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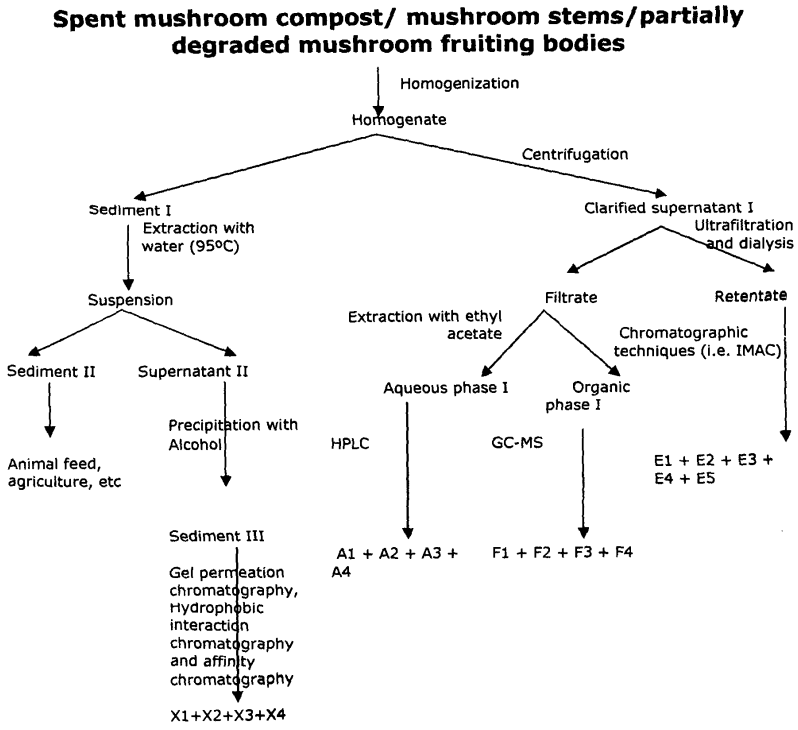
Remarks:
A request for correction of the title and the application text has been filed pursuant to Rule 139 EPC. A decision on the request will be taken during the proceedings before the Examining Division (Guidelines for Examination in the EPO, A-V, 3.).

(54) **Process for simultaneous extraction and purification of fine chemicals from spent mushroom compost, mushroom stems and partially degraded mushroom fruiting bodies**

(57) The aim of this process consists of simultaneous extraction and purification of fine chemicals (i.e enzymes, protein - bound polysaccharides, lectins, alkaloids, antibiotics and terpenes) from spent mushroom compost, mushroom stems or partially degraded mushroom fruiting bodies. Several mushroom strains can be used for this purpose such as *Agaricus bisporus, Pleurotus ostreatus, Lentinula edodes, Cordyceps sinensis, Grifola frondosa, Ganoderma lucidium, Poria cocus, Polyporus umbelatus, Hericium erinaceus, Auricularia auricular* and *Coriolus versicolor.*

Figure 1



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Description

FIELD OF THE INVENTION

[0001] This invention relates to a process of simultaneous extraction and purification of enzymes, protein-bound polysaccharides, lectins, terpenes, alkaloids and antibiotics from spent mushroom compost, mushroom stems and partially degraded mushroom fruiting bodies.

BACKGROUND OF THE INVENTION

[0002] During the last decades, there has been a strong concern regarding the environmental pollution due to large quantities of agro-industrial by-products that are disposed in the environment. However, these agro-industrial by-products such as lignocellulosic wastes, corn cobs, sugar cane bagasse and coffee pulp can be used as low-cost carbohydrate sources for growth of fungi for production of suitable fine chemicals for food, textile, detergent and pharmaceutical industries. Fungal solid-state fermentation technology has been used for centuries for bioconversion of agro-industrial wastes by using basidiomycete strains, namely in mushroom production.

The mushroom growing industry generates about 200 million ton/year of spent mushroom compost which also contains mushroom stems and partially degraded mushroom fruiting bodies. Since this huge waste material is not suitable to be re-utilized in mushroom production, it is either used as garden fertilizer or deposited in landfill which results in environmental pollution and public health problems according to several reports from World Health Organization (WHO). Therefore, the present invention involves a novel process for simultaneous extraction and purification of fine chemicals such as enzymes, free polyssacharides, protein-bound polysaccharides, lectins, terpenes, antibiotics and alkaloids from spent mushroom compost, mushroom stems and partially degraded mushroom fruiting bodies. This process will also contribute to health and environmental policies because it makes use of an agricultural waste material as raw material for novel processes.

SUMMARY OF THE INVENTION

[0003] The aim of this process consists of simultaneous extraction and purification of fine chemicals (i.e enzymes, protein - bound polysaccharides, lectins, antibiotics, alkaloids and terpenes) from spent mushroom compost, mushroom stems and partially degraded mushroom fruiting bodies. Several mushroom strains can be used for this purpose such as *Agaricus bisporus*, *Pleurotus ostreatus, Lentinula edodes, Cordyceps sinensis, Grifola frondosa, Ganoderma lucidium, Poria cocus, Polyporus umbelatus, Hericium erinaceus, Auricularia auricular* and *Coriolus versicolor*. These raw materials can be used for simultaneous extraction and purification of laccase, tyrosinase, pectinases, superoxide dismutase, xylanase, protease, cellulases, intracellular and extracellular protein-bound polysaccharides, lectins, antibiotics and terpenes. As far as enzymes, lectins and protein-bound polysaccharides are concerned, they can be separated and purified by gel filtration chromatography and immobilized metal affinity chromatography. On the other hand, several secondary metabolites including terpenes can be extracted either from the extracellular fluid or intracellular biomass by using suitable organic solvents such as methanol, acetone and ethyl acetate. Such secondary metabolites can be separated and purified by GC-MS and HPLC

DESCRIPTION OF THE DRAWING

[0004]

Fig. 1 is a scheme illustrating the simultaneous extraction and purification of fine chemicals from spent mushroom compost, mushroom stems and partially degraded mushroom fruiting bodies.

DETAILED DESCRIPTION OF THE INVENTION

[0005] The process for simultaneous extraction and purification of fine chemicals from wastes of mushroom production is presented in Figure 1.

Example 1

[0006] 100g (wet weight) of spent mushroom compost/ mushroom stems/degraded mushroom fruiting bodies was homogeneized with 3 volumes of 50 mM phosphate buffer pH 7.0 by using a Waring blendor for 15 min. at 4°C. The suspension was centrifuged at 5000 xg for 10 min. and the clear supernatant (Supernatant I) was concentrated by ultrafiltration with P10 membrane. The retentate was applied to a column packed with epoxy- activated Sepharose 6B- IDA- Cu(II) which was previously equilibrated with 20 mM phosphate buffer pH 7.0 containing 1 M NaCl. The column was washed with the same buffer system and adsorbed proteins were eluted with a linear gradient of imidazole (0- 75 mM) in the same buffer system. The column fractions were analysed for protein, enzyme activity, anti-bacterial and anti-viral activities. The filtrate (I) was extracted with equal volume of ethyl acetate by partitioning in a separating funnel (solvent-solvent extraction) and the organic extract/organic phase (I) was evaporated to dryness *in vacuo*. The dried extract was reconstituted in minimal amount of 50% aqueous methanol and were purified by GC-MS and HPLC. The purified fractions were tested for anti-bacterial and anti-viral activities. The aqueous extract/aqueous phase (I) was applied to an HPLC column for separation of secondary metabolites. The analytical column used was a Nucleosil 100-7 C-18 (250 x 4.6 mm i.d.) with a guard column of the same material (CC 8/4 Nucleosil 100-5

C18). Methanol and water (1:1) were used as mobile phase at a flow rate of 1 ml/min at 25°C.

[0007] The sediment (I) from Figure 1 was resuspended with one volume of water, heated at 100°C for 2 hours and the suspension was centrifuged at 5000xg for 5 min. The supernatant (II) was precipitated with four volumes of ethanol and the sediment (III) was redissolved in minimum volume of water. The free and protein-bound polysaccharides were separated and purified by gel-permeation chromatography on a column packed with Sephacryl HR-100 which was previously equilibrated with 20 mM phosphate buffer pH 7.0. Free and protein-bound polysacharides were eluted from the column with the same buffer system at a flow rate of 30 ml/h and column fractions were analysed by FTIR, UV-VIS and NMR as well as for anti-tumour, anti-viral, anti-fungal and anti-bacterial activities. The sediment (II) was used either as garden fertilizer or in animal feed.

Example 2

[0008] 100g (wet weight) of spent mushroom compost/ mushroom stems/degraded mushroom fruiting bodies was homogeneized with 4 volumes of 20 mM phosphate buffer pH 6.0 by using a Waring blendor for 10 min. at 4°C. The suspension was centrifuged at 5000 xg for 10 min. and the clear supernatant (Supernatant I) was concentrated by ultrafiltration with P10 membrane. The retentate was applied to a column packed with epoxy- activated Sepharose CL 6B- IDA- Cu(II) which was previously equilibrated with 20 mM phosphate buffer pH 6.0 containing 1M NaCl. The column was washed with the same buffer system and adsorbed proteins were eluted with a linear gradient of imidazole (0-50 mM) in the same buffer system. The column fractions were analysed for protein, enzyme activity, anti-bacterial and anti-viral activities. The filtrate (I) was extracted with equal volume of ethyl acetate by partitioning in a separating funnel (solvent-solvent extraction) and the organic extract (I) was evaporated to dryness *in vacuo*. The dried extract was reconstituted in minimal amount of 20% aqueous methanol and were purified by GC-MS and HPLC. The purified fractions were tested for anti-bacterial and anti-viral activities. The aqueous extract (I) was applied to an HPLC column for separation of secondary metabolites.The analytical column used was a Nucleosil 100-7 C-18 (250 x 4.6 mm i.d.) with a guard column of the same material (CC 8/4 Nucleosil 100-5 C18). Methanol and water (0.5: 1) was used as mobile phase at a flow rate of 1 ml/min at 25°C.

[0009] The sediment (I) from Figure 1 was resuspended with one volume of water, heated at 95°C for 3 hours and the suspension was centrifuged at 5000xg for 5 min. The supernatant (II) was precipitated with four volumes of ethanol and the sediment (III) was redissolved in minimum volume of water. The free and protein-bound polysaccharides were separated and purified by gel-permeation chromatography on Sephacryl HR-300 which was previously equilibrated with 20 mM phosphate buffer pH 6.0. Free and protein-bound polysacharides were eluted from the column with the same buffer system at a flow rate of 35 ml/h and column fractions were analysed by FTIR, UV-VIS and NMR as well as for anti-tumour, anti-viral, anti-fungal and anti-bacterial activities. The sediment (II) was used either as garden fertilizer or in animal feed.

Methods

[0010]

1. Preparation of chromatographic matrices

Epoxy-activated agarose gel containing 1,4-butanedioldiglycidyl ether as spacer arm was prepared as described in the literature. Under the conditions selected, the agarose matrix contained 30 μmole of epoxide groups/ml of sedimented gel. Subsequently, epoxy-activated agarose was reacted with iminodiacetic acid (IDA) as the chelating agent and the stationary phase thus obtained was thoroughly washed with water and kept at 4°C in 0.01% (w/v) sodium azide solution.

2. Enzyme assays

Total polygalacturonase or pectinase activity was assayed by incubating either the culture supernatant or column fractions, for 1 hour at 50°C, with 0.5 % (wt/vol.) polygalacturonic acid in 50 mM citrate buffer pH 4.8. The enzyme activity was determined from a calibration curve by using galacturonic acid as a standard. Reducing sugars in the reaction mixture were determined by the dinitrosalicylic acid method .One enzyme unit is defined as the amount of enzyme required for formation of 1 μmole of reducing sugars per minute at 50°C. Laccase (EC 1.10.3.2) was assayed by using o-dianisidine as a substrate. Assays were performed in 0.2M sodium acetate buffer at pH 4.5 containing 17mM o-dianisidine and the oxidized product was read at 450nm as described in the literature; One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmole substrate per min. Total xylanase (EC 3.2.1.8) activity was assayed by incubating the extract, for 1 hour at 50°C, with 0.5 % (w/v.) birchwood xylan in 0.05 M citrate buffer pH 4.8. The increase in reducing sugars in the reaction mixture was determined by the dinitrosalicylic acid method by using xylose as standard. All other enzyme assays were carried out as described in the literature.

3. Free and protein-bound polysaccharides

Free and protein-bound polysaccharides were assayed by phenol-sulphuric acid for total sugar content. Subsequently, these samples were analysed by using HPLC with a UV and RI detectors and a GPC column (Shodex) with a mobile phase containing deionised water at a flow rate of 1.0 ml/min at 25°C.

4. Protein assay
Total protein was determined by Bradford method by using BSA as a protein standard.
5. Biological activity of secondary metabolites and polysaccharides
Antibacterial Activity. The organic and aqueous extracts were studied for antibacterial activity against four bacteria: *Bacillus cereus, Bacillus megaterium, Sarcina lutea* and *Staphylococcus aureus*. The agar disc diffusion protocol was used for antibacterial assay. Sterile filter paper disc of 6 mm in diameter were loaded with 250 μg/disc and 150 μg/disc of the sample extract and were dried under laminar air flow cabinet. Standard antibiotic, streptomycin was used as a positive control. The loaded discs were placed in petri dish (90 mm in diameter) containing sterile nutrient agar medium inoculated with test microorganisms.
Assay of anti-proliferative activity on tumor cell lines. The anti-proliferative activity of the purified secondary metabolite was determined as follows. The cell lines L1210, M1, and HepG2 were purchased from ATCC which were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/L streptomycin, and 100 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO2. Cells in exponential growth phase were seeded into each well of a 96-well culture plate and incubated for 3 h before addition of the secondary metabolite. Incubation was carried out for another 48 h. Radioactive precursor was then added to each well and incubated for 6 h. The cultures were then harvested by a cell harvester. The incorporated radioactivity was determined by liquid scintillation counting.
Assay of secondary metabolite for antifungal activity. The assay for antifungal activity was performed by using sterile Petri dishes containing 10 ml potato dextrose agar. Sterile paper discs, 0.625 cm in diameter, were placed at a distance of 1 cm from the rim of the mycelial colony. An aliquot of the secondary metabolite was added to a disc. Incubation of the petri dish was carried out at 23 °C for 72 h until mycelial growth had enveloped peripheral disks containing the control and had formed crescents of inhibition around discs with antifungal samples. Three fungal species, *Fusarium oxysporum*, *Botrytis cinerea* and *Mycosphaerella arachidicola*, were examined in the assay. The antifungal protein red kidney bean lectin was used as a positive control.
Assay of inhibitory activity toward HIV reverse transcriptase. The assay for HIV reverse transcriptase inhibition activity was conducted in a nonradioactive ELISA kit from Boehringer-Mannheim. The inhibition assay was performed as described in the protocol included with the kit. The inhibitory activity of the secondary metabolite was calculated as percent inhibition as compared to a control without the secondary metabolite. *A. bisporus* lectin and red kidney bean lectin were used as positive controls.
6. Analytical techniques- Several physico-chemical techniques were used for identification of free and protein-bound polysaccharides as well as terpenes, alkaloids and antibiotics such as FTIR, UV-VIS, GC-MS and NMR.

Claims

1. Simultaneous extraction and purification of enzymes, lectins, free and protein- bound polysaccharides and secondary metabolites including terpenes, alkaloids and antibiotics from spent mushroom compost, mushroom stems and partially degraded mushroom fruiting bodies.

2. Such mushroom strains can be *Agaricus bisporus, Pleurotus ostreatus, Lentinula edodes, Cordyceps sinensis, Grifola frondosa, Ganoderma lucidium, Poria cocus, Polyporus umbelatus*, *Hericium erinaceus, Auricularia auricular* and *Coriolus versicolor*.

3. A process for chromatographic separation of pyranose dehydrogenase, xylanase, pectinase, protease and laccase from spent mushroom compost of *Agaricus bisporus*.

4. A process for chromatographic separation of tyrosinase, superoxide dismutase and protein-bound polysaccharides from partially degraded mushroom fruiting bodies.

5. Isolation and separation of extracellular free and protein-bound polysaccharides from spent mushroom compost by extraction, precipitation and gel-filtration chromatography.

6. Isolation of intracellular free and protein-bound polysaccharides from stem mushrooms by extraction, precipitation and gel-filtration chromatography.

7. Isolation of intracellular free and protein-bound polysaccharides from degraded mushroom fruiting bodies by extraction, precipitation and gel-filtration chromatography.

8. A process for separation of terpenes, alkaloids and antibiotics with biological activities by extraction and chromatographic techniques from spent mushroom compost.

9. A process for separation of terpenes, alkaloids and antibiotics with biological activities by extraction and chromatographic techniques from stem mushrooms.

10. A process for separation of terpenes, alkaloids and antibiotics with biological activities by extraction and chromatographic techniques from partially degraded mushroom fruiting bodies.

Amended claims in accordance with Rule 137(2) EPC.

1. Simultaneous extraction and purification of enzymes, lectins, free and protein- bound polysaccharides and secondary metabolites including terpenes, alkaloids and antibiotics from either spent mushroom compost, mushroom stems or partially degraded mushroom fruiting bodies.

2. Such mushroom strains can be *Agaricus bisporus, Pleurotus ostreatus, Lentinula edodes, Cordyceps sinensis, Grifola frondosa, Ganoderma lucidium, Poria cocus, Polyporus umbelatus, Hericium erinaceus, Auricularia auricular* and *Coriolus versicolor.*

3. A process for chromatographic separation of pyranose dehydrogenase, xylanase, pectinase, protease and laccase from spent mushroom compost of *Agaricus bisporus.*

4. A process for chromatographic separation of tyrosinase, superoxide dismutase and protein-bound polysaccharides from partially degraded mushroom fruiting bodies.

5. Isolation and separation of extracellular free and protein-bound polysaccharides from spent mushroom compost by extraction, precipitation and gel-filtration chromatography.

6. Isolation of intracellular free and protein-bound polysaccharides from stem mushrooms by extraction, precipitation and gel-filtration chromatography.

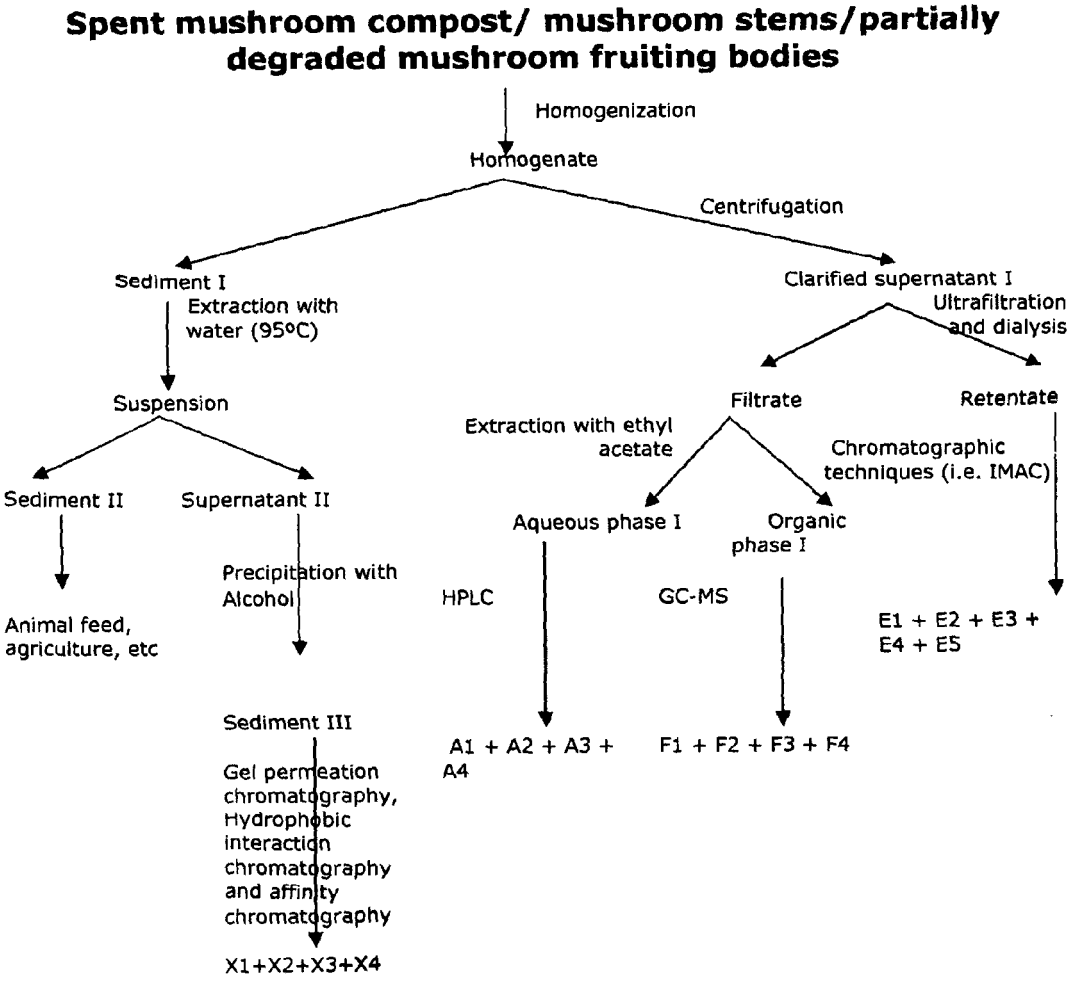
7. Isolation of intracellular free and protein-bound polysaccharides from degraded mushroom fruiting bodies by extraction, precipitation and gel-filtration chromatography.

8. A process for separation of terpenes, alkaloids and antibiotics with biological activities by extraction and chromatographic techniques from spent mushroom compost.

9. A process for separation of terpenes, alkaloids and antibiotics with biological activities by extraction and chromatographic techniques from stem mushrooms.

10. A process for separation of terpenes, alkaloids and antibiotics with biological activities by extraction and chromatographic techniques from partially degraded mushroom fruiting bodies.

Figure 1


Spent mushroom compost/ mushroom stems/partially degraded mushroom fruiting bodies
Homogenization
Homogenate
Centrifugation
Sediment I
Extraction with water (95°C)
Suspension
Sediment II
Supernatant II
Animal feed, agriculture, etc
Precipitation with Alcohol
Sediment III
Gel permeation chromatography, Hydrophobic interaction chromatography and affinity chromatography
X1+X2+X3+X4
Clarified supernatant I
Ultrafiltration and dialysis
Filtrate
Retentate
Extraction with ethyl acetate
Chromatographic techniques (i.e. IMAC)
Aqueous phase I
Organic phase I
HPLC
GC-MS
A1 + A2 + A3 + A4
F1 + F2 + F3 + F4
E1 + E2 + E3 + E4 + E5

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 07 39 8019

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	WASSER S P: "Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharidess" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER VERLAG, BERLIN, DE, vol. 60, 10 September 2002 (2002-09-10), pages 258-274, XP002402437 ISSN: 0175-7598	5-7	INV. C12P1/02
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Y	BALL ANDREW S ET AL: "The recovery of lignocellulose-degrading enzymes from spent mushroom compost" BIORESOURCE TECHNOLOGY, vol. 54, no. 3, 1995, pages 311-314, XP002484502 ISSN: 0960-8524 * the whole document *	1-10	

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	17 June 2008	Roscoe, Richard

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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EUROPEAN SEARCH REPORT

Application Number
EP 07 39 8019

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
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Y	WO 2007/018095 A (TAKARA BIO INC [JP]; ENDO HIROSHI; SAKAKIBARA HITOSHI; HATTORI YASUSHI) 15 February 2007 (2007-02-15) * the whole document * -----	1-10	
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			TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

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Munich	17 June 2008	Roscoe, Richard

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 07 39 8019

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17-06-2008

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82