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Declarations under Rule 4.17:

— as to the applicant's entitlement to claim the priority of the
earlier application (Rule 4.17(iii))

(54) Title: METHOD OF DEWATERING POST FERMENTATION FLUIDS

(57) Abstract: The present invention relates to a method of dewatering post fermentation fluids in a starch to ethanol process. More particularly the invention relates to use of a nuclease enzyme for separation of whole stillage into an insoluble fraction and a supernatant fraction. In a specific embodiment the present invention relates to a method of dewatering whole stillage comprising the steps of: i) subjecting whole stillage to one or more nuclease enzymes; ii) separating the material into an insoluble fraction and a supernatant fraction.



TITLE: METHOD OF DEWATERING POST FERMENTATION FLUIDS**Reference to sequence listing**

This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to methods for enzymatic dewatering insolubles in pre- or post-fermentation fluids or streams.

BACKGROUND OF THE INVENTION

Fermentation products, such as ethanol, are produced by first degrading starch-containing material into fermentable sugars by liquefaction and saccharification and then converting the sugars directly or indirectly into the desired fermentation product using a fermenting organism. Liquid fermentation products are recovered from the fermented mash (often referred to as "beer mash"), e.g., by distillation, which separate the desired fermentation product from other liquids and/or solids. The remaining fraction, referred to as "whole stillage" (WS), is dewatered and separated into a wet pellet (insoluble or "solid") and a supernatant ("liquid") phase, e.g., by centrifugation. The solid phase is referred to as "wet cake" (or "wet distiller's grains" (WDG)) and the liquid phase is referred to as "thin stillage". Dewatered wet cake is dried to provide "Distillers Dried Grains" (DDG) used as nutrient in animal feed. Thin stillage is typically evaporated to provide condensate and syrup or may alternatively be recycled directly to the slurry tank as "backset" (B). Condensate may either be forwarded to a methanator before being discharged or may be recycled to the slurry tank. The syrup consisting mainly of limit dextrins and non-fermentable sugars may be blended into DDG or added to the wet cake before drying to produce DDGS (Distillers Dried Grain with Solubles).

US patent application no. 2005/0079270 A1 discloses a method of dewatering corn stillage solids comprising adding to the solids an anionic copolymer comprising acrylic acid sodium salt, meth acrylic acid sodium salt or 2-acrylamido-2-methyl-1-propanesulfonic acid sodium salt to form a mixture of water and coagulated and flocculated solids; and separating the water from the coagulated and flocculated solids using a dewatering device.

Dewatering of whole stillage or other post-fermentation fluids is energy demanding and may consume up to one-third or higher of the energy requirement of a plant producing ethanol or a similar fermentation product. The energy uses are inversely correlated to the hydration capacity of the solubles and insolubles in WS. Thus, there is a need for improving processes involved in dewatering of whole stillage and it is of interest to explore enzymatic dehydration

('dewatering') of post-fermentation fluids, so that their processing may use less energy. Similar enzymatic dehydrations may be applied to other pre- or post-fermentation fluids (streams) of corn ethanol production or other crop processing's, such as the steep liquor of corn wet milling.

SUMMARY OF THE INVENTION

The invention provides in a first aspect an enzymatic method of dewatering the whole stillage comprising the steps of

- i) subjecting whole stillage to one or more nuclease enzymes;
- ii) separating the material into an insoluble fraction and a supernatant fraction.

In a second aspect the present invention relates to a use of a nuclease enzyme for separation of whole stillage into an insoluble fraction and a supernatant fraction.

In a third aspect the present invention relates to a use of a nuclease enzyme for dewatering insolubles in pre- or post-fermentation fluids or streams apart from whole stillage

DETAILED DESCRIPTION OF THE INVENTION

DESCRIPTION OF THE INVENTION

The object of the present invention is to provide an enzymatic method of dewatering whole stillage.

The present inventors have surprisingly found that subjecting whole stillage to nuclease (DNase, or phosphodiesterase) enzymes improves the solid-liquid separation and thereby decreases the water content in the wet cake after centrifugation compared to a corresponding method carried out without the presence of enzyme. Enzymes used for degrading whole stillage components include nucleases, carbohydrases such as alpha-amylase, glucoamylase, cellulase and/or hemicellulase, such as xylanase and beta-glucanase, pectinase, esterase, and protease, or a mixture thereof. Examples 3-5 show that subjecting whole stillage to one or more nucleases decreases the percentage of water in wet cake after centrifugation. This is advantageous as the energy cost of drying the wet cake is reduced when producing DDG or DDGS. The cost of transporting the wet cake from one place to another is also reduced. Further, the need for maintenance and repair of centrifuges, dryers and other equipment used is also reduced. All in all, the production cost is reduced.

Therefore, the first aspect the invention relates to a method of dewatering whole stillage comprising the steps of:

- i) subjecting whole stillage to one or more nuclease enzymes,
- ii) separating the material into an insoluble (wet cake) fraction and a supernatant (thin stillage) fraction.

Step i) and ii) may be carried out simultaneously or sequentially.

Whole Stillage and Production of Fermentation products

The method of the invention may be used on whole stillage derived from production of any suitable fermentation product, preferably a liquid fermentation product. The feedstock for producing the fermentation product may be any starch-containing material, preferably starch-containing plant material, including: tubers, roots, whole grain; and any combination thereof. The starch-containing material may be obtained from cereals. Suitable starch-containing material includes corn (maize), wheat, barley, cassava, sorghum, rye, potato, or any combination thereof. Corn is the preferred feedstock, especially when the fermentation product is ethanol. The starch-containing material may also consist of or comprise, e.g., a side stream from starch processing, e.g., C₆ carbohydrate containing process streams that may not be suited for production of syrups. Whole stillage typically contains about 10-15 wt-% dry solids. Whole stillage components include fiber, hull, germ, oil, nucleic acid, and protein components from the starch-containing feedstock and fermenting microorganisms.

Production of a fermentation product is typically divided into the following main process stages:

- a) Reducing the particle size of starch-containing material and pre-separation of certain components, e.g., by dry or wet milling;
- b) Cooking the starch-containing material in aqueous slurry to gelatinize the starch,
- c) Liquefying the gelatinized starch-containing material in order to break down the starch (by hydrolysis) into maltodextrins (dextrans);
- d) Saccharifying the maltodextrins (dextrans) to produce low molecular sugars (e.g., DP₁₋₂) that can be metabolized by a fermenting organism;
- e) Fermenting the saccharified material using a suitable fermenting organism directly or indirectly converting low molecular sugars into the desired fermentation product;

f) Recovering the fermentation product, e.g., by distillation in order to separate the fermentation product from the fermentation mash, recovering or recycling various co-, by-, or side-products, such as stillage or steep liquor

As also explained in the "Background"-section above whole stillage is a by-product consisting of liquids and solids remaining after recovery (e.g. by distillation) of a desired fermentation product from fermented mash (beer mash). According to the invention the fermentation product may be any fermentation product, including alcohols (e.g., ethanol, methanol, butanol, 1,3-propanediol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, gluconic acid, gluconate, succinic acid, 2,5-diketo-D-gluconic acid); ketones (e.g., acetone); amino acids (e.g., glutamic acid); gases (e.g., H₂ and CO₂), and more complex compounds, including, for example, antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, B₁₂, beta-carotene); and hormones. Fermentation is also commonly used in the consumable alcohol (e.g., beer and wine), dairy (e.g., in the production of yogurt and cheese), leather, and tobacco industries. In a preferred embodiment the fermentation product is a liquid, preferably an alcohol, especially ethanol.

The whole stillage contemplated according to the invention may be the side-product resulting from a fermentation product production process including above mentioned steps a) to f). However, the whole stillage may also be the side-product resulting from other fermentation product production processes based on starch-containing starting material.

Dewatering of Whole Stillage

Dewatering of whole stillage, in order to remove a significant portion of the liquid/water, may according to the invention (step ii) be done using any suitable separation technique, including centrifugation, pressing and filtration. In an embodiment the whole stillage is heated to a temperature of about 20-60°C or around the optimum of the enzyme(s) in question. The pH is the range from 3-10, preferably pH 4-7, or around the optimum of the enzyme(s) in question. In general the enzymatic treatment of whole stillage is carried out under condition suitable for the enzyme(s) in question.

In a preferred embodiment the dewatering is carried out by centrifugation. Preferred centrifuges in industry today are decanter type centrifuges, preferably high speed decanter type centrifuges. An example of a suitable centrifuge is the NX 400 steep cone series from Alfa Laval which is a high-performance decanter.

In another preferred embodiment, the separation is carried out using other conventional separation equipment such as a plate/frame filter press, belt filter press, screw press, gravity thickeners and deckers, or similar equipment.

Drying of Wet Cake

After the wet cake, containing about 30-35 wt-% dry solids, has been dewatered it may be dried in a drum dryer, spray dryer, ring drier, fluid bed drier or the like in order to produce DDG. DDG is a valuable feed ingredient for livestock, poultry and fish. It is preferred to provide DDG with a content of less than about 10-12 wt-% moisture to avoid mold and microbial breakdown and increase the shelf life. Further, high moisture content also makes it more expensive to transport DDG. The wet cake (insoluble fraction) is preferably dried under conditions that do not denature proteins in the wet cake. The wet cake may be blended with syrup separated from the thin stillage fraction and dried into DDGS.

The nuclease enzymes applied in step (i) of the method of the invention are in one embodiment selected from (a) phosphodiesterase, (b) polynucleotidase or nucleodepolymerase, (c) endonuclease or exonuclease, or (d) DNase or RNase.

More particularly the nuclease enzyme used in step i) is selected from the group consisting of members of EC 3.1.11.- (such as EC 3.1.11.1 to 6), EC 3.1.12.-, EC 3.1.13.-, EC 3.1.14.-, EC 3.1.15.-, EC 3.1.16.-, EC 3.1.21.- (such as EC 3.1.21.1 to 4), EC 3.1.22.-, EC 3.1.25.-, EC 3.1.26.-, EC 3.1.27.-, EC 3.1.3.-, EC 3.1.30.-, EC 3.1.31.-, EC 3.1.4.-, EC 3.1.99.-, family or a mixture thereof.

Nuclease enzyme may be derived from any suitable source. In a particular embodiment the nuclease enzyme is derived from a bacterium, e.g., *Bacillus*, such as *B. subtilis* or *B. licheniformis*, or from a fungus, e.g., *Aspergillus*, such as *A. oryzae*, or from a plant, e.g., mung bean, or from an animal source, e.g., bovine spleen.

In further aspects, the present invention also relates to a use of a nuclease enzyme for separation of whole stillage into an insoluble fraction and a supernatant fraction.

In particular, the separation may be done by centrifugation or filtration.

Apart from the applying the nucleases enzyme in dewatering of whole stillage it is also envisioned to use nuclease treatment for dewatering insolubles in pre- or post-fermentation fluids or

streams apart from whole stillage.

The present invention is further disclosed in the below list of preferred embodiments.

Embodiment 1. A method of dewatering whole stillage comprising the steps of:

- i) subjecting whole stillage to one or more nuclease enzymes;
- ii) separating the material into an insoluble fraction and a supernatant fraction.

Embodiment 2. The method of embodiment 1, wherein step i) and ii) are carried out simultaneously or sequentially.

Embodiment 3. The method of embodiments 1 or 2, further comprising a step iii) of drying the insoluble fraction.

Embodiment 4. The method of any of embodiments 1-3, wherein separation in step ii) is carried out by centrifugation, preferably a decanter centrifuge.

Embodiment 5. The method of any of embodiments 1-3, wherein separation in step ii) is carried out by filtration, preferably using a filter press, a screw press, a plate-and-frame press, a gravity thickener or decker.

Embodiment 6. The method of any of embodiments 1-5, wherein step i) is carried out at a temperature in the range from 20-60°C.

Embodiment 7. The method of any of embodiments 1-6, wherein step i) is carried out at a pH in the range from 3-10, preferably 4-7.

Embodiment 8. The method of any of embodiments 1-7, wherein the whole stillage is derived from a process of producing a fermentation product, preferably a liquid fermentation product.

Embodiment 9. The method of any of embodiments 1-8, wherein the whole stillage is derived from a process of producing a fermentation product from starch-containing material.

Embodiment 10. The method of embodiment 9, wherein the starch-containing material is a cereal.

Embodiment 11. The method of embodiment 9, wherein the starch-containing material is selected from the group consisting of corn, wheat, barley, cassava, sorghum, rye, potato, or any

combination thereof.

Embodiment 12. The method of embodiment 11, wherein the starch-containing material is corn.

Embodiment 13. The method of any of embodiments 8-12, wherein the fermentation product is an alcohol, preferably ethanol.

Embodiment 14. The method of any of embodiments 1-13, wherein the nuclease enzyme(s) used in step i) is(are) (a) phosphodiesterase, (b) polynucleotidase or nucleodepolymerase, (c) endonuclease or exonuclease, (d) DNase or RNase.

Embodiment 15. The method of any of embodiments 1-14, wherein the nuclease enzyme used in step i) is selected from the group consisting of members of EC 3.1.11.- (such as EC 3.1.11.1 to 6), EC 3.1.12.-, EC 3.1.13.-, EC 3.1.14.-, EC 3.1.15.-, EC 3.1.16.-, EC 3.1.21.- (such as EC 3.1.21.1 to 4), EC 3.1.22.-, EC 3.1.25.-, EC 3.1.26.-, EC 3.1.27.-, EC 3.1.3.-, EC 3.1.30.-, EC 3.1.31.-, EC 3.1.4.-, EC 3.1.99.-, family or a mixture thereof.

Embodiment 16. The method of embodiment 15, wherein the nuclease enzyme is derived from a bacterium, e.g., *Bacillus*, such as *B. subtilis* or *B. licheniformis*, or from a fungus, e.g., *Aspergillus*, such as *A. oryzae*, or from a plant, e.g., mung bean, or from an animal source, e.g., bovine spleen.

Embodiment 17. A use of a nuclease enzyme for separation of whole stillage into an insoluble fraction and a supernatant fraction.

Embodiment 18. The use of embodiment 17, wherein the separation is done by centrifugation or filtration.

Embodiment 19. A use of nuclease treatment for dewatering insolubles in pre- or post-fermentation fluids or streams apart from whole stillage.

The present invention is further illustrated in the following examples.

EXAMPLES

Example 1: Methods of evaluating the dewatering of the insolubles in the post-fermentation fluids

The extent of hydration of the insolubles in the post-fermentation fluids (suspensions) was evaluated according to the procedures described below.

Post-fermentation fluids, including whole stillages (obtained after ethanol distillation from the fermented materials) and backsets (obtained after partial removal of the insolubles from whole stillages) were sampled from seven dry milling corn ethanol plants. They were measured for pH with a Beckman ϕ 32 pH meter and a Thermo Scientific Orion 8175BNWP glass pH electrode, as well as dried matters (non-volatiles) content with a Denver Instruments IR60 moisture analyzer set at 130°C.

The weight of the original and treated whole stillages, backsets, centrifuged wet pellets, and supernatants are measured with a Mettler AB204-S balance, in polypropylene tubes by weight differences. Changes in levels of centrifuged wet pellets, equal to the ratios of the pellets' weight over the suspensions' weights, are used to evaluate the (de)hydration of the insolubles.

One mL of whole stillage or backset are pipetted into pre-weighed 1.7-mL microcentrifuge tubes, and the tubes with the samples are weighed. The tubes are centrifuged at 21130 *g* for 5 minutes. The supernatants are pipetted into other pre-weighed 1.7-mL microcentrifuge tubes, and the tubes with the supernatants are weighed. The tubes with the centrifuged pellets are weighed. The supernatants are pipetted back to the pellets to reconstitute the whole stillage or backset suspensions, and the tubes with the reconstituted suspensions are weighed. The net weights of the suspensions, supernatants, and pellets, as well as the pellet level as w% of the suspensions, are calculated from these weighing results. The loss from pipetting transfer of the supernatants is $(0.17 \pm 0.04)\%$.

Example 2: Preparation of nuclease polypeptides

Aspergillus oryzae Nuc A/B polypeptide (disclosed as SEQ ID NO: 1 and the mature polypeptide disclosed as SEQ ID NO: 2) may be prepared as described in WO2015155350. *Bacillus licheniformis* Nuc B polypeptide (SEQ ID NO: 5 and the mature polypeptide SEQ ID NO: 6) may be produced as disclosed in WO2011098579.

Bacillus subtilis Nuc B polypeptide (SEQ ID NO: 3 and the mature polypeptide of SEQ ID NO: 4) has previously been disclosed, e.g., in WO2014081884 and WO2014087011 and may be prepared according to known methods in the art.

Example 3: Effect of mixture of nuclease polypeptides on the pellets of whole stillage or backset

The effect of *A. oryzae*, *B. licheniformis*, and *B. subtilis* nuclease polypeptides on the relative weight of the pellet to that of the whole stillage or backset was determined as described below.

Mixtures of the three nuclease peptides were applied at 0, 1.0 (0.33 for each), and 10.0 (3.3 for each) mg/L levels to the whole stillages and backsets, and the capped reaction tubes were gently shaken (on a rocking platform at 1.5 rpm) at room temperature (23°C). After 4 and 27 hours, the weights of the reaction suspensions, centrifuged pellets and supernatants were determined as described in Example 1. The pellet levels relative to the values before the reaction started were determined as a dehydration measurement.

Various extents of decrease in the pellet level were observed when the nucleases were reacted with various whole stillages and backsets, at two different doses and under two different times. Table 1 lists the data for the whole stillage #3 and backset #7. The decrease in pellet level is attributable to dehydration of the pellets by the nucleases.

Table 1

	Nuclease mix, mg/L	Pellet level relative to pre-reaction level	
		4 hours of reaction	27 hours of reaction
Whole stillage #3	0	0.94	0.96
	1	0.89	0.88
	10	0.85	0.84
Backset #7	0	1.13	1.05
	1	0.97	0.92
	10	1.00	0.92

Example 4: Effect of nuclease polypeptides on the pellets of whole stillage

The effects of *A. oryzae*, *B. licheniformis*, and *B. subtilis* nuclease polypeptides on the relative weight of the pellet to that of the whole stillage or backset were determined similarly as described in Example 3, except the modifications noted below.

The nuclease peptides were applied individually at 0, 1, and 10 mg/L levels to the whole stillage #3, and the reactions were carried out for 5 and 71 hours. The pellet levels after 71 hours of reaction were compared to the values after 5 hours of reaction, as a dehydration measurement.

Various extents of decrease in the pellet level were observed when the nucleases were reacted with the whole stillage.

Table 2

Nuclease	Nuclease, mg/L	71-hour pellet level relative to 5-hour level
None	0	1.01
<i>A. oryzae</i>	1	0.96
SEQ ID NO: 2	10	1.00
<i>B. licheniformis</i>	1	0.96
SEQ ID NO: 6	10	0.93
<i>B. subtilis</i>	1	0.96
SEQ ID NO: 4	10	0.93

Example 5: Effect of nuclease polypeptides on the pellets of whole stillage

The effects of *A. oryzae*, *B. licheniformis*, and *B. subtilis* nuclease polypeptides on the relative weight of the pellet to that of the whole stillage or backset were determined similarly as described in Examples 3 and 4, except the modifications noted below.

The nuclease peptides were applied individually at 0, 1, and 10 mg/L levels to the whole stillage #3, as well as in mixtures at 3 (1 for each) and 30 (10 for each) mg/L. The reactions were carried out for 4 and 27 hours. The pellet levels relative to the values before the reaction started were determined as a dehydration measurement.

Various extents of decrease in the pellet level were observed when the nucleases were reacted with the whole stillage. As shown in Table 3, the *B. subtilis* nuclease was more active than the *B. licheniformis* and *A. oryzae* nuclease in decreasing the pellet level, and contributed the most to the activity of the nuclease mixtures.

Table 3

Nuclease	Nuclease, mg/L	Pellet level relative to pre-reaction level	
		4 hours of reaction	27 hours of reaction
None	0	0.93	0.92
<i>A. oryzae</i>	1	0.94	0.94
	10	0.93	0.92
<i>B. licheniformis</i>	1	0.90	0.93
	10	0.88	0.90
<i>B. subtilis</i>	1	0.91	0.89
	10	0.86	0.85
Mixture (1:1:1)	3	0.86	0.86
	30	0.85	0.84

The overall results demonstrated that nuclease polypeptide could decrease the pellet level in whole stillage and backset, indicating the dehydration of the pellet.

The invention described and claimed herein is not to be limited in scope by the specific aspects herein disclosed, since these aspects are intended as illustrations of several aspects of the invention. Any equivalent aspects are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

CLAIMS

1. A method of dewatering whole stillage comprising the steps of:
 - i) subjecting whole stillage to one or more nuclease enzymes;
 - ii) separating the material into an insoluble fraction and a supernatant fraction.
2. The method of claim 1, wherein separation in step ii) is carried out by centrifugation, preferably a decanter centrifuge.
3. The method of claim 1, wherein separation in step ii) is carried out by filtration, preferably using a filter press, a screw press, a plate-and-frame press, a gravity thickener or decker.
4. The method of any of claims 1-3, wherein the whole stillage is derived from a process of producing a fermentation product, preferably a liquid fermentation product.
5. The method of any of claims 1-4, wherein the whole stillage is derive from a process of producing a fermentation product from starch-containing material.
6. The method of claim 5, wherein the starch-containing material is a cereal.
7. The method of claims 5, wherein the starch-containing material is selected from the group consisting of corn, wheat, barley, cassava, sorghum, rye, potato, or any combination thereof.
8. The method of claim 7, wherein the starch-containing material is corn.
9. The method of any of claims 4-8, wherein the fermentation product is an alcohol, preferably ethanol.
10. The method of any of claims 1-9, wherein the nuclease enzyme(s) used in step i) is(are) (a) phosphodiesterase, (b) polynucleotidase or nucleodepolymerase, (c) endonuclease or exonuclease, (d) DNase or RNase.
11. The method of any of claims 1-10, wherein the nuclease enzyme used in step i) is selected from the group consisting of members of EC 3.1.11.- (such as EC 3.1.11.1 to 6), EC 3.1.12.-, EC 3.1.13.-, EC 3.1.14.-, EC 3.1.15.-, EC 3.1.16.-, EC 3.1.21.- (such as EC 3.1.21.1 to 4), EC 3.1.22.-, EC 3.1.25.-, EC 3.1.26.-, EC 3.1.27.-, EC 3.1.3.-, EC 3.1.30.-, EC 3.1.31.-, EC 3.1.4.-, EC 3.1.99.-, family or a mixture thereof.

12. The method of claim 11, wherein the nuclease enzyme is derived from a bacterium, e.g., *Bacillus*, such as *B. subtilis* or *B. licheniformis*, or from a fungus, e.g., *Aspergillus*, such as *A. oryzae*, or from a plant, e.g., mung bean, or from an animal source, e.g., bovine spleen.
13. A use of a nuclease enzyme for separation of whole stillage into an insoluble fraction and a supernatant fraction.
14. The use of claim 13, wherein the separation is done by centrifugation or filtration.
15. A use of nuclease treatment for dewatering insolubles in pre- or post-fermentation fluids or streams apart from whole stillage.

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/036490

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12P7/10 C12N9/22
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12P C12N

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

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

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/096707 A1 (GEN ELECTRIC [US]) 27 June 2013 (2013-06-27) [0006] - [0007], [0011], [0035], [0038] -----	1-14
X	WO 2012/084225 A1 (DIREVO IND BIOTECHNOLOGY GMBH [DE]; MILOS KLAUDIJA [DE]; KOEHLER STEFF) 28 June 2012 (2012-06-28) page 5, paragraph 6 page 11, paragraph 5 page 12, paragraph 5 page 14, paragraph 6 - page 15, paragraph 1 page 28, line 29 - line 32 ----- -/--	1-9, 11-14



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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International application No
PCT/US2017/036490

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FAN LÜ ET AL: "Enzyme disintegration with spatial resolution reveals different distributions of sludge extracellular polymer substances", BIOTECHNOLOGY FOR BIOFUELS, vol. 9, no. 1, 3 February 2016 (2016-02-03), XP055401670, DOI: 10.1186/s13068-016-0444-y -----	1-14
A	WO 2016/089816 A1 (LAKEVIEW NUTRITION LLC [US]) 9 June 2016 (2016-06-09) -----	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/036490

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-14

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-14

A method of dewatering whole stillage comprising the steps of:

- i) subjecting whole stillage to one or more nuclease enzymes;
- ii) separating the material into an insoluble fraction and a supernatant fraction.

2. claim: 15

A use of nuclease treatment for dewatering insolubles in pre- or post-fermentation fluids or streams apart from whole stillage.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2017/036490

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013096707 A1	27-06-2013	AU 2012358382 A1	17-07-2014
		CN 104010973 A	27-08-2014
		EP 2794491 A1	29-10-2014
		US 2013161255 A1	27-06-2013
		WO 2013096707 A1	27-06-2013
WO 2012084225 A1	28-06-2012	EP 2655644 A1	30-10-2013
		US 2013330791 A1	12-12-2013
		US 2015259633 A1	17-09-2015
		WO 2012084225 A1	28-06-2012
WO 2016089816 A1	09-06-2016	CA 2968871 A1	09-06-2016
		CN 107208122 A	26-09-2017
		EP 3227453 A1	11-10-2017
		WO 2016089816 A1	09-06-2016