GRANULAR MICROBIAL FORMULATION

Field of invention

The present invention relates to a granular microbial formulation and method of producing the formulation. More particularly, but not exclusively, the invention relates to a self-aggregated granular microbial formulation and method of producing the formulation under aseptic conditions, the formulation for use in the treatment of wastewater or other contaminated aqueous bodies.

Background of invention

Industrialisation and agricultural activities have led to the release of a variety of synthetic organic compounds into the water which spread rapidly throughout the environment. Many of these organic pollutants, even in small concentrations, adversely affect life forms, and create serious environmental threats.

Toxicity, carcinogenicity, and mutagenicity are the most critical biological properties of a potential pollutant. Removal of these xenobiotics and hazardous materials from wastewaters and contaminated aqueous bodies has become a major issue in recent years largely due to change in regulatory strategy. Biotechnological options are being sought for the purpose. New approaches for the treatment of water and wastewater containing xenobiotics are the use of specific microbial culture developed either by conventional enrichment and mutation or by genetic engineering and suitable reactor designs. A prevailing belief has been that, over time, the proper indigenous microbes will populate in a treatment plant and become acclimated to the waste. This approach, however, may not be sufficient to accumulate enough cells which can degrade the specific xenobiotic.

In addition, water and wastewater treatment systems regularly receive shock loads containing heavy doses of detergents, sanitizing agents, fats, oil, grease, chemicals and fibrous compounds. An influx of these complex substances can adversely affect the delicate balance of naturally occurring microorganisms in the water and wastewater treatment system, rendering the system ineffective. When indigenous microbial

populations within the system are non-existent or out of balance, the end result is an inefficient system. Wastewater treatment systems devoid of this natural biological process are typically plagued by sludge build-ups, tank deposits, fouled drain fields, backups and anaerobic odour problems.

US 6,383,379 discloses a method of preparing a microbial culture for wastewater treatment to deal with these problems. This method consisted of the steps of supplying an aerated material derived from an aeration tank to a bioreactor and cultivating microorganisms existing in the aerated material by adding a special culture medium. In this invention, indigenous microorganisms in wastewater treatment systems were selected in a separate bioreactor using a special culture media. One of main shortcomings for this invention is that one aeration tank needs to be installed and linked with treatment system. In addition, the tank needs to be operated continuously regardless of system operation in effective or ineffective condition.

One of other solutions is the bioaugmentation of selected microbial culture into process streams in case that these biological treatment systems are ineffective. Bioaugmentation is addition of microbes to contaminated water, materials or sites. The microbial culture available commercially for bioaugmentation is classified into a solid type formulation and a liquid type formulation. The solid type microbial culture may be prepared by enrichment cultivating a microorganism, inoculating the microorganism to a cereal medium, cultivating, hot-drying and crushing. Otherwise, the solid type microbial culture may be prepared by cultivating an inoculum in a liquid medium, freeze drying the cultured material and mixing the freeze dried material with a rice straw, rice bran, sawdust, fallen leaves or cereal. In a different way, the solid type microbial culture may be prepared by cultivating an inoculum in a liquid medium, adding a surfactant to the liquid medium and spray drying the cultured material.

The liquid type microbial culture may be prepared by enrichment cultivating a microorganism in a liquid medium, adding a silicon oil and nonionic surfactant to the liquid medium. Of the two types of microbial cultures (liquid type microbial culture and solid type microbial culture), each suffers from drawbacks. For example, the solid type microbial culture has a relatively long preservation time. However, it has a relatively long

activation time. The liquid type microbial culture has a relatively short activation time. However, it has a relatively short preservation time.

The cell immobilization techniques may afford a great of potential of improving the quality of microbial formulation. The immobilization methods of microorganisms in which various natural or synthetic materials are used to bring about the immobilization of microorganisms, have received attentions in recent years. US 6,153,416 discloses one entrapment type immobilization method employing calcium alginate-polyethylene glycol-polyethylene imide for immobilization of microbial cells. US 6,406,882 discloses another immobilization method employing coconut fiber for immobilization of microbial cells. Cell immobilization may provide protection against substrate toxicity under high toxic substrate concentrations. In addition, these immobilization methods may extend storage time. However, these immobilization methods require additional materials as support carriers; this directly decreases biomass concentrations of microbial formulations.

Therefore, there is continued interest in the development of new microbial formulation for bioaugmentation in treatment of water and wastewater streams, water and wastewater bodies, ground-waters and other contaminated aqueous bodies to enhance the process efficiency and reduce operation cost.

Summary of invention

In accordance with a first aspect of the invention, there is provided a method of producing a granular microbial formulation for the treatment of wastewater or other contaminated aqueous bodies, the method comprising the steps of:

(a) isolating a plurality of different microorganisms;
(b) mixing the plurality of different microorganisms in a reactor;
(c) feeding a liquid media into the reactor;
(d) supplying the reactor with an oxygen-containing gas;
(e) allowing a biomass of the plurality of different microorganisms to settle;
(f) discharging and replacing a portion of the liquid media in the reactor; and
(g) harvesting the microbial formulation for storage.

Preferably, the granular microbial formulation is self-aggregated and the method does not require a support material for the aggregation of the microbial formulation. Advantageously, the present invention does away with various natural or synthetic support substrates or materials to bring about the aggregation or immobilisation of the microorganisms.

Preferably, the plurality of microorganisms are nature-occurring and non-pathogenic. Still more preferably, the plurality of microorganisms is selected from the group consisting of: *Propioniferax*-like, *Rhodococcus erythropolis*, *Xenophilus azovorans*, *Comamonas* sp., *Hydrogenophaga palleronii*, *Zoogloea ramigera*, *Pseudomonas putida* and *Gordonia* sp.

Preferably, the steps (c) to (f) is a single reaction cycle, the reaction cycle being repeated a plurality of times to produce the granular microbial formulation of predetermined physical properties.

Preferably, the method is carried out under sterile conditions. More preferably, the reactor is autoclaved at 120°C for 25 minutes. The liquid media is fed into the reactor through an autoclaved membrane filter with a pore size not larger than 0.2 μm. The oxygen-containing gas is supplied into the reactor through an autoclaved membrane filter with a pore size not larger than 0.3 μm.

Preferably, the granular microbial formulation is stored between 4 to 25°C.

In accordance with a second aspect of the invention, there is provided a granular microbial formulation formed by a method according to the first aspect of the invention wherein the formulation comprises a plurality of microorganisms capable of cell-to-cell self aggregation without a support material.

Preferably, the plurality of microorganisms is selected from the group consisting of: *Propioniferax*-like, *Rhodococcus erythropolis*, *Xenophilus azovorans*, *Comamonas* sp., *Hydrogenophaga palleronii*, *Zoogloea ramigera*, *Pseudomonas putida* and *Gordonia* sp. Still preferably, the microbial formulation comprises nature-occurring and non-pathogenic microorganisms.

Preferably, the average particle size of the granules is between 100 to 6,000 µm.

Preferably, the formulation has a well-defined appearance and visible with the naked eye as separate entities after settling.

Advantageously, the formulation of the present invention may be used in bioaugmentation in the treatment of water and wastewater streams, water and wastewater bodies, ground-waters and other contaminated aqueous bodies.

Bioaugmentation can be defined as the application of selected microorganisms to enhance the microbial populations of an operating waste treatment facility to improve the efficiency or lower the operating costs.

Successful bioaugmentation requires total system management which includes the control of environmental conditions, proper application, process monitoring and maintenance of desired population diversity. Microorganisms are the primary decomposers of all organic pollutants, and thus are essentially the driving force. Typical bioaugmentation products are blends of naturally occurring microbes selected on the basis of accelerated reproduction rates and their ability to perform specific functions, such a good floc forming abilities to enhance settling or their exceptional performance at eliminating a wide range of recalcitrant compounds prevalent in the wastewater.

Advantageously, the granular microbial formulation developed in the present invention possesses high catabolism capacity, a relatively short activation time and a relatively long preservation time, and easy separation from effluent to avoid washout.

Brief Description of Figures

In order that the present invention may be fully understood and readily put into practical effect, there shall now be described by way of non-limitative examples only preferred embodiments of the present invention, the description being with reference to the accompanying illustrative figures.

In the figures:

Figure 1 is an image analysis picture of the strain PG-08 autoaggregates;

Figure 2 is a scanning electron microscopy picture of the strain PG-08 autoaggregates;

Figure 3 is a schematic drawing of the system for carrying out the method according to an embodiment of the present invention;

Figure 4 is a picture image of biomass harvested according to the method of the present invention, the biomass harvested on day 5;

Figure 5 is a picture image of biomass harvested according to the method of the present invention, the biomass harvested on day 28;

Figure 6 is a graph showing the comparison of specific phenol degradation rate between the strain PG-03 and a granular microbial formulation according to an embodiment of the invention;

Figure 7 is a graph showing the effect of storage on activity of a granular microbial formulation according to an embodiment of the invention; and

Figure 8 is a graph showing effect of bioaugmentation of self-aggregated granular microbial formulation.

Detailed Description of the Preferred Embodiments

By "self-aggregated" (when referred to the present formulation), it is meant the microorganisms are aggregated together into a cluster without the aid of any additional support material.

By "support material", it is meant any natural or synthetic material used to bring about aggregation or immobilisation of microorganisms.

The present invention relates to development of a self-aggregated granular microbial formulation from nature-occurring and non-pathogenic bacteria without the need for inert support materials. Those bacteria were isolated from activated sludge flocs or aerobic granules. The mineral medium, used for bacteria isolation and development of microbial formulation, contained (per litre) 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of NaCl, 0.02 g $FeCl_3 \cdot 6H_2O$, 0.01 g $CaCl_2$, phosphate buffer (1.35 g of KH_2PO_4 and 1.65 g of K_2HPO_4), and 1 ml of microelement solution. The composition of the microelement solution was: $FeCl_3 \cdot 6H_2O$, 1.5 g L^{-1}, H_3BO_3, 0.15 g L^{-1}, $CuSO_4 \cdot 5H_2O$, 0.03 g L^{-1}, KI, 0.03 g L^{-1}, $MnCl_2 \cdot 4H_2O$, 0.12 g L^{-1}, $Na_2MoO_4 \cdot 2H_2O$, 0.06 g L^{-1}, $ZnSO_4 \cdot 7H_2O$, 0.12 g L^{-1}, $CoCl_2 \cdot 6H_2O$, 0.15 g L^{-1}. The medium was sterilised by autoclaving for 20 min at 121°C. The phenol solution was filter-sterilised and added to the autoclaved medium. While this invention has been illustrated with reference to specific media and carbon source, it is understood by a person skilled in the art that any other culture media and carbon source can be used.

Portions of activated biomass were added to 15 mL of above isolation medium and aseptically mixed in a sterilised beaker in order to detach the activated biomass. The supernatant was then serially diluted with medium (10^1- to 10^7-fold dilutions), and 150 µl of each dilution was spread onto agar plates containing isolation medium (supplemented with 500 mg phenol per litre) solidified with 1.2% Bacto Agar (Difco, Detroit, USA). The plates were inverted and incubated in a 25°C incubator (Sanyo, Japan), and monitored over 4 weeks. Visible colonies were observed after 1 week of incubation.

Pure cultures of bacteria were isolated by cycles of replating onto mineral medium/phenol agar plates. Purity was confirmed by examination with both light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan) and scanning electron microscopy (SEM). For observations with SEM, bacterial cultures were prepared by first fixing for 1–4 h in 4% (vol/vol) glutaraldehyde. Fixed samples were washed 20 min three times with 0.10 M sodium cacodylate buffer, dehydrated in a graded ethanol series (25, 50, 75, 95, and 100% vol/vol) and dried with a critical point dryer (VG Microtech, East Grinstread, West Sussex, UK). Dried samples were then sputtercoated with gold to increase their electrical conductivity, at 20 mA in a high vacuum (2.8×10^{-6} Torr), and the samples was observed with a Stereoscan 420 (Leica Cambridge Instruments).

These bacterial isolate were then phylogeneticly identified according to 16S rRNA gene sequencing. A whole cell direct lysis PCR amplification method was used to amplify the 16S rRNA gene of isolates as described previously (Maszenan et al., "Three isolates of novel polyphosphate-accumulating Gram-positive cocci, obtained from activated sludge, belong to a new genus, *Tetrasphaera* gen. nov., and description of two new species, *Tetrasphaera japonica* sp. nov. and *Tetrasphaera australiensis* sp. nov.", International Journal of Systemic Evolution and Microbiology, 2000, 50:593-603). The nearly full-length 16S rRNA gene was amplified by PCR with forward primer Eubac27F and reverse primer Universal 1492R1 [Lane DJ, 1991. 16S/23S rRNA sequencing, p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Son, Ltd., Chichester, England]. The PCR products were purified with Qiagen PCR purification kit (Qiagen, Germany) according to manufacturer's instructions, and stored at -20°C before sequencing. The 16S rRNA gene sequence of the isolates were determined using the ABI model 310A DNA sequencer (Applied Biosystems, Perkin-Elmer) and the ABI PRISM® BigDye™ Terminator Ready Reaction Cycle Sequencing Kit (version 3.0) (Applied Biosystems, Perkin-Elmer). The sequences were compiled and aligned using the BioEdit program (Tom Hall, Department of Microbiology, North Carolina State University). The probable identity of the query sequence was determined using the BLAST algorithm (Altschul et al., "Basic local alignment search tool", Journal of Molecular Biology 1990, 215:403-410) with the database maintained by National Centre for Biotechnology Information (NCBI) (National Institutes of Health, USA). The final data set contained sequences of isolates and closely related organisms obtained from the Ribosomal Database Project (RDP) [Maidak et al., "The RDP- II (Ribosomal Database Project)", Nucleic Acids Research 2001, 29:173-174] or GenBank, and consisted of homologous sites at which the sequences were unambiguously aligned.

Eight bacterial strains were selected for preparation of self-aggregated granular microbial formulation. The three strains, *Zoogloea ramigera* BS-01, *Pseudomonas putida* BS-02, and *Gordonia* sp. BS-03, were isolated from activated sludge taken from a wastewater treatment plant. The other five strains, *Propioniferax*-like PG-02, *Rhodococcus erythropolis* PG-03, *Xenophilus azovorans* PG-05, *Comamonas* sp. PG-08, *Hydrogenophaga palleronii* PG-10, were isolated from aerobic granules (Jiang et al.,

"Bacterial diversity and function of aerobic granules engineered in a sequencing batch reactor for phenol degradation", Applied and Environmental Microbiology, 2004, 70: 6767-6775). Although the species level of the two strains BS-03 and PG-08 was not determined, the two strain *Gordonia* sp. BS-03 and *Comamonas* sp. PG-08 were most related to *Gordonia amicalis* and *Comamonas nitrativorans* species, respectively, according to comparison of 16S rRNA sequencing. All these strains are not pathogens, as there is no report of infections in man, animals and plants by these bacterial species.

Based on Gram-staining experiment, the three strains *Gordonia* sp. BS-03, *Propioniferax*-like PG-02 and *Rhodococcus erythropolis* PG-03 belong to Gram-positive bacteria. The other five strains are Gram-negative bacteria. Autoaggregation properties of the eight strains were measured using the spectrophotometric method. The eight strains were individually grown on the mineral medium including 0.25 g phenol per litre. Liquid cultures were incubated at 25°C in a shaker with 150 rpm. At the exponential stationary phase, cells were harvested by centrifugation and washed twice in carbon substrate-free mineral medium. The final cell pellet was re-suspended in the medium and dispersed in a Mini BeadBeater (Biospec Products, OK, USA) at 4600 rpm for 1 min, followed by dilution with medium to give an optical density (OD) at 600 nm of 0.5 units, determined using a UV-visible spectrophotometer (Perkin-Elmer, USA). For the autoaggregation assay, 8 mL of the final culture suspensions was transferred to tubes and then allowed to stand at room temperature (20 ± 2°C) for 30 min. The supernatant with a volume of 1 mL was carefully pipetted into a micro-cuvette and the optical density was measured at 600 nm. The autoaggregation index for a strain (x) was calculated by comparing initial OD ($OD_{(x,initial)}$) and OD value after a standing time of 30 min ($OD_{(x,\ 30)}$), as follows:

$$\text{Autoaggregation index (\%)} = \frac{OD_{(x,initial)} - OD_{(x,30)}}{OD_{(x,initial)}} \times 100$$

The autoaggregation indices for the eight bacterial strains were shown in **Table 1**. Of the eight strains, the strain PG-08 possessed the strongest autoaggregation ability. Autoaggregation took place when PG-08 was cultivated in a shaking incubator in phenol medium and cell aggregates with a compact structure and a mean size of 0.45 mm were formed as shown by image analysis microscopy (**Figure 1**) and scanning electron microscopy (**Figure 2**). Cell aggregates of the strain PG-08 possessed good settleability

as these aggregates settled immediately to the bottom of the reagent bottle after shaking was ceased for only one minute. Therefore, the cell aggregates of the strain PG-08 can serve as biocarriers for immobilization of other bacterial cells.

Table 1 Autoaggregation indices for strains

Strain	BS-01	BS-02	BS-03	PG-02	PG-03	PG-05	PG-08	PG-10
Autoaggregation Index (%)	68.7	25.2	12.3	36.2	0.2	7.7	95.5	1.3

To develop the self-aggregated granular microbial formulation, the eight strains were cultivated separately in batch systems in the mineral medium including 0.25 g phenol per litre. Other microorganisms suitable for use in the present invention may be added or substituted where such microorgamisms comprise at least the following characteristics: nature-occurring, and lack of pathogenicity. In the exponential-growth phase, cells for each strain were harvested through centrifugation. Biomass of all the eight strains with almost amount for each strain was mixed and introduced into the autoclaved column-type glass reactor R1 (1) with a working volume of 4 litres as shown in **Figure 3**. The reactor R1 (1) was operated in a sequential model controlled by a timer (2) with a cycle time ranging from 24 to 8 hours. The liquid media was fed into the reactor (1) by a feeding pump (3) through an autoclaved membrane filter with a pore size of 0.2 μm (4). An oxygen-containing gas was supplied into the reactor by a gas pump (5) through an autoclaved membrane filter with a pore size of 0.3 μm (6). Effluent was drawn at the middle port of the reactor by a pump (7). The media filling and effluent discharge time were each 20 min, the settling time was varied from 2 h to 15 min and the remainder was the reaction time. To avoid contamination of other microorganisms into the reactor, gas-out pipe was shut by an electron-controlled valve (8) when gas supply was stopped. In addition, a control reactor R2 (not shown), which had the same geometrical configuration, was set up for negative control. There is no difference between the operation in the two reactors R1 and R2, except no biomass was inoculated into the reactor R2. The liquid in the reactor R2 was taken regularly and spread into R2A agar plates. No biomass growth was found in the control reactor R2.

The effluent was analyzed for phenol and the biomass sample for mixed liquor suspended solids (SS), and specific oxygen utilization rate (SOUR) by following a standard method (APHA, 1995, Standard methods for the examination of water and waste water, 19th edn, Washington DC: American Public Health Association). The biomass size was measured by a laser particle size analysis system (MasterSizer Series 2600; Malvern Instruments Ltd, Malvern, Worcestershirw, UK) when the size was less than 1880 μm or an image analysis system (Quantimer 500 Image Analyser; Leica Cambridge Instruments GmbH, 4 Nubloch, Germany) when the size was greater than 1880 μm. Microbial observation was conducted by using either microscopy or image analysis (IA). The evolution of granular microbial formulation in the reactor R1 was monitored by means of optical microscopy, an IA technique and SEM during the reactor operation fed with phenol as sole carbon substrate. The physical characteristics and microbial activity of sludge in terms of SVI, settling velocity, size, SOUR, and strength were also determined during the experiments.

Formation of self-aggregated granular microbial formulation

After five days of operation, granular microbial formulation was observed in the R1 (**Figure 4**). Granular microbial formulation at this stage had a compact and dense structure with a clear boundary. The formation of granular microbial formulation was initiated through cell-to-cell self-aggregation as natural or synthetic materials were not added. Biomass concentration in R1 on day five was low and about 0.2 g SS L^{-1}. With reactor operation, biomass concentration increased gradually and reached 4.2 g SS L^{-1} on day 42. Granular microbial formulation existed in R1 as predominance after day 28. The image picture of granular microbial formulation on day 28 was shown in **Figure 5**. SEM showed that the granular microbial formulation had a smooth outer surface with a compact and regular appearance. These microorganisms are embedded in an extracellular polymeric matrix. Since then, biomass was taken daily from the reactor for storage in room temperature. The reactors could be operated stably for a period of four months.

Physical characteristics of self-aggregated granular microbial formulation

The characteristics of the fresh sample of the granular microbial formulation taken from the reactor on day 60 were determined and shown in **Table 2**:

TABLE 2 Characteristics of fresh and storage granular microbial formulation

Characteristics of fresh and storage granular microbial formulation				
	Fresh sample	45 day storage sample at 25 °C	180 day storage sample at 4 °C	180 day storage sample at 25 °C
Average diameter (mm)	1.6	1.5	1.52	1.47
Settling velocity (m h^{-1})	26.2	25.8	25.2	24.7
Biomass density (g L^{-1})	28.6	28.7	27.2	26.5

The microbial products had a mean diameter of 1.6 mm, with a range of 0.5-3.0 mm. The mature microbial formulation cultivated in the reactor R1 was found to have very good settleability. The settling velocity was as high as 26.2 m h^{-1}, a dry biomass density reached to 28.6 g L^{-1}. High settling velocity and biomass density of granular microbial formulation would result from the denser microbial structure. In biological systems, biomass serves as the biocatalyst for the degradation of organic matter. Obviously, a high retainable biomass in the reactor would ensure a faster and more efficient removal of organic pollutants, while excellent settling cultures are essential for the good functioning of biological treatment systems.

Phenol degradation capacity of granular microbial formulation

Specific phenol degradation rates under an initial phenol concentration of 250 mg L^{-1} among granular microbial formulation and single bacterial strains were compared in **Figure 6.** Experiments were performed in triplicate in 500 ml serum bottles. Each bottle contained 100 ml phenol media. Granular microbial formulation and individual strains were introduced into the medium respectively, and all reagent bottles were capped immediately and incubated on a 150 rpm orbital shaker in the dark at 25°C. Phenol concentrations were assayed at regular intervals during incubation. The specific phenol degradation rates were calculated by performing a linear regression on initial curves of

cell phenol disappearance, respectively, against time. Biomass concentrations for bacterial strains were determined by filtering the cell suspension through a 0.2 µm filter and drying the filter and cells to a constant weight for 24 h at 80°C. Among the eight bacterial strains, the strain PG-03 possessed the highest specific phenol degradation rate and was 0.15 g phenol g SS^{-1} h^{-1}. In comparison, the specific phenol degradation rate of the strain PG-03 was much less than that of the granular microbial formulation developed in this invention. The granular microbial formulation had a specific phenol degradation rate of 1.52 g phenol g SS^{-1} h^{-1}, ten times higher than that of the strain PG-03 (**Figure 6**).

Effect of preservation in room temperature

To investigate the effect of preservation on the self-aggregated granular microbial formulation, the granular microbial formulation sample taken from the reactor R1 on day 60 and stored in 1 x phosphate buffer solution (PBS) in room temperature (25°C) or 4°C. After 45 days and 180 days of storage, the physical characteristics and activity were measured. Results showed that the storage either in between the temperatures of 4°C to 25°C did not affect negatively the physical characteristics of the granular microbial formulation in terms of diameter, settling velocity and biomass density as was shown earlier in **Table 2**. There was no significant difference in these parameters between fresh and storage samples.

Oxygen uptake by the biomass was measured to monitor changes in biomass activity after storage. For specific oxygen utilization rate (SOUR) determinations, samples with known VSS concentrations were filled to overflowing in standard BOD (biochemical oxygen demand) bottles. Dissolved oxygen (DO) levels were measured with a DO meter (YSI 5000) at time intervals of 10 seconds over a 15 minute period or until DO became limiting, whichever occurred earlier. The oxygen consumption rate in mg l^{-1} min^{-1} was determined from the slope of the line of best fit to a plot of DO against time. SOUR was calculated by dividing the oxygen consumption rate by the VSS concentration. A phenol concentration of 250 mg L^{-1} was used in the SOUR assays. SOUR for fresh biomass sample was 130 mg DO g VSS^{-1} h^{-1} (**Figure 7**). The value of SOUR for the 45 day storage biomass sample reached to 8.9 mg DO g VSS^{-1} h^{-1}. The metabolic activity could be quickly recovered. After activating the storage biomass for

one day, the value of SOUR increased to 45 mg DO g VSS^{-1} h^{-1} (**Figure 7**). The value of SOUR reached to 120.6 mg DO g VSS^{-1} h^{-1} after three days of activation (**Figure 7**). The biomass still maintained activity after 180 days of storage. Although storage temperatures did not affect the stability of the self-aggregated granular microbial formulation, the sample stored in 4°C had higher activity than that stored in 25°C. While the value of SOUR was 87.6 g VSS^{-1} h^{-1} after three days of activation for storage sample in 25°C, the value of SOUR reached to 112.3 mg DO g VSS^{-1} h^{-1} after three days of activation for storage sample in 4°C.

Bioaugmentation of self-aggregated granular microbial formulation

Experiment about the phenol biodegradation by bioaugmentation of self-aggregated granular microbial formulation was carried out at a concentration of 1000 mg phenol L^{-1}. Four autoclaved 500 mL reagent bottles which served as batch systems were run in parallel at the same time with 100 mL of the mineral medium in each bottle. Four bottles were inoculated with activated sludge at a concentration of 2 g SS L^{-1}. In the four bottles, two bottles were bioaugmented with self-aggregated granular microbial formulation at 10% of activated sludge biomass (w/w), and the other two bottles were used as control without bioaugmentation. Prior to bioaugmentation, the granular microbial formulation after 180 day storage at 4°C was taken out and activated for only one day. All reagent bottles were covered with aluminium foil to prevent photo-oxidation. Then, these bottles were incubated at 150 rpm at 25°C. Oxygen concentrations were maintained above 5.5 mg L^{-1} through regular intake of air passed through two sterile 0.2-μm-pore size filters. Culture samples were regularly taken for measurement of phenol concentrations as shown in **Figure 8**. It was clear that bioaugmentation of the self-aggregated granular microbial formulation significantly improved phenol biodegradation.

Based on above illustrations, main advantages for the self-aggregated granular microbial formulation developed in the present invention include as following:

1. The formulation is formed through cell-to-cell self aggregation. Natural or synthetic support materials are not required for formation of the self-aggregated granular microbial formulation.
2. The self-aggregated granular microbial formulation possesses compact physical structure. The compact structure can protect microorganisms in the microbial

formulation from inhibition from toxic substrates at high concentrations through concentration gradient that develops within the granular microbial formulation because of diffusional resistance. Therefore, the granular microbial formulation can maintain high catabolic activity under a condition of high toxic/inhibition waste concentrations.

3. The self-aggregated granular microbial formulation possesses good settleability, and can be easily retained in biological treatment systems after bioaugmentation.
4. The self-aggregated granular microbial formulation can maintain physical structure and stability even after 180 day storage.
5. The activity of self-aggregated granular microbial formulation can be quickly recovered even after 180 day storage.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purpose of illustration, it will be apparent to those skilled in the art that certain of the details described herein can be varied considerably without departing from the basic principles of the invention. It is therefore intended that the appended claims encompass such modifications and enhancements.

CLAIMS

1. A method of producing a granular microbial formulation for the treatment of waste water and other contaminated aqueous bodies, the method comprising the steps of:

(a) isolating a plurality of different microorganisms;

(b) mixing the plurality of different microorganisms in a reactor;

(c) feeding a liquid media into the reactor;

(d) supplying the reactor with an oxygen-containing gas;

(e) allowing a biomass of the plurality of different microorganisms to settle;

(f) discharging and replacing a portion of the liquid media in the reactor; and

(g) harvesting the granular microbial formulation for storage.

2. The method according to claim 1, wherein the granular microbial formulation is self-aggregated and the method does not require a support material for the aggregation of the microbial formulation.

3. The method according to any one of claims 1 or 2, wherein the plurality of microorganisms is nature-occurring and non-pathogenic.

4. The method according to claim 3, wherein the plurality of microorganisms is selected from the group consisting of: *Propioniferax*-like, *Rhodococcus erythropolis*, *Xenophilus azovorans*, *Comamonas* sp., *Hydrogenophaga palleronii*, *Zoogloea ramigera*, *Pseudomonas putida* and *Gordonia* sp.

5. The method according to any one of the preceding claims, wherein the steps (c) to (f) is a single reaction cycle, the reaction cycle being repeated a plurality of times to produce the granular microbial formulation of predetermined physical properties.

6. The method according to any one of the preceding claims, wherein the method is carried out under sterile conditions.

7. The method according to claim 6, wherein the reactor is autoclaved at 120°C for 25 minutes.

8. The method according to claim 6, wherein the liquid media is fed into the reactor through an autoclaved membrane filter with a pore size not larger than 0.2 µm.

9. The method according to claim 6, wherein the oxygen-containing gas is supplied into the reactor through an autoclaved membrane filter with a pore size not larger than 0.3 µm.

10. The method according to any one of the preceding claims, wherein the granular microbial formulation is stored in temperature between 4°C and 25°C.

11. A granular microbial formulation formed by a method according to any one of claims 1 to 10, wherein the formulation comprises a plurality of microorganisms capable of cell-to-cell self aggregation without a support material.

12. The formulation according to claim 11, wherein the plurality of microorganisms is selected from the group consisting of: *Propioniferax*-like, *Rhodococcus erythropolis*, *Xenophilus azovorans*, *Comamonas* sp., *Hydrogenophaga palleronii*, *Zoogloea ramigera*, *Pseudomonas putida* and *Gordonia* sp.

13. The formulation according to any one of claims 11 or 12, wherein the average particle size of the granules is between 100 to 6,000 µm.

14. The formulation according to claim 11, wherein the microbial formulation comprises nature-occurring and non-pathogenic microorganisms.

15. The formulation according to any one of claims 11 to 14, wherein the formulation has a well-defined appearance and visible with the naked eye as separate entities after settling.