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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 February 2004 (12.02.2004)

PCT

(10) International Publication Number
WO 2004/013054 A1

(51) International Patent Classification[7]: C02F 3/34, C07K 16/12, C12N 9/00

(21) International Application Number: PCT/EP2003/007495

(22) International Filing Date: 10 July 2003 (10.07.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0217417.5 27 July 2002 (27.07.2002) GB

(71) Applicant *(for all designated States except US)*: **THE QUEEN'S UNIVERSITY OF BELFAST** [GB/GB]; University Road, Belfast BT7 1NN, Northern Ireland (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LARKIN, Michael, James** [GB/GB]; Queen's University of Belfast, University Road, Belfast BT7 1NN, Northern Ireland (GB). **CONNERY, Nichola, Louise** [GB/GB]; Queen's University of Belfast, University Road, Belfast Bt7 1NN, Northern Ireland (GB).

(74) Agent: **MURGYTROYD & COMPANY**; Scotland House, 165-169 Scotland Street, Glasgow G5 8PL (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/013054 A1

(54) Title: A METHOD OF CONTROLLING MICROTHRIX PARVICELLA PROLIFERATION OF WASTE TREATMENT PLANTS

(57) Abstract: A method of controlling Microthrix parvicella proliferation in a waste treatment plant comprises the step of inactivating one or more Microthrix parvicella growth factors in the waste, wherein the growth factors are selected from the group consisting of: gamma-butyrolactone and Rpf protein. The or each growth factors is inactivated by including in the waste a bacterial isolate which uses the or each growth factor as a carbon source and/or nitrogen source, typically a sole carbon source. Alternatively, one or more of the growth factors may be inactivated by adding to the waste an enzyme preparation which functions to specifically cleave the or each growth factor. Suitably, an antibody preparation, which includes one or more antibodies which specifically target and inactivate one or more of the growth factors, is included in the waste being treated. A composition for carrying out the methods of the invention is also described.

A METHOD OF CONTROLLING MICROTHRIX PARVICELLA PROLIFERATION OF WASTE TREATMENT PLANTS

Introduction

This invention relates to a method of controlling the proliferation of Microthrix parvicella in waste treatment plants, and particularly in activated sludge waste treatment plants.

"Microthrix parvicella" is a filamentous bacterium, belonging to the order Actinomycetales. This bacterium is the major causative organism of bulking and foaming in activated sludge waste-water treatment plants in Europe (Eikelboom, 1977; Rossetti et al, 1994; Kristensen et al, 1994), Australia (Seviour et al, 1994), South Africa (Blackbeard et al, 1986), and the USA (Richard et al, 1982). This organism is frequently found in vast numbers in waste treatment systems, where it causes solids separation problems. This problem is manifested as several major operational problems

that can have serious environmental and economic repercussions. These include; reduced final effluent quality (through poor settling i.e., bulking, which in turn leads to worsened effluent quality due to loss of biomass, which normally reduces carbonaceous matter, and BOD levels); quantity of solids going to landfill or for incineration; load exertion on pumps and a potential biohazard through the production of foams, containing high numbers of potentially pathogenic bacteria which can aerosolise. These problematic manifestations can readily result in the affected waste-water treatment plant in question failing to meet the effluent standards set by the relevant government authorities. In some countries, financial penalisation for the plant operator can be costly.

One of the major issues that has been raised regarding the proliferation of this microorganism is it's extremely slow-growth when in pure laboratory culture. This is in stark contrast to the apparent abundance of the organism in waste treatment systems encountering a Microthrix parvicella-induced bulking event.

The concept of cell-cell signalling among bacteria is now a generally accepted phenomenon. Since the 1960's, the role of N-acyl homoserine lactones (AHL's) as quorum-sensing molecules has been observed amongst many species of Gram-negative bacteria. More recently, it has come to light that signalling molecules may play a role in the growth

of Gram-positive bacteria (such as the Actinomycetales). The signalling molecules which may affect the onset of secondary metabolism processes and the growth characteristics of the Actinomycetales are comprised of a family of compounds containing a gamma -butyrolactone ring (similar to the AHL's) and small proteins regarded as Rpf-like proteins (resuscitation promoting factor-like proteins).

Amongst the genus Streptomyces, the most widely studied gamma-butyrolactone-containing molecule is A-Factor (2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone). This molecule, and a range of other butyrolactone-containing compounds (acylated at the C3 position with acyl moieties of variable size and composition), do not affect growth rate but markedly affect secondary metabolism events involved in morphogenesis (Nodwell and Losick, 1996) and antibiotic production (Bibb, 1996). A-factor is a low molecular weight regulatory compound produced by S. griseus which induces antibiotic production and sporulation. The compound is notable in that it is effective in extremely low concentrations in the nM range (Horinouchi and Beppu, 1992).

The other group of signalling molecules noted for some members of this group of bacteria are the Rpf-like proteins. The Rpf protein was discovered as a factor produced by actively growing Micrococcus luteus cells, which, in picomolar concentrations, will resuscitate "dormant" cells which have

undergone a period of starvation (Mukamolova et al, 1998). Again the effect is not noted to increase growth rate per se. The protein is ~ 17kDa in size and more recently, genes encoding proteins similar to Rpf have been found throughout a cohort of Actinomycetes, including Mycobacterium tuberculosis, M. leprae, M. bovis, M. smegmatis, Corynebacterium glutamicum and several species of Streptomyces (Mukamolova et al, 1998).

Current control strategies for Microthrix parvicella -induced activated sludge bulking involves some of the following methods. The addition of chemical chelating agents, polyelectrolytes, which act by physically sequestering activated sludge flocs, thus enhancing the compaction and sedimentation rate of the activated sludge. Oxidising agents such as permanganate and chlorine may be used to "bleach" the whole sludge biomass. Operational conditions may be altered to waste higher volumes of sludge, thereby reducing the total biomass in the system. The problem with these methods for controlling sludge bulking is that they are not only empirical, but extremely non-specific. Indeed, the use of aggressive oxidising agents or "bleaches" not only "kills off" the majority of the biomass, but also results in elevated doses of these agents in the resulting effluent. The use of polyelectrolytes does not remove Microthrix, the causative organism, and it is returned to the oxidation ditch with the return activated sludge (RAS).

It is an object of the invention to overcome at least some of the above problems by specifically targeting molecules that are growth factor and enhance the growth of Microthrix parvicella.

Statements of Invention

According to the invention, there is provided a method of controlling Microthrix parvicella proliferation in a composition, suitably waste in a waste treatment plant, comprising the step of inactivating one or more Microthrix parvicella growth factors in the composition, wherein the growth factors are selected from the group consisting of: gamma-butyrolactone and Rpf protein. In this specification, the term "inactivating" should be understood to include degrading, cleaving, chemically modifying, and other means of rendering the growth factor inactive. The term "gamma-butyrolactone" should be understood to include gamma-butyrolactone containing molecules and related compounds such as those having a butyrolactone ring. The term "Rpf protein" should be understood to include Rpf-like proteins. The term "composition" should be understood as primarily relating to a waste in a water treatment plant, however it should not be restricted to this meaning. As such, the method of the invention could be equelly applied to the treatment of seawater, fresh water, rivers, lakes, canals, resevoirs etc.

Thus, in one embodiment of the invention, one or more of the growth factors may be inactivated by including in the waste a bacterial isolate which uses one of the growth factors as a carbon source and/or nitrogen source, typically a sole carbon source. Bacterial strains which use gamma-butyrolactone as a carbon source are likely to occur commonly in nature and may be selected from the group of bacteria including Mycobacterium vaccae which grows on gamma-butyrolactone (Hamamura et al, 1999) and isolated strains such as Rhodococcus rhodochrous NCIMB 13064 which utilises this compound as a sole carbon source (Curragh et al, 1994) and other similar strains isolated from waste-water treatment systems. Bacterial strains which use peptides as carbon and nitrogen source through the action of protease enzymes are very common in nature (Rao and Deshpande 1998). Bacterial strains that utilise Rpf protein as a carbon and or nitrogen source may be selected from a wide variety of these known protease producing bacterial strains. Typically, a combination of bacterial isolates selected from the one or more of the above two groups may be added to the waste.

In an alternative embodiment of the invention, or in addition to the above, one or more of the growth factors may be inactivated by adding to the waste an enzyme preparation which functions to specifically cleave the or each growth factor. Non specific and or specific microbial proteases to cleave Rpf and Rpf-like proteins could be used in conjunction with

esterases to reverse the internal cyclic structure of the gamma-butyrolactone ring and thus inactivate it. A further possible method of inactivating one or more of the growth factors is to include in the waste, antibodies which specifically target and inactivate one or more of the growth factors. Such antibodies may be monoclonal or polyclonal antibodies. Antibody production is a commonly known methodology (Heddy Zola, 2000) and has been applied to detect specific microorganisms in waste-waters (e.g. Brigmon et al, 1995). The technique of developing monoclonal antibodies to very specifically bind to and detect small molecules in water samples is established (Knopp, 1995) and has been developed further by use of the phage display technique for the production of antibodies (Hall et al 1997). Monoclonal antibodies have been developed, for example, to specifically bind to, and thus detect, small molecules as chemical pollutants; for example hormone disrupting chemicals such as estrogen and related molecules (Goda et al, 2000). The sensitivity of this method with respect to such small molecules has been improved by coupling the antibodies to magnetic particles (Matsunaga et al, 2003). Such methodology could be employed to bind to and remove or inactivate the target molecules that act as growth factors for Microthrix parvicella.

Chemical selectors may be employed to inactivate the gamma-butyrolactone based on chemical modification of the gamma-butyrolactone ring. Areas or

"selectors", typically upstream from the main aeration basin of a waste-water treatment plant, which provide conditions that favour such modification would be employed. These conditions could include excess hydroxyl (OH^-) ions (for example, by increasing the pH) to incur hydroxide ion catalysis of the internal ester bond. Alternatively, by introducing either an acid or a base (alkali), general acid or general base catalysis of the ester bond would occur, by attacking the bond from the slightly negatively charged oxygen atom or the slightly positively charged carbon atom, respectively. These chemical modifications would inactivate the factor and result in the production of a linear carboxylic acid.

The invention also relates to a composition for inactivating Microthrix parvicella growth factors comprising one or more inactivating agents selected from the group consisting of:
a bacterial preparation which uses the growth factor as a carbon and/or nitrogen source, typically a sole carbon source; an enzyme preparation which specifically cleaves the growth factor; and an antibody preparation which specifically targets and inactivates the growth factor,
wherein the at least one growth factor is selected from the group consisting of: gamma-butyrolactone and Rpf protein.

The invention also relates to a composition for inactivating Microthrix parvicella growth factors

consisting essentially of one or more inactivating agents selected from the group consisting of: a bacterial preparation which uses the growth factor as a carbon source, typically a sole carbon source; an enzyme preparation which specifically cleaves the growth factor; and an antibody preparation which specifically targets and inactivates the growth factor, wherein the at least one growth factor is selected from the group consisting of: gamma-butyrolactone and Rpf protein. Any combination of the above mentioned inactivating agents is envisgaed.

The invention also relates to the use of a composition according to the invention in waste treatment plants to control the proliferation of Microthrix parvicella.

It is reasonable to suggest that both Rpf-like proteins and A-factor and A-factor homologues will be present in these waste treatment systems as the producing organisms are likely to present in the highly heterogeneous culture mix. Furthermore, they are known to be effective at extremely low concentrations. It has been shown that gamma-butyrolactone is produced as an end product metabolite resulting from the aerobic β-oxidation of chlorinated fatty acids (Curragh et al, 1994). Such substrates and this type of metabolic pathway occur in many waste treatment systems. Moreover, Microthrix parvicella has been associated with waste treatment systems loaded with high levels of fatty

acids (Andreasen and Neilsen, 1998); many of which are likely to be chlorinated due to the extensive use of hypochlorite disinfectants.

In light of the growing evidence of factors which can have effects at low concentrations on a number of the members of the Actinomycetales (of which Microthrix parvicvella is a member), the effect of gamma-butyrolactone, and sterile, spent culture supernatants from growing cells which produce these compounds, as well as Rpf protein, has been investigated using Microthrix parvicella laboratory cultures to establish that these alone and in combination may enhance growth.

Detailed Description of the Invention

The invention will be more clearly understood from the following description of some embodiments thereof, given by way of example only.

Materials and Methods

Microthrix parvicella RN1 (Rossetti et al. (1997) was obtained from Dr. Valter Tandoi (Water Research Institute, Rome). The growth medium (R2AM) used was a modified form of R2A (Reasoner and Geldreich (1985)), with the additions of (per litre medium): $CaCl_2.2H_2O$, 0.05g; cycloheximide, 20mg; NMS Trace Elements (Atlas (1995)), vitamins (Eikelboom (1968)). To test growth on sludge supernatants and spent culture media, 10X R2AM was supplemented with

centrifuged sludge, filter sterilised (0.2µm), (R2AMS) or filter sterilised (0.2µm) spent culture supernatants from M. luteus (NCIMB 13267) and Streptomyces griseus (NCIMB). Due to the paucity of growth and the "clumpy" nature of the filamentous bacteria, the organism was cultivated in sterile microtitre plate wells (total volume of 150µl/well) in the presence of a metabolic indicator, XTT (Sigma) at a final concentration of 0.25mM. Optical densities (620 nm) of wells were measured using SLT Labinstruments (Austria) Microtitre Plate Reader. Plates were incubated at 15°C (Knoop and Kunst, 1998).

Results and Discussion

The cell culture doubling times of Microthrix parvicella in situ, were not determined directly, but in batch culture, on R2AM medium (see materials and methods). It can be seen in Table 1 that the addition of low concentrations of gamma-butyrolactone more than halved the cell doubling times of Microthrix in pure culture. The addition of sterilised spent culture supernatants from Micrococcus luteus and Streptomyces griseus also reduced the cell culture doubling times to less than 5 days. The control medium, R2AM, without addition of any growth factor, is a complex medium in which carbon sources would not be limiting (see materials and methods). This yielded a cell culture doubling time of >20days, however doubling times as long as

>100days have been observed on this medium in other experiments (data not shown).

Table 1. Increase in growth rate observed as a decrease in doubling times of M. parvicella in the presence of gamma-butyrolactone and spent culture supernatants containing Rpf (M. luteus) and A-factor (S. griseus)

gamma-butyrolactone Concentration (mM)	Growth rate - Doubling Time (Days) R2AM medium
0	21
0.001	8
0.01	7
M. luteus spent culture supernatant	1-5
S. griseus spent culture supernatant	<2

It has been shown that the cell culture doubling time of Microthrix parvicella is significantly reduced (and thus growth rate increased) by the addition of these growth factors to the pure culture medium. These doubling times are equal to (and in the case of M. luteus and S. griseus spent supernatants, lesser than) the doubling times observed when grown on R2AMS (see materials and methods). Considering this data in the light of the

typical sludge age (roughly equivalent to the mean cell residence time of around 10 days) in a waste treatment plant which exhibits Microthrix parvicella bulking (depending on temperature and weather), the effect seen here would be the difference between a Microthrix parvicella bulking event occurring or not occurring.

A number of bacterial strains have been isolated which have been shown to utilise gamma-butyrolactone as a sole carbon source. Using the same method used to obtain these organisms, other isolates can be obtained which utilise A-factor Rpf and Rpf-like factors. These organisms can be employed to degrade, or "mop up", the signalling molecules in waste treatment systems in a number of ways. For example, ensuring a higher proportion of the growth factor degrading microorganisms are present in waste treatment plant will reduce the concentration of signalling molecules. Alternatively, the incorporation of "selectors" could be employed. Selectors are compartmentalised areas within the waste treatment system, usually upstream of the aeration basin. These areas contain an environment which should be unfavourable to adverse parameters or microorganisms within the activated sludge. These have previously been used to administer e.g, temperature shock, and regions of high food/microorganism ratio (F/M ratio) in an attempt to control bulking. By utilising this method in a novel way, extremely high numbers of these bacteria would be incorporated in selectors to degrade growth

factors in the input waste. Another control method of the invention is to apply an enzyme preparation to the waste treatment works. Using the isolates obtained, an enzyme preparation can be obtained to specifically cleave the signalling molecule in question, thus effectively removing it from the system. Specific antibodies can also be raised to similarly target and inactivate the signalling molecules. Using technology to raise specific monoclonal and polyclonal antibodies to our laboratory strain of Microthrix, antibodies which have a high specific affinity to these molecules could be administered, therefore blocking routes of entry to the cell and subsequent attachment to receptor proteins or target DNA.

There are a number of operational and economic advantages to all of the above methods for controlling Microthrix induced sludge bulking. Current control methods are expensive: large volumes of consumables (oxidising agents and polyelectrolytes) are continually purchased. The suggested methods here are inexpensive and are largely self-renewable. Current methods are non-specific, targeting all of the biomass, and also inhibiting/killing the floc-forming bacteria in the process. The method of the invention offers a much higher degree of specificity. Previous methods introduce toxic oxidising agents to the system and result in elevated levels of these in the final effluent; however, the methods of the invention

employ resources which are already present in the activated sludge biomass.

The invention is limited to the embodiments disclosed herein which may be varied without departing from the spirit of the invention.

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CLAIMS

1. A method of controlling Microthrix parvicella proliferation in a waste treatment plant comprising the step of inactivating one or more Microthrix parvicella growth factors in the waste, wherein the growth factors are selected from the group consisting of: gamma-butyrolactone and Rpf protein.

2. A method as claimed in Claim 1 in which the or each growth factors is inactivated by including in the waste a bacterial isolate which uses the or each growth factor as a carbon source and/or nitrogen source, typically a sole carbon source.

3. A method as claimed in Claim 2 in which the bacterial isolate which use gamma-butyrolactone as a carbon source are selected from the group comprising: bacteria including Mycobacterium vaccae; isolated strains such as Rhodococcus rhodochrous NCIMB 13064; and other similar strains isolated from waste-water treatment systems.

4. A method as claimed in any preceding Claim in which one or more of the growth factors may be inactivated by adding to the waste an enzyme preparation which functions to specifically cleave the or each growth factor.

5. A method as claimed in Claim 4 in which the enzyme preparation comprises non specific and or

specific microbial proteases to cleave Rpf and Rpf-like proteins.

6. A method as claimed in Claim 5 in which the enzyme preparation further includes esterases to reverse the internal cyclic structure of the gamma-butyrolactone ring and thus inactivate it.

7. A method as claimed in any preceding Claim in which an antibody preparation, which includes one or more antibodies which specifically target and inactivate one or more of the growth factors, is included in the waste being treated.

8. A method as claimed in Claim 7 in which the antibody preparation includes magnetic particles to which the antibodies are coupled.

9. A method as claimed in any preceding Claim in which growth factors in the waste are inactivated by means of chemical modification of the gamma-butyrolactone ring.

10. A composition for inactivating Microthrix parvicella growth factors comprising one or more inactivating agents selected from the group consisting of:
a bacterial preparation which uses the growth factor as a carbon and/or nitrogen source, typically a sole carbon source;
an enzyme preparation which specifically cleaves the growth factor; and an antibody preparation which

specifically targets and inactivates the growth factor,
wherein the at least one growth factor is selected from the group consisting of: gamma-butyrolactone and Rpf protein.

11. A composition for inactivating Microthrix parvicella growth factors consisting essentially of one or more inactivating agents selected from the group consisting of:
a bacterial preparation which uses the growth factor as a carbon source, typically a sole carbon source;
an enzyme preparation which specifically cleaves the growth factor; and
an antibody preparation which specifically targets and inactivates the growth factor,
wherein the at least one growth factor is selected from the group consisting of: gamma-butyrolactone and Rpf protein. Any combination of the above mentioned inactivating agents is envisgaed.

12. Use of a composition according to either Claim 10 or 11 in waste treatment plants to control the proliferation of Microthrix parvicella.

INTERNATIONAL SEARCH REPORT

PCT/EP 03/07495

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C02F3/34 C07K16/12 C12N9/00

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)
IPC 7 C02F C07K C12N

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

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

EPO-Internal, BIOSIS, MEDLINE, COMPENDEX, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 55624 A (YOUNG MICHAEL ;KELL DOUGLAS B (GB); UNIV WALES (GB); YOUNG DANIELL) 10 December 1998 (1998-12-10) page 3 -page 7 page 18 -page 19, line 36	10,11
A	page 23 -page 25, line 39 page 38, line 10 - line 30; claims 1-22	1-9,12
X	CURRAGH HELEN ET AL: "Haloalkane degradation and assimilation by Rhodococcus rhodochrous NCIMB 13064" MICROBIOLOGY (READING), vol. 140, no. 6, 1994, pages 1433-1442, XP001155768 cited in the application page 1439 -page 1441	1-12

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	FUJITA MASANORI ET AL: "An enzyme-linked immunosorbent assay for detection of linear alkylbenzene sulfonate: Development and field studies" ENVIRONMENTAL SCIENCE AND TECHNOLOGY, vol. 32, no. 8, 15 April 1998 (1998-04-15), pages 1143-1146, XP002259489 ISSN: 0013-936X abstract ---	1-12
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