Title: MICROORGANISMS AND METHODS FOR THE BIOSYNTHESIS OF AROMATICS, 2,4-PENTADIENOATE AND 1,3-BUTADIENE

Abstract: The invention provides non-naturally occurring microbial organisms having a toluene, benzene, p-toluic, terephthalate, (2-hydroxy-3-methyl-4-oxobutoxy)phosphonate, (2-hydroxy-4-oxobutoxy)phosphonate, benzoate, styrene, 2,4-pentadienoate, 3-butenol-1 or 1,3-butadiene pathway. The invention additionally provides methods of using such organisms to produce toluene, benzene, p-toluic, terephthalate, (2-hydroxy-3-methyl-4-oxobutoxy)phosphonate, (2-hydroxy-4-oxobutoxy)phosphonate, benzoate, styrene, 2,4-pentadienoate, 3-butenol-1 or 1,3-butadiene.
## Classification Of Subject Matter

INV. C12P5/00 C12P5/02 C12P7/40

**ADD.**

According to International Patent Classification (IPC) or to both national classification and IPC

## Fields Searched

Minimum documentation searched (classification system followed by classification symbols)

C12P

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, WPI Data, BIOSIS

## Documents Considered To Be Relevant

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim no.</th>
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</thead>
</table>
| X        | FISCHER-ROMERO C ET AL: "Tol umonas auensis gen. nov., sp. nov., a tol uene-producing bacterium from anoxic sediments of a freshwater lake."
the whole document | 1-16 |
Whole document, especially claim 1 | 1-16 |

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
- "E" earlier document but published on or after the international filing date
- "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)
- "O" document referring to an oral disclosure, use, exhibition or other means
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- "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
- "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
- "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
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Date of the actual completion of the international search: 11 November 2011

Date of mailing of the international search report: 13/03/2012

Name and mailing address of the ISA:
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Authorized officer:
Kools, Patrick

Form PCT/ISA/210 (second sheet) (April 2005)
<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
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</table>
| A        | SELMER ET AL: "p-Hydroxyphenyl acetate decarboxylase from Clostridium difficile. A novel glycyl radical enzyme catalyzing the formation of p-cresol."
EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 268, no. 5, 1 March 2001 (2001-03-01), pages 1363-1372, XP055011607, ISSN: 0014-2956
Whole document, especially table 1 | 1-16 |
| A        | SCHNEIDER SABINE ET AL: "Anaerobic metabolism of L-phenylalanine via benzoyl-CoA in the denitrifying bacterium Thauera aromatica".
the whole document | 1-16 |
| A        | HUGHES E J L ET AL: "EVIDENCE FOR ISOFUNCTIONAL ENZYMES IN THE DEGRADATION OF PHENOL TO TOLUATE AND P TOLUATE AND P CRESOL VIA CATECHOL META CLEAVAGE PATHWAYS IN ALCALIGENES-EUTROPHUS".
JOURNAL OF BACTERIOLOGY, vol. 158, no. 1, 1984, pages 79-83, XP002663267, ISSN: 0021-9193
the whole document | 1-16 |
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. □ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   1-16

Remark on Protest

□ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.

□ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

□ No protest accompanied the payment of additional search fees.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. **claims**: 1-16

   Non-natural microorganisms having a toluene pathway comprising at least one exogenous sequence encoding a toluene pathway enzyme, where in the pathway involved the stepwise degradation of phenylalanine to toluene. Method for producing toluene, comprising culturing said microorganisms.

2. **claims**: 17-24

   Non-natural microorganisms having a benzene pathway comprising at least one exogenous sequence encoding a benzene pathway enzyme, wherein the pathway comprises a phenylalanine benzene lyase. Method for producing toluene, comprising culturing said microorganisms.

3. **claims**: 25-44

   Non-natural microorganisms having a styrene pathway comprising at least one exogenous sequence encoding a styrene pathway enzyme, wherein the pathway selected from two alternative pathways comprising 5 or 6 specific enzymes. Method of producing styrene, comprising culturing said microorganisms.

4. **claims**: 45-54

   Non-natural microorganisms having a 1,3-butadiene pathway comprising at least one exogenous sequence encoding a 1,3-butadiene pathway enzyme selected from 6 different decarboxylases. Method for producing 1,3-butadiene, comprising culturing said microorganisms.

5. **claims**: 55-59

   Non-natural microorganisms having a (2-hydroxy-4-oxobutoxy)phosphonate pathway comprising at least one exogenous sequence encoding a (2-hydroxy-4-oxobutoxy)phosphonate pathway enzyme, selected from a specific group. Method for producing (2-hydroxy-4-oxobutoxy)phosphonate, comprising culturing said microorganisms.

6. **claims**: 60-77

   Non-natural microorganisms having a benzoate pathway comprising at least one exogenous sequence encoding a
benzoate pathway enzyme, selected from a specific group of eight enzymes. Method for producing benzoate, comprising cultivating said microorganism.

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7. claims: 78-103

Non-natural microorganism having a benzene pathway comprising at least one exogenous sequence encoding a benzene pathway enzyme, selected from a specific group of pathways and enzymes. Method for producing benzene, comprising cultivating said microorganism.

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8. claims: 104-129

Non-natural microorganism having a toluene pathway comprising at least one exogenous sequence encoding a toluene pathway enzyme, selected from a specific group of enzymes. Method for producing toluene, comprising cultivating said microorganism.

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9. claims: 130-170, 203-211, 223-232

Non-natural microorganism having a 2,4-pentadienoate pathway comprising at least one exogenous sequence encoding a 2,4-pentadienoate pathway enzyme selected from eight different pathways. Method for producing 2,4-pentadienoate, comprising cultivating said microorganism.

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10. claims: 171-183, 189-202, 213-222, 242, 243

Non-natural microorganism having a 1,3-butadiene pathway comprising at least one exogenous sequence encoding a 1,3-butadiene pathway enzyme selected from different pathways all including a 3-hydroxypent-4-enoate decarboxylase. Method for producing 1,3-butadiene, comprising cultivating said microorganism.

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11. claims: 184-188, 233-241

Non-natural microorganism having a 1,3-butadiene pathway comprising at least one exogenous sequence encoding a 1,3-butadiene pathway enzyme, and able to produce 3-buten-1-ol. Method for producing 3-buten-1-ol, comprising cultivating said microorganism.

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<th>Publication date</th>
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<tr>
<td>WO 2009111513 Al</td>
<td>11-09-2009</td>
<td>CA 2728285 Al</td>
<td>11-09-2009</td>
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<td>EP 2285948 Al</td>
<td>23-02-2011</td>
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<td>US 2011008861 Al</td>
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