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## (54) Title: A METHOD FOR RAPID START-UP OF MICROBIAL GRANULATION IN WASTEWATER BIOTREATMENT PROCESS

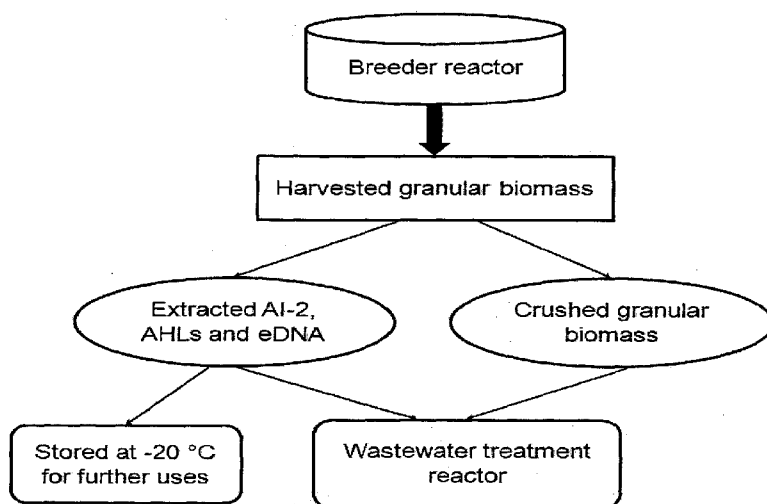


Fig. 1

(57) Abstract: There is disclosed herein a method of accelerating the growth of aerobic granules, comprising the step of adding: (a) one or more aggregating signalling compounds or precursors thereof; and/or (b) crushed aerobic granular biomass, to an activated sludge, wherein: (i) the amount of the one or more aggregating signalling compounds is equivalent to an amount extracted from an aerobic granular biomass that is about 1 wt% to about 10 wt % of the total estimated dry biomass in the activated sludge; and (ii) the amount of the crushed aerobic granular biomass is in an amount of from about 1 wt% to about 10 wt % of the total estimated dry biomass of the activated sludge.



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## **A Method For Rapid Start-Up Of Microbial Granulation In Wastewater Biotreatment Process**

### **Background of the Invention**

The listing or discussion of a prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

Aerobic granules, a special microbial agglomeration, have been reported to treat various types of wastewaters efficiently, with the advantages of excellent settleability, high biomass concentration, and consequently higher loading rates for the system. Aerobic granules develop from flocculent sludge to compact aggregates, granular sludge, and finally mature aerobic granules. However, the exact mechanisms involved in the successive stages of the aerobic granulation process have not yet been adequately explained. The relatively long start-up period and instability of aerobic granules hinder its broad application in wastewater treatment.

Initiation of microbial aggregation is closely related to population size and biomass density. The quorum sensing (QS) ability of bacteria functions through the secretion and detection of signalling molecules, and is regulated by cell density. When the concentration of signalling molecules reaches a threshold level, bacteria respond collectively to gene expression and synchronize behaviors on a population-wide scale. Therefore, in order to initiate microbial aggregation, the first step will be to improve biomass density through external mechanical force and appropriate operating conditions. Operation strategies for initiating the granulation process have been identified as selection pressures including hydraulic shear force, high loading, and settling time. However, it is not yet possible to reproducibly form these aerobic granules on the scale and under the conditions typically found in a wastewater reactor.

Given this, the current applications of QS are typically concentrated on promoting or preventing biofilm formation.

5 Signalling molecules that can mediate QS systems have been described, such as the acyl homoserine lactone (AHL), oligopeptides and autoinducer-2 (AI-2). In particular, the AHL-mediated QS system has been investigated in conjunction with biofilm formation. It has been demonstrated that AHL-mediated QS is important for regulation of gene expression for extracellular polymeric substances (EPS) and extracellular DNA (eDNA), which are the essential components for the formation of microbial biofilms. Autoinducer-2 (AI-2), which is  
10 secreted by both Gram-negative and Gram-positive bacteria, is also known as a universal signalling molecule that modulates QS during biofilm formation.

The functions of QS and eDNA are well known in pure culture and simple mixed-species bacterial communities under well controlled conditions. However, making use of QS and eDNA in natural and engineered environments, e.g. activated sludge systems where many  
15 different types of species co-exist in conjunction with sporadic physical and chemical interference remains one of the greatest challenges. Limited information is currently available on or the role of QS in real multi-species cultures for wastewater treatment and water reclamation.

## 20 **Summary of Invention**

In a first aspect of the invention, there is provided a method of accelerating the growth of aerobic granules, comprising the step of adding:

- (a) one or more aggregating signalling compounds or precursors thereof; and/or
  - (b) crushed aerobic granular biomass,
- 5 to an activated sludge, wherein:

(i) the amount of the one or more aggregating signalling compounds is equivalent to an amount extracted from an aerobic granular biomass that is about 1 wt% to about 10 wt% of the total estimated dry biomass in the activated sludge; and

(ii) the amount of the crushed aerobic granular biomass is in an amount of from about 1 wt% to about 10 wt% of the total estimated dry biomass of the activated sludge.

In embodiments of the invention, when the method uses one or more aggregating signalling compounds, said compounds are selected from one or more of the group consisting of extracellular deoxyribose nucleic acid, acyl homoserine lactones and autoinducer-2.

In further embodiments of the invention, when the method uses a precursor of aggregating signalling compounds, said precursor is dihydroxy-2,3-pentanedione.

In yet further embodiments of the invention, when the method uses one or more aggregating signalling compounds, the method may further comprise the step of extracting the one or more aggregating signalling compounds from an aerobic granular biomass. For example, the one or more aggregating signalling compounds may be extracted from the aerobic granular biomass by:

- (a) suspending the aerobic granular biomass in a liquid to generate a mixture;
- (b) homogenising the mixture, optionally by sonication;
- (c) concentrating the biomass in the mixture, optionally by centrifugation; and
- (d) separating the mixture into a liquid comprising the one or more aggregating signalling compounds and biomass, optionally wherein the liquid comprising the one or more aggregating signalling compounds and biomass is added directly to the activated sludge or is stored at -20°C or below for up to at least 3 months (e.g. 6 months, 1 year, 2 years) before being added to the activated sludge.

In yet further embodiments of the invention, step (a) above may be preceded by harvesting the aerobic granular biomass from a breeder reactor containing a mother liquor, comprising a liquid and the aerobic granular biomass, by concentrating the aerobic granular biomass in the mother liquor and separating the aerobic granular biomass from the liquid, optionally  
5 wherein the concentration step comprises centrifugation of the mother liquor. For example, the breeder reactor may be a sequencing batch reactor, optionally wherein the volume of the breeder reactor is 1% to 10% of the volume of the activated sludge.

In further embodiments of the invention, the breeder reactor may initially comprise a  
10 precursor activated sludge and is operated under high selection pressure conditions to generate a mother liquor comprising a liquid and the aerobic granular biomass. For example, the high selection pressure conditions may comprise a settling time of 2 to 30 minutes and/or a high shear force and optionally wherein the precursor activated sludge further comprises self-aggregating microbial species with high hydrophobicity.

15 In yet further embodiments of the invention, when the method uses the crushed aerobic granular biomass, the crushed aerobic granular biomass may be prepared by obtaining (or provided) an aerobic granular biomass and mechanically crushing it, optionally the mechanical crushing is achieved by the use of a mechanical beater or a blender. For  
20 example, before the crushed aerobic granular biomass is added to the activated sludge, the crushed aerobic granular biomass is suspended in a liquid and homogenised, optionally by sonication, optionally wherein the crushed aerobic granular biomass is concentrated by removal of the liquid.

25 In yet further embodiments, the aerobic granular biomass may be obtained (or provided) by harvesting the aerobic granular biomass from a breeder reactor containing a mother liquor, comprising a liquid and the aerobic granular biomass, by concentrating the aerobic granular

biomass in the mother liquor, optionally the concentration step comprises centrifugation of the mother liquor and separation of the aerobic granular biomass from the liquid. For example, the breeder reactor is a sequencing batch reactor, optionally wherein the volume of the breeder reactor is 1%-10% of the volume of the activated sludge.

5

In further embodiments, the breeder reactor initially comprises a precursor activated sludge and is operated under high selection pressure conditions to generate a mother liquor comprising a liquid and the aerobic granular biomass. For example, the high selection pressure conditions may comprise a settling time of 2 to 30 minutes and/or a high shear force, optionally, the precursor activated sludge may further comprise self-aggregating microbial species with high hydrophobicity.

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In still further embodiments, the activated sludge is contained within a wastewater reactor, optionally wherein the wastewater reactor is a sequencing batch reactor or a membrane bioreactor.

15

In yet still further embodiments, the activated sludge is a flocculated activated sludge.

## Figures

The invention will now be described in further detail below, with the aid of the following figures.

20

Fig. 1 Schematic outline of method for accelerating growth of aerobic granules in wastewater treatment reactor

Fig. 2 Graph showing the enhancement of cell motility by the addition of AI-2

Fig. 3 Development of microbial colonies/aggregates on a substrate with and without exposure to AI-2

25

Fig. 4 AI-2 in suspended activated sludge and aerobic granules

Fig.5 AHLs in suspended activated sludge and aerobic granules

Fig.6 eDNA in suspended activated sludge and aerobic granules

### Description

- 5 It is believed that with a better understanding of the cellular communication that is associated with granulation, it will be possible to control the granule formation and maturation process.

Described herein are processing steps that may be taken that will enable one to expedite the  
10 process of aerobic granule formation in a wastewater treatment plant in a repeatable manner. The correlation between hydraulic shear force, high loading, and settling time and cell density, quorum sensing and initiation of granulation process shall be used.

The comprehensive understanding of the relationship between QS and biofilms will enable  
15 operators to better control the development of microbial aggregation for different endpoint purposes, i.e. to promote or inhibit the formation of such microbial aggregates.

The method described herein leverages on QS biology and eDNA to regulate the development of microbial granules from flocculent activated sludge. It establishes a system  
20 for generating signalling molecules and their application for the rapid start-up of the granulation process in a wastewater reactor containing activated sludge. It links engineering operating parameters with process enhancement and microbial activities, and draws a correlation between structural formation of microbial granules, QS signal expression and eDNA. The granular biomass and/or signals dosing method described herein deploys QS  
25 signals to direct expression of EPS during the granulation process.

The overall method involves:

- (1) use of operating strategies and specific reactor configuration to increase microbial cell density;
- (2) identification of signalling molecules and eDNA;
- (3) aerobic granular biomass and/or signals extraction and/or addition; and
- 5 (4) use of the granular biomass and/or signal types (e.g. signalling molecules), and/or eDNA and dosage to initiate aerobic granule formation (e.g. in a full-scale wastewater treatment reactor).

10 It will be appreciated that, in order to achieve the desired acceleration in aerobic granule formation, the final step is all that is necessary.

The method disclosed herein essentially involves the external addition of identified QS signals and eDNA to activated sludge systems so that agglomeration leading to granulation will be greatly accelerated. The method allows the process of granulation to be reduced by  
15 up to 80% in time compared to the conventional process. This will significantly shorten the overall process start-up period and hence will help to reduce operational costs. The method can also be used to remediate disaggregated granules or poorly settling sludge when this occurs and so provides for better long-term system stability and process recoveries.

20 Granular biomass will be first cultivated in a smaller breeder reactor which can be operated under high selection pressure conditions. That is, conditions that favour the formation of aerobic granules. The granular biomass developed in the breeder reactor will serve as a source of signalling molecules (e.g. AHLs and AI-2) and eDNA for promoting biomass granulation in the wastewater treatment reactor as illustrated in Figure 1.

25

- (a) Breeder reactor: The breeder reactor can be a small sequencing batch reactor (SBR) with a volume of up to 10% of the wastewater treatment reactor. It will be operated at

a shorter settling time than is usual (e.g. 2-30 minutes), which has been proven to be the most effective selection pressure for aerobic granulation. Rapid formation of granular biomass in the breeder reactor may also be achieved by augmenting the culture with self-aggregating microbial species with high hydrophobicity. A shorter settling time may be accomplished by stopping the air supply and all of the mechanical mixing devices.

(b) Wastewater treatment reactors: can be SBRs or be in a membrane bioreactor configuration (MBR), these reactors are operated with a short settling time of 2-30 minutes and/or high hydraulic shear force in terms of a superficial upflow air velocity of 0.3-3.6 cm/s compared to conventional systems. A good settleability of sludge is required before dosing granular biomass and/or signals into the SBR or MBR to promote agglomeration.

(c) Identification and quantification of signalling molecules and eDNA: *In situ* acyl-homoserine lactones (AHLs) and AI-2 generated by the microbial consortia cultivated in the breeder reactor are extracted and identified by mass spectroscopy (MS) and confirmed and quantified by MS/MS and bioassay methods. The AHLs are expected to range from C4 to C14 which are produced by microbial communities at different stages of the granulation process. AI-2 can be detected and quantified using the AI-2 reporter, *Vibrio harveyi* BB170. eDNA in granular biomass drawn from the breeder reactor can be extracted and measured by using PicoGreen dsDNA Quantification Kit. It will be appreciated that such tests are not necessary for the operation of the process. However, such tests can be used to quantify the amount of signalling molecules and eDNA.

(d) Delivery methods of signalling molecules to wastewater treatment reactors (Figure 1):

**Method 1 - Dosing of extracted signalling molecules and eDNA**

The granular biomass from the breeder reactor will be concentrated (e.g. centrifuged).

5 The harvested biomass is resuspended in fresh water, and then homogenized by a physical method such as sonication. The homogenized biomass is further concentrated (e.g. centrifuged) for recovering centrate (or supernatant) which is rich in signalling molecules of AHLs and AI-2 and eDNA. The centrate (or supernatant) with signalling molecules can be directly added to the wastewater treatment reactor to promote  
10 microbial aggregation, while it can also been stored at -20°C for at least 3 months. Storage allows for massive production of signalling molecules and eDNA-rich supernatant for applications in various wastewater treatment systems, e.g. improving sludge settleability in the activated sludge process, and reducing membrane biofouling in a MBR through promoting sludge agglomeration and aggregation. While the supernatant  
15 can be used as is, it should be noted that the use of one or more of the signalling compounds or eDNA in a sufficient dose would be sufficient to catalyse the agglomeration process.

**Method 2 – Direct dosing of crushed granular biomass**

20 Granular biomass harvested from the breeder reactor will first be crushed mechanically and crushed granular biomass further subjected to sonication for homogenisation. The homogenised mixture may then be concentrated by evaporation of the liquid. For mechanical crushing of granular biomass can be with a mechanical beater or blender. Such pretreated biomass will be directly added to the wastewater treatment reactor to  
25 promote microbial aggregation.

It will be appreciated, that to obtain the granular biomass from the breeder reactor for use in either of methods 1 and 2, a precursor activated sludge is treated under high selection pressure, as discussed herein.

5 (e) Dosage: Dosage of extracted signalling molecules and eDNA may be preceded by screening methods. Typically efficacy of the supplemented signalling molecules and eDNA shall first be indicated by positive impact on the amount of EPS produced by the microbial communities and subsequently by impact on microbial floc size. With respect to direct addition of crushed granular biomass, dosage of crushed biomass  
10 drawn from the breeder reactor can be evaluated in terms of biomass concentration and volume against the biomass in the wastewater treatment reactor. It will be appreciated that the exact amount of the agents described above as catalysing the agglomeration will vary according to the microbial community and the characteristics of the wastewater in question.

15 The method disclosed herein shall have application where there is interest in wastewater treatment with well agglomerated sludge to the extent of granule formation. Current state-of-the-art wastewater treatment with granular biomass often faces unstable performance caused by disintegration of agglomerated biomass. The method disclosed herein is intended  
20 to enable wastewater treatment plants achieve faster start-ups and thereafter to maintain stable well-agglomerated biomass. The method can be used in both sewage and industrial wastewater treatment, and at new plants and existing facilities. The method can also serve to reduce the foot-prints of land area at new plants and expand treatment capacity at existing facilities (by increasing MLSS concentration) and hence avoiding need for substantial  
25 additional investments for infrastructure. The method shall also have application where there is interest in control of membrane biofouling in MBRs with well flocculated sludge to the extent of granular biomass. Membrane biofouling is one of the most challenging hurdles in

current application of the MBR for wastewater treatment and water reclamation. By addition of extracted signalling molecules of AI-2 and AHLs and eDNA into the MBR, this invention will enable biosludge to quickly flocculate and thereafter to reduce microbial attachment onto membrane surface and consequently reduce incidence of membrane fouling.

5

## Experimental Section

### Example 1

Fig. 2 shows that in a pure culture of *Escherichia coli*, the cell motility after addition of 4,5-dihydroxy-2,3-pentanedione (DPD; an AI-2 precursor) in the range of 0.5 to 10  $\mu$ M was increased by 6-17 fold, as compared to the control. As a result, the initial aggregation rate increased accordingly, leading to a rapid microbial aggregation. This confirms that bacterial motility drives bacteria to come together to form aggregates.

### Example 2

Aerobic granules form through bacterium-to-bacterium adhesion and are considered as a special type of biofilm. In order to further confirm the findings obtained in the pure culture (Example 1), AI-2 was first immobilized on a solid surface which was then put into contact with mixed-species bacteria for 1 hr. The microscopic images in Fig. 3 clearly show that large microbial colonies/aggregates were rapidly developed with exposure to AI-2 as compared to the control without AI-2. This provides visual evidence that the addition of a signalling molecule to culture media can lead to rapid microbial aggregation.

### Example 3

Figs. 4 to 6 show that AI-2, AHLs and eDNA are essential to achieving aerobic granulation, and also shows that a high-concentration of such signalling molecules (AI-2, AHLs) and eDNA were present in the aerobic granules cultivated in the breeder reactor. It is expected that signalling molecules (AI-2 and AHLs) and eDNA extracted from or present in granules

that is equivalent to at least 1 - 10% of biomass in the wastewater treatment reactor will be needed to initiate rapid microbial aggregation in the wastewater treatment reactor.

**Claims**

1. A method of accelerating the growth of aerobic granules, comprising the step of adding:

- (a) one or more aggregating signalling compounds or precursors thereof; and/or
- (b) crushed aerobic granular biomass,

to an activated sludge, wherein:

(i) the amount of the one or more aggregating signalling compounds is equivalent to an amount extracted from an aerobic granular biomass that is about 1 wt% to about 10 wt% of the total estimated dry biomass in the activated sludge; and

(ii) the amount of the crushed aerobic granular biomass is in an amount of from about 1 wt% to about 10 wt% of the total estimated dry biomass of the activated sludge.

2. The method of Claim 1, wherein, when the method uses one or more aggregating signalling compounds, said compounds are selected from one or more of the group consisting of extracellular deoxyribose nucleic acid, acyl homoserine lactones and autoinducer-2.

3. The method of Claim 1, wherein, when the method uses a precursor of aggregating signalling compounds, said precursor is dihydroxy-2,3-pentanedione.

4. The method of Claim 1 or Claim 2, wherein, when the method uses one or more aggregating signalling compounds, the method further comprises the step of extracting the one or more aggregating signalling compounds from an aerobic granular biomass.

5. The method of Claim 4, wherein the one or more aggregating signalling compounds are extracted from the aerobic granular biomass by:

- (a) suspending the aerobic granular biomass in a liquid to generate a mixture;

- (b) homogenising the mixture, optionally by sonication;
- (c) concentrating the biomass in the mixture, optionally by centrifugation; and
- (d) separating the mixture into a liquid comprising the one or more aggregating signalling compounds and biomass.

6. The method of Claim 5, wherein the liquid comprising the one or more aggregating signalling compounds - is added directly to the activated sludge or is stored at -20°C or below for up to at least 3 months before being added to the activated sludge.

7. The method of Claim 5 or Claim 6, wherein, step (a) of Claim 5 is preceded by harvesting the aerobic granular biomass from a breeder reactor containing a mother liquor, comprising a liquid and the aerobic granular biomass, by concentrating the aerobic granular biomass in the mother liquor and separating the aerobic granular biomass from the liquid, optionally wherein the concentration step comprises centrifugation of the mother liquor.

8. The method of Claim 7, wherein the breeder reactor is a sequencing batch reactor, optionally wherein the volume of the breeder reactor is about 1% to 10% of the volume of the activated sludge.

9. The method of Claim 7 or Claim 8, wherein the breeder reactor initially comprises a precursor activated sludge and is operated under high selection pressure conditions to generate a mother liquor comprising a liquid and the aerobic granular biomass.

10. The method of Claim 9, wherein the high selection pressure conditions comprises a settling time of 2 to 30 minutes and/or a high shear force.

11. The method of Claim 10, wherein the precursor activated sludge further comprises self-aggregating microbial species with high hydrophobicity.
12. The method of any one of the preceding claims, wherein when the method uses the crushed aerobic granular biomass, the crushed aerobic granular biomass is prepared by obtaining an aerobic granular biomass and mechanically crushing it, optionally the mechanical crushing is achieved by the use of a mechanical beater or a blender.
13. The method of Claim 12, wherein, before the crushed aerobic granular biomass is added to the activated sludge, the crushed aerobic granular biomass is suspended in a liquid and homogenised, optionally by sonication, optionally wherein the crushed aerobic granular biomass is concentrated by removal of the liquid.
14. The method of Claim 12 or Claim 13, wherein the aerobic granular biomass is obtained by harvesting the aerobic granular biomass from a breeder reactor containing a mother liquor, comprising a liquid and the aerobic granular biomass, by concentrating the aerobic granular biomass in the mother liquor, optionally the concentration step comprises centrifugation of the mother liquor and separation of the aerobic granular biomass from the liquid.
15. The method of Claim 14, wherein the breeder reactor is a sequencing batch reactor, optionally wherein the volume of the breeder reactor is 1%-10% of the volume of the activated sludge.
16. The method of Claim 14 or Claim 15, wherein the breeder reactor initially comprises a precursor activated sludge and is operated under high selection pressure conditions to generate a mother liquor comprising a liquid and the aerobic granular biomass.

17. The method of Claim 16, wherein the high selection pressure conditions comprises a shorter settling time of 2 to 30 minutes and/or a high shear force.
18. The method of Claim 16 or Claim 17, wherein the precursor activated sludge further comprises self-aggregating microbial species with high hydrophobicity.
19. The method of any one of the preceding claims, wherein the activated sludge is contained within a wastewater reactor.
20. The method of Claim 19, wherein the wastewater reactor is a sequencing batch reactor or a membrane bioreactor.
21. The method of any one of the preceding claims, wherein the activated sludge is a flocculated activated sludge.

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## Figures

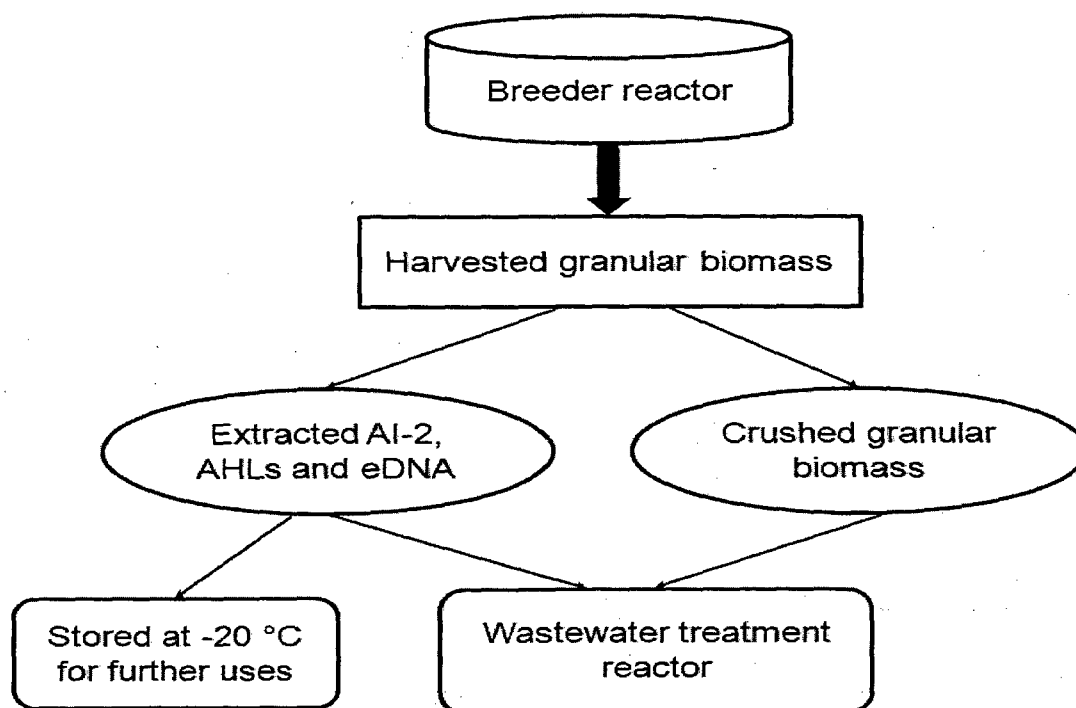


Fig. 1

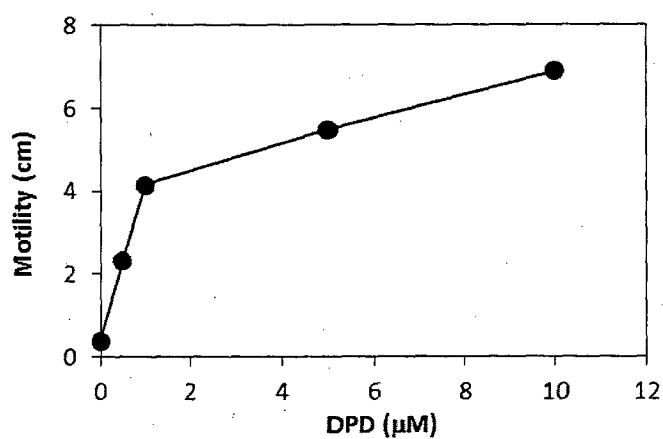


Fig. 2

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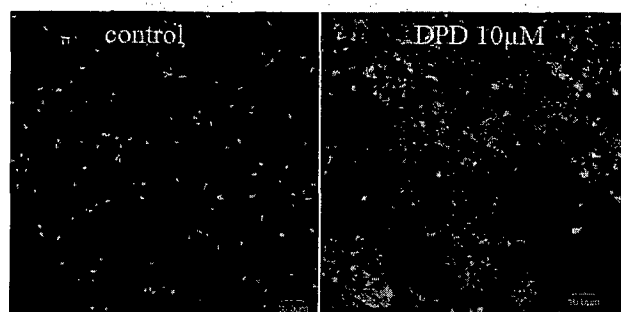


Fig. 3

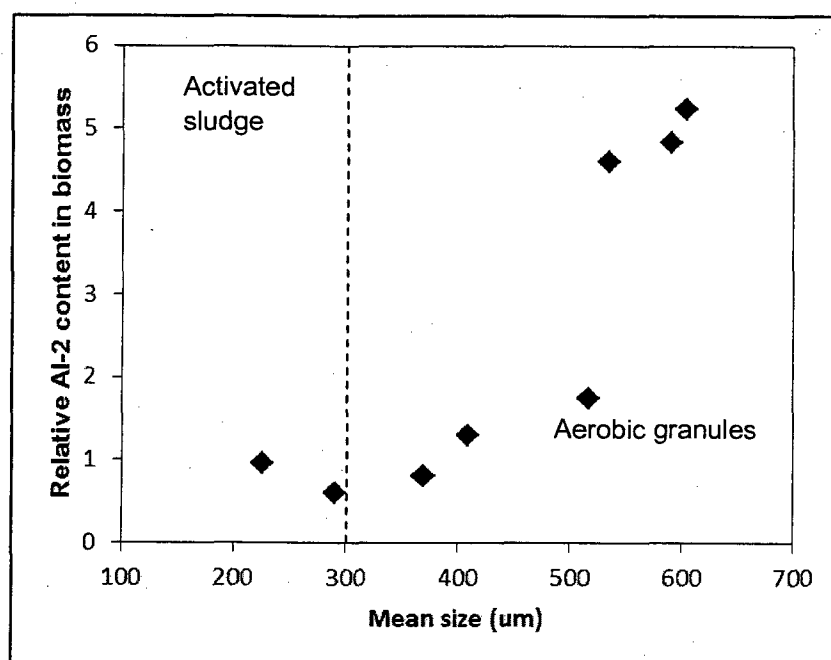


Fig. 4

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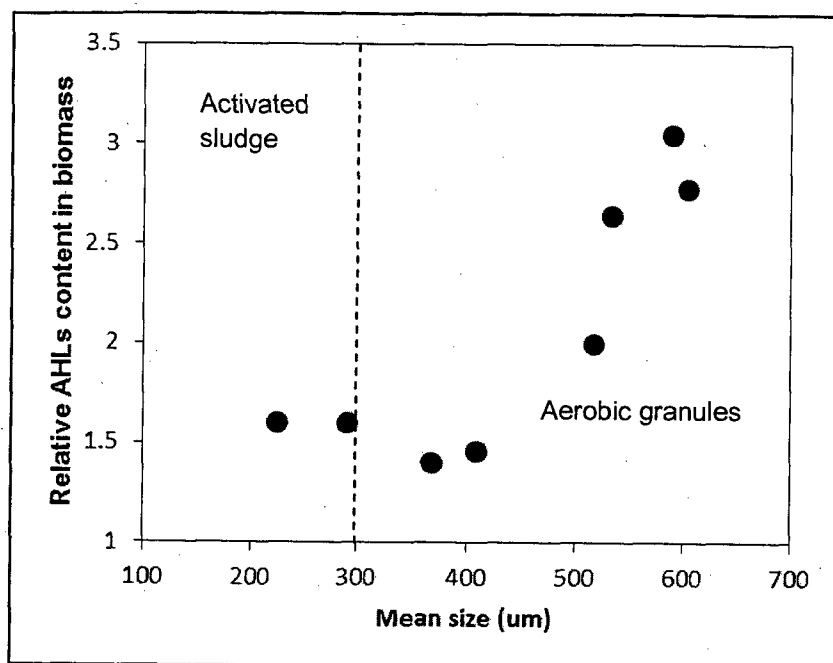


Fig. 5

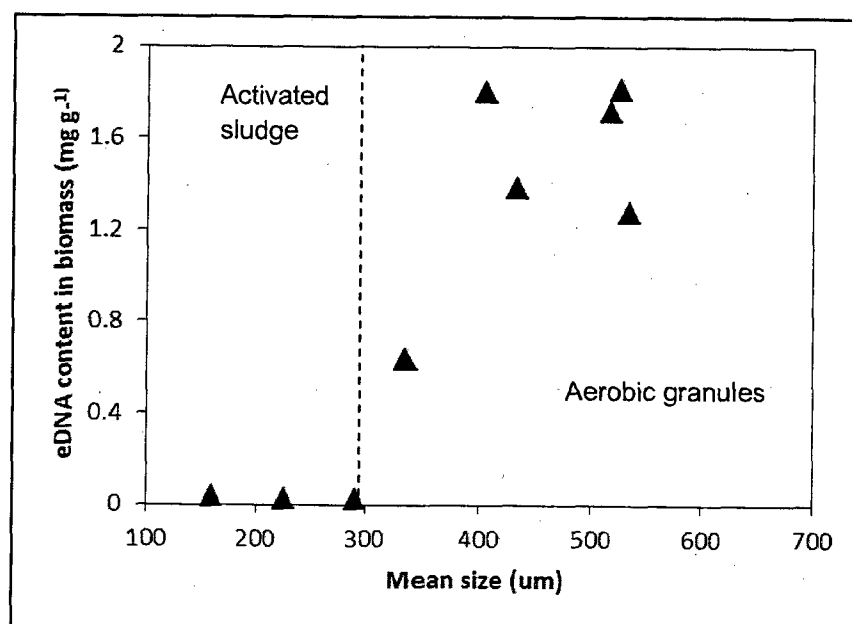


Fig. 6

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2013/000538

## A. CLASSIFICATION OF SUBJECT MATTER

C12N 1/00 (2006.01) C02F 3/12 (2006.01) C02F 9/14 (2006.01) C02F 11/02 (2006.01)

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)

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)

EPODOC and WPI/IC/CN C02F1 or C02F3 or C02F9 or C02F11 or C12N1 or C12N11 or C12N7 and Keyword(s) (aerobic biogranul+, N\_acylhomoserine, grow+, (activated 2w sludge), amount, crush+) &amp; like terms.

Cluster - SPATEN-English full text databases and Keyword(s) (aerobic biogranul+, N\_acylhomoserine, grow+, (activated 2w sludge), amount, crush+) &amp; like terms.

GooglePatent search and keywords ("aerobic granules" "signaling molecules")

GoogleScholar search and keywords (seed "activated sludge" "quorum sensing" wastewater growth adding granules)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------|-----------------------|
|           | Documents are listed in the continuation of Box C                                  |                       |

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| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | PCT/SG2013/000538             |
| Category*                                             | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | Relevant to claim No.         |
| X                                                     | <p>"Enhanced Phenol Biodegradation and Aerobic Granulation by Two Coaggregating Bacterial Strains", HE-LONG JIANG, JOO-HWA TAY, ABDUL MAJID MASZENAN, AND STEPHEN TIONG-LEE TAY, VOL. 40, 6137-6142, NO. 19, 2006 ENVIRONMENTAL SCIENCE &amp; TECHNOLOGY. Retrieved from the Internet &lt;URL: <a href="http://download.bioxon.com.cn/view/upload/month_0805/20080504_a4055030f913749d915cIKDfb30cGdO.attach.pdf">http://download.bioxon.com.cn/view/upload/month_0805/20080504_a4055030f913749d915cIKDfb30cGdO.attach.pdf</a>&gt;</p> <p>See sections: Title, Introduction, Bioaugmentation in Sequencing Batch Reactors (SBRs), Results and Discussion.</p> | 1b, 4-11, 19-21               |
| X                                                     | <p>WO 2011/106848 A1 (ENVIRONMENTAL BIOTECHNOLOGY CRC PTY LIMITED) 09 September 2011</p> <p>See the abstract; page 1 lines 24-35, page 2 lines 1-34, page 5 lines 20-25, page 6 lines 3-4, page 7 lines 16-32, page 24 lines 4-8; and claims 1-24.</p>                                                                                                                                                                                                                                                                                                                                                                                                        | 1c, 12-21                     |
|                                                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                               |

Form PCT/ISA/210 (fifth sheet) (July 2009)

## INTERNATIONAL SEARCH REPORT

International application No.  
**PCT/SG2013/000538****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:  
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
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 Supplemental Box for Details**

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 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.:

**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.

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| INTERNATIONAL SEARCH REPORT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | International application No.<br><b>PCT/SG2013/000538</b> |
| <b>Supplemental Box</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                           |
| <p><b>Continuation of: Box III</b></p> <p>This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.</p> <p>This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:</p> <p>□ Independent claim 1a and dependent claims are directed to a method of accelerating the growth of aerobic granules comprising the step of adding: (a) one or more aggregating signaling compounds or precursors thereof; and (b) crushed aerobic granular biomass, to an activated sludge, wherein: (i) the amount of the one or more aggregating signaling compounds is equivalent to an amount extracted from an aerobic granular biomass that is about 1 wt% to about 10 wt% of the total estimated dry biomass in the activated sludge; and (ii) the amount of the crushed aerobic granular biomass is in an amount of from about 1 wt% to about 10 wt% of the total estimated dry biomass of the activated sludge. The feature of adding to an activated sludge the synergistic effect of both (a) an aggregating signaling compound and (b) crushed aerobic granular biomass is specific to this group of claims.</p> <p>□ Independent claim 1b and dependent claims are directed to a method of accelerating the growth of aerobic granules comprising the step of adding one or more aggregating signaling compounds or precursors thereof to an activated sludge, wherein the amount of the one or more aggregating signaling compounds is equivalent to an amount extracted from an aerobic granular biomass that is about 1 wt% to about 10 wt% of the total estimated dry biomass in the activated sludge. The feature of adding to an activated sludge an aggregating signaling compound is specific to this group of claims.</p> <p>□ Independent claim 1c and dependent claims are directed to a method of accelerating the growth of aerobic granules comprising the step of adding crushed aerobic granular biomass, to an activated sludge, wherein the amount of the crushed aerobic granular biomass is in an amount of from about 1 wt% to about 10 wt% of the total estimated dry biomass of the activated sludge. The feature of adding to an activated sludge crushed aerobic granular biomass is specific to this group of claims.</p> <p>PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.</p> <p>When there is no special technical feature common to all the claimed inventions there is no unity of invention.</p> <p>In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied <i>a priori</i>.</p> |                                                           |
| Form PCT/ISA/210 (Supplemental Box) (July 2009)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                           |

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|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------|-----------------------------------------------------------|-------------------------|
| <b>INTERNATIONAL SEARCH REPORT</b><br>Information on patent family members                                                                                                                                                                                            |                         | International application No.<br><b>PCT/SG2013/000538</b> |                         |
| This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information. |                         |                                                           |                         |
| <b>Patent Document/s Cited in Search Report</b>                                                                                                                                                                                                                       |                         | <b>Patent Family Member/s</b>                             |                         |
| <b>Publication Number</b>                                                                                                                                                                                                                                             | <b>Publication Date</b> | <b>Publication Number</b>                                 | <b>Publication Date</b> |
| WO 2011/106848 A1                                                                                                                                                                                                                                                     | 09 Sep 2011             | CA 2828775 A1                                             | 09 Sep 2011             |
|                                                                                                                                                                                                                                                                       |                         | CN 103068746 A                                            | 24 Apr 2013             |
|                                                                                                                                                                                                                                                                       |                         | EP 2542507 A1                                             | 09 Jan 2013             |
|                                                                                                                                                                                                                                                                       |                         | US 2013075327 A1                                          | 28 Mar 2013             |
|                                                                                                                                                                                                                                                                       |                         | WO 2011106848 A1                                          | 09 Sep 2011             |
| <b>End of Annex</b>                                                                                                                                                                                                                                                   |                         |                                                           |                         |
|                                                                                                                                                                                                                                                                       |                         |                                                           |                         |
| Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.<br>Form PCT/ISA/210 (Family Annex)(July 2009)                                                                                               |                         |                                                           |                         |