Title: BACTERIA FOR THE DEGRADATION AND MODIFICATION OF FATS, OILS AND GREASE

Abstract: The invention discloses several Gram-positive microorganisms that effectively and efficiently degrade fats, oils and grease. A composition comprising said microorganism and a method for degrading fatty acids and grease are also disclosed.
Bacteria for the Degradation and Modification of Fats, Oils and Grease

Field of the Invention

The invention discloses several Gram-positive microorganisms that effectively and efficiently degrade fats, oils and greases. A composition comprising said microorganism and a method for degrading fatty acids and grease are also disclosed. More particularly, the present invention is related to providing a non-pathogenic, spore-forming Gram-positive, lipophilic bacterial strain that produces extracellular lipase and also efficiently oxidizes or degrades fatty acids and grease. The invention is further related to a formulation comprising said Gram-positive organism.

Background of the Invention

Fats, oils and greases (FOGs) in wastewater create problems including the production of foul odours, the blockage of sewer lines and may interfere with the proper operation of sewage treatment works. Removal of FOG from wastewater is thus critically important to ensure that wastewater is disposed of efficiently and economically. One of the major mechanisms of sewer line blockage due to oil is the conversion of oil to a greasy-like substance. When oil was incubated with the wastewater bacteria, an opaque, semi-solid sticky material was formed. Some of this material stuck to the sides of the culture vessels. The composition of the fatty acids was found to be different from the original oil. Production of the semi-solid material was suggested to be a consequence of microbial action and this is likely to source of the well-documented problem of sewer and pipeline blockage caused by FOGs (Chao & Yang, 1981, Treatment of Wool Scouring Wastewater, J Water Pollution Control Fed., .53:311-317).

Most food service establishments are required to have a device that prevents grease from flowing directly from the kitchen or food preparation area into the sewer or to an on-site waste disposal system. Commonly called grease traps, these devices function to physically prevent oils and grease from flowing directly into the sanitary sewer and to store the separated grease solid for eventual solid waste disposal.
Many municipalities place restrictions and surcharges based on the biological oxygen demand (BOD), and oil and grease (O&G) levels in the effluents from grease traps. In addition to wastewater treatment costs and surcharges, the grease solids from the traps must also be periodically removed and disposed. The food service establishment then faces two recurring charges for wastewater treatment, one a municipal treatment cost and secondly a grease disposal cost.

However, the frequency of pumping the accumulated grease solids can be quite variable, ranging from several weeks to several months. If traps are not cleaned on a regular basis, grease clogs may occur causing wastewater to back up into the food preparation area causing malodours and requiring the establishment to close until the problem is corrected. In addition to providing physical means to trap O&G, grease traps can function to biologically mediate a reduction of BOD and O&G in the bulk liquid resulting in cleaner effluent wastewater. This reduction of BOD and O&G is dependent upon the hydraulic retention time, which is dependent on the size of the grease trap and wastewater flow. Other factors that affect biological activity within a grease trap include pH, temperature and whether or not the facility practices bioaugmentation.

Bioaugmentation, the addition of commercial bacterial products that increase the biological activity in the system, has been used to reduce the BOD and O&G in the effluents from grease traps. This has helped to reduce surcharges that the food establishments must pay to municipalities for wastewater services. Additionally, bioaugmentation has been used to decrease the pumping frequency of grease traps, to keep drain lines open and to reduce malodours. In addition to grease traps, bioaugmentation has also been used to help remove grease from lift stations, drain lines, septic tanks and other situations where grease accumulation can cause flow problems and malodours. Bioaugmentation products can be either liquid or dry. Because of ease of handling, liquid products are generally preferred and added by a liquid metering pump drawing on a container that is replenished on a periodic basis. Strains used in bioaugmentation of grease applications produce an important extracellular enzyme, lipase. This enzyme hydrolyzes and breaks the ester bond between the glycerol backbone and the fatty acid moieties making up the grease.
The glycerol is relatively easily disposed by biodegradation, however the fatty acids are difficult to degrade and can persist causing pH drops, clogging and malodours.

When Gram-negative microorganisms are used for bioaugmentation in liquid products, they are present as vegetative cells and as such, they may be killed by chemicals, such as surfactants and preservatives, which are often used in such formulations. Therefore, products containing Gram-negative organisms cannot contain biocides and surfactants. Then, unpreserved liquid products may develop severe malodours from microbial contaminants growing in the product. Some of these contaminants may be undesirable in a food service environment. Furthermore, unpreserved products may also suffer from decreased shelf life and efficacy. Clearly, while Gram-negative microorganisms have an advantage in fatty acid degradation, their use in residential and food service products have serious drawbacks.

Dry Gram-negative products, on the other hand, do have a slight advantage of improved shelf life over liquid Gram-negative formulations. However, this advantage is only marginal and varies significantly from strain to strain. Disadvantages of dry products include contaminating dust and difficulty in handling dry materials. Although dry products can be rehydrated with water and applied like liquid products, the disadvantages of using unpreserved liquids containing Gram-negative microorganisms still apply to rehydrated dry materials. Many Gram-negative microorganisms are known to have the ability to biodegrade fatty acids generated by the action of lipase. This ability to oxidize and degrade fatty acids is generally not found in Gram-positive, spore-forming microorganisms, specifically members of the genus Bacillus.

Accordingly, there is a need to develop bioaugmented formulations that can effectively and efficiently degrade or oxidize fats, oil and grease without causing malodours or other undesirable conditions, such as occurs with Gram-negative organisms.

Specifically, there is a need to find non-pathogenic, spore-forming Gram-positive, lipophilic bacterial strain that produces extracellular lipase and efficiently oxidizes or breaks down fatty acids and grease. Heretofore, such a Gram-positive
organism and a formulation containing the same have not been identified or produced.

Some of the previous arts which focussed on the use of microorganisms for the treatment of fat, oil or greases waste are described as follow:


WO200294181-A by Tisinger J L, Paone D A, Leder J, Drahos DJ, Pacne DA, Brahos DJ, Tisinger J, Paone D, Drahos D, Tisinger L, Paone A and Drahos J (2003) - use a novel Bacillus megaterium SB3112 (A) having the ATCC deposit number PTA-3142 for degrading fatty acids and grease flowing into a sewer or to an on-site waste disposal system.


material; Bacillus subtilis, B. brevis, B. licheniformis, B. thuringiensis, B. cereus and/or B. tumilus and Pseudomonas aeruginosa, P. fluorescens, P. putida, P. syringae, P. mallei, P. diminuta, P. vesicularis and/or P. pickettii.

Brief Description of the Drawings

Figure 1 is the comparison of palm oil-degrading efficiency between the commercial product and individual strains of Bacillus spp. locally isolated.

Figure 2 is 16sRNA gene sequence for Bacillus cereus Strain Dr.Y135 with accession number of EF121823.

Figure 3 is 16sRNA gene sequence for Bacillus sp. strain SeAG 1 Y135 with accession number of EU828795.

Figure 4 is 16sRNA gene sequence for Uncultured Bacillus sp. clone A.rzi Y135 with accession number of EU835195.

Figure 5 is 16sRNA gene sequence for Bacillus amyloliquefaciens strain RXZ Y135 with accession number of EU835195.

Summary of the Invention

It is, therefore, an object of the present invention to provide a non-pathogenic, spore-forming Gram-positive, lipophilic bacterial strain that produces extracellular lipase and also efficiently hydrolyzes or degrades fatty acids and grease or a mixture of fatty acid and grease.

It is a further object of the present invention to provide a composition comprising a non-pathogenic, spore-forming Gram-positive, lipophilic bacterial strain that produces extracellular lipase and efficiently hydrolyzes or degrades fats, oils and grease.
An additional object of the present invention is to provide a method for degrading fatty acid and grease using a Gram-positive strain of *Bacillus* spp. Yet another object of the present invention is to enhance the biodegrading activity of Gram-positive strain of *Bacillus* species.

Various other objects and advantages of the present invention will become evident from a brief description of the drawings and detailed description of the invention.

Additional advantages and novel features of the invention will be set forth in part in the description that follows, and in part will become more apparent to those skilled in the art upon examination of the following or upon learning by practice of the invention.

**Detailed Description of the Invention**

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The embodiment describes herein is not intended as limitations on the scope of the invention.

The present invention relates to Gram positive microorganisms, *Bacillus* spp. The disclosed *Bacillus* species is effective for degradation of fats, oils and greases. The above and various other objects and advantages of the present invention are achieved by biologically pure cultures Gram-positive microorganism, *Bacillus* spp. The said microorganisms with their 16srRNA gene sequence submitted to Genbank with the accession numbers of EF121823, EU828795, EU835195 and EU835195.

It should be understood that unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the methods and materials described
herein are preferred. Unless mentioned otherwise, the techniques employed or contemplated herein are standard methodologies well known to one of ordinary skill in the art. The materials, methods and examples are only exemplary and not limiting.

The term "biodegradation", "biodegraded", or "biodegrading" as used herein means that the substrate is broken down, oxidized or degraded by the microorganism. And the term "activity enhancement" as used herein means that the biodegradation activity of the microorganism is increased by the presence or addition of a particular component, said component being designated as "activity enhancer", or "activator".

By utilizing a Gram-positive, spore-forming organism that can oxidize fatty acids, one can obtain the advantages of an improved preserved liquid product. Unlike Gram-negative containing products, the preserved, spore-based Gram-positive containing product can contain preservatives and surfactants to aid in the biodegradation of fats, oils and greases (FOGs), because the spores are relatively resistant to biocides and surfactants. Furthermore, these products may also contain micronutrients promoting the growth of the microorganisms. Thus, a Gram-positive product comprising lipase-producing, fatty acid degrading, spore-forming microorganism in a preserved liquid formulation offers various advantages required for efficacious degradation of oil and grease.

Liquid products formulated in accordance with the present invention for grease traps, or other similar uses where fatty acid or grease needs to be degraded, may also contain in addition to surfactants, biocides, growth promoting non-toxic amounts of inorganic nutrients and micronutrients, certain activity enhancers, stabilizers, viscosifiers and the like.

Other inclusions in the formulation are exemplified below:
(A) Other microorganisms may be selected from the group consisting of the genera Acinetobacter, Aspergillus, Azospirillum, Burkholderia, Bacillus, Ceriporiopsis, Enterobacter, Escherichia, Lactobacillus, Paenibacillus, Paracoccus, Pseudomonas, Rhodococcus, Syphingomonas, Streptococcus, Thiobacillus, Trichoderma and Xanthomonas.
(B) Within *Bacillus* genera, the microorganism may be selected from the group consisting of *Bacillus subtilis*, *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus thuringiensis* and a combination thereof.

(C) The preservative is selected from the group consisting of 1,2-benzisothiazolin-3-one; 5-chloro-2-methyl-4-isothiazolin-3-one, 2-methyl-4-isothiazolin-3-one; quaternium-15; phenol; sodium o-phenylphenate; o-phenylphenol; 6-acetoxy-2,4-dimethyl-2-hydroxyethyl)-a-triazine; chlorhexidine; p-hydroxybenzoic acid or its methyl, propyl, or butyl esters; benzoic, ascorbic, citric, or sorbic acid; imidazolidinyl urea; diazolidinyl urea; dimethylol dimethyldantoin; methylene bisthiocyanate; 2-bromo-2-nitropropane-1,3-diol; 1,2-benzisothiazoline-3-one; methyl anthranilate and a mixture thereof.

(D) The surfactant is selected from the group consisting of trideceth-3; 3 mole ethylene oxide adduct of a linear, primary C12-14 alcohol; 7 mole ethylene oxide adduct of a linear, primary C12-14 alcohol; sodium lauryl sulfate; ammonium lauryl sulfate; dodecyl benzene sulfonic acid; ammonium lauryl sulfate; sodium xylene sulfonate; sodium lauryl sulfate; cocamide diethanolamine; lauramine oxide; sodium alphasulfo methyl C12-18 ester and disodium alphasulfo C12-18 fatty acid salt; sodium dodecylbenzene sulfonate; alkyl polyglycoside; nonylphenoxypoly (ethylenoxy) ethanol, branched; nonylphenoxypoly (ethylenoxy) ethanol, branched; alkoxylated linear alcohol; blend of ethoxylates of linear, primary 12-14 carbon number alcohol; octylphenoxypolyethoxyethanol absorbed on magnesium carbonate; sodium dodecylbenzene sulfonate and isopropyl alcohol; poe (6) tridecyl alcohol; poly (oxy-1, 2-ethanediyl), alpha (nonylphenyl)-omega hydroxy, branched, and a mixture thereof.

(E) The range (v/v) in which various components may be included in the composition in accordance with the present invention are as follows

(i) *Bacillus* spp. ranging from about 1×10^6 to about 1×10^6 CFU/ml
(ii) glycerol ranging from about 0.01% to about 10%
(iii) surfactant ranging from 0.1 to 10%
(iv) preservative ranging from 1 ppm to 1.0%
(v) color ranging from 0.02% to 1%
(vi) fragrance ranging from 0.02% to 1.0%
(vii) viscosifier ranging from 0.05% to 5%

Additional features of the present invention are provided in the following examples, which should not be construed as limiting the claims in any way.

Example 1

Gravimetric analysis of fat/oil degradation

Experiments were conducted in 250-ml conical flasks stoppered with cotton wool to encourage aseptic conditions. All flasks contained 1 ml of fat/oil and 100 ml of enriched nutrient medium (yeast extract (0.2 g/l), glucose (0.1 g/l), potassium nitrate (1 g/l), magnesium sulphate-7 hydrate (0.2 g/l), disodium hydrogen orthophosphate (0.1 g/l), calcium chloride-2-hydrate (0.01 g/l), manganese sulphate-1-hydrate (0.01 g/l), ferric ammonium citrate (0.005 g/l) or standard nutrient medium (yeast extract (0.1 g/l), glucose (0.025 g/l), potassium nitrate (1 g/l), magnesium sulphate-7 hydrate (0.2 g/l), disodium hydrogen orthophosphate (0.1 g/l), calcium chloride-2-hydrate (0.01 g/l), manganese sulphate-1-hydrate (0.01 g/l), ferric ammonium citrate (0.005 g/l)). A commercial oil-degrading supplement, Microblaze, in the form of block was initially chipped using sterile spatula to give approximately 1 cm³ block having an approximate spore count of 1 x 10⁶ CFU bacteria and were directly added into the oil growth medium. Bacillus spp. strains were initially grown overnight at 30°C on an orbital shaker (250 rpm) and were added into the flask to a final concentration of 1 x 10⁶ CFU/ml. Controls were prepared in the same manner but without the addition of the supplement. The flasks were incubated for 21 or 28 days at 30°C with shaking at 130 rev/min before the remaining lipids were extracted. All treatments and controls were prepared in triplicate.

Lipids were extracted by transferring the contents of the flasks into separating funnels and adding 40 ml of dichloromethane. The funnels were shaken vigorously, allowed to settle and the organic phase transferred to a Florence flask. If emulsification had occurred, separation of the two phases was achieved by
Centrifugation at 4500 g for 2–10 min assisted by the addition of a few drops of saturated sodium chloride solution. The remaining aqueous phase was re-extracted a further 3 times and the solvent phases pooled together and dried using a rotary evaporator. The dried oil was redissolved in a few ml of dichloromethane, transferred to a vial containing anhydrous sodium sulphate and then filtered through Whatman number 6 filter paper into a pre-weighed vial. The solvent was evaporated under oxygen free nitrogen at 40°C and the weight of oil determined.

Example 2

Microbial strains for degrading FOGs

The bacterial sources; Bacillus amyloliquefaciens strain RXZ (EU835195), Bacillus pumilus Strain Lubn2 (EU851976), Bacillus cereus Strain DRY135 (DQ 851857), Bacillus sp. strain SeAG 1 (EU828795) were obtained from the Dept. of Biochemistry Culture Collection, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. Values in parentheses indicate Genbank Accession number. Benchmarking product, BioAccess, was obtained from and contained several undisclosed Bacillus strains;

Example 3

BioAccess product properties

BioAccess product properties are as follow:

- Type: Multiple Bacillus spp.
- Form: 99+ Spores
- Spore Count: 100 million CFU/ml
- Gram-Negative Bacteria: N/A (Biosafety Level 1)
- Appearance: Blue Liquid
- Fragrance: Mint
- pH: 6.0 – 7.0
- Flash Point: N/A
• Specific Gravity: 1.0 – 1.1
• Shelf Life: One Year at 21 °C (70 °F)

Oil degradation efficiency

Figure 1 showed the comparison of palm oil-degrading efficiency between the commercial product (BioAccess) product and individual strains of *Bacillus* spp. locally isolated. The degradation was carried out at 30 °C since this is the normal environmental temperatures of Malaysian climate. Only *Bacillus* sp. strain SeAG 1 gave significantly higher reduction in palm oil than the commercial Bioaccess consortium (p<0.05). The rest of the strains gave no significant difference in terms of palm oil degradation capacity compared to the commercial BioAccess consortium (p>0.05). However, the combination of all of the local strains gave significantly higher degradation of palm oil compared to the commercial BioAccess consortium (p<0.05).
Claims:

1. A biologically pure culture of *Bacillus amyloliquefaciens* strain RXZ Y135 with accession number of EU835195

2. A biologically pure uncultured *Bacillus* sp. clone *A.rzi* Y135 with accession number of EU835195

3. A biologically pure culture of *Bacillus cereus* Strain Dr.Y135 with accession number of EF121823

4. A biologically pure culture of *Bacillus* sp. strain SeAG 1 Y135 with accession number of EU828795

5. A bacterial composition comprising *Bacillus amyloliquefaciens* in as in claim 1, uncultured *Bacillus* sp. clone *A.rzi* as in claim 2, *Bacillus cereus* as in claim 3 and *Bacillus* sp. strain SeAG 1 Y135 as in claim 4, having all of the characteristics of strains RXZ Y135, *A.rzi*, Dr.Y135 and strain SeAG 1 Dr.Y135, respectively.

6. The component selected from claim 5 consisting of a non-toxic nutrient formulation, surfactant, activator, preservative, filler, stabilizer, fragrance, viscosifier, enzymes, and a combination thereof.

7. The composition of claim 5, wherein the bacteria are selected from the group consisting of *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus cereus* and *Bacillus* sp. and a combination thereof.

8. The composition of claim 5, wherein the components are *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus cereus* and *Bacillus* sp. ranging from about $1 \times 10^5$ to about $1 \times 10^9$ CFU/ml

9. The composition of claim 5 comprising of surfactant ranging from 0.1% to about 10% (w/v)
10. The composition of claim 5 comprising of preservative ranging from 1 ppm to 1.0% (v/v)

11. The composition of claim 5 comprising of color ranging from 0.02% to 1% (v/v)

12. The composition of claim 5 comprising of fragrance ranging from 0.02% to 1.0% (v/v)

13. The composition of claim 5 comprising of viscosifier ranging from 0.05% to 5% (v/v).
Figure 1
LOCUS: EF121823  711 bp  DNA  linear  BCT
12-DEC-2006
DEFINITION: Bacillus cereus strain Dr.Y135 16S ribosomal RNA gene, partial sequence.
ACCESSION: EF121823
VERSION: EF121823.1  GI:119068000
KEYWORDS:
SOURCE: Bacillus cereus
ORGANISM: Bacillus cereus, Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus; Bacillus cereus group.
REFERENCE: 1 (bases 1 to 711)
AUTHORS: Shukor,Y., Gusmanizar,N, and Syed,A.
TITLE: Direct Submission
JOURNAL: Submitted (12-NOV-2006) Biochemistry, Universiti Putra Malaysia, Faculty of Biotechnology and Biomolecular Sciences, Serdang, Selangor 43000, Malaysia
FEATURES: Location/Qualifiers
source 1..711
 /organism="Bacillus cereus"
 /mol_type="genomic DNA"
 /strain="Dr.Y135"
/db_xref="taxon:1396"
 rRNA 1..711
 /product="16S ribosomal RNA"

ORIGIN:
1  tggtagtccc acacgtaaa cggtagtgc taagttgtag aggattccg cccctttagt
61  gcggaagta aacgattaa cactcgcgcgt ggaggagtcg ggcgeagaag ccgaaatca
121  agaagttgc ggaggcgcgc acacgcggagt ggaggcttcg ttaatgtga agcaacgcg
181  agaaccctac caggtcttgca cactctctgaa aacccctaga gataaggcctctctcgcg
241  acagacagct caggtgttgc atgggttcg tcagccgctc tcggagatg tggatagaag
301  tccgcgacag aagcgcaccc tggatcttgag tggatcacat taagttgagc actctaaagt
361  gcggtgctcg gacaaagccag aggaagttgc ggatgctgct aacatcatgt ggcgctttgt
421  acctggctct cactcctggct aacttcagac gttcagagct cgcggctgga gcgggttgga
481  gcctctctctacttctgctcgc ggattttcctc ctcgtggagg gtagcctctcctctcgtg
541  tggatctcgt caagtgctg gacacgctc gacagtccag ctcggcggcc gctctctcgtc
601  acacgccgct cccagcagcag tggatctcgtc ctcctctccctc ctcgcttgac aacatcatgt
661  gagcgcgcct ctcgcttgac gacatcgtccttgag tgcgatagac tggatcagag catccttga

Figure 2
LOCUS: EU828795 1281 bp DNA linear BCT
06-AUG-2008
DEFINITION: Bacillus sp. SeAG 1 16S ribosomal RNA gene, partial sequence.
ACCESSION: EU828795
VERSION: EU828795
KEYWORDS: 
SOURCE: Bacillus sp. SeAG 1
ORGANISM: Bacillus sp. SeAG 1
Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE: 1 (bases 1 to 1281)
TITLE: Direct Submission
JOURNAL: Submitted (17-JUN-2008) Biochemistry, Faculty of Biotechnology and Molecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, 43400, Malaysia
FEATURES: Location/Qualifiers
source 1..1281
/orrganism="Bacillus sp. SeAG 1"
/mol_type="genomic DNA"
/strain="SeAG 1"
/db_xref="taxon:547401"
rRNA <1..1281
/product="16S ribosomal RNA"

ORIGIN

1 tgcggccagct cttatgagtt aagcgcggag ggtttagtaa cagtttgtt aaacctgctgcacgtgctgat
61 aagacgaggg ttaactcgggg aacccgcggc taaaacctgga taacattttg aacccgcggtgg
121 ttagtaagt agacgcgcagct cggcgtgctgca cttatggagtt gacccgcgtgca gctagtcagtaa
gcgttcttctc gagtggag cggcgagtt nonagcagtt gctgagtt gctgagtt gctgagtt gctgagtt
241 ccgactctgg cagtcgacgtc cggcggagc cggcggagc cggcggagc cggcggagc cggcggagc
301 cggcgcgcgc gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt
361 aaactcggag cggcgcgcgc gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt
421 tcctcggagc cggcgcgcgc gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt
481 gttgccggc gcggcgcgcgc gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt
541 gcgcggcgag cggcgcgcgc gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt
601 tctggaatgc tctgcgcggc ggaacctgagtt tctggaatgc tctgcgcggc ggaacctgagtt tctggaatgc tctggaatgc
661 tctggaatgc tctgcgcggc ggaacctgagtt tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc
721 cggcgcgcgc gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt
781 gtaacctgagtt tctggaatgc tctgcgcggc ggaacctgagtt tctggaatgc tctggaatgc tctggaatgc tctggaatgc
841 cggcgcgcgc gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt gcgtgagtt
901 gtaacctgagtt tctggaatgc tctgcgcggc ggaacctgagtt tctggaatgc tctggaatgc tctggaatgc tctggaatgc
961 gtaacctgagtt tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc
1021 gcgcaacctgagtt tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc
1081 gcgcaacctgagtt tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc
1141 gcgcaacctgagtt tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc
1201 gcgcaacctgagtt tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc
1261 gcgcaacctgagtt tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc tctggaatgc

Figure 3
LOCUS: EU835195 1306 bp DNA linear ENV 22-JUL-2008
DEFINITION: Uncultured *Bacillus* sp. clone *A.rzi* 16S ribosomal RNA gene, partial sequence.
ACCESSION: EU835195
VERSION: EU835195.1 GI:194295612
KEYWORDS: ENV.
SOURCE: uncultured *Bacillus* sp.
ORGANISM: uncultured *Bacillus* sp.
Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus; environmental samples.
REFERENCE: 1 (bases 1 to 1306)
AUTHORS: Othman,A.R., Shukor,M.Y. and Syed,M.A.
TITLE: Direct Submission
JOURNAL: Submitted (13-JUN-2008) Biotechnology and Biomolecular Science, Universiti Putra Malaysia, Serdang, Seri Kembangan, Selangor 43400, Malaysia
FEATURES: Location/Qualifiers
source 1..1306
/organism="uncultured Bacillus sp."
/mol_type="genomic DNA"
/isolation_source="dumping area"
/db_xref="taxon:83428"
/clone="A.rzi"
/environmental_sample
/country="Malaysia: Pangkor"
/rRNA
<1..>1306
/product="16S ribosomal RNA"

ORIGIN
1 caacctgctc gngtgatgtt tgggcggagcc ctggagtacag cggcgggtta cctgctgctgta
d1 naangagat ccggcgggct aactccgcaat aacctggtc tggctggttgc cctccggcctgtc
t21 teccatcatt atgtgtgcctc tgtacccccc ctcagggctg aacctcggggg cattattaca
t18 tggctgctgctc ctggggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t24 tggctgctgctc ctggggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t30 caacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t36 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t41 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t48 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t54 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t60 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t66 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t72 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t78 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t84 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t90 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
t96 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
102 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
108 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
114 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
120 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc
126 cccacctggtct ggctggttgc cctccggcctgtc tcctcgtgcgt tggctggttgc cctccggcctgtc

Figure 4
LOCUS: EU835195  1306 bp  rRNA linear  BCT 13-JUN-2008
DEFINITION: Bacillus amyloliquifaciens.
ACCESSION: EU835195
VERSION:
KEYWORDS:
SOURCE: Bacillus amyloliquifaciens
ORGANISM: Bacillus amyloliquifaciens
   Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE: 1 (bases 1 to 1306)
AUTHORS: othman,Ar., shukor,My. and syed,Ma.
TITLE: unpublished
JOURNAL: Unpublished
REFERENCE: 2 (bases 1 to 1306)
AUTHORS: othman,Ar., shukor,My. and syed,Ma.
TITLE: unpublished
JOURNAL: Unpublished
REFERENCE: 3 (bases 1 to 1306)
AUTHORS: othman,Ar., shukor,My. and syed,Ma.
TITLE: Direct Submission
JOURNAL: Submitted (13-JUN-2008) Biotechnology and Biomolecular Science, Universiti Putra Malaysia, Serdang, Selangor 43400, Malaysia
COMMENT: BankIt Comment: supersalyaman_85@yahoo.com.
FEATURES:
   Location/Qualifiers
      source 1..1306 /organism="Bacillus amyloliquifaciens"
      /mol_type="rRNA"
      /db_xref="taxon:1390"

BASE COUNT: 316 a 308 c 400 g 272 t 10 others
ORIGIN:
1 cacacctgtctntgtatgtttcgtccggagcggtggaagaacgctggatgccgggtttgagtttaacctggtggtgtgctttggtttggttggtttggttggtttggttggtttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggttggtttggt tg}
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. C12N1/20  C12R1/07  C12R1/085  C02F3/34

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)

C12N  C12R  C02F

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

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

EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, WPI Data

**C. 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>
</tr>
</thead>
</table>
| X        | DATABASE BIOSIS [Online]
BIOSCIENCES INFORMATION SERVICE,
PHILADELPHIA, PA, US; 2001,
ARCHAMBAULT J G ET AL: "Development of a
Bacillus consortium for digestion of food
waste in grease trap system"
XP002537501
Database accession no. PREV200200251470
abstract
-----                                             | 1,5-13 |
| X        | WO 02/094181 A2 (TISINGER JESSI LIND [US];
PAONE DOMENIC A [US]; LEDER JONATHAN [US];
D) 28 November 2002 (2002-11-28)
the whole document
-----                                             | 1,5-13 |
9 January 2001 (2001-01-09)
the whole document
-----                                             | 1,5-13 |

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 doubt 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

  "P" document published prior to the international filing date but later than the priority date claimed

  "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.

  "S" document member of the same patent family

**Date of the actual completion of the international search**

16 July 2009

**Date of mailing of the international search report**

23/10/2009

**Name and mailing address of the ISA/Authorized officer**

European Patent Office, P.B. 5818 Patentlaan 2
NL – 2280 HV Rijswijk
Tel. (+31-70) 340-2240,
Fax (+31-70) 340-3016

Lejeune, Robert
**INTERNATIONAL SEARCH REPORT**

**Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

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).

**Box No. III  Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

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 (completely); 5-13 (partially)

**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(completely); 5-13(partially)
   Bacillus amyoliquefaciens strain RXZ Y135, a bacterial composition comprising it.

2. claims: 2(completely); 5-13(partially)
   Bacillus sp. clone A.rzi, a bacterial composition comprising it.

3. claims: 3(completely); 5-13(partially)
   Bacillus cereus strain Dr. Y135, a bacterial composition comprising it.

4. claims: 4(completely); 5-13(partially)
   Bacillus sp. strain SeAG 1 Y135, a bacterial composition comprising it.
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td>WO 02094181</td>
<td>28-11-2002</td>
<td>AT 346910 T</td>
<td>15-12-2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 2002303532 B2</td>
<td>07-07-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2447749 A1</td>
<td>28-11-2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DE 60216448 T2</td>
<td>20-09-2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 1392218 A2</td>
<td>03-03-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ES 2278026 T3</td>
<td>01-08-2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 2004528852 T</td>
<td>24-09-2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MX PA03010552 A</td>
<td>08-09-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NZ 530191 A</td>
<td>29-07-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2003008382 A1</td>
<td>09-01-2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2005036990 A1</td>
<td>17-02-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2003049832 A1</td>
<td>13-03-2003</td>
</tr>
<tr>
<td>US 6171847</td>
<td>09-01-2001</td>
<td>NONE</td>
<td></td>
</tr>
</tbody>
</table>