrate); even more preferably in a dosage of 0.050 - 2.000 % (w/w; enzyme product vs. dry organic mass substrate); and most preferably in a dosage of 0.100 - 1.500 % (w/w; enzyme product vs. dry organic mass substrate). It is also preferred that the one or more enzyme is added in a dosage of 0.001 - 5.000 mg enzyme protein per g dry organic mass substrate, VS; preferably in a dosage of 0.005 - 4.000 mg enzyme protein per g dry organic mass substrate, VS; more preferably in a dosage of 0.010 - 3.000 mg enzyme protein per g dry organic mass substrate, VS; more preferably in a dosage of 0.020 - 2.000 mg enzyme protein per g dry organic mass substrate, VS; and most preferably in a dosage of 0.040 - 1.800 mg enzyme protein per g dry organic mass substrate, VS.

Proteases

Any protease or proteolytic enzyme suitable for use under alkaline conditions can be used. Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically or genetically modified mutants are included. The protease may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270.

Preferred commercially available protease enzymes include those sold under the trade names Everlase™, Kannase™, Alcalase™, Savinase™, Primase™, Durazym™, and Esperase™ by Novozymes A/S (Denmark), those sold under the tradename Maxatase, Maxacal, Maxapem, Properase, Purafect and Purafect OXP by Genencor International, and those sold under the tradename Opticlean and Optimase by Solvay Enzymes.

Hemicellulolytic enzymes

Any hemicellulase suitable for use in hydrolyzing hemicellulose, may be used. Preferred hemicellulases include pectate lyases, xylanases, arabinofuranosidases, acetyl xylan esterase, ferulic acid esterase, glucuronidases, endo-galactanase, mannases, endo or exo arabinases, exo-galactanses, and mixtures of two or more thereof. Preferably, the hemicellulase for use in the present invention is an endo-acting hemicellulase, and more preferably, the hemicellulase is an endo-acting hemicellulase which has the ability to hydrolyze hemicellulose under basic conditions of above pH 7, preferably pH 7-10.

In an embodiment the hemicellulase is a xylanase. In an embodiment the xylanase may preferably be of microbial origin, such as of fungal origin (e.g., *Trichoderma*, *Meripilus*, *Humicola*, *Aspergillus*, *Fusarium*) or from a bacterium (e.g., *Bacillus*). In a preferred embodiment the xylanase is derived from a filamentous fungus, preferably derived from a strain of *Aspergillus*, such as *Aspergillus aculeatus*; or a strain of *Humicola*, preferably *Humicola lanuginosa*. The xylanase may preferably be an endo-1,4-beta-xylanase, more preferably an endo-1,4-beta-xylanase of GH10 or GH11. Examples of commercial xylanases include SHEARZYME® 200L, SHEARZYME® 500L, BIOFEED WHEAT®, and PULPZYME™ HC (from Novozymes) and GC 880, SPEZYME® CP (from Genencor Int).

The hemicellulase may be added in an amount effective to hydrolyze hemicellulose, such as, in amounts from about 0.001 to 0.5 wt.-% of total solids (TS), more preferably from about 0.05 to 0.5 wt.-% of TS.

Xylanases may be added in the amounts of 1.0-1000 FXU/kg dry solids, preferably from 5-500 FXU/kg dry solids, preferably from 5-100 FXU/kg dry solids and most preferably from 10-100 FXU/kg dry solids.

Xylanases may alternatively be added in amounts of 0.001-1.0 g/kg DS substrate, preferably in the amounts of 0.005-0.5 g/kg DS substrate, and most preferably from 0.05-0.10 g/kg DS substrate.

Pectolytic enzymes (or Pectinases)

Any pectinolytic enzyme that can degrade the pectin composition of plant cell walls may be used in practicing the present invention. Suitable pectinases include, without limitation, those of fungal or bacterial origin. Chemically or genetically modified pectinases are also encompassed. Preferably, the pectinase used in the invention are recombinantly produced and are mono-component enzymes.

Pectinases can be classified according to their preferential substrate, highly methyl-esterified pectin or low methyl-esterified pectin and polygalacturonic acid (pectate), and their reaction mechanism, beta-elimination or hydrolysis. Pectinases can be mainly endo-acting, cutting the polymer at random sites within the chain to give a mixture of oligomers, or they may be exo-acting, attacking from one end of the polymer and producing monomers or dimers. Several pectinase activities acting on the smooth regions of pectin are included in the classification of enzymes provided by Enzyme Nomenclature (1992), e.g., pectate lyase (EC 4.2.2.2), pectin lyase (EC 4.2.2.10), polygalacturonase (EC 3.2.1.15), exo-polygalacturonase (EC 3.2.1.67), exo-polygalacturonate lyase (EC 4.2.2.9) and exo-poly-alpha-galacturonosidase (EC 3.2.1.82).

In embodiments the pectinase is a pectate lyase. Pectate lyase (EC 4.2.2.2) enzymatic activity as used herein refers to catalysis of the random cleavage of alpha-1,4-glycosidic

linkages in pectic acid (also called polygalacturonic acid) by transelimination. Pectate lyases are also termed polygalacturonate lyases and poly(1,4-a-D-galacturonide) lyases.

The Pectate lyase is an enzyme which catalyses the random cleavage of a-1,4-glycosidic linkages in pectic acid (also called polygalacturonic acid) by transelimination. Pectate lyases also include polygalacturonate lyases and poly(1,4-a-D-galacturonide) lyases.

Examples of preferred pectate lyases are those that have been cloned from different bacterial genera such as *Erwinia*, *Pseudomonas*, *Klebsiella*, *Xanthomonas* and *Bacillus*, especially *Bacillus licheniformis* (US patent application 6,124,127), as well as from *Bacillus subtilis* (Nasser et al. (1993) FEBS Letts. 335:319-326) and *Bacillus* sp. YA-14 (Kim et al. (1994) Biosci. Biotech. Biochem. 58:947-949). Purification of pectate lyases with maximum activity in the pH range of 8-10 produced by *Bacillus pumilus* (Dave and Vaughn (1971) J. Bacteriol. 108:166-174), *B. polymyxa* (Nagel and Vaughn (1961) Arch. Biochem. Biophys. 93:344-352), *B. stearothermophilus* (Karbassi and Vaughn (1980) Can. J. Microbiol. 26:377-384), *Bacillus* sp. (Hasegawa and Nagel (1966) J. Food Sci. 31:838-845) and *Bacillus* sp. RK9 (Kelly and Fogarty (1978) Can. J. Microbiol. 24:1 164-1 172) have also been described.

A preferred pectate lyase may be obtained from *Bacillus licheniformis* as described in US patent application 6,124,127.

Other pectate lyases could be those that comprise the amino acid sequence of a pectate lyase disclosed in Heffron et al., (1995) Mol. Plant-Microbe Interact. 8: 331-334 and Henrissat et al., (1995) Plant Physiol. 107: 963-976.

A single enzyme or a combination of pectate lyases may be used. A preferred commercial pectate lyase preparation suitable for the invention is BioPrep® 3000 L available from Novozymes A/S.

Mannanases

In the context of the present invention a mannanase is a beta- mannanase and defined as an enzyme belonging to EC 3.2.1 .78.

Mannanases have been identified in several *Bacillus* organisms. For example, Talbot et al., Appl. Environ. Microbiol., Vol.56, No. 11, pp. 3505-3510 (1990) describes a beta-mannanase derived from *Bacillus stearothermophilus* having an optimum pH of 5.5-7.5. Mendoza et al., World J. Microbiol. Biotech., Vol. 10, No. 5, pp. 551-555 (1994) describes a beta-mannanase derived from *Bacillus subtilis* having an optimum activity at pH 5.0 and 55°C. JP-03047076 discloses a beta-mannanase derived from *Bacillus* sp., having an optimum pH of 8-10. JP-63056289 describes the production of an alkaline, thermostable beta-mannanase. JP-08051975 discloses alkaline beta-mannanases from alkalophilic *Bacillus* sp. AM-001. A purified mannanase from *Bacillus amyloliquefaciens* is disclosed in WO 97/1 1164. WO 94/25576 discloses an enzyme from *Aspergillus aculeatus*, CBS 101 .43, exhibiting mannanase activity

and WO 93/24622 discloses a mannanase isolated from *Trichoderma reesei*.

The mannanase may be derived from a strain of the genus *Bacillus*, such as the amino acid sequence having the sequence deposited as GENESEQP accession number AAY54 122 or an amino acid sequence which is homologous to this amino acid sequence. A suitable commercial mannanase preparation is Mannaway® produced by Novozymes A/S.

Ferulic esterases

In the context of the present invention a ferulic esterase is defined as an enzyme belonging to EC 3.1.1.73.

A suitable ferulic esterase preparation can be obtained from *Malabrancea*, e.g., from *P. cinnamomea*, such as e.g. a preparation comprising the ferulic esterase having the amino acid sequence shown in SEQ ID NO:2 in European patent application number 07121322.7, or an amino acid sequence which is homologous to this amino acid sequence.

Another suitable ferulic esterase preparation can be obtained from *Penicillium*, e.g., from *P. aurantioghseum*, such as e.g. a preparation comprising the ferulic esterase having the amino acid sequence shown in SEQ ID NO:2 in European patent application number 0815469.7, or an amino acid sequence which is homologous to this amino acid sequence. A suitable commercial preparation comprising ferulic esterase activity is NOVOZYM® 342 L produced by Novozymes A/S.

Alkaline endo-glucanases

The term "endoglucanase" means an endo-1,4-(1,3;1,4)-beta-D-glucan 4-glucanohydrolase (E.C. No. 3.2.1.4), which catalyses endo-hydrolysis of 1,4-beta-D-glycosidic linkages in cellulose, cellulose derivatives (such as carboxymethyl cellulose and hydroxyethyl cellulose), lichenin, beta-1,4 bonds in mixed beta-1,3 glucans such as cereal beta-D-glucans or xyloglucans, and other plant material containing cellulosic components. Alkaline endo-glucanases are endo-glucanases having activity under alkaline conditions.

In a preferred embodiment endoglucanases may be derived from a strain of the genus *Trichoderma*, preferably a strain of *Trichoderma reesei*; a strain of the genus *Humicola*, such as a strain of *Humicola insolens*; or a strain of *Chrysosporium*, preferably a strain of *Chrysosporium lucknowense*.

In a preferred embodiment endoglucanases may be derived from a strain of the genus *Bacillus akibai*.

In an embodiment the alkaline endo-glucanase composition is one of the commercially available products CAREZYME®, ENDOLASE® and CELLUCLEAN® (Novozymes A/S, Denmark). The enzyme may be applied in a dosage of 1-100 g/kg cellulose.

Acid cellulolytic Activity

The term "acid cellulolytic activity" as used herein are understood as comprising enzymes having cellobiohydrolase activity (EC 3.2.1.91), e.g., cellobiohydrolase I and/or cellobiohydrolase II, as well as endo-glucanase activity (EC 3.2.1.4) and/or beta-glucosidase activity (EC 3.2.1.21) having activity at pH below 6.

The cellulolytic activity may, in a preferred embodiment, be in the form of a preparation of enzymes of fungal origin, such as from a strain of the genus *Trichoderma*, preferably a strain of *Trichoderma reesei*, a strain of the genus *Humicola*, such as a strain of *Humicola insolens*; or a strain of *Chrysosporium*, preferably a strain of *Chrysosporium lucknowense*.

In preferred embodiment the cellulolytic enzyme preparation contains one or more of the following activities: endoglucanase, cellobiohydrolases I and II, and beta-glucosidase activity.

In a preferred embodiment cellulolytic enzyme preparation is a composition disclosed in WO2008/151079, which is hereby incorporated by reference. In a preferred embodiment the cellulolytic enzyme preparation comprising a polypeptide having cellulolytic enhancing activity, preferably a family GH61A polypeptide, preferably those disclosed in WO 2005/074656 (Novozymes). The cellulolytic enzyme preparation may further comprise beta-glucosidase, such as beta-glucosidase derived from a strain of the genus *Trichoderma*, *Aspergillus* or *Penicillium*, including the fusion protein having beta-glucosidase activity disclosed in co-pending application US 60/832,511 (Novozymes). In a preferred embodiment the cellulolytic enzyme preparation may also comprises a CBH II enzyme, preferably *Thielavia terrestris* cellobiohydrolase II (CEL6A). In another preferred embodiment the cellulolytic enzyme preparation may also comprise cellulolytic enzymes; preferably those derived from *Trichoderma reesei* or *Humicola insolens*.

The cellulolytic enzyme composition may also comprise a polypeptide having cellulolytic enhancing activity (GH61A) disclosed in WO 2005/074656; an *Aspergillus oryzae* beta-glucosidase fusion protein (WO 2008/057637), and cellulolytic enzymes derived from *Trichoderma reesei*.

The cellulolytic enzyme composition may also comprise an *Aspergillus aculeatus* GH10 xylanase (WO 94/021785) and a *Trichoderma reesei* cellulase preparation containing *Aspergillus fumigatus* beta-glucosidase (WO 2005/047499) and *Thermoascus aurantiacus* GH61A polypeptide (WO 2005/074656).

The cellulolytic enzyme composition may also comprise an *Aspergillus aculeatus* GH10 xylanase (WO 94/021785) and/or a *Trichoderma reesei* cellulase preparation.

In another preferred embodiment the cellulolytic composition comprising a polypeptide having cellulolytic enhancing activity (GH61A) disclosed in WO 2005/074656; an *Aspergillus oryzae* beta-glucosidase fusion protein (WO 2008/057637), *Thielavia terrestris* cellobiohydrolase II (CEL6A), and cellulolytic enzymes preparation derived from *Trichoderma*

reesei.

In an embodiment the cellulolytic enzyme composition is the commercially available product CELLUCLAST™ 1.5L, CELLUZYME™, Cellic™ CTec, Cellic™ CTec2, Cellic™ HTec, Cellic™ HTec2 (all Novozymes A/S, Denmark) or ACCELLARASE™ 1000 (Genencor Int, Inc., USA).

The cellulolytic activity may be dosed in the range from 0.1-100 FPU per gram total solids (TS), preferably 0.5-50 FPU per gram TS, especially 1-20 FPU per gram TS.

Cellulase Activity Using Filter Paper Assay (FPU assay)

The process is disclosed in a document entitled "Measurement of Cellulase Activities" by Adney, B. and Baker, J. 1996. Laboratory Analytical Procedure, LAP-006, National Renewable Energy Laboratory (NREL). It is based on the IUPAC process for measuring cellulase activity (Ghose, T.K., Measurement of Cellulase Activities, Pure & Appl. Chem. 59, pp. 257-268, 1987. The process is carried out as described by Adney and Baker, 1996, supra, except for the use of a 96 well plates to read the absorbance values after color development, as described below:

Enzyme Assay Tubes:

- A rolled filter paper strip (#1 Whatman; 1 X 6 cm; 50 mg) is added to the bottom of a test tube (13 X 100 mm).
- To the tube is added 1.0 mL of 0.05 M Na-citrate buffer (pH 4.80).
- The tubes containing filter paper and buffer are incubated 5 min. at 50° C ($\pm$ 0.1° C) in a circulating water bath.
- Following incubation, 0.5 mL of enzyme dilution in citrate buffer is added to the tube. Enzyme dilutions are designed to produce values slightly above and below the target value of 2.0 mg glucose.
- The tube contents are mixed by gently vortexing for 3 seconds.
- After vortexing, the tubes are incubated for 60 mins. at 50° C ($\pm$ 0.1° C) in a circulating water bath.
- Immediately following the 60 min. incubation, the tubes are removed from the water bath, and 3.0 mL of DNS reagent is added to each tube to stop the reaction. The tubes are vortexed 3 seconds to mix.

Blank and Controls:

- A reagent blank is prepared by adding 1.5 mL of citrate buffer to a test tube.
- A substrate control is prepared by placing a rolled filter paper strip into the bottom of a test tube, and adding 1.5 mL of citrate buffer.
- Enzyme controls are prepared for each enzyme dilution by mixing 1.0 mL of citrate

buffer with 0.5 mL of the appropriate enzyme dilution.

- The reagent blank, substrate control, and enzyme controls are assayed in the same manner as the enzyme assay tubes, and done along with them.

5 Glucose Standards:

- A 100 mL stock solution of glucose (10.0 mg/mL) is prepared, and 5 mL aliquots are frozen. Prior to use, aliquots are thawed and vortexed to mix.

- Dilutions of the stock solution are made in citrate buffer as follows:

G1 = 1.0 mL stock + 0.5 mL buffer = 6.7 mg/mL = 3.3 mg/0.5 mL

10 G2 = 0.75 mL stock + 0.75 mL buffer = 5.0 mg/mL = 2.5 mg/0.5 mL

G3 = 0.5 mL stock + 1.0 mL buffer = 3.3 mg/mL = 1.7 mg/0.5 mL

G4 = 0.2 mL stock + 0.8 mL buffer = 2.0 mg/mL = 1.0 mg/0.5 mL

- Glucose standard tubes are prepared by adding 0.5 mL of each dilution to 1.0 mL of citrate buffer.

- 15
- The glucose standard tubes are assayed in the same manner as the enzyme assay tubes, and done along with them.

Color Development:

- Following the 60 min. incubation and addition of DNS, the tubes are all boiled together for 5 mins. in a water bath.

- 20
- After boiling, they are immediately cooled in an ice/water bath.
 - When cool, the tubes are briefly vortexed, and the pulp is allowed to settle. Then each tube is diluted by adding 50 microL from the tube to 200 microL of ddH₂O in a 96-well plate. Each well is mixed, and the absorbance is read at 540 nm.

- 25
- #### Calculations (examples are given in the NREL document)
- A glucose standard curve is prepared by graphing glucose concentration (mg/0.5 mL) for the four standards (G1-G4) vs. A₅₄₀. This is fitted using a linear regression (Prism Software), and the equation for the line is used to determine the glucose produced for each of the enzyme assay tubes.

- 30
- A plot of glucose produced (mg/0.5 mL) vs. total enzyme dilution is prepared, with the Y-axis (enzyme dilution) being on a log scale.

- A line is drawn between the enzyme dilution that produced just above 2.0 mg glucose and the dilution that produced just below that. From this line, it is determined the enzyme dilution that would have produced exactly 2.0 mg of glucose.

- 35
- The Filter Paper Units/mL (FPU/mL) are calculated as follows:

FPU/mL = 0.37/ enzyme dilution producing 2.0 mg glucose

Cellulolytic Enhancing Activity

The term "cellulolytic enhancing activity" is defined herein as a biological activity that enhances the hydrolysis of the substrate material by proteins having cellulolytic activity. For purposes of the present invention, cellulolytic enhancing activity is determined by measuring the increase in reducing sugars or in the increase of the total of cellobiose and glucose from the hydrolysis of the substrate material, e.g., pre-treated substrate material by cellulolytic protein under the following conditions: 1-50 mg of total protein/g of cellulose in PCS (pre-treated corn stover), wherein total protein is comprised of 80-99.5% w/w cellulolytic protein/g of cellulose in PCS and 0.5-20% w/w protein of cellulolytic enhancing activity for 1-7 day at 50°C compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS).

The term "polypeptide having cellulolytic enhancing activity" means a GH61 polypeptide that catalyzes the enhancement of the hydrolysis of a cellulosic material by enzyme having cellulolytic activity. For purposes of the present invention, cellulolytic enhancing activity is determined by measuring the increase in reducing sugars or the increase of the total of cellobiose and glucose from the hydrolysis of a cellulosic material by cellulolytic enzyme under the following conditions: 1-50 mg of total protein/g of cellulose in PCS, wherein total protein is comprised of 50-99.5% w/w cellulolytic enzyme protein and 0.5-50% w/w protein of a GH61 polypeptide having cellulolytic enhancing activity for 1-7 days at a suitable temperature, e.g., 50°C, 55°C, or 60°C, and pH, e.g., 5.0 or 5.5, compared to a control hydrolysis with equal total protein loading without cellulolytic enhancing activity (1-50 mg of cellulolytic protein/g of cellulose in PCS). In a preferred aspect, a mixture of CELLUCLAST® 1.5L (Novozymes A/S, Bagsvaerd, Denmark) in the presence of 2-3% of total protein weight *Aspergillus oryzae* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* according to WO 02/095014) or 2-3% of total protein weight *Aspergillus fumigatus* beta-glucosidase (recombinantly produced in *Aspergillus oryzae* as described in WO 2002/095014) of cellulase protein loading is used as the source of the cellulolytic activity. The polypeptides having cellulolytic enhancing activity enhance the hydrolysis of the substrate material catalyzed by proteins having cellulolytic activity by reducing the amount of cellulolytic enzyme required to reach the same degree of hydrolysis preferably at least 0.1-fold, more at least 0.2-fold, more preferably at least 0.3-fold, more preferably at least 0.4-fold, more preferably at least 0.5-fold, more preferably at least 1-fold, more preferably at least 3-fold, more preferably at least 4-fold, more preferably at least 5-fold, more preferably at least 10-fold, more preferably at least 20-fold, even more preferably at least 30-fold, most preferably at least 50-fold, and even most preferably at least 100-fold.

In a preferred embodiment the hydrolysis and/or fermentation is carried out in the

presence of a cellulolytic enzyme in combination with a polypeptide having enhancing activity. In a preferred embodiment the polypeptide having enhancing activity is a family GH61A polypeptide. WO 2005/074647 discloses isolated polypeptides having cellulolytic enhancing activity and polynucleotides thereof from *Thielavia terrestris*. WO 2005/074656 discloses an isolated polypeptide having cellulolytic enhancing activity and a polynucleotide thereof from *Thermoascus aurantiacus*. U.S. Published Application Serial No. 2007/0077630 discloses an isolated polypeptide having cellulolytic enhancing activity and a polynucleotide thereof from *Trichoderma reesei*.

10 Alpha-Amylase

According to the invention any alpha-amylase may be used, such as of fungal, bacterial or plant origin. In a preferred embodiment the alpha-amylase is an acid alpha-amylase, e.g., acid fungal alpha-amylase or acid bacterial alpha-amylase. The term "acid alpha-amylase" means an alpha-amylase (E.C. 3.2.1.1) which added in an effective amount has activity optimum at a pH in the range of 3 to 7, preferably from 3.5 to 6, or more preferably from 4 to 5.

Bacterial Alpha-Amylase

According to the invention a bacterial alpha-amylase is preferably derived from the genus *Bacillus*.

20 In a preferred embodiment the *Bacillus* alpha-amylase is derived from a strain of *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus subtilis* or *Bacillus stearothermophilus*, but may also be derived from other *Bacillus* sp. Specific examples of contemplated alpha-amylases include the *Bacillus licheniformis* alpha-amylase shown in SEQ ID NO: 4 in WO 99/19467, the *Bacillus amyloliquefaciens* alpha-amylase SEQ ID NO: 5 in WO 99/19467 and the *Bacillus stearothermophilus* alpha-amylase shown in SEQ ID NO: 3 in WO 99/19467 (all sequences hereby incorporated by reference). In an embodiment the alpha-amylase may be an enzyme having a degree of identity of at least 60%, preferably at least 70%, more preferred at least 80%, even more preferred at least 90%, such as at least 95%, at least 96%, at least 97%, at least 98% or at least 99% to any of the sequences shown in SEQ ID NOS: 1, 2 or 3, respectively, in WO 99/19467.

30 The *Bacillus* alpha-amylase may also be a variant and/or hybrid, especially one described in any of WO 96/23873, WO 96/23874, WO 97/41213, WO 99/19467, WO 00/60059, and WO 02/10355 (all documents hereby incorporated by reference). Specifically contemplated alpha-amylase variants are disclosed in US patent nos. 6,093,562, 6,297,038 or US patent no. 6,187,576 (hereby incorporated by reference) and include *Bacillus stearothermophilus* alpha-amylase (BSG alpha-amylase) variants having a deletion of one or two amino acid in positions R179 to G182, preferably a double deletion disclosed in WO 1996/023873 - see e.g., page 20,

lines 1-10 (hereby incorporated by reference), preferably corresponding to delta(181-182) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO:3 disclosed in WO 99/19467 or deletion of amino acids R179 and G180 using SEQ ID NO:3 in WO 99/19467 for numbering (which reference is hereby incorporated by reference). Even more preferred are Bacillus alpha-amylases, especially Bacillus stearothermophilus alpha-amylase, which have a double deletion corresponding to delta(181-182) and further comprise a N193F substitution (also denoted 1181* + G182* + N193F) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO:3 disclosed in WO 99/19467.

In an embodiment the bacterial alpha-amylase is dosed in an amount of 0.0005-5 KNU per g DS, preferably 0.001-1 KNU per g DS, such as around 0.050 KNU per g DS.

Fungal Alpha-Amylase

Fungal alpha-amylases include alpha-amylases derived from a strain of the genus *Aspergillus*, such as, *Aspergillus oryzae*, *Aspergillus niger* and *Aspergillus kawachii* alpha-amylases.

A preferred acidic fungal alpha-amylase is a Fungamyl-like alpha-amylase which is derived from a strain of *Aspergillus oryzae*. According to the present invention, the term "Fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high identity, i.e. at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or even 100% identity to the mature part of the amino acid sequence shown in SEQ ID NO: 10 in WO 96/23874.

Another preferred acid alpha-amylase is derived from a strain *Aspergillus niger*. In a preferred embodiment the acid fungal alpha-amylase is the one from *Aspergillus niger* disclosed as "AMYA_ASPNG" in the Swiss-prot/TrEMBL database under the primary accession no. P56271 and described in WO 89/01969 (Example 3 - incorporated by reference). A commercially available acid fungal alpha-amylase derived from *Aspergillus niger* is SP288 (available from Novozymes A/S, Denmark).

Other contemplated wild-type alpha-amylases include those derived from a strain of the genera *Rhizomucor* and *Meripilus*, preferably a strain of *Rhizomucor pusillus* (WO 2004/055178 incorporated by reference) or *Meripilus giganteus*.

In a preferred embodiment the alpha-amylase is derived from *Aspergillus kawachii* and disclosed by Kaneko et al. J. Ferment. Bioeng. 81:292-298(1996) "Molecular-cloning and determination of the nucleotide-sequence of a gene encoding an acid-stable alpha-amylase from *Aspergillus kawachii*."; and further as EMBL:#AB008370.

The fungal alpha-amylase may also be a wild-type enzyme comprising a starch-binding domain (SBD) and an alpha-amylase catalytic domain (i.e., non-hybrid), or a variant thereof. In an embodiment the wild-type alpha-amylase is derived from a strain of *Aspergillus kawachii*.

An acid alpha-amylases may according to the invention be added in an amount of 0.001 to 10 AFAU/g DS, preferably from 0.01 to 5 AFAU/g DS, especially 0.3 to 2 AFAU/g DS or 0.001 to 1 FAU-F/g DS, preferably 0.01 to 1 FAU-F/g DS.

5 Commercial Alpha-Amylase Products

Preferred commercial compositions comprising alpha-amylase include MYCOLASE™ from DSM (Gist Brocades), BAN™, TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME™ X, LIQUOZYME™ SC and SAN™ SUPER, SAN™ EXTRA L (Novozymes MS) and CLARASE™ L-40,000, DEX-LO™, SPEZYME™ FRED, SPEZYME™ AA, and SPEZYME™ DELTA AA
 10 (Genencor Int.), and the acid fungal alpha-amylase sold under the trade name SP288 (available from Novozymes A/S, Denmark).

Carbohydrate-Source Generating Enzyme

The term "carbohydrate-source generating enzyme" includes glucoamylase (being
 15 glucose generators), beta-amylase and maltogenic amylase (being maltose generators) and also pullulanase and alpha-glucosidase. A carbohydrate-source generating enzyme is capable of producing a carbohydrate that can be used as an energy-source by the fermenting organism(s) in question, for instance, when used in a process of the invention for producing a fermentation product, such as methane. The generated carbohydrate may be converted directly
 20 or indirectly to the desired fermentation product, preferably methane. According to the invention a mixture of carbohydrate-source generating enzymes may be used. Especially contemplated mixtures are mixtures of at least a glucoamylase and an alpha-amylase, especially an acid amylase, even more preferred an acid fungal alpha-amylase. The ratio between acid fungal alpha-amylase activity (FAU-F) and glucoamylase activity (AGU) (i.e., FAU-F per AGU) may in
 25 an embodiment of the invention be between 0.1 and 100, in particular between 2 and 50, such as in the range from 10-40.

Glucoamylase

A glucoamylase used according to the invention may be derived from any suitable
 30 source, e.g., derived from a microorganism or a plant. Preferred glucoamylases are of fungal or bacterial origin, selected from the group consisting of *Aspergillus* glucoamylases, in particular *Aspergillus niger* G1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102), or variants thereof, such as those disclosed in WO 92/00381, WO 00/04136 and WO 01/04273 (from Novozymes, Denmark); the *A. awamori* glucoamylase disclosed in WO 84/02921,
 35 *Aspergillus oryzae* glucoamylase (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or variants or fragments thereof. Other *Aspergillus* glucoamylase variants include variants with enhanced thermal stability: G137A and G139A (Chen et al. (1996), Prot. Eng. 9, 499-505); D257E and

D293E/Q (Chen et al. (1995), Prot. Eng. 8, 575-582); N182 (Chen et al. (1994), Biochem. J. 301, 275-281); disulphide bonds, A246C (Fierobe et al. (1996), Biochemistry, 35, 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al. (1997), Protein Eng. 10, 1199-1204.

Other glucoamylases include *Athelia rolfsii* (previously denoted *Corticium rolfsii*) glucoamylase (see US patent no. 4,727,026 and (Nagasaka, Y. et al. (1998) "Purification and properties of the raw-starch-degrading glucoamylases from *Corticium rolfsii*, Appl Microbiol Biotechnol 50:323-330), *Talaromyces glucoamylases*, in particular derived from *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (US patent no. Re. 32,153), *Talaromyces duponti*, *Talaromyces thermophilus* (US patent no. 4,587,215).

Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP 135,138), and *C. thermohydrosulfuricum* (WO 86/01831) and *Trametes cingulata*, *Pachykytospora papyracea*; and *Leucopaxillus giganteus* all disclosed in WO 2006/069289; or *Peniophora rufomarginata* disclosed in PCT/US2007/066618; or a mixture thereof. Also hybrid glucoamylase are contemplated according to the invention. Examples of the hybrid glucoamylases are disclosed in WO 2005/045018. Specific examples include the hybrid glucoamylase disclosed in Table 1 and 4 of Example 1 (which hybrids are hereby incorporated by reference).

Contemplated are also glucoamylases which exhibit a high identity to any of above mention glucoamylases, i.e., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or even 100% identity to the mature enzymes sequences mentioned above.

Commercially available compositions comprising glucoamylase include AMG 200L; AMG 300 L; SANTM SUPER, SANTM EXTRA L, SPIRIZYMETM PLUS, SPIRIZYMETM FUEL, SPIRIZYMETM B4U and AMGTM E (from Novozymes A/S); OPTIDEXTM 300 (from Genencor Int.); AMIGASETM and AMIGASETM PLUS (from DSM); G-ZYMETM G900, G-ZYMETM and G990 ZR (from Genencor Int.).

Glucoamylases may in an embodiment be added in an amount of 0.0001-20 AGU/g DS, preferably 0.001-10 AGU/g DS, especially between 0.01-5 AGU/g DS, such as 0.1-2 AGU/g DS.

Biological treatment

Microorganisms for additional biological treatment or pre-treatment may be selected among bacteria, yeasts or fungi, or mixtures thereof. The microorganisms or mixtures of two or more microorganisms may provide for an improved methane production in the anaerobic fermentation step of the biogas production process. Preferred examples of microorganisms according to the invention includes strains of the genus: *Bacillus*, *Pseudomonas*, *Enterobacter*, *Rhodococcus*, *Acinetobacter*, and *Aspergillus* such as *Bacillus licheniformis*, *Pseudomonas*

putida, *Enterobacter dissolvens*, *Pseudomonas fluorescens*, *Rhodococcus pyridinivorans*, *Acinetobacter baumannii*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Pseudomonas plecoglossicida*, *Pseudomonas pseudoacaligenes*, *Pseudomonas antarctica*, *Pseudomonas monteillii*, *Pseudomonas mendocina*, *Bacillus subtilis*, *Aspergillus niger* and *Aspergillus oryzae* and any combinations or two or more thereof.

Particular preferred strains include: *Bacillus subtilis* (NRRL B-50136), *Pseudomonas monteillii* (NRRL B-50256), *Enterobacter dissolvens* (NRRL B-50257), *Pseudomonas monteillii* (NRRL B-50258), *Pseudomonas plecoglossicida* (ATCC 31483), *Pseudomonas putida* (NRRL B-50247), *Pseudomonas plecoglossicida* (NRRL B-50248), *Rhodococcus pyridinivorans* (NRRL 50249), *Pseudomonas putida* (ATCC 49451), *Pseudomonas mendocina* (ATCC 53757), *Acinetobacter baumannii* (NRRL B-50254), *Bacillus pumilus* (NRRL B-50255), *Bacillus licheniformis* (NRRL B-50141), *Bacillus amyloliquefaciens* (NRRL B-50151), *Bacillus amyloliquefaciens* (NRRL B-50019), *Pseudomonas mendocina* (ATCC 53757), *Pseudomonas monteillii* (NRRL B-50250), *Pseudomonas monteillii* (NRRL B-50251), *Pseudomonas monteillii* (NRRL B-50252), *Pseudomonas monteillii* (NRRL B-50253), *Pseudomonas antarctica* (NRRL B-50259), *Bacillus amyloliquefaciens* (ATCC 55405), *Aspergillus niger* (NRRL 50245), and *Aspergillus oryzae* (NRRL 50246).

The skilled person will appreciate how to determine suitable amounts of these preferred strains in uses according to the invention, using well known techniques. In preferred embodiments the strains are added in amounts in the range of 1.0×10^6 to 5.0×10^9 CFU/g.

As examples of particular preferred microorganisms or mixtures of two or more microorganisms can be mentioned:

- A mixture containing: *Bacillus subtilis* (NRRL B-50136; 1.1×10^9 CFU/g), *Pseudomonas monteillii* (NRRL B-50256; 0.6×10^9 CFU/g), *Enterobacter dissolvens* (NRRL B-50257; 0.6×10^9 CFU/g), *Pseudomonas monteillii* (NRRL B-50258; 0.8×10^9 CFU/g), *Pseudomonas fluorescens* (ATCC 31483; 0.8×10^9 CFU/g), *Pseudomonas putida* (NRRL B-50247; 0.4×10^9 CFU/g), *Pseudomonas plecoglossicida* (NRRL B-50248; 0.4×10^9 CFU/g), *Rhodococcus pyridinivorans* (NRRL 50249; 0.8×10^9 CFU/g), *Pseudomonas putida* (ATCC 49451, 0.4×10^9 CFU/g), *Pseudomonas mendocina* (ATCC 53757; 0.8×10^9 CFU/g), and *Acinetobacter baumannii* (NRRL B-50254; 0.2×10^9 CFU/g);
- A mixture containing: *Bacillus subtilis* (NRRL B-50136; 1.6×10^9 CFU/g), *Bacillus pumilus* (NRRL B-50255; 0.2×10^9 CFU/g), *Bacillus amyloliquefaciens* (NRRL B-50141; 0.2×10^9 CFU/g), *Bacillus amyloliquefaciens* (NRRL B-50151; 0.2×10^9 CFU/g), *Bacillus amyloliquefaciens* (NRRL B-50019; 0.2×10^9 CFU/g), *Pseudomonas monteillii* (NRRL B-50256; 0.2×10^9 CFU/g), *Enterobacter dissolvens* (NRRL B-50257; 0.3×10^9 CFU/g), *Pseudomonas monteillii* (NRRL B-50258; 0.8×10^9 CFU/g), *Pseudomonas*

plecoglossicida (ATCC 31483; 0.7×10^9 CFU/g), *Pseudomonas putida* (NRRL B-50247; 0.2×10^9 CFU/g), *Pseudomonas plecoglossicida* (NRRL B-50248; 0.2×10^9 CFU/g), *Rhodococcus pyridinivorans* (NRRL 50249; 0.3×10^9 CFU/g), *Pseudomonas putida* (ATCC 49451; 0.2×10^9), *Pseudomonas mendocina* (ATCC 53757; 0.3×10^9 CFU/g), *Pseudomonas monteilli* (NRRL B-50250; 0.1×10^9 CFU/g), *Pseudomonas monteilli* (NRRL B-50251; 0.1×10^9 CFU/g), *Pseudomonas monteilli* (NRRL B-50252; 0.1×10^9 CFU/g), *Pseudomonas monteilli* (NRRL B-50253; 0.1×10^9 CFU/g), and *Pseudomonas antarctica* (NRRL B-50259; 0.2×10^9 CFU/g); and

- A mixture containing: *Bacillus subtilis* (NRRL B-50136; 3.5×10^9 CFU/g), *Bacillus amyloliquefaciens* (ATCC 55405; 1.0×10^9 CFU/g), *Pseudomonas antarctica* (NRRL B-50259; 0.2×10^9 CFU/g), *Aspergillus niger* (NRRL 50245; 0.8×10^9 CFU/g), and *Aspergillus oryzae* (NRRL 50246; 0.8×10^9 CFU/g).

Further, the microorganism or mixture of two or more microorganisms commercially available from Novozymes Biological Inc. under the trade names: BI-CHEM ABR-Hydrocarbon, BI-CHEM DC 1008 CB and Manure Degradar are also suitable.

The incubation under aerobic conditions may be performed as batch process, fed batch process or continuous process. In a batch process the container is filled, a suitable inoculum of the microorganisms is added and the process proceeding for a desired time. In a fed batch process a initial volume of substrate material is added into the container, typically 25-75% of the total operational volume of the container, a suitable inoculum of the microorganism is added and the process is proceeding until a certain conversion and/or cell density is reached where additional feed in form of substrate material is added at a suitable rate and the process is continued until the container is full and optionally for an additional time without additional feed. In a continuous process the process is started by adding the material into the container and a suitable inoculum of the microorganism is added, when a desired cell density is reached a stream of the composition in the container is removed and simultaneously a stream of the material is added to the container so that the volume remains essentially constant and the process is continued in principle as long as desired. It may even be possible to use a combination of these techniques. These techniques are known within the art and the skilled person will appreciate how to find suitable parameters for a particular process depending on the particular dimensions and properties of the container.

Means for aeration are well known in the art and it is within the capabilities of the skilled person to select suitable means for aeration for the present invention. Usually aeration is performed by blowing atmospheric air through the composition typically via one or more tube(s) or pipe(s) located in the lower part of the container said one or more tube(s) or pipe(s) is/are provided with holes at regular intervals to provide for an even distribution of the air in the composition. Other means for aerating may also be used according to the invention.

The rate of aeration during the aerobic fermentation step is selected to provide for a convenient growth rate of the microorganisms. Rate of aeration may be measured in volume air per volume ferment per minute (v/v/m) and usually aeration in the range of 0.01 v/v/m to 10 v/v/m is suitable, preferably 0.05 v/v/m to 5 v/v/m, more preferred 0.1 v/v/m to 2 v/v/m, more preferred 0.15 v/v/m to 1.5 v/v/m and most preferred 0.2 v/v/m to 1 v/v/m.

The duration of this step will be decided taking into account that on one side the incubation under aerobic conditions should be continued for a sufficient long time to make a satisfactory part of the lignocellulosic soluble and available for the following microbial or biological process, on the other side the aerobic step should not be extended so long that a too large fraction of the fibre fraction is combusted. Usually the aerobic fermentation is continued for 5 to 30 days, preferably from 7 to 25 days, more preferred from 10 to 20 days and most preferred around 15 days. It has been found that using such an incubation period a suitable high fraction of the lignocellulosic fibres is converted into a form that can be converted in a following microbial or biological process.

The temperature in this step should be selected taking into account the particular requirements of the microorganism or mixture of two or more microorganisms used according to the invention. Usually the temperature is selected in the range of 20°C to 70°C, preferably in the range of 30°C to 60°C, more preferred in the range of 40°C to 50°C.

The method according to the invention increases the degradability of the substrate material making it more accessible for a following microbial or biological process such as for example a biogas production process leading to a higher yield than would have been possible without the method of the invention.

The incubation under aerobic conditions is continued until the degradability of the lignocellulosic fibres has been increased in a satisfactory extent so that a considerable high fraction of lignocellulosic fibres has been made accessible for a following microbial or biological process.

When lignocellulosic fibres have been made accessible according to the present invention the accessible fibres or part thereof will be available for the following microbial or biological process, meaning that the accessible fibres or part thereof can be converted in the following microbial or biological process. Thus, it can be determined if the accessibility of lignocellulosic fibres have been increased by the method of the invention by performing a following microbial or biological process on the material treated according to the invention and comparing the yield of said following microbial or biological process with a corresponding following microbial or biological process using same material comprising lignocellulosic fibres but without the method of the invention.

Using biogas formation as an example of a following microbial or biological process it can be determined if the method for treatment according to the invention increases the

accessibility of a material comprising lignocellulosic fibres. A material comprising lignocellulosic fibres can be treated using a method of the invention, followed by a usual anaerobic biogas forming process and the yield of the biogas using the material comprising lignocellulosic fibres treated according to the invention can be determined and compared with the same biogas forming process but without the method of the invention. If the yield of biogas is higher using the method of the invention, according to the invention, the accessibility of the lignocellulosic fibres has increased. The skilled person will appreciate that the increased accessibility according to the invention can be determined in other ways using different following microbial or biological methods.

The method according to the invention may be used in connection with any microbial or biological process where it is desired to achieve an increased utilisation of the material. In one embodiment a method of the invention relates to the production of methane. In this embodiment the production of methane may be conducted as a two step process comprising a microbiological aerobic step and/or an enzymatic pre-treatment followed by a process for biogas production. In another embodiment the production of methane may be conducted as a two step process comprising a microbiological aerobic step and/or a pre-treatment followed by a process for biogas production with a simultaneous enzymatic treatment before or during the biogas production process. In principle, any process for biogas formation as known within the art may be used herein.

In another embodiment, the production of methane may be conducted as a process comprising a first process for biogas formation, followed by a microbiological aerobic step and/or an enzymatic treatment, again followed by a second process for biogas formation.

The amount of methane obtained by the process depends on the composition of the material, which again depends on the animals from which the manure is derived, the feed they are given etc.; but typically an amount of methane of approximately 225 ml CH₄/g VS is achieved in the first anaerobic fermentation. The second anaerobic fermentor typically provides at least 10%, preferably at least 25%, more preferred at least 30%, more preferred at least 35%, more preferred at least 40%, more preferred at least 45%, even more preferred at least 50%, most preferred at least 55% and in a particular preferred embodiment at least 60% of the amount of biogas obtained in the first anaerobic fermentation.

Pre-treatment:

The term "pre-treatment" is intended to include any suitable treatment of the material prior to the actual biogas producing step. The substrate material, which may simply be manure, may be pre-treated in any suitable way. The pre-treatment is carried out before or at the same time as the enzymatic hydrolysis. The purpose of the pre-treatment is to reduce the particle size, separate and/or release cellulose; hemicellulose and/or lignin and in this way

increase the rate of hydrolysis. Pre-treatment processes such as wet-oxidation and alkaline pre-treatment targets lignin, while dilute acid and auto-hydrolysis targets hemicellulose. Steam explosion is an example of a pre-treatment that targets lignin.

The pre-treatment step may be a conventional pre-treatment step using techniques well known in the art, such as, milling or wet milling. In a preferred embodiment pre-treatment takes place in a slurry of substrate material and water. The substrate material may during pre-treatment be present in an amount between 10-80 wt.-%, preferably between 20-70 wt.-%, especially between 30-60 wt.-%, such as around 50 wt.-%.

Chemical, Mechanical and/or Biological Pre-treatment

The substrate material may according to the invention be chemically, mechanically and/or biologically pre-treated before hydrolysis in accordance with the process of the invention. Mechanical pre-treatment (often referred to as "physical"- pre-treatment) may be carried out alone or may be combined with other pre-treatment processes.

Preferably, the chemical, mechanical and/or biological pre-treatment is carried out prior to the hydrolysis. Alternatively, the chemical, mechanical and/or biological pre-treatment may be carried out simultaneously with hydrolysis, such as simultaneously with addition of one or more hydrolyzing enzymes, and/or other enzyme activities, to release fermentable sugars, such as glucose and/or maltose.

Chemical Pre-treatment

The term "chemical pre-treatment" refers to any chemical pre-treatment which promotes the separation and/or release of cellulose, hemicellulose and/or lignin. Examples of suitable chemical pre-treatments include treatment with; for example, dilute acid, lime, alkaline, organic solvent, ammonia, sulfur dioxide, carbon dioxide. Further, wet oxidation and pH-controlled hydrothermolysis are also considered chemical pre-treatment.

Other pre-treatment techniques are also contemplated according to the invention. Cellulose solvent treatment has been shown to convert about 90% of cellulose to glucose. It has also been shown that enzymatic hydrolysis could be greatly enhanced when the lignocellulose structure is disrupted. Alkaline H_2O_2 , ozone, organosolv (uses Lewis acids, $FeCl_3$, $Al_2(SO_4)_3$ in aqueous alcohols), glycerol, dioxane, phenol, or ethylene glycol are among solvents known to disrupt cellulose structure and promote hydrolysis (Mosier et al. Bioresource Technology 96 (2005), p. 673-686).

Alkaline chemical pre-treatment with base, e.g., $NaOH$, Na_2CO_3 , $NaHCO_3$, $Ca(OH)_2$, lime hydrate, ammonia and/or KOH or the like, is also within the scope of the invention. Pre-treatment processes using ammonia are described in, e.g., WO 2006/1 10891, WO 2006/1 1899, WO 2006/1 1900, WO 2006/1 10901, which are hereby incorporated by reference. Also the Kraft

pulping process as described for example in "Pulp Processes" by Sven A. Rydholm, page 583-648. ISBN 0-89874-856-9 (1985) might be used. The solid pulp (about 50% by weight based on the dry wood chips) is collected and washed before the enzymatic treatments.

Wet oxidation techniques involve use of oxidizing agents, such as: sulphite based oxidizing agents or the like. Examples of solvent pre-treatments include treatment with DMSO (Dimethyl Sulfoxide) or the like. Chemical pre-treatment is generally carried out for 1 to 60 minutes, such as from 5 to 30 minutes, but may be carried out for shorter or longer periods of time dependent on the material to be pre-treated.

Other examples of suitable pre-treatment processes are described by Schell et al. (2003) Appl. Biochem and Biotechn. Vol. 105-108, p. 69-85, and Mosier et al. Bioresource Technology 96 (2005) 673-686, and US publication no. 2002/0164730, which references are hereby all incorporated by reference.

Mechanical Pre-treatment

The term "mechanical pre-treatment" refers to any mechanical (or physical) pre-treatment which promotes the separation and/or release of cellulose, hemicellulose and/or lignin from substrate material. For example, mechanical pre-treatment includes various types of milling, irradiation, steaming/steam explosion, and hydrothermolysis.

Mechanical pre-treatment includes comminution (mechanical reduction of the size). Comminution includes dry milling, wet milling and vibratory ball milling. Mechanical pre-treatment may involve high pressure and/or high temperature (steam explosion). In an embodiment of the invention high pressure means pressure in the range from 300 to 600 psi, preferably 400 to 500 psi, such as around 450 psi. In an embodiment of the invention high temperature means temperatures in the range from about 100 to 300°C, preferably from about 140 to 235°C. In a preferred embodiment mechanical pre-treatment is carried out as a batch-process, in a steam gun hydrolyzer system which uses high pressure and high temperature as defined above. A Sunds Hydrolyzer (available from Sunds Defibrator AB (Sweden) may be used for this.

In a preferred embodiment the substrate material is subjected to a irradiation pre-treatment. The term "irradiation pre-treatment" refers to any pre-treatment by microwave e.g. as described by Zhu et al. "Production of ethanol from microwave-assisted alkali pre-treated wheat straw" in Process Biochemistry 41 (2006) 869-873 or ultrasonic pre-treatment, e.g., as described by e.g. Li et al. "A kinetic study on enzymatic hydrolysis of a variety of pulps for its enhancement with continuous ultrasonic irradiation", in Biochemical Engineering Journal 19 (2004) 155-164. Preferably, the substrate material prior to step (a) or (b) has been subjected to a microwave and/or an ultrasonic irradiation treatment.

In another preferred embodiment, the substrate material or the slurry is homogenized;

preferably by milling, wet-milling, grinding or wet-grinding prior to or during step (a) or prior to step (b).

Combined Chemical and Mechanical Pre-treatment

In a preferred embodiment the substrate material is subjected to both chemical and mechanical pre-treatment. For instance, the pre-treatment step may involve dilute or mild acid treatment and high temperature and/or pressure treatment. The chemical and mechanical pre-treatments may be carried out sequentially or simultaneously, as desired.

In a preferred embodiment the pre-treatment is carried out as a dilute and/or mild acid steam explosion step. In another preferred embodiment pre-treatment is carried out as an ammonia fiber explosion step (or AFEX pre-treatment step).

In yet another preferred embodiment, a base is added to the substrate material or the slurry prior to or while it is being homogenized; preferably the base is NaOH, Na_2CO_3 , NaHCO_3 , $\text{Ca}(\text{OH})_2$, lime hydrate, ammonia and/or KOH.

Biological pre-treatment

The term "biological pre-treatment" refers to any biological pre-treatment which promotes the separation and/or release of cellulose, hemicellulose, and/or lignin from the substrate material. Known biological pre-treatment techniques involve applying lignin-solubilizing microorganisms (see, for example, Hsu, T.-A., 1996, Pretreatment of biomass, in Handbook on Bioethanol: Production and Utilization, Wyman, C. E., ed., Taylor & Francis, Washington, DC, 179-212; Ghosh, P., and Singh, A., 1993, Physicochemical and biological treatments for enzymatic/microbial conversion of lignocellulosic biomass, Adv. Appl. Microbiol. 39: 295-333; McMillan, J. D., 1994, Pretreating lignocellulosic biomass: a review, in Enzymatic Conversion of Biomass for Fuels Production, Himmel, M. E., Baker, J. O., and Overend, R. P., eds., ACS Symposium Series 566, American Chemical Society, Washington, DC, chapter 15; Gong, C. S., Cao, N. J., Du, J., and Tsao, G. T., 1999, Ethanol production from renewable resources, in Advances in Biochemical Engineering/Biotechnology, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Olsson, L., and Hahn-Hagerdal, B., 1996, Fermentation of lignocellulosic hydrolysates for ethanol production, Enz. Microb. Tech. 18: 312-331; and Vallander, L., and Eriksson, K.-E. L., 1990, Production of ethanol from lignocellulosic materials: State of the art, Adv. Biochem. Eng./Biotechnol. 42: 63-95).

EXAMPLES

Example 1: Pig manure co-digested with straw

In order to test the effect of enzymes addition on the continuous anaerobic co-digestion of pig manure and straw, two pilot scale anaerobic digester were started and operated for about 2 months. The enzymes were tested for their ability to provide an improved biogas yield and/or increased rate of biogas production.

5 The experiment was performed in two 30 m³ continuously stirred digesters with an average hydraulic retention time (HRT) of 15 days and a process temperature of 53 degree Celsius (thermophilic conditions). The digesters were fed 4 times a day and biogas production was measured continuously by industrial gas meters (differential pressure meters, Yokogawa, EJX1 10A). The digesters were fed 2 tonnes of pig manure/straw mixture every day. As the
10 manure contained straw, an initial maceration was necessary to liquify the substrate material and avoid clogging of the pipes. One of the digesters served as a reference digester (R2) and to the other digester (R1) was added a mixture of 5 different commercial enzyme products (all from Novozymes); the enzymes are shown in table 1. The experiment was conducted during a two month period from 15.03.2010 to 20.05.2010, totally 4 HRT. In the experiment 800 gram of
15 enzyme mix was added daily with an automatic dosing system. The mix consisted of 160 gram of each commercial enzyme product, except for the protease which was added with a separate pump. The amount of enzymes mixture was estimated to be approximately 0.77% of the daily dosing of substrate VS.

20 Table 1: Specification of enzymes used in the test

No	Main enzyme activity	Product Name	Enzyme dosage ¹	Enzyme dosage ²
1	Protease	Alcalase 2.5L	0.154%	0.074
2	Alpha-Amylase	Termamyl 300 L	0.154%	0.068
3	Cellulase	FiberCare D	0.154%	0.246
4	Cellulase	Novozym 342	0.154%	0.068
5	Pectate Lyase	BioPrep 3000 L	0.154%	0.042

¹) weight% of product vs. dry organic mass substrate

²) mg enzyme protein vs. dry organic mass substrate (mg EP/g VS)

In order to follow the digesters' performance, the raw substrate and effluents of each
25 digester were sampled on a weekly basis. The main characteristics (TS, VS, COD soluble, total-N, ammonia, pH and volatile fatty acids) were determined on the effluent samples. Standard analyses were performed as described in the art (Standard Methods for the Examination of Water and Wastewater, Eaton et al., Amer Public Health Assn; 21, October 15, 2005). In short, total solids (TS) were determined by drying at 105 degree Celsius until no
30 further weight change occurred. Ash was determined in a muffle furnace by heating the sample

to 550 degree Celsius in a crucible until no further weight change occurred. Volatile solids (VS) were calculated by subtracting total solids with ash content.

Chemical oxygen demand (COD) was measured by the potassium dichromate method. The pH was measured by calibrated industrial pH electrode installed on each digester. Volatile fatty acids (VFA) (C2-C5) were determined by means of gas chromatograph (Hewlett Packard 6850A) with flame ionization detector (FID). The column was an HP-INNOWax, 30 m x 0,25 mm x 0,25 μ m. The carrier gas was He. The temperature of the column was gradually increased from 110°C to 220 degree Celsius at the rate of 10 degree Celsius per min.

Total-N was analyzed by using a Kjell-Foss 16,200 autoanalyzer. Total ammonia nitrogen (TAN) was measured based on following principle: in strongly alkaline solution ammonium nitrogen is present almost entirely as ammonia, which reacts with hypochlorite ions to form monochloramine. This in turn reacts with a substituted phenol to form a blue indophenols derivative that is determined colorimetrically.

Samples of biogas produced were taken once a week and gas composition (methane and carbon dioxide concentration) was analyzed by GC using a Perkin Elmer Clarus 500 gas chromatograph equipped with a Thermal Conductivity Detector and a Turbomatrix 16 Headspace auto sampler. Methane and CO₂ were isolated using a 12' x 1/8" Hayesep Q 80/100 Column, He was used as the carrier gas at 30 ml min⁻¹, and the injection port, oven, and detector temperature were 110, 40, and 150 degree Celsius respectively.

In figure 1 the biogas production from the two digesters during the test is illustrated. A total extra biogas yield of 22 % was recorded with enzyme addition and there was no significant difference between the gas qualities in the two digesters. In average the CH₄ concentration in the two digesters have been 65.15% by enzymes treatment (R2) and 65.35% in the reference (R1).

In the period with enzyme treatment, the load has in average been 2000 kg per day with a VS concentration of 5.2%, providing a biogas yield with enzyme treatment that was 47 m³/day in contrast to 39 m³/day without enzymes, corresponding to respectively 0.451 m³ biogas/kg VS (0.29 m³ CH₄/kg VS) and 0.375 m³ biogas/kg VS (0.24 m³ CH₄/kg VS), respectively.

During the experiment, the dry-matter and volatile solids were measured in the raw manure and in the digestate from the two digesters. From the differences measured in biogas production, lower VS concentration (i.e. more efficient degradation of organic matter) should be expected in the digester where enzymes had been added. However, it was not possible to observe a difference in the VS concentration. The reason for this might be explained by sampling error or accumulation of VS in the top of the digester as foam.

In figure 2, the concentrations of VFA in the two digesters are illustrated. In general there is only a slight difference between the two digesters. In the period with enzyme addition there is general a minor higher concentration of VFA in the digester with added enzymes, which points

to improved hydrolysis and acidogenesis from the addition of enzymes.

Example 2: Pig manure co-digested with low amounts of straw

This test was performed as in example 1, but with two differences: (a) lower amounts of straw were used this time; and (b) the maceration of substrate was left out (not necessary). In addition, two enzyme dosages were tested in separate timeperiods:

- 13-09-2010 to 01-10-2010 (1 HRT): 80 g of enzyme dosing (0.077% enzymes).
- 01-10-2010 to 20-10-2010 (1 HRT): 800 g of enzyme dosing (0.77% enzymes).

In figure 3 the biogas production from the two digesters during the test is illustrated. In the first dosage-period an extra gas yield of 4.6% was recorded with enzyme addition. In this experiment the digester that was used for enzymes in the first trial was now used as reference digesters and vice versa. Both digesters were operated at steady state conditions for a period of three months between the first and second experiment in order to ensure no residual effect of enzymes in the reference reactor.

In the period with enzyme treatment the load was in average 2,000 kg per day with a VS concentration of 5.3% providing a biogas yield with enzyme treatment of 45.7 m³/day versus 43.7 m³/day without treatment, corresponding to respectively 0.431 m³ biogas/kg VS (0.28 m³ CH₄/kg VS) and 0.412 m³ biogas/kg VS (0.27 m³ CH₄/kg VS)

In figure 4, the concentrations of VFA in the two digesters in test 2 are illustrated. In the period with enzyme addition there is a slightly higher concentration of VFA in the digester with added enzymes than observed in the first experiment.

Both the first and second experiments showed a higher gas yield by addition of enzymes. However, there is a large difference in the results from the two periods. In the first period with enzyme treatment the yield was 0.29 m³ CH₄/kgVS in the enzyme treated digester and 0.24 m³ CH₄/kgVS in the reference digester. In the second experiment the result from the enzyme treated digester was almost the same as in the first experiment (0.28 m³ CH₄/kg VS) but the reference was in this case higher than in the first period (0.27 m³ CH₄/kg VS) and the effect of the treatment thus much lower.

In the first and second experiments an extra yield of respectively 22% and 5.3% was recorded. The extra gas yield in the first period didn't result in a lower VS concentration in the digester, which would be expected in the end of the experiment. The reason for this might be explained by sampling error or accumulation of VS in the top of the digester.

The only difference between the running circumstances between the two experiments was that the manure in the first experiment contained more straw and an initial maceration was necessary to avoid clogging of the pipes. In the second experiment this maceration was not necessary.

Example 3: Pig manure co-digested with maize silage and added cellulase or enzymes mix.

The aim of this experiment was to test the effect of enzymes in the biogas production from pig manure co-digested with maize silage. The enzymes were added directly into a corresponding digestion reactor and were tested for improved biogas yield and/or increased rate of biogas production.

In order to test the effect of enzyme addition on the continuous anaerobic co-digestion of pig manure and maize silage, three laboratory scale anaerobic digesters were started and operated for 103 days.

Each experimental set-up of these three semi-continuous completely stirred tank reactor (CSTR) tests consisted of stainless steel laboratory scale reactors with a working volume of 5 liters plus 1 liter headspace. The premixed substrate was stored at 4 degree Celsius for long term and was fed to a reservoir at ambient temperature prior to dosing into the reactors. The substrate was periodically mixed in the reservoir and mechanically pre-treated by a shredder pump, which delivered the substrate to the digester. As the feeding was performed, the same volume of fermented material was removed through an overflow via U-shaped tubing in order to maintain a gas-tight closure and constant working volume. The hydraulic retention time (HRT) was 20 days, the test was performed at 37 degree Celsius (mesophilic digestion) and the VS ratio of the substrates was 80% maize silage and 20% fresh manure.

One digester was used as reference (no enzymes addition) throughout the experimental period and the other 2 reactors were used as experimental reactors (enzyme reactors). To start the fermentation, the reactors were fed with sludge from a working biogas plant that uses a mixture of pig manure and maize silage as input material at mesophilic temperature. After 10 days the addition of the designed substrate was started at a low loading rate and the loading rate was increased during the next 10 days. The reactors were fed with substrate once per day. When the steady state conditions were reached and stable daily biogas production had been observed, the enzymes were added once a day directly to the digesters using a syringe through a silicon rubber septum after the daily feeding cycle. In this way the risk of enzyme wash out from the digesters was minimized.

A commercial cellulase preparation (FiberCare D, Novozymes) and an enzymes cocktail of four commercial enzyme preparations were tested. The enzymes cocktail consisted of 4 commercial enzymes preparations (all from Novozymes): a protease (Alcalase 2.5L, type DX), an alpha-amylase (Termamyl 300L), a cellulase (FiberCare D) and a lipase (Lipolase 100L). To the first enzyme reactor (R1) the cellulase was added in an amount corresponding to 0.01g/gTS or 1.6mg enzyme protein per gram TS (total solids) fed, and in the second enzyme reactor (R2) the enzyme cocktail was tested in an amount corresponding to 0.04g/gTS as shown in Table 2.

Table 2: Specification of enzymes used in the test

No	Activity	Product Name	Enzyme dosage ¹	Enzyme dosage ²
1	Protease	Alcalase 2.5L	1%	0.48
2	Alpha-Amylase	Termamyl 300 L	1%	0.44
3	Cellulase	FiberCare D	1%	1.6
4	Lipase	Lipolase 100L	1%	0.19

1): weight% of product on dry mass substrate

2): mg enzyme protein on g dry mass substrate

5 Temperature, pH, and redox potential were monitored continuously. Calibrated electrodes to measure pH and redox potential were inserted into the fermentors through a wall in sealed sockets.

10 Ultrapure nitrogen gas was used to purge the system at the beginning of the experiment. The evolved gas left the fermentor through flexible neoprene tubing connected to the top plate. Gas volume was measured using direct mass flow controllers (DMFC, Brooks) attached to each gas exit port. Composition of the biogas was measured by taking 250 micro liters aliquots from the headspace and injecting the gas mixture into a gas chromatograph (Shimadzu GC-2010) equipped with a Carboxen 1010 column and a TCD detector. Ultrapure nitrogen was used as carrier gas. Samples were taken twice a week from each digester for analysis.

15 In order to follow the digesters' performance, the raw substrate and effluents of each digester were collected on a weekly basis. The main characteristics (TS, VS, COD soluble, total-N, ammonia, pH and volatile fatty acids) were determined on the effluents. Standard analyses were performed as described in the art (Standard Methods for the Examination of Water and Wastewater, Eaton et al., Amer Public Health Assn; 21, October 15, 2005). In short, 20 volatile fatty acids were determined by HPLC (Hitachi Elite, equipped with ICSep ICECOREGEL 64H column and refractive index detector L2490) using the following parameters: solvent 0.1 N H₂SO₄, flow rate 0.8 ml/min, column temperature 50 degree Celcius, detector temperature 41 degree Celcius.

25 Total nitrogen (TN) was determined with a Teledyne Tekmar Apollo 9000 automatic TOC instrument. This apparatus burns the biomass at 730 degree Celcius and measures the released COx and NOx by infrared absorption.

Total solids (TS) were determined by drying at 105 degree Celsius until no further weight change occurred. Ash was determined in a muffle furnace by heating the sample to 550 degree Celsius in a crucible until no further weight change occurred. Volatile solids (VS) were

calculated by subtracting total solids with ash content.

The ammonium ion concentrations of the liquid samples were determined using a colorimetric method. Briefly, in strongly alkaline solution ammonium nitrogen is present almost entirely as ammonia, which reacts with hypochlorite ions to form monochloramine. This in turn reacts with a substituted phenol to form a blue indophenols derivative that is determined colorimetrically. Samples were filtered through a filter paper and then through a 0.2 μm pore size sterile syringe type filter in order to remove suspended solids.

In figures 5 and 6 the biogas productions from the three digesters during the test are illustrated. During the start-up period of each digester the biogas production increased as the organic loading rate of the reactors increased gradually. Following the start-up period a stable daily biogas production phase was reached in each digester. The performance of each individual reactor varied to a certain degree, which has been observed in similar experimental settings and is probably due to fluctuations in the structure of the microbiological community of any given digester and substrate heterogenic properties.

The results show significant improvement in biogas production in the case of cellulase treatment (Figure 5) and using the enzyme cocktail (Figure 6). Another way of presenting the biogas yield over a longer period is to plot the accumulated gas production and the accumulated fed VS into the reactor. The mean biogas yield over the period is then equal to the slope of the linear regression made on each series of data. The biogas yield during enzyme addition (1 HRT) in the enzyme added reactors and the control reactor are shown in Table 3. Clear biogas yield improvements was observed during days 85-103 when the cellulase and enzymes cocktail was added to digester 1 and 2, respectively (table 3). This corresponds to about 43% for digester 1 and 27% for digester 2, when compared to the reference digester. If the results of enzymes addition are compared to the biogas yield from each reactor during one preceding retention time (days 62-84) a 46% improvement was achieved with the addition of the enzyme cocktail and 17% with addition of the cellulase alone. Earlier experience with the reactors has hinted at a 20% margin of error on gas production. An increase of 20-43% is therefore a significant improvement of biogas yield. In table 3, a general overview of the accumulated biogas production of the three continuous reactors is shown.

Table 3: Overview of biogas production of the 3 digesters during the test period

Parameter	Digester 1	Digester 2	Digester 3
Enzyme Addition	Cellulase	Cellulase Protease Alpha-Amylase Lipase	No enzymes Control

Operation (days)	103	103	103
Enzyme addition (days)	85-103	85-103	No
Biogas production days 85-103 (L/day)	17.5 ± 2.07	15.4 ± 3.2	12.3 ± 2.1
Biogas Yield days 85-103 (L/g TS fed)	0.69	0.61	0.48
Biogas Yield days 62-84 (L/g TS fed)	0.59	0.42	0.48

Following an initial period, the digesters were stabilized at 18-20g/l (1.8-2% TS). The values of organic material content followed the dry material content values and the ratios of VS/TS were about 0.65. Ammonia accumulation was not observed. During the start-up period, when the substrate input started the levels of acetate and propionate increased sharply, but the microbiological system adjusted itself quickly and the level of these indicator volatile acids dropped to normal and stayed there for the rest of the experiment in all three cases.

Example 4: Pig manure co-digested with a defined amount of wheat straw pellets and added enzymes

The aim of this experiment was to test the effect of enzymes in the biogas production from pig manure co-digested with a defined amount of wheat straw pellets.

Two experimental setups were used. Each setup consisted of two CSTR reactors with 5 L capacity and a working volume 3.5 L. In setup 1, the reactors (R1 and R2) were fed with pig manure having a TSA/S concentration of 6.8%/5.3%. In setup 2, the reactors (R3 and R4) were fed with a mixture of pig manure (as in setup 1) mixed with wheat straw pellets in a ratio of 189:11 (5.5% wheat straw on weight to weight basis). The wheat straw pellets and the mixture had a TSA/S concentration of 91.5%/86.9% and 11.5%/9.8%, respectively. The reactors were fed 5 times a week (Monday to Friday) and had a HRT of 20 days. All reactors were continuously stirred and operated at 55°C. After a stabilization period of 21 days, R2 and R4 was added the same mixture of 5 different commercial enzyme products (1% of TS in feedstock) also used in example 1 and 2. R1 and R3 was added an inactivated enzymes blend (heat treatment at 121°Cmin). Addition of enzymes was done on a regular basis immediately after each feeding of the reactors.

Analysis: A sample of the reactor content for analysis was taken from all reactors 11 days after enzyme addition had started (day 32 of the experiment). The content of lignin, cellulose and hemicelluloses was determined by measuring lignin, acid detergent fiber (ADF), and neutral detergent fiber (NDF) according to Van Soest, et al. 1991 (Van Soest, P. J., Robertson, J. B., Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch

polysacchariodes in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597.)

Ammonia-N/total-N content was determined using standard methods (APHA 1995, Standard Methods for the Examination of Water and Waste Water, 19th ed. American Public Health Association, USA). The protein content was determined by multiplying the difference
5 between Total-N and ammonia-N by a factor of 6.25. Biogas production was measured daily.

The biogas production of the reactors is illustrated in figure 7 and summarized in table 4.
Table 4: Overview of biogas production of the 4 digester during the test period

Parameter	R1	R2	Difference R2 to R1	R3	R4	Difference R4 to R3
Enzyme Addition	Inactive blend	Active blend		Inactive blend	Active blend	
Operation (days)	41	41		41	41	
Enzyme addition (days)	21-41	21-41		21-41	21-41	
Biogas production days 14-20 (L/day)	3.63 ± 1.16	3.57 ± 1.05	-1.6%	6.94 ± 1.55	7.16 ± 1.70	+3.1%
Biogas Yield days 35-41 (L/day)	3.17 ± 1.16	3.41 ± 1.05	+7.7%	6.67 ± 1.19	7.31 ± 1.65	+9.6%

A rather large deviation in the daily production was observed due to the fact that the
10 reactors were only fed Monday to Friday. Nevertheless, a higher methane production was seen on average in both setups when comparing reactors added active enzyme to reactors with inactive enzymes. The biogas data was supported by the composition analysis. TS and VS reduction was higher when active enzymes were added and especially the pool of cellulose was affected by the enzyme blend. This shows that the cellulases were an important component of
15 the enzymes blend.

Example 5: Pig manure co-digested with a defined amount of wheat straw pellets and added enzymes

This test was performed in the pilot reactors described in example 1 and 2, but with a defined amount of wheat straw pellets in the feedstock as in example 4 (pig manure mixed with wheat straw pellets in a ratio of 189:1 1). The enzymes were dosed to one reactor from day 1 in a concentration of 1% of TS in the feedstock. The biogas output of the reactors is illustrated in figure 8. The first test period lasted from day 1 to day 25. In this period the loading was 1.5 tons/day and the HRT was set to 20 days. The biogas production during this period was 25.8 m³/t substrate for the reference reactor and 26.1 m³/t substrate for the test reactor. The second test period lasted from day 26 to the end of the experiment. Here the loading was increased to 2.25 tons/day resulting in a HRT of approximately 13.3 days. A clear difference in the biogas output between the two reactors was seen during this period. Thus, the reference reactor produced an average of 25.1 m³/t substrate while the test reactor to which enzymes were added, produced an average of 29.0 m³/t substrate. This corresponds to a difference of 15.5%.

Example 6: Pig manure co-digested with a defined amount of wheat straw pellets and added cellulases, beta-glucanase and xylanase

This experiment was carried out in order to show that an improvement in the biogas production could be obtained by adding cellulases, beta-glucanase and xylanase; thus avoiding the use of the protease, alpha-amylase and pectate lyase as described in example 1. Five lab-scale reactors (R1, R2, R3, R4, R5), as described in example 4 was used. The reactors were fed Monday to Friday with a mixture of pig manure and wheat straw pellets in a ratio of 189:1 1. The digestion temperature was 55°C and HRT was 15 days. Enzymes was added to R2, R3, R4 and R5 following a stabilization period of 21 days. The enzymes consisted of Fibercare D (cellulase) Novozym 342 (cellulase) and Ultraflo L (a Beta-glucanase with cellulase- and xylanase-activities as well. All enzymes are commercial products from Novozymes. Figure 9 shows the difference in biogas production between the test reactors and the control reactors. Values are given in % for the last week of the stabilization period (day 15-21) and three weeks following addition of enzymes. All test reactors had a lower biogas production during the stabilization period ranging from -7.7% to -0.1%. An immediate improvement in biogas production in the test reactors (R2/R3/R4/R5 compared to R1) was seen when enzymes was added, with the effect lasting for at least two weeks.

CLAIMS

1. A biogas production process comprising the steps of providing a substrate comprising manure, and:

(a) adding one or more enzyme to the substrate, and then adding the substrate with the one or more enzyme to a biogas digester; or

(b) adding the substrate to a digester tank and adding one or more enzyme to the tank

2. The process of claim 1, wherein the one or more enzyme is selected from the group consisting of an amylolytic enzyme, a lipolytic enzyme, a proteolytic enzyme, a cellulolytic enzyme, an oxidoreductase and a plant cell-wall degrading enzyme.

3. The process of claim 2, wherein the one or more enzyme is selected from the group consisting of aminopeptidase, alpha-amylase, amyloglucosidase, arabinofuranosidase, arabinoxylanase, beta-glucanase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, ferulic acid esterase, deoxyribonuclease, endo-cellulase, endo-glucanase, endo-xylanase, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannanase, mannosidase, oxidase, pectate lyase, pectin lyase, pectin trans-eliminase, pectin ethylesterase, pectin methylesterase, pectinolytic enzyme, peroxidase, protease, phytase, phenoloxidase, polygalacturonase, polyphenoloxidase, proteolytic enzyme, rhamnogalacturonan lyase, rhamnogalacturonase, rhamnogalacturonase, ribonuclease, SPS-ase, transferase, transglutaminase, xylanase and xyloglucanase.

4. The process of any of claims 1 - 3, wherein the one or more enzyme comprises 2 - 10 different enzymes.

5. The process of any of claims 1- 4, wherein the one or more enzyme is added in a dosage of 0.001 - 5.000 % (w/w; enzyme product vs. dry organic mass substrate); preferably in a dosage of 0.005 - 4.000 % (w/w; enzyme product vs. dry organic mass substrate); more preferably in a dosage of 0.010 - 3.000 % (w/w; enzyme product vs. dry organic mass substrate); even more preferably in a dosage of 0.050 - 2.000 % (w/w; enzyme product vs. dry organic mass substrate); and most preferably in a dosage of 0.100 - 1.500 % (w/w; enzyme product vs. dry organic mass substrate).

6. The process of any of claims 1- 4, wherein the one or more enzyme is added in a

dosage of 0.001 - 5.000 mg enzyme protein per g dry organic mass substrate, VS; preferably in in a dosage of 0.005 - 4.000 mg enzyme protein per g dry organic mass substrate, VS; more preferably in a dosage of 0.010 - 3.000 mg enzyme protein per g dry organic mass substrate, VS; more preferably in a dosage of 0.020 - 2.000 mg enzyme protein per g dry organic mass substrate, VS; and most preferably in a dosage of 0.040 - 1.800 mg enzyme protein per g dry organic mass substrate, VS.

7. The process of any of claims 1 - 6, wherein the substrate is homogenized; preferably by milling, wet-milling, grinding or wet-grinding prior to or during step (a) or prior to step (b).

8. The process of claim 7, wherein a base is added to the substrate prior to or while it is being homogenized; preferably the base is NaOH, Na_2CO_3 , NaHCO_3 , $\text{Ca}(\text{OH})_2$, lime hydrate, ammonia and/or KOH.

9. The process of any of claims 1 - 8, wherein the content of manure in the substrate is adjusted by continuous or stepwise addition of manure during step (a) or (b).

10. The process of any of claims 1 - 9, wherein the manure constitutes above 15% wt-% DS, preferably above 20% wt-% DS, preferably above 25% wt-% DS, preferably above 30 wt-% DS, preferably above 35 wt-% DS, , preferably above 40 wt-% DS, , preferably above 45 wt-% DS, , preferably above 50 wt-% DS, , preferably above 55 wt-% DS, , preferably above 60 wt-% DS, , preferably above 65 wt-% DS, more preferably above 70 wt-% DS of the substrate.

11. The process of any of claims 1 - 10, wherein the substrate is degraded at a pH in the range from 7 to 10; preferably from 8 to 9; most preferably at around 8.5.

12. The process of any of claims 1 - 11, wherein the substrate is degraded at a temperature in the range from 20-70°C, preferably 30-60°C, and more preferably 40-50°C.

13. The process of any of claims 1 - 12, wherein the substrate prior to step (a) or (b) has been subjected to a microwave and/or an ultrasonic irradiation treatment.

14. The process of any of claims 1 - 13, wherein the substrate has been chemically, mechanical and/or biologically treated prior to step (a) or (b).

15. The process of any of claims 1-14, wherein the manure is porcine manure and/or cattle manure.

16. The process of any of claims 1-15, wherein the step

(a) adding one or more enzyme to the substrate, and then adding the substrate with the one or more enzyme to a biogas digester tank; or

5 (b) adding the substrate to a digester tank and adding one or more enzyme to the tank is followed by a fermentation step.

17. The process according to claim 16, wherein the fermentation step is an anaerobic fermentation step.

10

Figure 1

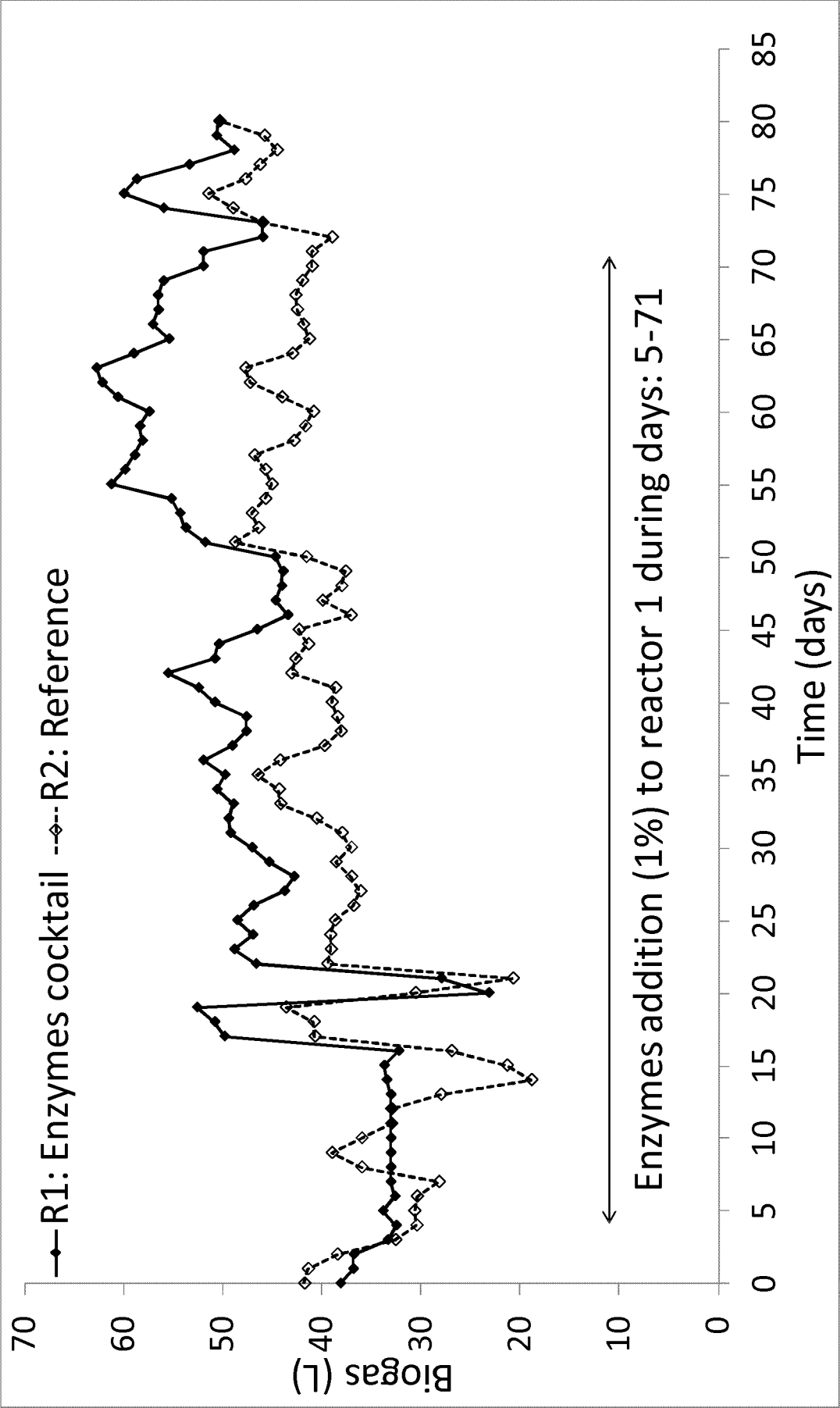


Figure 2A

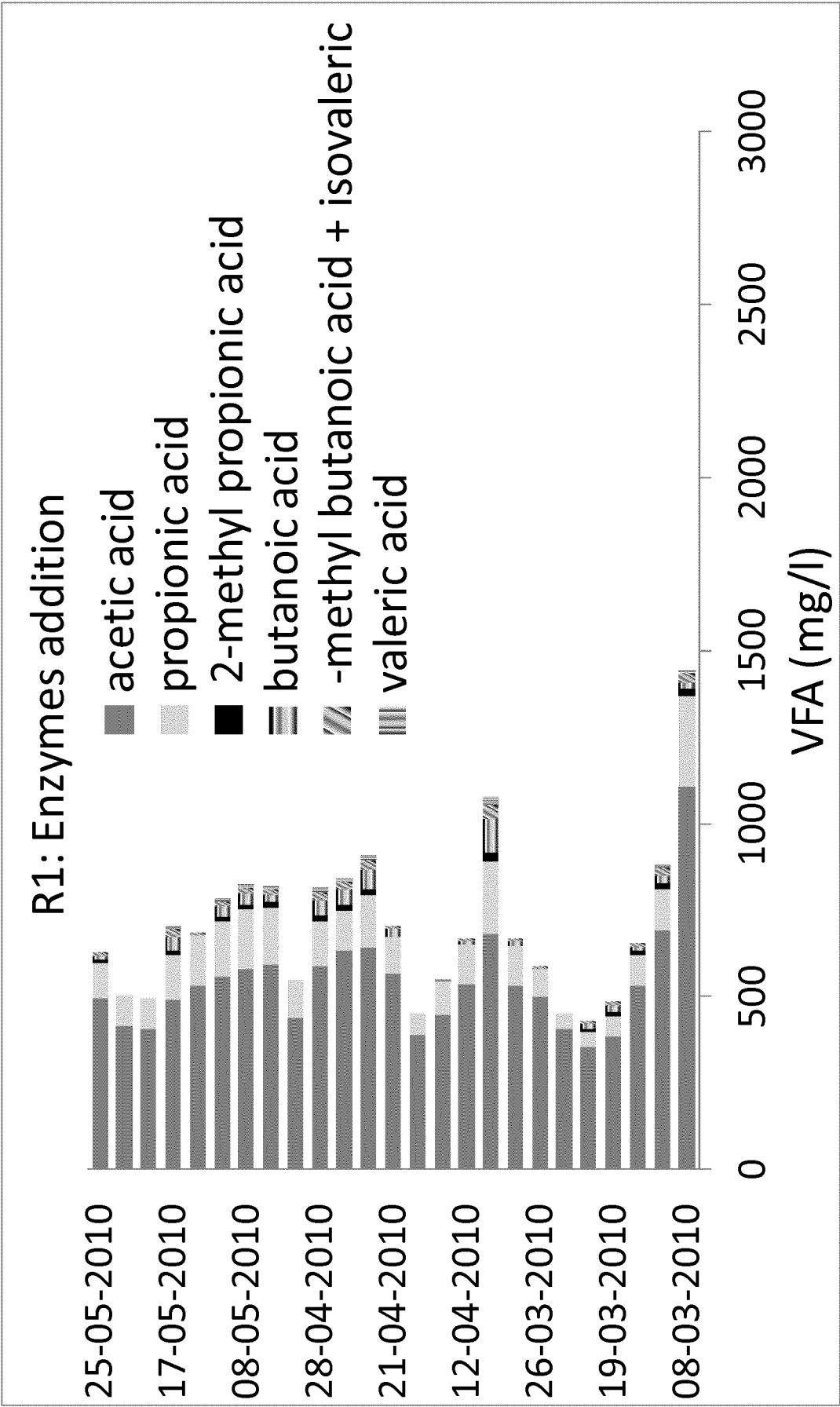


Figure 2B

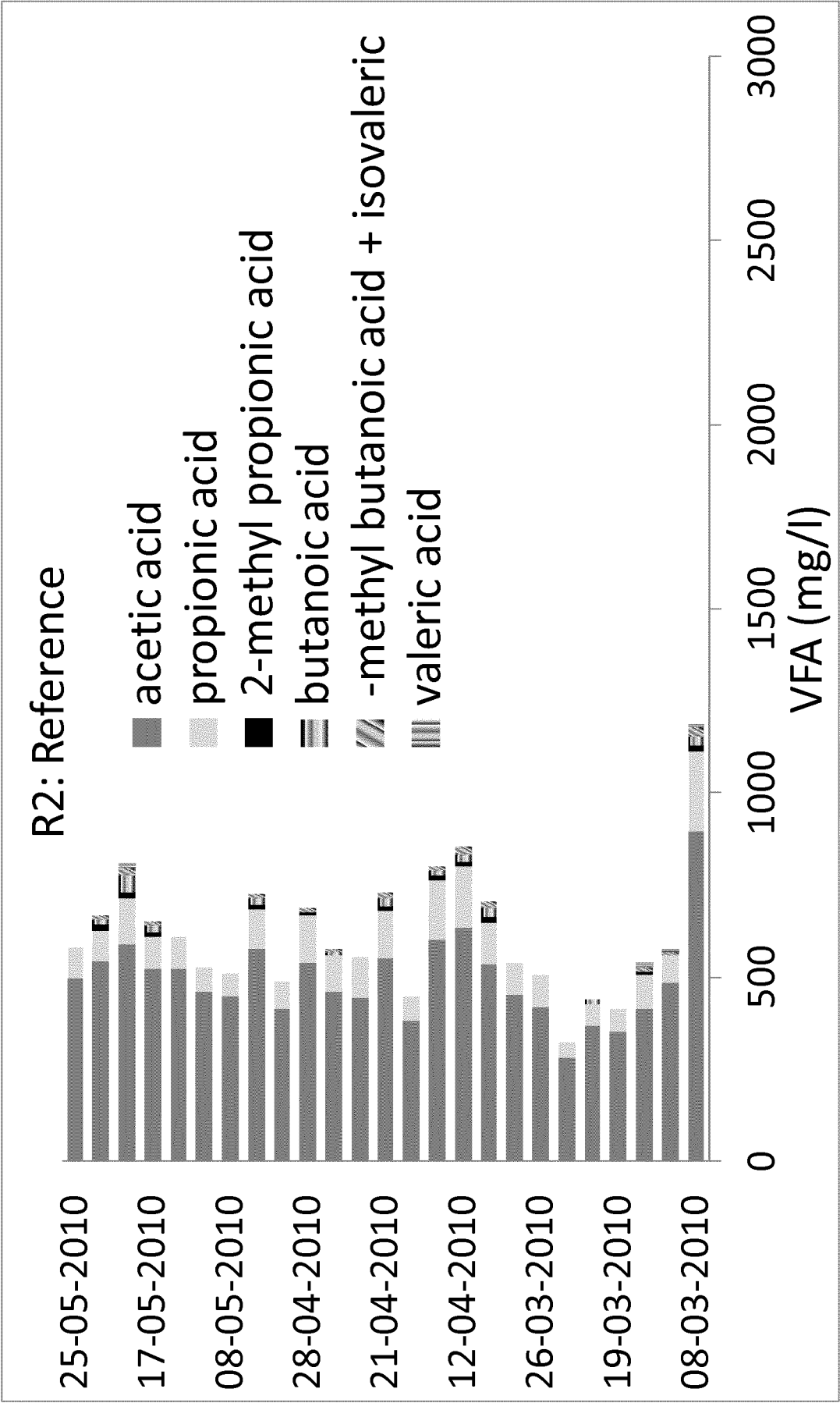


Figure 3

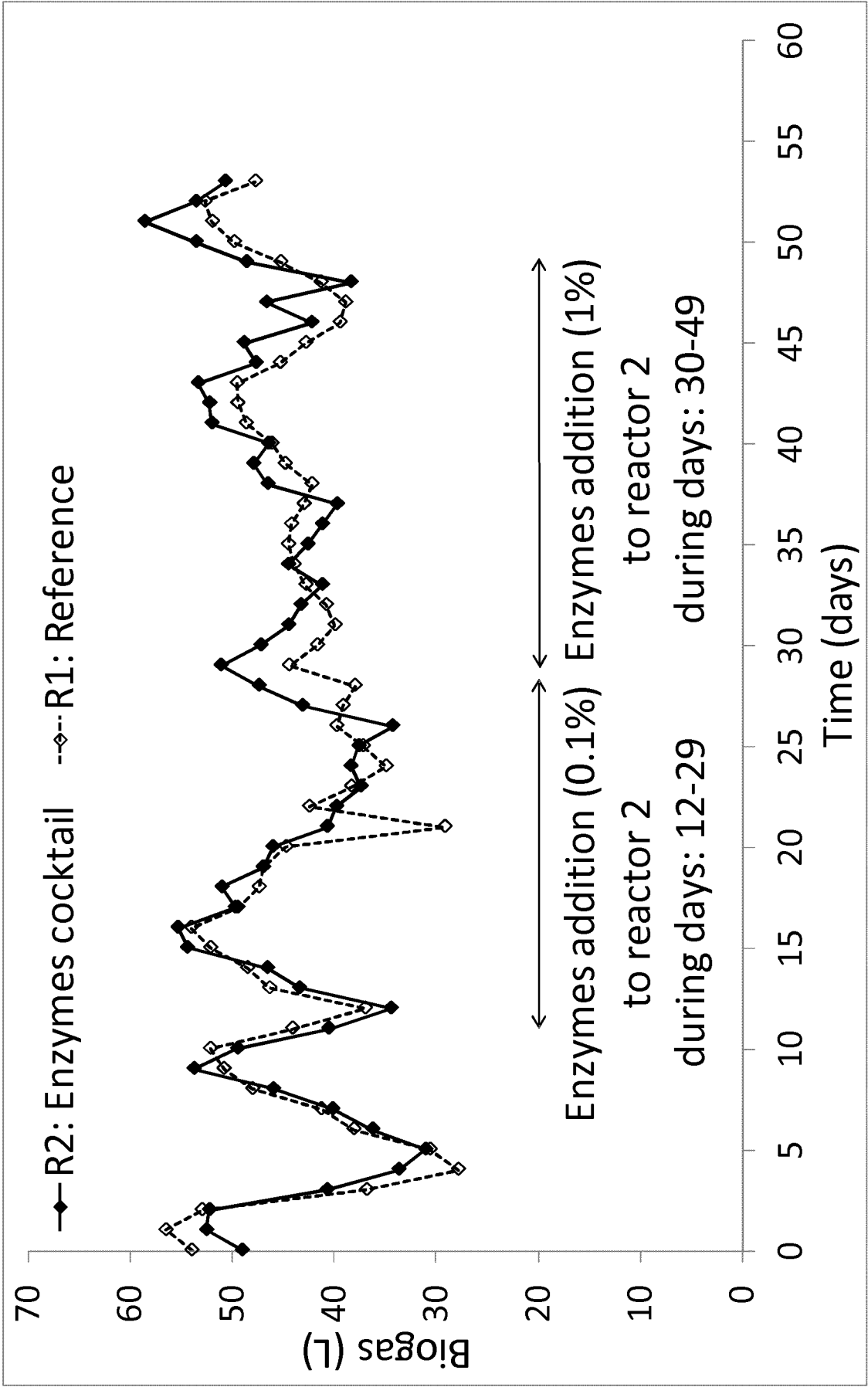


Figure 4A

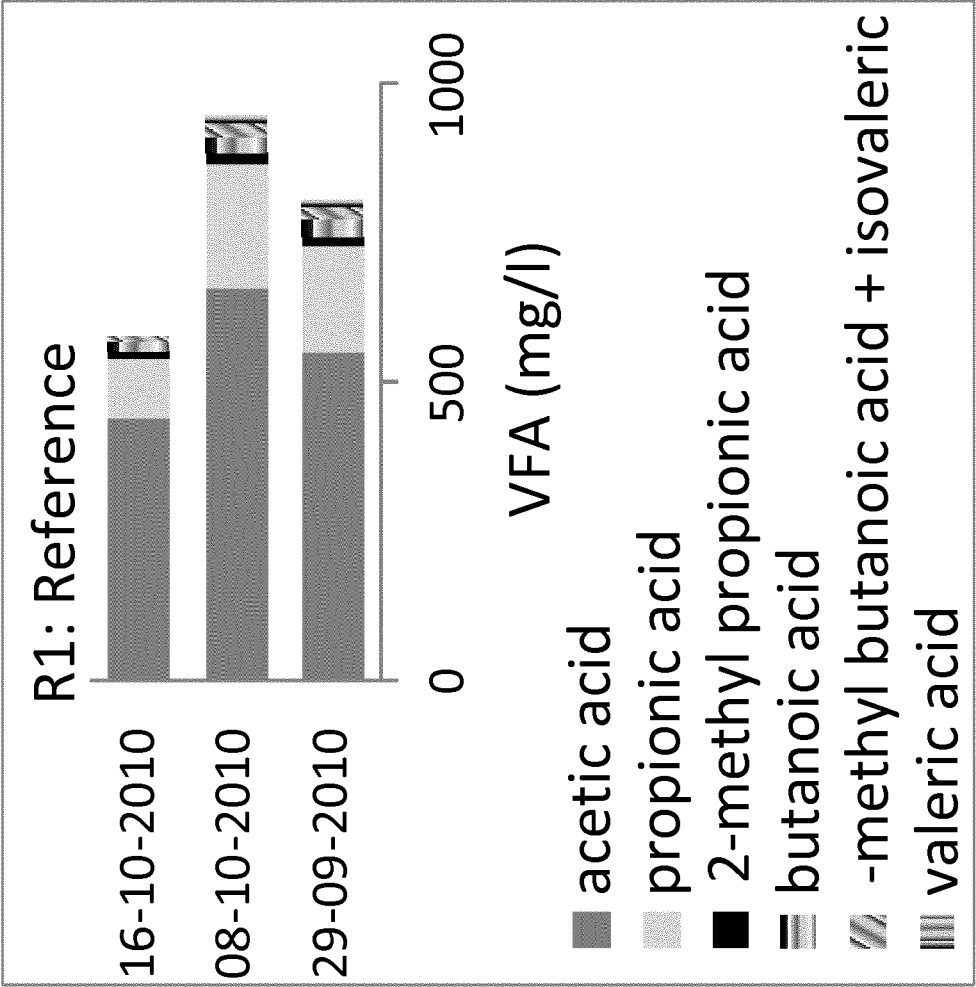


Figure 4B

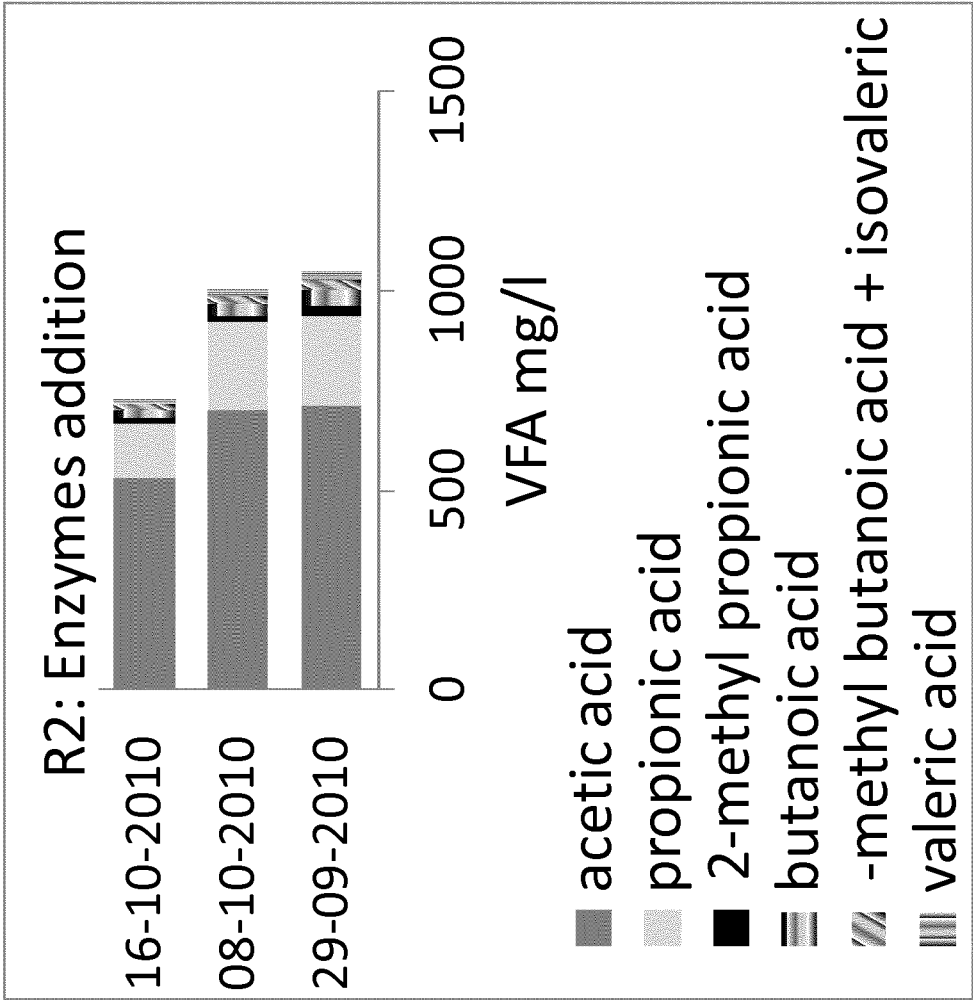


Figure 5

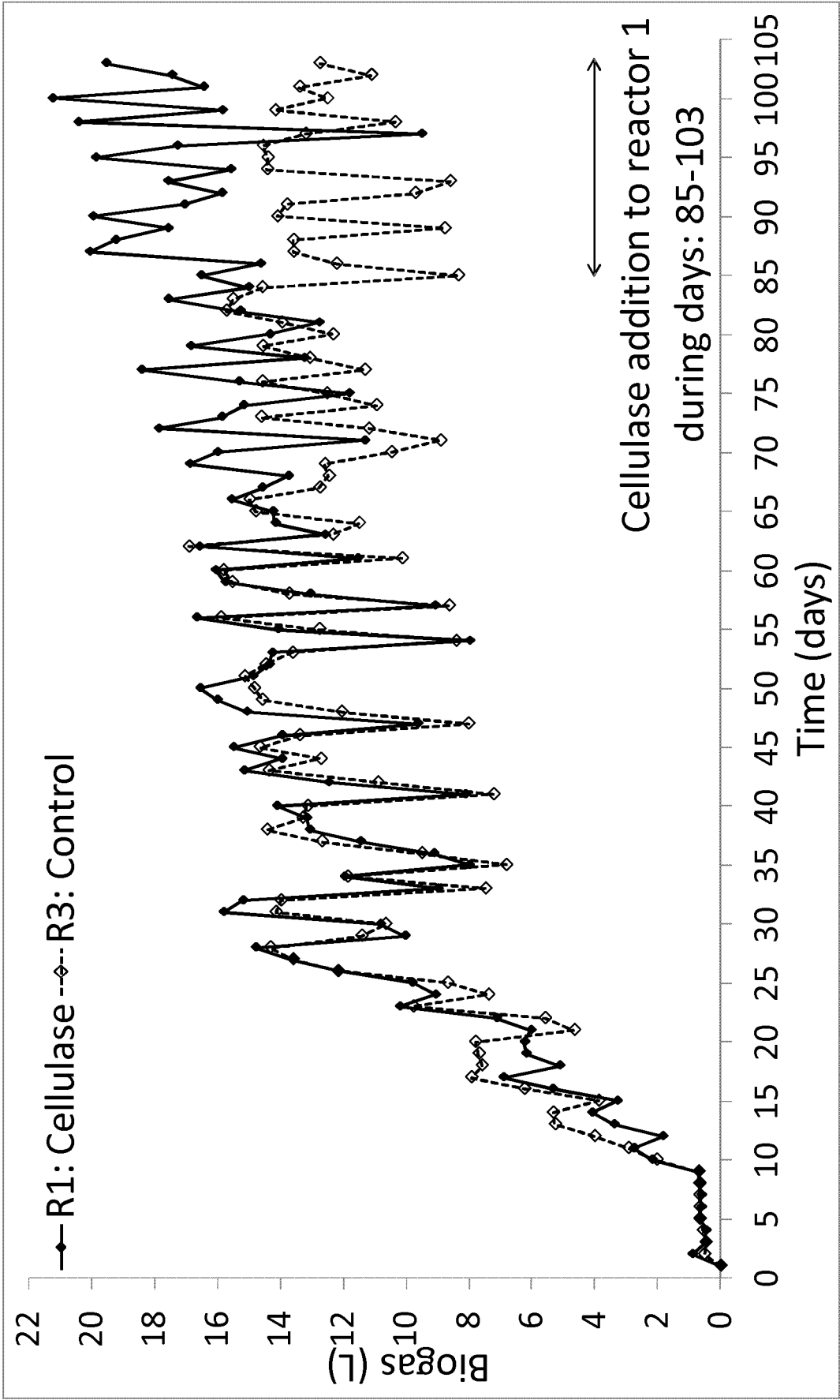


Figure 6

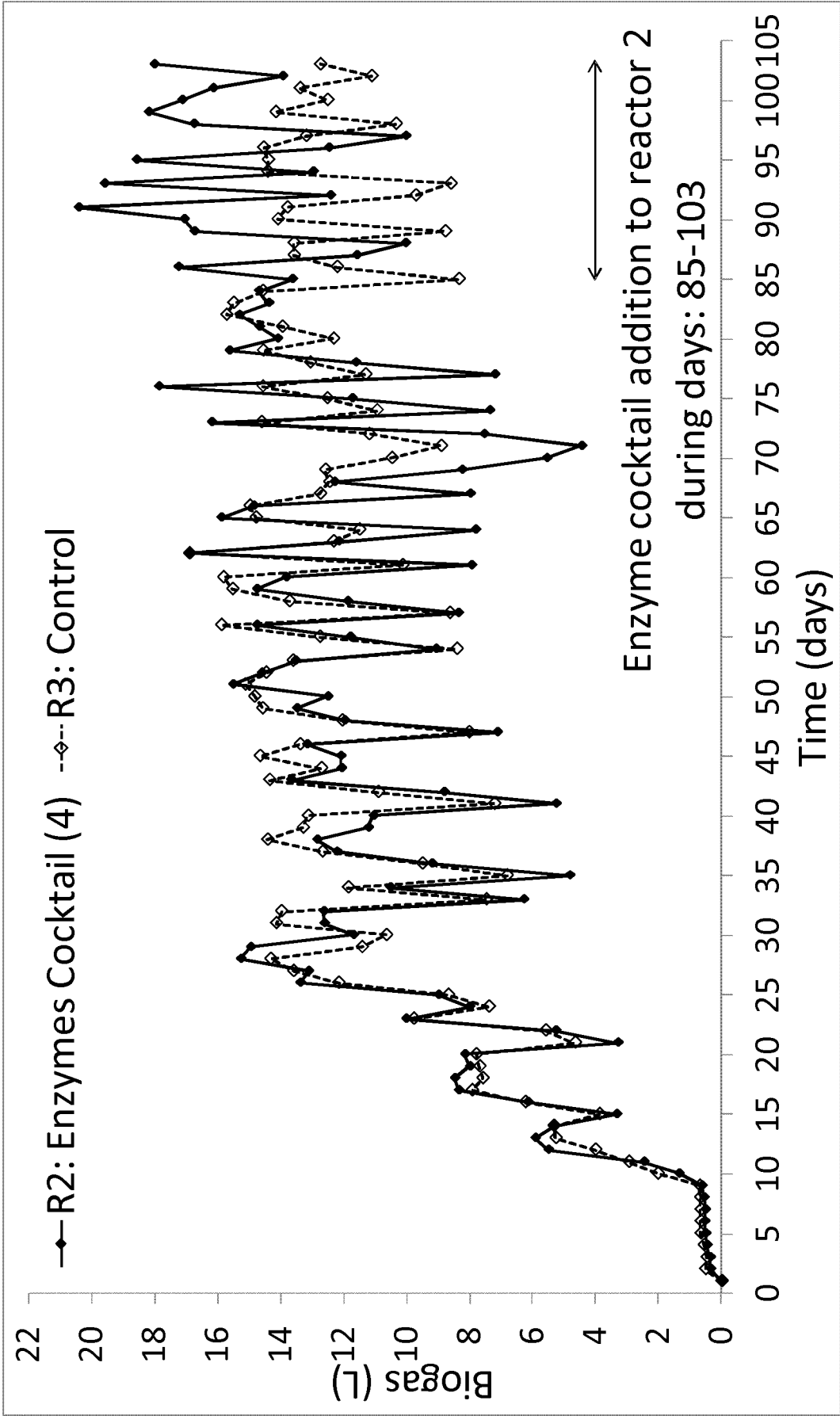


Figure 7

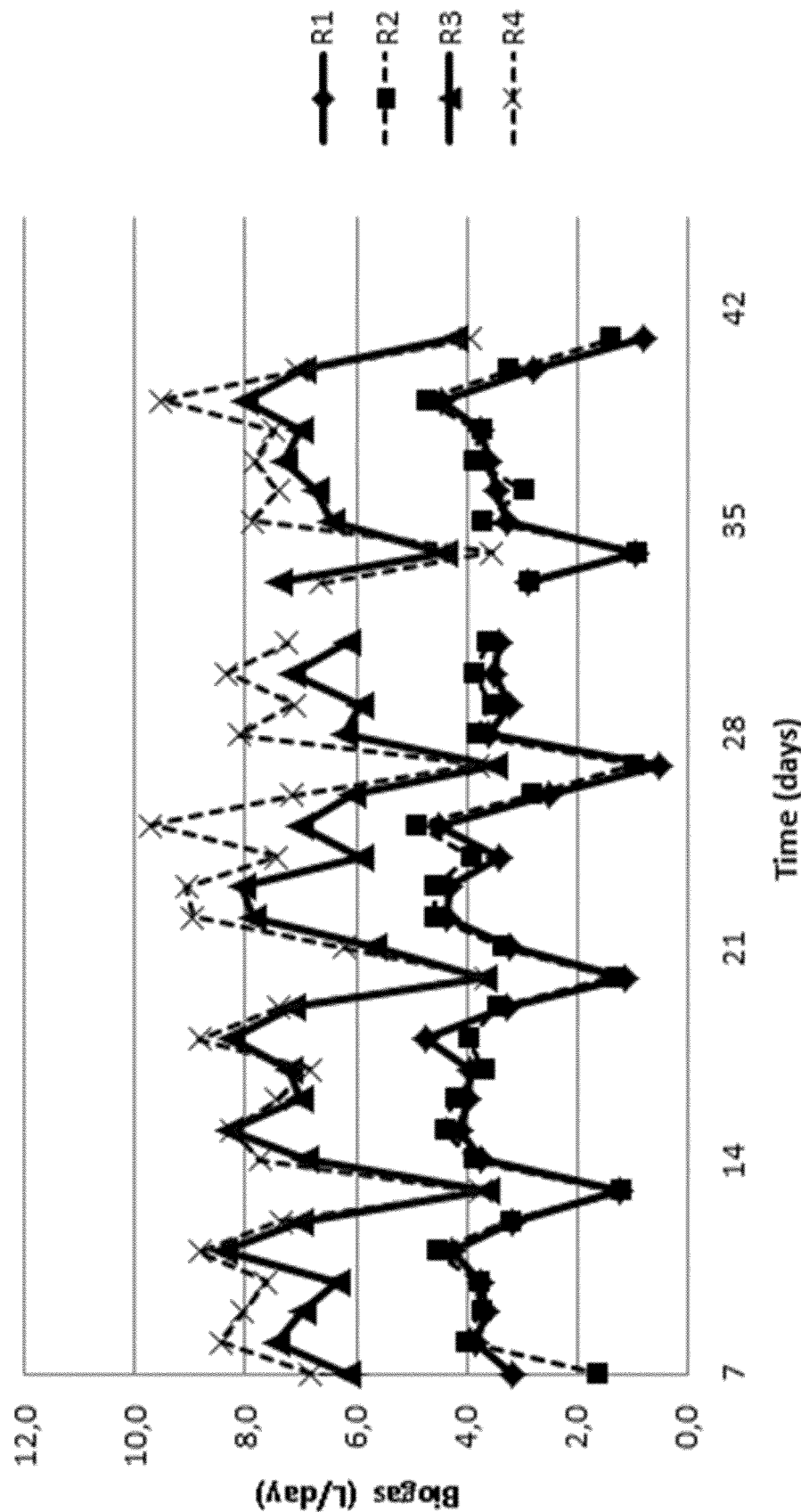


Figure 8a

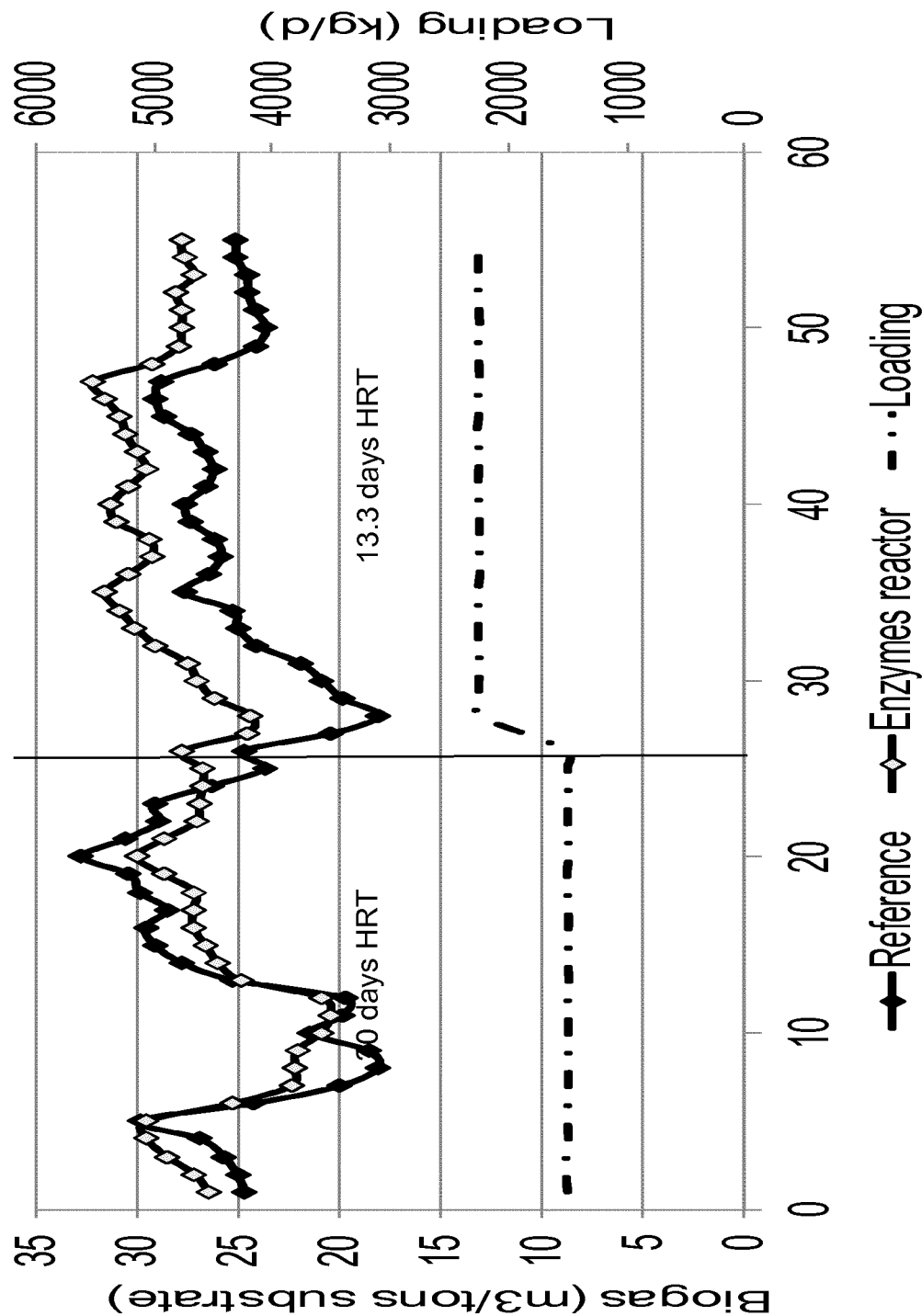


Figure 8b

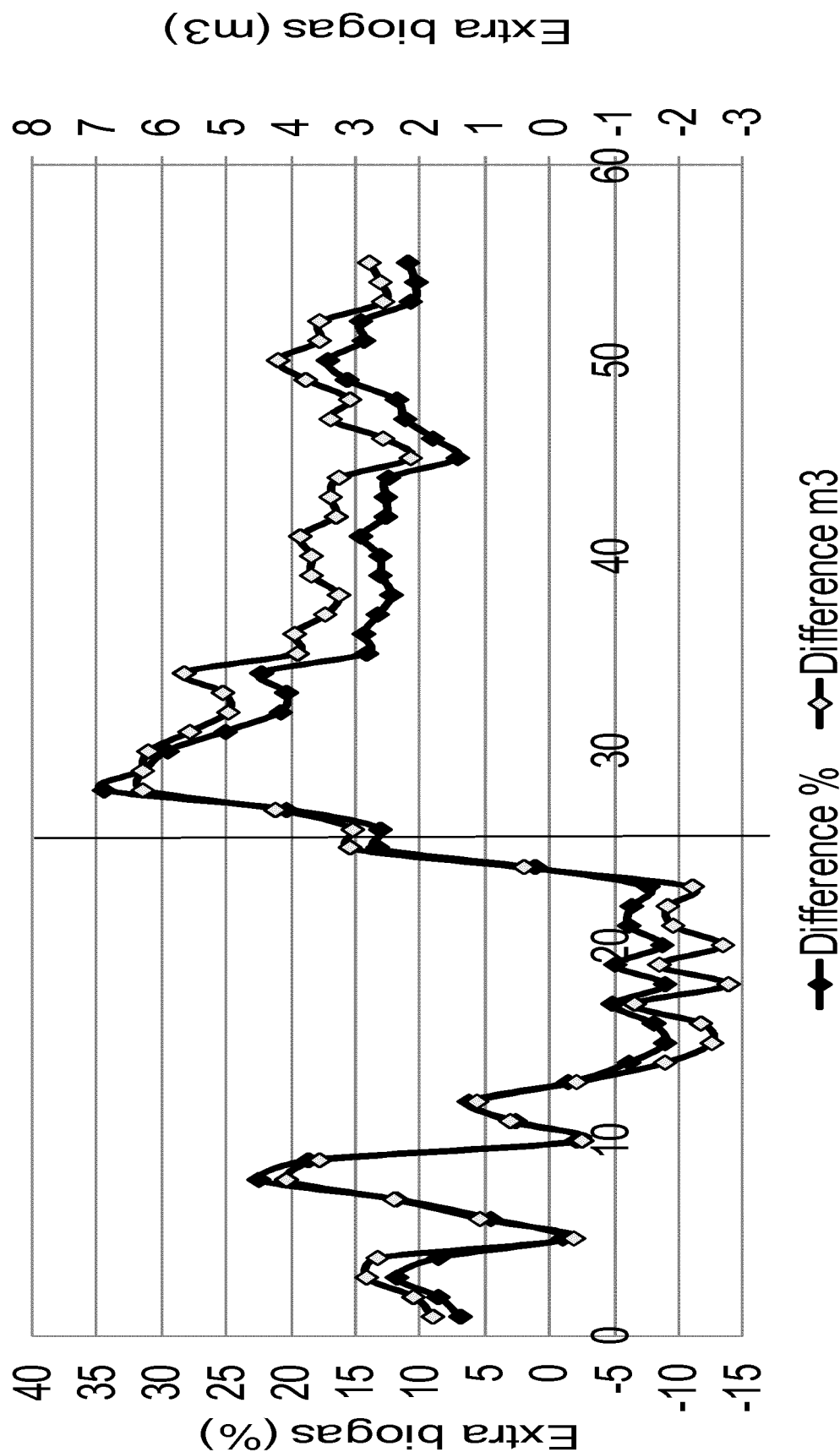


Figure 9

