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(54) **Microalgae granules.**

(57) The invention is in the field of production of phototrophic microbial cultures, such as algae. In particular, the invention is directed to the cultivation and subsequent harvesting of algae from the dispersion they grow in. In accordance with the invention a starting culture of phototrophic microorganisms is subjected to selective pressure in a growth medium, followed by removing non-settling cultures from said medium, while allowing cultures that settle inside said medium to remain in said medium.

NL C 2012273

P103484NL00

Title: Microalgae granules

The invention is in the field of production of phototrophic microbial cultures, such as algae. In particular, the invention is directed to the cultivation and subsequent harvesting of algae from the dispersion they grow in.

Microalgae (also referred to as microphytes) are microscopic algae, typically found in freshwater and marine systems. The term 'microalgae' covers in this respect both microalgae (eukaryotic photosynthetic microbes) and bacteria (oxygenic photosynthetic bacteria, such as cyanobacteria), as well as all other phototrophic microbial cultures. Microalgae are species which exist individually, or in chains or groups. Depending on the species, their sizes can range from about one micrometer (μm) to a few hundreds of micrometers.

Over the last few decades an extensive amount of research has been carried out in an attempt to develop biofuels and other biobased products from sustainable resources. A variety of different biomasses from different sources have been researched for the production of different biofuels including biodiesel, bio-ethanol, biogas, bio-hydrogen, bio-oil and bio-syngas. Biofuel sources such as sugar based ethanol and palm oil (or other agrocrops such as soybean, rapeseed and sunflower) were found to have the disadvantages that they compete with food crops and impact biodiversity and nature.

The use of aquatic phototrophic microbial cultures, in particular algae, particularly microalgae, is generally seen as more environmentally sound because primary production with algae can be more efficient than with higher plants. In particular the farming of algae enables higher areal yields and provides the possibility of using non-arable land or salt water environments.

Although microalgae are promising as a durable source for fuels and other valuable chemical components, they are hard to separate from the aqueous medium they grow in.

It is an object to obtain microalgae cultures in the form of particles that show improved separability properties. These particles are also referred to herein as microalgae granules.

It was found that this and other objects can be met by a process for obtaining granules of phototrophic microorganisms. This process comprises subjecting a starting culture of phototrophic microorganisms to selective pressure in a growth medium, and removing non-settling cultures from said medium, while allowing cultures that settle inside said medium to remain in said medium.

Although the invention is described with particular reference to microalgae, it is to be understood that it can be applied to other phototrophic microorganisms, such as other types of algae cultures as well.

Microalgae granules in accordance with the present invention offer superb properties is solid/liquid separation. The selection is based on ability to settle ("settleability"). In accordance with the invention algae are grown in a reactor (or an environment further referred to as reactor) and are allowed to settle for a certain time. The reactor can be of any shape and size commonly encountered in the art, and may for instance be in the form of an open pond. The non-settled algae cultures are removed and the settled algae cultures remain in the system. The cultures that are removed are typically in the form of free cells or flocs. Repeating this over various cycles leads to selection of microalgae, which form the granules in accordance with the present invention. The selection of the proper algae cultures may also be done for instance by using a fluidized bed, wherein the upward water flow is adjusted so that the non-settling or slowly-settling cultures are washed out over the top of the bed, whereas the settling cultures remain in the system.

The invention thus provides the application of selective pressure to such an extent that microalgae granules are produced, which are characterized by a very high settling velocity.

Growing microorganisms in granules has been described for instance in WO-A-2004/024638, incorporated herein by reference. This document however does not teach or suggest to apply this approach to phototrophic microorganisms, such as algae. WO-A-2004/024638 relates to a method for the treatment of waste water comprising an organic nutrient. According to the WO-A-2004/024638, the waste water is in a first step fed to sludge granules, after the supply of the waste water to be treated the sludge granules are fluidized in the presence of an oxygen-comprising gas, and in a third step, the sludge granules are allowed to settle in a settling step. This makes it possible to effectively remove not only organic nutrients but optionally also nitrogen compounds and phosphate.

WO-A-2013/012329, incorporated herein by reference, describes a method for producing an open phototrophic culture with improved storage compound production capability. This known process comprises subjecting a starting culture to selective pressure, thus giving a competitive advantage to storage compound producing species, by subjecting said starting culture to a cycle of alternating dark phases and light phases and providing limitation of availability of essential growth nutrients in one or more of said light phases.

Typical settling velocities previously reported for microalgae range from 0.5 to 2 cm/minute (see for instance Van Den Hende *et al.* (Biotechnology and Bioengineering **108**(2011)549-58) and Valigore *et al.* (Water Research **46**(2012)2957-64)).

In accordance with the present invention it is possible to have granules showing a settling velocities of more than 10 cm/min, preferably more than 15 cm/min, even more preferably more than 18 cm/min, for instance 20-100 cm/min. As a consequence of the process of the invention,

the algae cultures of the present invention have to settle much faster and will become granules rather than flocs. Flocs are different from granules in that flocs are more loosely clumped together, have a more open structure and therefore settle slower.

Figure 1 shows an example of an algae culture developing in accordance with the present invention after various points in time.

The first solid liquid separation of microalgae after cultivation is costly. Algae need light and in practice microalgae cultures are known to be light limited at concentrations exceeding about 1-5 g/dm^3 (the concentration of algae becomes too high to permit light throughout the medium). As a result, the product stream is obtained as a very diluted stream.

In the art, the first solid liquid separation is commonly carried out by means of centrifugation, filtration or similar techniques. In accordance with the present invention these can be replaced by fast, low cost and low energy consuming settling, which relies on gravity alone. The granules of the present invention also have improved separation properties for use in equipment operated by centrifugal forces.

Surprisingly it was found possible to produce algae granules having a dimension of about 0.02 to 2 mm (largest dimension, which correspond to the diameter for spherical particles). Preferably the granules have a diameter of 100-500 μm, *e.g.* about 200 μm. Typically the granules are spherical or almost spherical. Typically each granule comprises 1000 to 10000 algae cells.

The processes of the present invention have an advantage in that they do not require the sterilization of equipment and incoming and outgoing flows. The selective mechanisms ensures the functionality of the process is maintained. Other microorganisms, such as bacteria may be present in the granules as well. It is possible that these other microorganisms and algae live in symbiosis, for instance because bacteria may add to the mechanical stability of the granules, while the algae provide

the energy required for growth. It is also surprising that the method of the invention results in granules having such a high settling velocity, which by result may lead to a short settling time of several minutes or less, *e.g.* 45 seconds, depending on the design of the reactor.

The invention can be used for either high-value compounds or low-value compounds usually produced in bulk. The invention may find use in pure or mixed cultures, xenic or axenic cultures, both with fresh or marine water or variations thereof.

Commercial application could be an alternative for the solid-liquid separation steps now used in algae cultivation. It could also be used as a step before the solid-liquid separation step now used to decrease the liquid flow at least a factor ten at conceptually lower costs.

Example

A photobioreactor with microalgae flocs was subjected to the following conditions: a settling phase of 2 minutes, a settling distance to stay in the system of 60 centimeter, a solid retention time of 12 days. Within seven cycles microalgae granules were formed. Figure 1 shows free cells (day 0), flocs (day 6, 26, 69) and granules (day 75).

Conclusies

1. Werkwijze voor het verkrijgen van korrels van fototrofe micro-organismen, hetwelk werkwijze de stappen omvat van het onderwerpen van een startcultuur van fototrofe micro-organismen aan selectieve druk in een groeimedium, en het verwijderen van niet-bezinkende culturen uit het genoemde medium, terwijl culturen die bezinken binnen het genoemde medium in het genoemde medium mogen blijven.

2. Werkwijze volgens conclusie 1, waarbij de genoemde fototrofe micro-organismen algen zijn, bij voorkeur microalgen.

3. Werkwijze volgens één der voorgaande conclusies, die een cyclus van stappen omvat van het verwijderen van niet-bezinkende biomassa, totdat de genoemde culturen korrels vormen.

4. Werkwijze volgens één der voorgaande conclusies, die een cyclus van stappen omvat waarbij bezinkende biomassa selectief wordt behouden over niet-bezinkende biomassa, tot de genoemde culturen korrels vormen.

5. Werkwijze volgens één der voorgaande conclusies, die een cyclus van stappen omvat waarbij biomassa met een hogere bezinkingssnelheid selectief wordt behouden over biomassa met een lagere bezinkingssnelheid, tot de genoemde culturen korrels vormen.

6. Werkwijze volgens één der voorgaande conclusies, waarbij er naast fototrofe micro-organismen andere micro-organismen aanwezig zijn in de genoemde korrels.

7. Werkwijze volgens één der voorgaande conclusies, waarbij de genoemde niet-bezinkende culturen in de vorm van vlokken zijn.

8. Werkwijze volgens één der voorgaande conclusies, waarbij de genoemde korrels worden onderworpen aan condities die de productie van opslagverbindingen bevorderen.

9. Werkwijze volgens één der voorgaande conclusies die een deel vormt van een afvalwaterverwerkingswerkwijze.

10. Werkwijze volgens één der voorgaande conclusies die een deel vormt van de productie van op maat gemaakte chemische producten of andere verbindingen met een hoge waarde.

11. Korrel die kan worden verkregen door de werkwijze volgens één der voorgaande conclusies.

12. Korrel volgens de voorgaande conclusie, met een bezinkingssnelheid van ongeveer 20 – 100 cm/minuut.

13. Korrel volgens conclusie 11 of 12 met een densiteit van ongeveer 1030 – 1100 g/dm^3.

14. Korrel volgens één der conclusies 11 tot en met 13 met een grootste dimensie van 0,02 tot 2 mm.

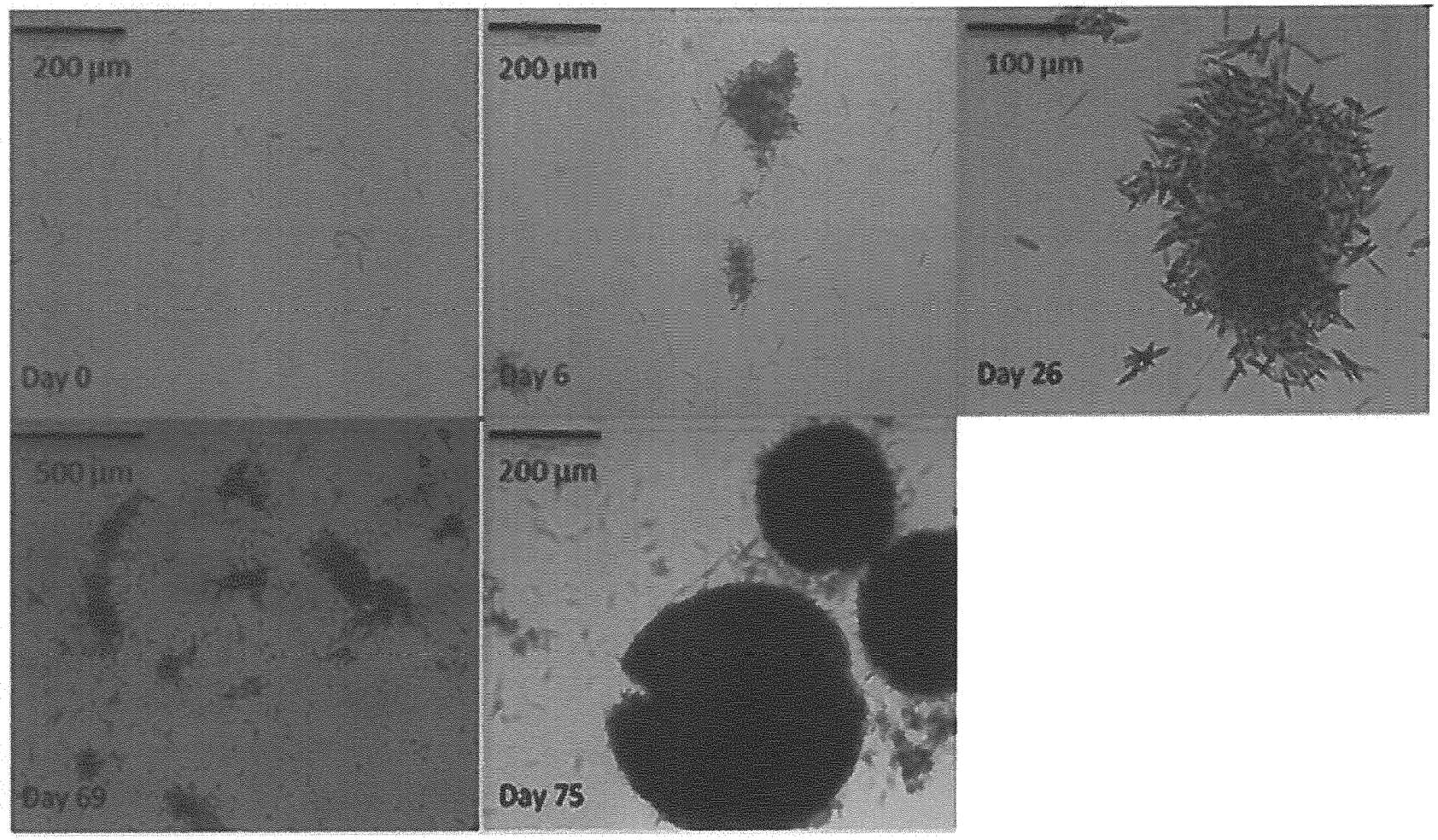

Fig. 1

SAMENWERKINGSVERDRAG (PCT)

RAPPORT BETREFFENDE NIEUWHEIDSONDERZOEK VAN INTERNATIONAAL TYPE

IDENTIFICATIE VAN DE NATIONALE AANVRAGE	KENMERK VAN DE AANVRAGER OF VAN DE GEMACHTIGDE
	P103484NL00
Nederlands aanvraag nr. **2012273**	Indieningsdatum **14-02-2014**
	Ingeroepen voorrangsdatum
Aanvrager (Naam) **Technische Universiteit Delft, et al**	
Datum van het verzoek voor een onderzoek van internationaal type **12-04-2014**	Door de Instantie voor Internationaal Onderzoek aan het verzoek voor een onderzoek van internationaal type toegekend nr. **SN 61791**

I. CLASSIFICATIE VAN HET ONDERWERP (bij toepassing van verschillende classificaties, alle classificatiesymbolen opgeven)

Volgens de internationale classificatie (IPC)

C12N1/12 **C12N1/36** **C02F3/32**

II. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimumdocumentatie

Classificatiesysteem	Classificatiesymbolen
IPC	**C02F** **C12N**

Onderzochte andere documentatie dan de minimum documentatie, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

III.	☐	**GEEN ONDERZOEK MOGELIJK VOOR BEPAALDE CONCLUSIES**	(opmerkingen op aanvullingsblad)
IV.	☐	**GEBREK AAN EENHEID VAN UITVINDING**	(opmerkingen op aanvullingsblad)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2012273

A. CLASSIFICATIE VAN HET ONDERWERP
INV. C12N1/12 C12N1/36 C02F3/32
ADD.

Volgens de Internationale Classificatie van octrooien (IPC) of zowel volgens de nationale classificatie als volgens de IPC.

B. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte miminum documentatie (classificatie gevolgd door classificatiesymbolen)
C02F C12N

Onderzochte andere documentatie dan de mimimum documentatie, voor dergelijke documenten, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

Tijdens het onderzoek geraadpleegde elektronische gegevensbestanden (naam van de gegevensbestanden en, waar uitvoerbaar, gebruikte trefwoorden)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, WPI Data

C. VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	"Elucidating Novel Algal-Sludge Granules for Wastewater Treatment and Biomethane Feedstock Generation", National Science Foundation , 2 juli 2013 (2013-07-02), XP002730620, Gevonden op het Internet: URL:http://www.nsf.gov/awardsearch/showAward?AWD_ID=1335816 [gevonden op 2014-10-01] * het gehele document *	1-14
A	US 2010/264094 A1 (SCHWARTZ GREGORY [US] ET AL) 21 oktober 2010 (2010-10-21) * het gehele document *	1-14

-/--

[X] Verdere documenten worden vermeld in het vervolg van vak C.

[X] Leden van dezelfde octrooifamilie zijn vermeld in een bijlage

° Speciale categorieën van aangehaalde documenten

"A" niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft

"D" in de octrooiaanvrage vermeld

"E" eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven

"L" om andere redenen vermelde literatuur

"O" niet-schriftelijke stand van de techniek

"P" tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur

"T" na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvrage, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding

"X" de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur

"Y" de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht

"&" lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

Datum waarop het onderzoek naar de stand van de techniek van internationaal type werd voltooid	Verzenddatum van het rapport van het onderzoek naar de stand van de techniek van internationaal type
3 oktober 2014	

Naam en adres van de instantie	De bevoegde ambtenaar
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lejeune, Robert

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ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2012273

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
A	WO 98/37027 A1 (UNIV DELFT TECH [NL]; HEIJNEN JOSEPH JOHANNES [NL]; LOOSDRECHT MARINUS) 27 augustus 1998 (1998-08-27) * het gehele document *	1-14
A	ADAV S S ET AL: "Aerobic granular sludge: Recent advances", BIOTECHNOLOGY ADVANCES, deel 26, nr. 5, 1 september 2008 (2008-09-01), bladzijden 411-423, XP022851467, ELSEVIER PUBLISHING, BARKING, GB ISSN: 0734-9750, DOI: 10.1016/J.BIOTECHADV.2008.05.002 [gevonden op 2008-07-14] * bladzijde 418, linker kolom *	1-14
A	SARMAN GULTOM ET AL: "Review of Microalgae Harvesting via Co-Pelletization with Filamentous Fungus", ENERGIES, deel 6, nr. 11, 12 november 2013 (2013-11-12), bladzijden 5921-5939, XP055143554, ISSN: 1996-1073, DOI: 10.3390/en6115921 * het gehele document *	1-14
A	WO 2013/055887 A1 (UNIV MINNESOTA [US]; HU BO [US]; RUAN RONGSHENG R [US]; ZHANG JIANGUO) 18 april 2013 (2013-04-18) * het gehele document *	1-14

2

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Informatie over leden van dezelfde octrooifamilie

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2012273

In het rapport genoemd octrooigeschrift	Datum van publicatie	Overeenkomend(e) geschrift(en)	Datum van publicatie
US 2010264094 A1	21-10-2010	GEEN	
WO 9837027 A1	27-08-1998	AT 223356 T	15-09-2002
		DE 69807659 D1	10-10-2002
		DE 69807659 T2	28-05-2003
		DK 0964831 T3	06-01-2003
		EP 0964831 A1	22-12-1999
		ES 2182270 T3	01-03-2003
		NL 1005345 C2	24-08-1998
		PT 964831 E	31-12-2002
		US 6566119 B1	20-05-2003
		WO 9837027 A1	27-08-1998
WO 2013055887 A1	18-04-2013	GEEN	

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
SN61791	14.02.2014		NL2012273

International Patent Classification (IPC)
INV. C12N1/12 C12N1/36 C02F3/32

Applicant
Technische Universiteit Delft, et al

This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the application
- ☒ Box No. VIII Certain observations on the application

	Examiner
	Lejeune, Robert

Application number

WRITTEN OPINION

NL2012273

Box No. I Basis of this opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☐ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☐ furnished subsequently for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	8, 10
	No:	Claims	1-7, 9, 11-14
Inventive step	Yes:	Claims	
	No:	Claims	1-14
Industrial applicability	Yes:	Claims	1-14
	No:	Claims	

2. Citations and explanations

see separate sheet

WRITTEN OPINION

Box No. VIII Certain observations on the application

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Reference is made to the following documents:

D1 "Elucidating Novel Algal-Sludge Granules for Wastewater Treatment and Biomethane Feedstock Generation",
, 2 juli 2013 (2013-07-02),
Gevonden op het Internet:
URL:http://www.nsf.gov/awardsearch/showAward?AWD_ID=1335816
[gevonden op 2014-10-01]

D2 US 2010/264094 A1 (SCHWARTZ GREGORY [US] ET AL) 21 oktober 2010 (2010-10-21)

D3 WO 98/37027 A1 (UNIV DELFT TECH [NL]; HEIJNEN JOSEPH JOHANNES [NL]; LOOSDRECHT MARINUS) 27 augustus 1998 (1998-08-27)

D4 ADAV S S ET AL: "Aerobic granular sludge: Recent advances", BIOTECHNOLOGY ADVANCES, ELSEVIER PUBLISHING, BARKING, GB,
deel 26, nr. 5, 1 september 2008 (2008-09-01), bladzijden 411-423, XP022851467,
ISSN: 0734-9750, DOI: 10.1016/J.BIOTECHADV.2008.05.002
[gevonden op 2008-07-14]

Novelty

D1 describes the formation of large, dense bioaggregates composed of both microalgae and bacteria. These aggregates are naturally formed in the wastewater environment and can be used in a flow through bioreactor. This mode of operation is SBR (sequential batch reaction): repeating a cycle composed of a period of growth, followed by settling of the dense particles/aggregates and removal of part of the treated liquid (which does not comprise the settles aggregates). Applying an SBR operation in which algae comprising granules are obtained (as disclosed in D1)

comprises all the necessary steps of claim 1. Therefore, the subject matter of claim 1 (and, for essentially the same reasons claims 2-7, 9, 11-14) is not new in the light of D1.

Inventive step

The subject matter of claims 8 and 10 are new over D1 by the stipulation that storage compounds are desirable (claim 8) or high value chemical compounds are produced (claim 10). No inventive step step can be acknowledge for what are very basic applications of fermentation technology.

Concerning the concept of using selective pressure in order to obtain high density (settling in minutes instead of hours) is known in the prior art for obtaining aerobic granular sludge: In D4 (see lft hand column on page 418) the concept of washing out the suspended microorganism to select for high density granules is described. The present application merely applies this concept to produce granular sludge with algae - which cannot even rely on being new since D1 describes such granules.

D3 describes a method for obtaining high density aerobic sludge granules using the same steps as present in claim 1.

Re Item VIII

Clarity and Support

D2 describes a method to select for rapidly settling algal strain by repeating periods of mixing, followed by removal of the upper portion after having allowed the denser algae to settle. The obtained flocs comprise algae and other microorganisms. No granules are obtained (if this were the case, D2 would be cited against the novelty of claims 1-6). Claim 1 differs from D2 only in that "granules" (korrels) are obtained. Since D2 does not obtain granules, despite applying the same method as claim 1, one can only conclude that an essential technical feature is missing from claim 1.