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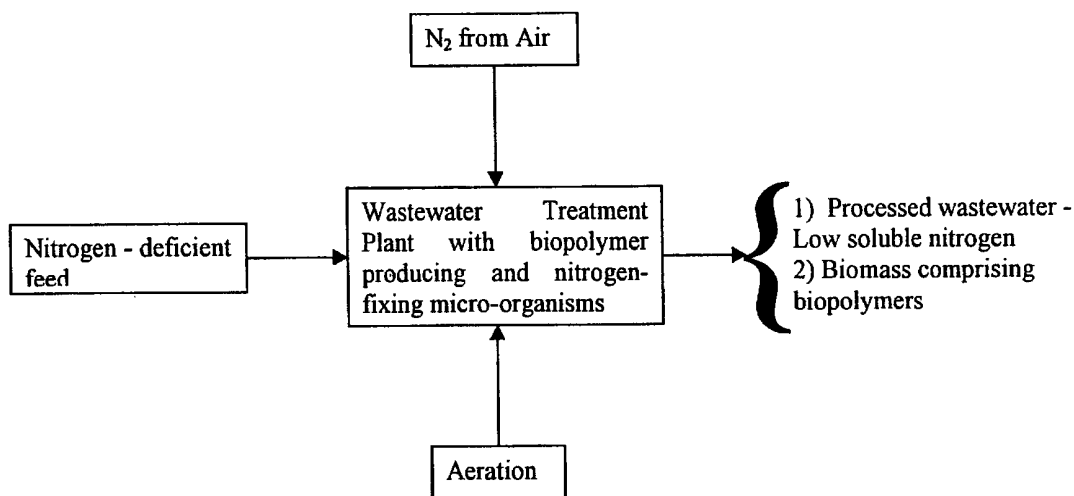
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(54) Title: PROCESS FOR PRODUCTION OF BIOPOLYMERS FROM NITROGEN DEFICIENT WASTEWATER



(57) Abstract: A process for producing biopolymers comprises passing nitrogen deficient wastewater through a treatment system comprising micro-organisms which grow aerobically in nitrogen deficient wastewater, and controlling the environment in the treatment system by maintaining a sufficiently stable dissolved oxygen level to thereby encourage the growth and/or activity of micro-organisms which produce the biopolymers, and recovering the biomass produced and the biopolymers.

PROCESS FOR PRODUCTION OF BIOPOLYMERS FROM NITROGEN DEFICIENT WASTEWATER

FIELD OF INVENTION

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The invention comprises a process for the production of biopolymers from nitrogen-deficient wastewater, such as wastewater from pulp and paper production.

BACKGROUND

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Aerobic biological processes are commonly used in the treatment of industrial wastewater, before discharge to the environment. In such processes micro-organisms consume wastewater contaminants, and are encouraged to proliferate in reactor systems under intensive aeration. A variety of process configurations are known, such as activated
15 sludge, moving bed biofilm reactors, membrane bioreactors, aerated lagoons and aerated stabilisation basins. Aerated lagoons and aeration stabilisation basins are examples of low rate biological treatment systems, whilst activated sludge and variant systems and moving bed biofilm reactors are high rate biological treatment systems.

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Pulp and paper mill wastewaters are typically deficient in nutrient nitrogen required to support bacterial growth for biological treatment. Conventionally activated sludge processes used in the pulp and paper industry are operated with the addition of nitrogen to the system, typically in the form of urea. Other industrial wastewaters can also be nitrogen-deficient. By "nitrogen-deficient wastewater" is meant wastewater for which, due to lack
25 of available nitrogen in the wastewater, an additional nitrogen source is required for the biological consumption of the biodegradable organic material present.

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It has also previously been proposed to use industrial wastewaters to produce biopolymers having economic value. For example Japanese patent application 3143397 discloses a
30 method for the production of a polyhydroxyalkanoate (PHA) wherein a carbon source is added to an activated sludge so as to raise microbial numbers. Denitrification of this culture is then required prior to its aerobic culture under nitrogen deficient conditions to

achieve the intracellular accumulation of polyhydroxybutyric acid. Japanese patent 2514131 requires wastewater to be treated to undergo an initial anaerobic acid fermentation so as to achieve a conversion of the organic content of the wastewater to carboxylic acids. The fermented wastewater then undergoes a nitrogen removal step prior to its use as a feed for nitrogen fixing micro-organisms. Application of this method for the production of polymers, such as PHAs accumulated in the nitrogen-fixing micro-organisms, is also disclosed. Both methods require removal of nitrogen in a separate step, prior to the use of the wastewater for the production of polymer.

SUMMARY OF INVENTION

The invention provides an improved or at least alternative process for the production of hydroxyalkanoic acids or their polymers from wastewaters.

In broad terms the invention comprises a process for producing biopolymers comprising passing nitrogen deficient wastewater through a treatment system comprising micro-organisms which grow aerobically in nitrogen deficient wastewater and controlling the environment in said treatment system by maintaining a sufficiently stable dissolved oxygen level to thereby encourage the growth and/or activity of micro-organisms which produce the biopolymers, and recovering biomass produced and the biopolymers.

Typically the dissolved oxygen level in the treatment system is monitored and aeration is controlled to maintain a substantially stable dissolved oxygen level. The pH, temperature, loading rate and any nutrient addition will generally be conventional.

Ideally the process is also operated to remove contaminants from the wastewater and the dissolved oxygen is maintained at a level which also optimises organic matter removal in relation to biomass or biopolymer yield.

Preferably the process also includes maintaining a sufficiently stable dissolved oxygen level in the treatment system to also encourage the growth and/or activity of nitrogen-fixing micro-organisms, so that nitrogen supplementation is not required.

Preferably the biopolymer produced comprises a hydroxyalkanoic acid or a polyester thereof. Preferably the polyhydroxyalkanoate polyester produced includes 3-hydroxybutyric acid and/or 3-hydroxyvalerate and/or 3-hydroxyhexanoate and/or other hydroxyalkanoic acid congeners.

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Preferably the process includes maintaining the dissolved oxygen level at a stable level selected to thereby select the polymer composition of the biopolymer(s) produced such as the relative proportion of congeners.

10 Typically the wastewater is pulp and paper wastewater but the process of the invention may alternatively be applied to any other nitrogen-deficient industrial wastewater.

Typically the nitrogen-deficient wastewater may have a biochemical oxygen demand (BOD) to nitrogen (N) ratio of greater than 100:5, more typically greater than 100:2, most
15 typically greater than 100:1.

Typically the treatment system is a high rate biological treatment system such as an activated sludge system.

20 The micro-organisms may be indigenous to the wastewater and the process conditions are controlled to encourage growth of the required isolates of species, or the wastewater may be seeded with biopolymer producing and/or nitrogen fixing micro-organisms. One or more of the micro-organisms may be immobilised or restricted to a solid support submerged or held within the wastewater being treated.

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BRIEF DESCRIPTION OF THE FIGURES

The invention is further described with reference to the accompanying figures which are referred to in the examples in which:

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Figure 1 schematically shows one form of the process of the invention,

Figure 2 is a photomicrograph of isolate 8 (PHA stain, 1000 x magnification) which is referred to in example 3, and

Figure 3 is a total ion chromatogram of ethyl ester derivatives of an extract from biomass from nitrogen deficient wastewater, produced as described in example 3, confirming production of PHA.

DETAILED DESCRIPTION

We have found that by maintaining a sufficiently stable dissolved oxygen level in an aerobic biological treatment system comprising as a media nitrogen deficient wastewater, the growth and accumulation of PHA or other biopolymer producing micro-organisms occurs and is encouraged. Nitrogen-deficient wastewater is supplied to a treatment system such as an activated sludge system, which is typically operated at a normal operating pH, phosphorus level and temperature to facilitate biological growth, and with monitoring of the dissolved oxygen level and aeration control to maintain the dissolved oxygen level at a substantially stable level. The dissolved oxygen level is controlled at a level which is optimum for production of the biopolymers and preferably also efficient removal of organic matter, whilst producing an acceptable biomass yield. The stability of the dissolved oxygen level is believed to be of greater significance than the specific level.

Some industrial wastewater such as pulp and paper wastewater typically has a low nitrogen content relative to the organic carbon content. Nitrogen may be added as urea or in another form. However in the process of the invention the micro-organisms may also obtain sufficient nitrogen for growth by fixation of atmospheric nitrogen. Maintaining a substantially stable dissolved oxygen level also encourages nitrogen-fixing micro-organisms.

Known processes for producing biopolymers from pulp and paper wastewater may require anaerobic fermentation before aerobic growth of the biopolymer producing micro-organisms. We have found that in the process of the invention micro-organisms from pulp

and paper wastewater can produce PHAs without the requirement for an anaerobic fermentation prior to the productive growth of PHA producing micro-organisms.

The process of the invention is effectively carried out in an activated sludge treatment system, but may potentially be applied to other forms of high rate system. With an activated sludge system, the dissolved oxygen level, coupled with organic loading, solids retention time, and food to micro-organism ratio, is selected to give the best production of biopolymers, such as PHAs, and optionally removal of organic matter (as measured by COD, BOD or TOC). An activated sludge system for example may comprise multiple tanks or zones operating under different conditions and in such a multi tank/zone system the process of the invention may operate in, for example, two separate environments each having a different but stable dissolved oxygen level. For example a first tank/zone may optimise organics removal while a second tank/zone may be used to optimise PHA production. Both tanks may be nitrogen-fixing environments. In addition a PHA producing, nitrogen-fixing system of the invention, whether as a single or multi-tank system, may be used in conjunction with and prior to a conventional non-nitrogen-fixing system to process nitrogen-deficient wastewater, without the need for supplementary nitrogen, to produce PHAs and processed wastewater having a low soluble nitrogen content.

We have also found that it is possible to control the polymer composition of PHA polymer produced and in particular the relative proportion of congeners, by selecting the dissolved oxygen level at which the process is run. The polymer composition may comprise hydroxybutyrate, hydroxyvalerate and other higher carbon chain hydroxy-aliphatic acids. Different proportions of congeners are produced by different dissolved oxygen levels. For example, we have found that a greater hydroxy-valerate fraction may be produced at lower dissolved oxygen concentrations and a lower hydroxy-valerate fraction maybe produced at higher dissolved oxygen concentrations.

Under the conditions of the process of the invention PHA producing and optionally also nitrogen-fixing micro-organisms will proliferate. However seeding of the treatment system with appropriate organisms is not excluded. Micro-organisms that contain

members known to fix nitrogen and/or produce PHAs which could be used to seed the treatment system include: Azotobacteraceae, Enterobacteraceae, Spirochaetaceae, Rhizobiaceae, Pseudomonadaceae, Rhodospirillaceae, Chromatiaceae, Bacillaceae, Methlococcaceae, *Beijerinckia* spp, *Derxia* spp, *Azospirillum* spp, *Xanthobacter* spp, *Azorhizobium* spp.

The above is not intended to be exhaustive and there may be other species of biopolymer producing and/or nitrogen-fixing organisms which may be equally or more effective in the process of the invention.

The invention is further illustrated by the following non-limiting examples:

Example 1

Production of intracellular biopolymers and removal of soluble organic material (COD(s)) was studied in a continuous stirred tank reactor (CSTR) system without recycle under three different feed substrate concentrations. The CSTR was operated under nitrogen fixing conditions of controlled dissolved oxygen. The BOD:N ratio was approximately 100:0.3.

Table 1 gives a summary of the main parameters obtained in this work for the three different feed substrate concentrations.

Table 1: Biomass N and treatment performance data obtained from CSTR

Parameter		Condition 1	Condition 2	Condition 3
Feed COD(s)	mg/L	450	840	630
HRT	d	0.5	0.5	0.5
Dissolved oxygen	%	30	30	30
Observed yield	mgTSS/mgCOD	0.18-0.26	0.14-0.19	0.14-0.19
COD(s) removed	mg/L	180-252	590-630	388-426
	%	44-53	72-74	63-66
Reactor biomass	mg/L	47-57	107-129	70-86

Parameter		Condition 1	Condition 2	Condition 3
N-fixation rate	mgN/mgTSS.d	0.09-0.16	0.13-0.15	0.12-0.15
Biomass N	mgN/mgTSS	0.04-0.07	0.06-0.07	0.06-0.07
Biomass P	mgP/mgTSS	.002-.007	.007-.013	0.070.011

Table 1 presents data on the nitrogen content of the biomass. For all feed substrate concentrations, the nitrogen content of the biomass was low (0.07 mg N/mg TSS or 7%) compared with 12%, the average composition of cell tissue (Pirt, 1975). The low values observed in this system were attributed to the intracellular storage of polymers resulting in an increased C:N ratio due to high cellular carbon content and low biomass nitrogen concentrations. This was confirmed with microscopic examination using a stain specific for the detection of PHAs.

Table 2: Description of isolates from a pulp and paper waste treatment system that tested positive for PHA production

	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Colony morphology on NFMSA agar	large, brown, irregular, raised, tough	yellow, round, smooth, glistening	bright yellow, dry, small, round	creamy - brown, round, smooth
Cell morphology (5 day old cultures on NFMS agar)	curved rods	dumbell rods	small dumbell rods	long dumbell rods
16S rDNA sequence identification	<i>Azorhizobium</i> spp.	<i>Xanthobacter</i> spp.	<i>Xanthobacter</i> spp.	<i>Xanthobacter</i> spp.
PHA stain	+	+	+	+
Ability to fix nitrogen	+	+	+	+

The PHA staining technique of (Gerhardt *et al.*, 1994) was used. Using this technique PHA granules stain darkly against a lighter background, as illustrated in Figure 2.

The above results evidence that PHA can be synthesised by micro-organisms indigenous to the treatment system with good treatment performance (as measured by COD(s) removal) in a nitrogen-fixing pulp and paper treatment system.

5 **Example 2**

A nitrogen fixing bioreactor treating a pulp and paper wastewater with a BOD:N ratio of approximately 100:0.8 was operated under nitrogen-fixing conditions. Samples from the mixed liquor were characterised and quantified using a transesterification ethyl/propyl-ester derivatisation and GC-MS technique.

A range of PHA contents between 1.2% to 11% dry weight of biomass were measured in the microbial biomass.

15 Figure 3 is a chromatogram showing the abundance of ethyl ester derivatives of an extract from the nitrogen fixing bioreactor. The PHA produced consisted of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate units. Benzoic acid was used as an internal standard. It has been observed that PHAs with different relative monomer compositions were produced under different bioreactor conditions.

20 **Example 3**

The following results were obtained from a single stage activated sludge pilot plant. The feed to the pilot plant was thermomechanical pulping wastewater (TMP) with a BOD:N ratio of 100:0.8 and a loading rate of BOD_5 $1.07 \text{ kg BOD}_5 \cdot \text{m}^{-3} \cdot \text{d}^{-1}$. Nitrogen was not supplemented. Dissolved oxygen was controlled at 14% in phase one of the experiment and 5% during phase two of the experiment.

Table 3: PHA production from a nitrogen-fixing activated sludge system treating pulp and paper wastewater

Nominal DO setting, %	DO level, % mean +/- 95 % CI	Total PHA production*, g/kg dry weight biomass	3-OH-butyrate, % total PHA*	3-OH-valerate, % total PHA*	valerate to butyrate ratio
14	13.79 +/- 0.02	41	83	17	0.20
	13.75 +/- 0.01	30	80	20	0.24
	13.76 +/- 0.04	68	83	17	0.20
	13.54 +/- 0.06	25	70	30	0.43
	mean	41	79	21	0.27
	95 % CI	19	6	6	0.10
5	4.46 +/- 0.02	44	47	53	1.15
	4.57 +/- 0.13	39	48	52	1.07
	4.53 +/- 0.04	40	70	30	0.43
	mean	41	55	45	0.88
	95 % CI	3	15	15	0.45

* hydroxybutyrate and hydroxyvalerate only congeners analysed in this example

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Biomass production rates: Pilot sludge discharge: 135.5 litres per cubic metre wastewater per day. Sludge dry weight concentration: 5 g/L wasted sludge

Sludge discharge rate: 677.5 g per cubic metre wastewater per day. PHA yield: 40 g/kg dry weight sludge

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PHA production rate: 27 g PHA per cubic metre wastewater per day

The results show that polyhydroxyalkanoates (PHAs) were produced from nitrogen deficient treatment system without nitrogen supplementation or the requirement of a nitrogen removal step. In this example, PHA composition was changed by selecting the dissolved oxygen concentration, with a greater hydroxyvalerate fraction being produced under lower dissolved oxygen concentrations. Hydroxyalkanoate monomeric composition determines the physical properties of the PHA polymer. For example, higher ratios of the C₅ - C₁₂ congeners lead to softer plastics. This example demonstrates that it is possible to

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alter the ratios of the congeners, such as hydroxybutyric and hydroxyvalerate acids and thereby control the physical properties of the polymer composition.

The foregoing describes the invention and examples thereof. Alterations and modifications as will be obvious to those skilled in the art are intended to be incorporated in the scope hereof.

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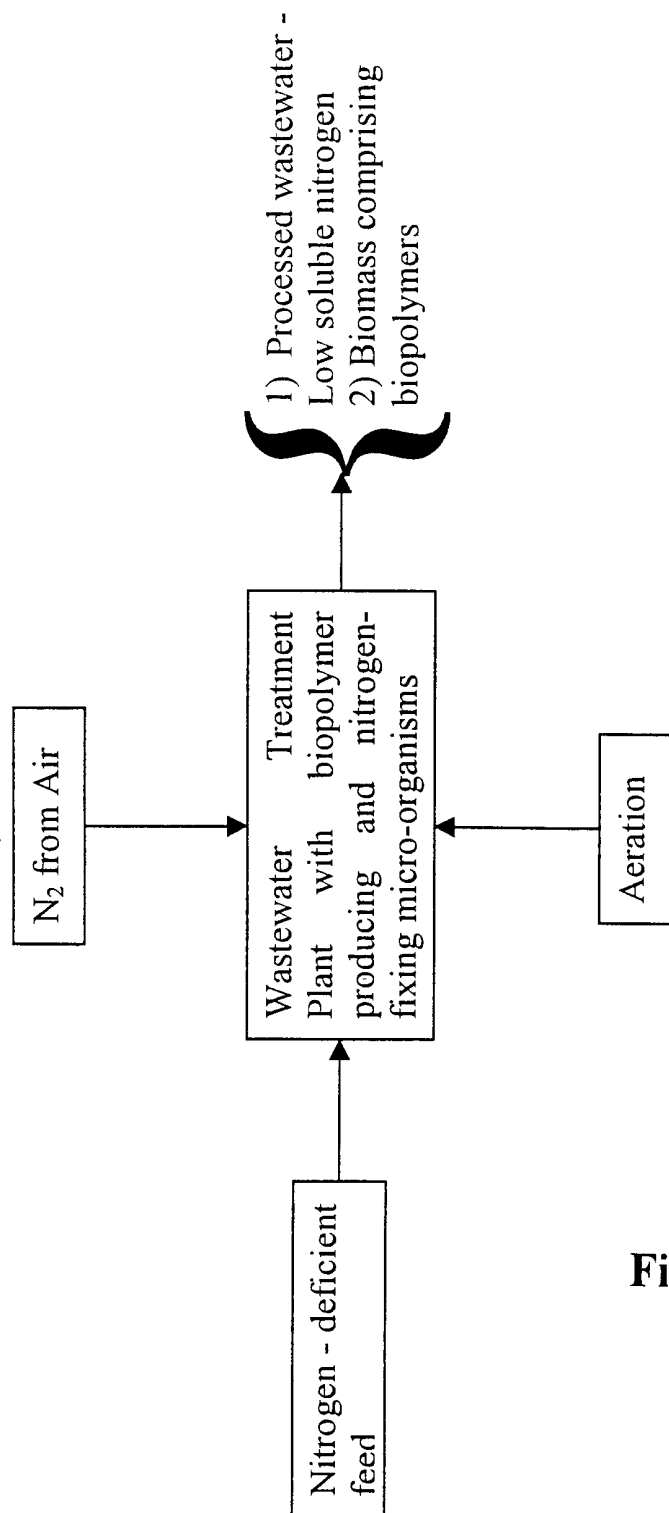
CLAIMS

1. A process for producing biopolymers comprising passing nitrogen deficient wastewater through a treatment system comprising micro-organisms which grow aerobically in nitrogen deficient wastewater, and controlling the environment in said treatment system by maintaining a sufficiently stable dissolved oxygen level to thereby encourage the growth and/or activity of micro-organisms which produce the biopolymers, and recovering the biomass produced and the biopolymers.
2. A process according to claim 1 including maintaining a sufficiently stable dissolved oxygen level in the treatment system to also encourage the growth and/or activity of nitrogen-fixing micro-organisms.
3. A process according to either claim 1 or claims 2 wherein the biopolymer produced comprises a hydroxyalkanoic acid or a polyester thereof.
4. A process according to claim 3 wherein the polyhydroxyalkanoate polyester produced includes 3-hydroxybutyric acid and/or 3-hydroxyvalerate and/or 3-hydroxyhexanoate and/or other hydroxyalkanoic acid congeners.
5. A process according to any one of claims 1 to 4 wherein the process excludes a transition from anaerobic to aerobic culture conditions or a requirement for a nitrogen supplementation or nitrogen removal step.
6. A process according to any one of claims 1 to 5 wherein the BOD:nitrogen ratio in the nitrogen-deficient wastewater is greater than 100:5.
7. A process according to any one of claims 1 to 5 wherein the BOD:nitrogen ratio in the nitrogen deficient wastewater is greater than 100:2.
8. A process according to any one of claims 1 to 5 wherein the BOD:nitrogen ratio in the nitrogen deficient wastewater is greater than 100:1.

9. A process according to any one of claims 1 to 8 wherein the wastewater is nitrogen deficient wastewater resulting from pulp or pulp and paper production.
10. A process according to any one of the preceding claims including controlling aeration in the treatment system to maintain said substantially stable dissolved oxygen level.
11. A process according to any one of the preceding claims including maintaining the dissolved oxygen level at a stable level selected to also achieve good treatment performance in relation to organic matter removal.
12. A process according to any one of the preceding claims including maintaining the dissolved oxygen level at a stable level selected to thereby select the polymer composition of the biopolymer(s) produced.
13. A process according to claim 12 including maintaining the dissolved oxygen level at a stable level selected to thereby select the relative proportions of congeners of a polyhydroxyalkanoate polymer produced.
14. A process according to either one of claims 12 and 13 including selecting between a lower dissolved oxygen concentration to increase the relative proportion of congeners and a higher dissolved oxygen concentration to decrease the relative proportion of congeners produced.
15. A process according to any one of the preceding claims wherein the treatment system is a high rate biological treatment system.
16. A process according to claim 15 wherein the high rate biological treatment system is an activated sludge system.
17. A process according to any one of the preceding claims wherein the treatment system includes one controlled zone in which the dissolved oxygen level is maintained at a level which optimises biopolymer production and another controlled zone in which the dissolved oxygen level is maintained at a level which optimises treatment performance in relation to organic matter removal.

18. A process according to any one of the preceding claims further including subsequently treating an outflow from the controlled environment through a conventional wastewater treatment plant including non-nitrogen-fixing organisms, without nitrogen supplementation.
- 5 19. A process according to any one of the preceding claims, which is carried out with nitrogen supplementation.
20. A process according to any one of the preceding claims wherein the controlled environment is seeded with biopolymer producing and/or nitrogen fixing micro-organisms.
- 10 21. Biopolymers produced by the process of any one of claims 1 to 20.

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**Figure 1**

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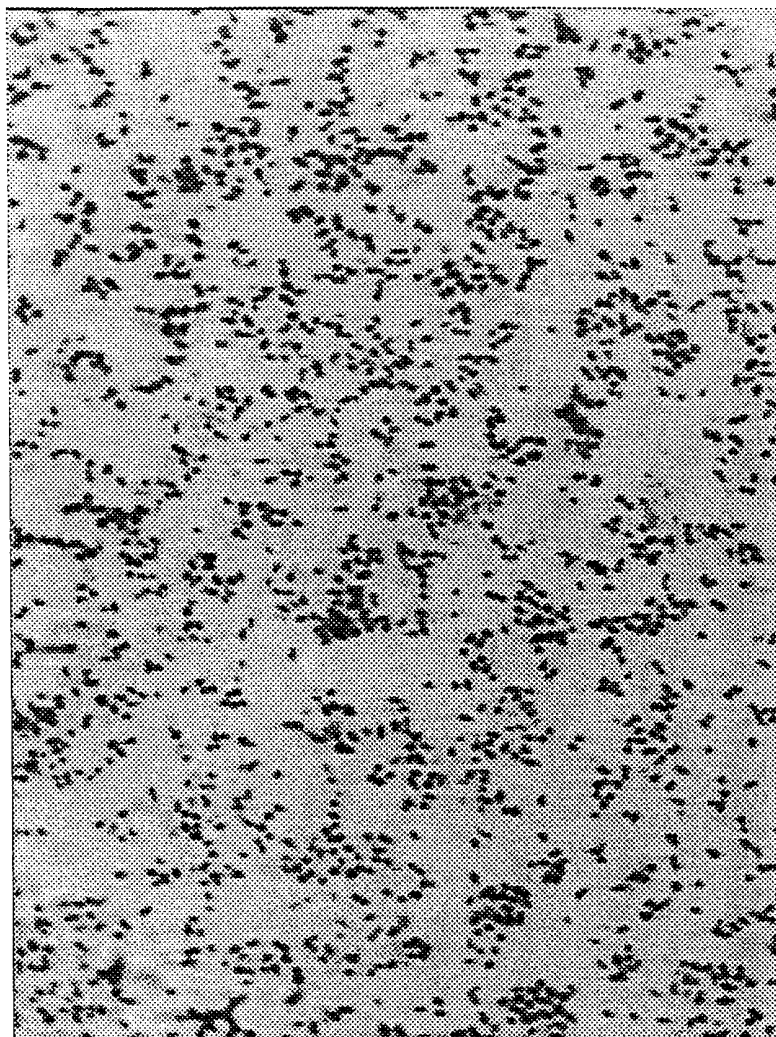
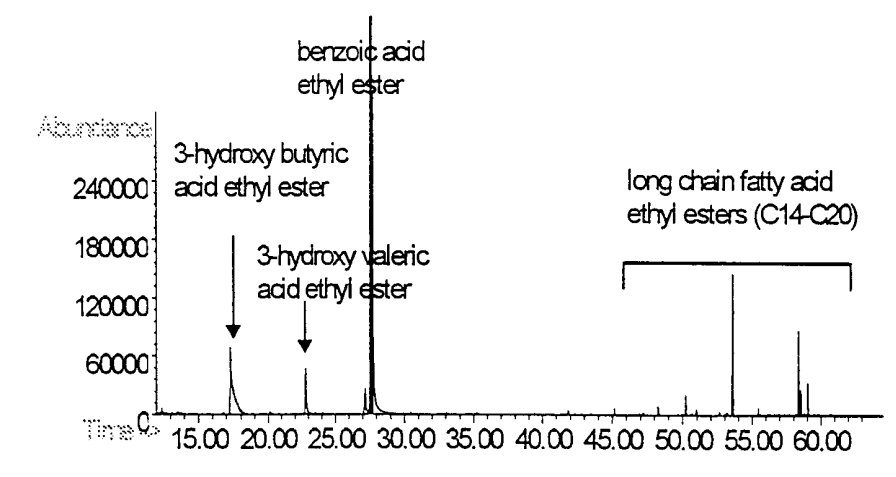


Figure 2

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**Figure 3**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ00/00234

A. CLASSIFICATION OF SUBJECT MATTERInt. Cl. ⁷: C12P 7/44, 7/46, C12S 3/08, C12R 1:64, 1:065

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)

See electronic databases below - Keyword search.

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)

Derwent WPAT, JAPIO; STN CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P.X	WO 00/52189 (Technische Universiteit Delft) 8 September 2000; see the example, claims in particular.	1, 3-4, 10-21
X	JP 3-143397 A2 (Taisei Corp.) 18 June 1991 & Chemical Abstracts, on line abstract no. 115: 678175; see the abstract and figure 1 in particular.	1-21
X.O	Applied Biochemistry & Biotechnology, vol. 77-79, 389-399, April 1999 (Twentieth Symposium on Biotechnology for Fuels and Chemicals, 1998), Chua, H. et al., Accumulation of Biopolymers in Activated Sludge Biomass; see the whole document.	1-21



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ00/00234

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X, O	Macromolecular Symposia, vol. 148, 415-424, December 1999 (7 th International Conference on Polymer Characterisation, University of North Texas, Denton, Texas, January 4-8, 1999). Yu, P.H. et al., Conversion of food industrial wastes into bioplastics with municipal activated sludge; See 'Results and Discussion' in particular.	1-21
X, Y	Journal of Biotechnology, vol. 65, 127-161, 1998, Braunegg, G. et al., Review article: Polyhydroxyalkanoates, biopolyesters from renewable resources: Physiological and engineering aspects; See parts 2.3.2, 2.3.3, 2.3.7, 2.4.1.2, 2.6.2, 2.8.1.1, 2.8.1.2 in particular.	1-21
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X, Y	Folia Microbiologica, vol. 43 (2), 177-181, 1998, Pal, S. et al., Nutritional and Cultural Conditions for Production of Poly-3-hydroxybutyric Acid by <i>Azotobacter chroococcum</i> ; See the whole document.	1-21
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Y	Mededelingen - Faculteit Landbouwkundige en Toegepast (Med.Fac.Landbouww.Univ.Gent), no. 61/4b, 2101-2108, 1996, Stante, L. et al., Production of Poly-Beta-Hydroxybutyrate By <i>Lamproedia</i> Spp. Isolated From Activated Sludge For Phosphorous Removal; See 'Introduction' and 'Conclusions' in particular.	1-21

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/NZ00/00234

This Annex lists the known "A" publication level 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.

Patent Document Cited in Search Report				Patent Family Member	
WO	0052189	AU	200029498	NL	1011431
JP	03143397				
					END OF ANNEX