

# Search for new cultured lipophilic bacteria in industrial fat-containing wastes

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**Abstract.** Fat-containing wastes that are generated as a result of industrial production of food products and are being accumulated in large quantities in wastewater and sewage treatment plants and present a serious environmental problem. Microorganisms that decompose various types of lipids may be potential candidates for creation of commercial bioformulations for fat destruction. The aim of the study was to obtain pure cultures of lipophilic bacteria from fat-containing wastes, to study their diversity and activity for the development of a biological product. As a result, 30 strains of different phylogenetic groups with lipolytic activity was obtained. The most isolated strains were represented by enterobacteria and pseudomonas members within the *Gammaproteobacteria*. Almost half of the isolated strains were closely related to conditionally pathogenic microorganisms such as *Serratia*, *Klebsiella* etc. Non-pathogenic strains and promising for biotechnology ones belonged to *Pseudomonas citronellolis*, *P. nitroreducens*, *P. synxantha*, *P. extremaustralis*, *Bacillus subtilis*, *B. amyloliquefaciens*, *Brevibacillus brevis* and *Microvirgula* sp.

## 1 Introduction

The ability of microorganisms to produce enzymes that hydrolyze fat, lipases and esterases, has the great potential for use in biotechnologies for the disposal of food waste and for the treatment of fat-containing wastewaters. Fat-containing wastes generated as a result of industrial production of food products being accumulated in large quantities in wastewater and sewage treatment plants and present a serious environmental problem [1]. Microorganisms that decompose various types of fats may be potential candidates for the creation of biological preparations for wastes destruction. It is known that many bacteria, yeasts and fungi are potential extracellular lipase producers [2]. Members of genus *Bacillus* and *Pseudomonas* are the most studied and widely used producers of bacterial lipases [3]. Isolation of new microorganisms, producing biotechnologically important enzymes such as lipase and esterase is of current interest, since these microorganisms can be effective for bioremediation of various types of waste, including those with a high content of oils and fats [4-5]. The aim of the study was to obtain pure cultures of lipophilic bacteria from fat-

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containing wastes of food industry, to study their diversity and activity for the development of a commercial biological product.

## 2 Materials and methods

In order to obtain strains with lipolytic activity, we used samples of wastewater and fat deposits from the grease trap of treatment facilities of a meat processing plant and a containment pond of a dairy plant in Tomsk, Russia. pH, redox potential (Eh) and temperature values were measured at the sampling sites in liquid phases. The measurements were carried out by a Hanna Instruments pH211 pH meter equipped with a temperature sensor, pH electrode and Eh measurement electrode. The content of potassium ions ( $K^+$ ), ammonium ( $NH_4^+$ ), phosphates ( $PO_4^{3-}$ ), nitrate ( $NO_3^-$ ) and nitrite ions ( $NO_2^-$ ) were also analyzed. Chemical analysis was carried out commercially on the basis of a specialized laboratory.

In order to obtain enrichments and pure cultures a selective mineral nutrient medium of the following composition we used the following (per liter):  $NH_4Cl$  – 0.625 g,  $CaCl_2$  – 0.0025 g,  $MnCl_2$  – 0.005 g,  $MgSO_4 \times 7H_2O$  – 0.05 g,  $FeSO_4 \times 5H_2O$  – 0.0025 g,  $NaCl$  – 1.25 g,  $Na_2HPO_4$  – 2.5 g,  $KH_2PO_4$  – 0.25 g; pH 7,5. 1 % of pork fat, 1% of vegetable oil or 0.8 % of milk fat was added to the base medium as a substrate for the growth of microorganism. The lipolytic properties of the enrichment and pure cultures were evaluated by the growth results in the glycerol-fuchsin Stern broth with vegetable oil or pork fat as an additional carbon source at the concentration of 1 %. To prepare the Stern broth 5 drops of a saturated alcoholic solution of basic fuchsin, 1 ml of glycerol and 2 ml of freshly prepared 10 % sodium sulfite solution were added to 100 ml of meat-peptone broth (MPB); pH 7.6.

For pure cultures isolation we used enrichments that showed the presence of lipolytic activity on the Stern broth. Then, pure cultures were isolated by serial dilutions according to the Drigalski method on Petri dishes in agarized mineral medium containing pork fat or vegetable oil as a single source of carbon. Pure cultures were isolated from separately lying colonies.

The phylogenetic affiliation of the isolated microorganism was determined based on the analysis of the 16S rRNA gene sequences fragments. DNA isolation from bacterial cultures was carried out by the phenol method with CTAB/NaCl [6]. Further, the obtained DNA products were used for PCR amplification of bacterial 16S rRNA genes with the universal bacterial primers 27F [7] and 1492R [8]. The composition of the PCR mixture was described previously [9], as well as the amplification regime [10].

Editing the sequenced nucleotide sequences was carried out by the sequence alignment editor BIOEDIT (<http://www.jwbrown.mbio.ncsu.edu>). The analysis of the obtained nucleotide 16S rRNA gene sequences was performed using the BLAST program in the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov>) and SILVA database classifier (<http://www.arb-silva/aligner/de>). To exclude chimeras, sequences were checked using the DECIPHER web-tool (<http://www2.decipher.codes/FindChimeras.html>). The obtained nucleotide sequences of 16S rRNA gene fragments were deposited in the GenBank database. Numbers are given in Tab. 2.

### 3 Results and Discussion

#### 1.1 Isolation of pure cultures with lipolytic activity

Sources for isolation of microorganisms with lipolytic properties were samples of fat-containing wastes from milk and meat processing industries and physico-chemical characteristics of liquid phases are showed in Table 1.

**Table 1.** Physical and chemical parameters of wastes in the sampling sites

|                       | pH   | Eh, mV | T °C  | NH <sup>4+</sup> (PL 2,0 <sup>1</sup> ) | K <sup>+</sup> (PL 50,0 <sup>2</sup> ) | NO <sup>3-</sup> (PL 45,0 <sup>2</sup> ) | NO <sup>2-</sup> (PL 45,0 <sup>2</sup> ) | PO <sub>4</sub> <sup>3-</sup> (PL 12,0 <sup>1*</sup> ) |
|-----------------------|------|--------|-------|---|--|--|--|--|
| Milk plant            | 9.48 | 45     | +18.8 | 3.80                                    | 22.97                                  | 4.66                                     | 0.31                                     | 34.14  |
| Meat processing plant | 5.72 | 8      | +20.4 | 31.90                                   | 54.6                                   | 9.82                                     | 0.27                                     | 122  |

<sup>1</sup> The threshold limit values (TLVs) are presented in accordance with the Decree of the Government of the Russian Federation on 07.29.2013N 644 (ed. on 26.07.2018) "On approval of the Rules for cold water supply and sanitation and on amendments to some acts of the Government of the Russian Federation"  
<sup>2</sup> TLVs in accordance with the Sanitary Regulations and Standards of the Russian Federation 2.1.4.1074-01  
 \* TLV for total phosphorus content

In total, we obtained 30 bacterial pure cultures, 19 strains were isolated from the grease trap of a meat processing plant, 11 strains were isolated from the containment pond wastewater of a milk plant. All bacterial strains were isolated using a mineral medium with animal fat, but also grew with the addition of vegetable and milk fats as single carbon sources. The lipophilic properties of the isolates were confirmed by growth on a diagnostic medium (Stern broth). A change in the color of the culture medium during the cultivation process demonstrates the ability of the strains to extracellular production of lipases. The ability of microorganisms to produce extracellular enzymes indicates the presence of adaptational mechanisms to extreme environmental conditions and the biotechnological potential [11].

#### 2.2 Phylogenetic analysis

The phylogenetic position of all obtained microorganisms was established using molecular methods. Analysis of 16S rRNA gene fragments showed that the isolates belong to *Proteobacteria* (classes *Gammaproteobacteria*, *Betaproteobacteria*) and *Firmicutes* (Table 2). Most isolates represented enterobacteria (11 strains) and pseudomonads (12 strains) of the class *Gammaproteobacteria*. Almost all analyzed fragments of DNA sequences 586-1436 bp long showed a high percentage of similarity (99.9-100%) with the closest validly described microorganisms.

Almost half of the isolates (14 strains) were related to conditionally pathogenic microorganisms belonging to risk group II according to German TRBA classification. Detected conditional pathogens included enterobacterial strains related to *Serratia marcescens* (strains A11 and A14), *Leclercia adecarboxylata* (strains B13 and B14), *Klebsiella pneumoniae* (strain A12), *Klebsiella huaxiensis* (strain BF12), *Enterobacter cloacae* (strain D152), *Raoultella ornithinolytica* (strains P1 and P2), *Morganella morganii* (strains B31 and B36). Strains B12 and B14 also appeared to be related to the conditional pathogenic organism, the sequences of which showed a 99.97% similarity with *Alcaligenes*

*faecalis* (Table 2). Most of the conditionally pathogenic organisms were obtained from the grease trap of a meat processing plant. Wastes from livestock and milk industries are often a source of pathogenic microorganisms of a similar species composition [12–13]. In addition to participation in metabolic processes such as hydrolysis and lipid modification, bacterial lipases can act factors of virulence in some phylogenetic groups of microorganisms [14–15], which explains the large number of pathogens among lipophiles.

**Table 2.** Phylogenetic position of isolated microorganisms

| №  | Isolate | GeneBank accession number | Sequence length | Similarity % | Nearest validly described organism (GeneBank Number) | Site of isolation    | Phylogenetic affiliation                                |
|--|---------|---------------------------|-----------------|--------------|--|----------------------|---|
| <b>Pure cultures from the grease trap of a meat processing plant</b> |         |                           |                 |              |  |                      |   |
| 1  | A11     | MT459407                  | 851 bp          | 100          | <i>Serratia marcescens</i> (CP041233)                | Pond water           | <i>Gammaproteobacteria</i> ;<br><i>Enterobacterales</i> |
| 2  | A12     | MT459439                  | 786 bp          | 100          | <i>Klebsiella pneumoniae</i> (DQ470485)              | Clinical samples     | <i>Gammaproteobacteria</i> ;<br><i>Enterobacterales</i> |
| 3  | A13     | MT437044                  | 1422 bp         | 99.93        | <i>Pseudomonas citronellolis</i> (KM210229.1)        | Active sludge        | <i>Gammaproteobacteria</i> ;<br><i>Pseudomonadales</i>  |
| 4  | A14     | MT459410                  | 451 bp          | 100          | <i>Serratia marcescens</i> (CP041233)                | Pond water           | <i>Gammaproteobacteria</i> ;<br><i>Enterobacterales</i> |
| 5  | BF11    | MT441542                  | 1436 bp         | 100          | <i>Pseudomonas extremaustralis</i> (LT629689)        | Antarctic pond water | <i>Gammaproteobacteria</i> ;<br><i>Pseudomonadales</i>  |
| 6  | BF12    | MT459440                  | 651 bp          | 100          | <i>Klebsiella huaxiensis</i> (CP036175)              | Human urine          | <i>Gammaproteobacteria</i> ;<br><i>Enterobacterales</i> |
| 7  | BF13    | MT459441                  | 620 bp          | 100          | <i>Leclercia adecarboxylata</i> (NR_104933)          | Drinking water       | <i>Gammaproteobacteria</i> ;<br><i>Enterobacterales</i> |
| 8  | BF14    | MT459448                  | 808 bp          | 99.88        | <i>Enterobacter ludwigii</i> (CP041062)              | Soil                 | <i>Gammaproteobacteria</i> ;<br><i>Enterobacterales</i> |
| 9  | B21     | MT491128                  | 770 bp          | 100          | <i>Pseudomonas synxantha</i> (KT767761)              | Raw milk             | <i>Gammaproteobacteria</i> ;<br><i>Pseudomonadales</i>  |
| 10   | B24     | MT491129                  | 745 bp          | 100          | <i>Pseudomonas synxantha</i> (KT767761)              | Raw milk             | <i>Gammaproteobacteria</i> ;<br><i>Pseudomonadales</i>  |
| 11   | B25     | MT491141                  | 764 bp          | 100          | <i>Pseudomonas synxantha</i> (KT767761)              | Raw milk             | <i>Gammaproteobacteria</i> ;<br><i>Pseudomonadales</i>  |
| 12   | B26     | MT491299                  | 744 bp          | 100          | <i>Pseudomonas synxantha</i> (KT767761)              | Raw milk             | <i>Gammaproteobacteria</i> ;<br><i>Pseudomonadales</i>  |
| 13   | B31     | MT472134                  | 766 bp          | 100          | <i>Morganella morganii</i> (KU598846)                | Mouse feces          | <i>Gammaproteobacteria</i> ;<br><i>Enterobacterales</i> |

|   |      |              |        |       |  |  |  |
|---|------|--------------|--------|-------|--|--|--|
| 14  | B34  | MT4769<br>67 | 673 bp | 100   | <i>Bacillus amyloliquefaciens</i> (CP000560)                       | No information                             | <i>Firmicutes; Bacilli; Bacillales</i>       |
| 15  | B36  | MT4769<br>66 | 771 bp | 100   | <i>Morganella morgani</i> (KU598846)                               | Mouse feces                                | <i>Gammaproteobacteria; Enterobacterales</i> |
| 16  | B37  | MT4769<br>21 | 768 bp | 100   | <i>Microvirgula aerodenitrificans/curvata</i> (CP028519/NR_153739) | Bioreactor / hydrocarbon contaminated soil | <i>Betaproteobacteria; Neisseriales</i>      |
| 17  | B12  | MT4769<br>11 | 754 bp | 99.87 | <i>Alcaligenes faecalis</i> (KF254754)                             | Mosquito intestines                        | <i>Betaproteobacteria; Burkholderiales</i>   |
| 18  | B14  | MT4769<br>14 | 772 bp | 99.87 | <i>Alcaligenes faecalis</i> (KF254754)                             | Mosquito intestines                        | <i>Betaproteobacteria; Burkholderiales</i>   |
| 19  | B10  | MT4769<br>02 | 738 bp | 100   | <i>Bacillus subtilis</i> (CP034484)                                | No information                             | <i>Firmicutes; Bacilli; Bacillales</i>       |
| <b>Pure cultures from containment pond wastewater of milk plant</b> |      |              |        |       |  |  |  |
| 20  | D13  | MT4599<br>31 | 907 bp | 100   | <i>Pseudomonas azelaica (nitroreducens)</i> (AM088475)             | Garden soil                                | <i>Gammaproteobacteria; Pseudomonadales</i>  |
| 21  | D14  | MT4644<br>64 | 618 bp | 100   | <i>Pseudomonas nitroreducens</i> (NR_114975)                       | Oil water                                  | <i>Gammaproteobacteria; Pseudomonadales</i>  |
| 22  | D151 | MT4644<br>66 | 800 bp | 100   | <i>Pseudomonas nitroreducens</i> (NR_114975)                       | Oil water                                  | <i>Gammaproteobacteria; Pseudomonadales</i>  |
| 23  | D152 | MT4651<br>16 | 587 bp | 100   | <i>Enterobacter cloacae</i> (MG274288)                             | Chickpea rhizosphere                       | <i>Gammaproteobacteria; Enterobacterales</i> |
| 24  | P1   | MT4654<br>56 | 596 bp | 100   | <i>Raoultella ornithinolytica</i> (KT767970)                       | Raw milk                                   | <i>Gammaproteobacteria; Enterobacterales</i> |
| 25  | P2   | MT4656<br>58 | 614 bp | 100   | <i>Raoultella ornithinolytica</i> (KT767970)                       | Raw milk                                   | <i>Gammaproteobacteria; Enterobacterales</i> |
| 26  | P3   | MT4658<br>47 | 601 bp | 100   | <i>Pseudomonas nitroreducens</i> (HM192780)                        | Soil                                       | <i>Gammaproteobacteria; Pseudomonadales</i>  |
| 27  | P4   | MT4721<br>29 | 902 bp | 100   | <i>Pseudomonas nitroreducens</i> (KX682023)                        | Agricultural soil                          | <i>Gammaproteobacteria; Pseudomonadales</i>  |
| 28  | P5   | MT4721<br>05 | 757 bp | 100   | <i>Pseudomonas nitroreducens</i> (NR_114975)                       | Oil water                                  | <i>Gammaproteobacteria; Pseudomonadales</i>  |
| 29  | M22  | MT4769<br>01 | 788 bp | 100   | <i>Bacillus subtilis</i> (CP034484)                                | No information                             | <i>Firmicutes; Bacilli; Bacillales</i>       |

|    |     |              |        |     |   |                   |  |
|----|-----|--------------|--------|-----|---|-------------------|--|
| 30 | 28Д | MT4768<br>51 | 785 bp | 100 | <i>Brevibacillus<br/>brevis</i><br>(AP008955) | No<br>information | <i>Firmicutes; Bacilli;<br/>Bacillales</i> |
|----|-----|--------------|--------|-----|---|-------------------|--|

The remaining pure cultures were related to microorganisms that are not pathogens of humans, animals or plants. Most of the strains belonged to the genus *Pseudomonas*, such as *P. nitroreducens* (strains P3-P5, D13, D14, D151), *P. synxantha* (strains B21, B24-B26), *P. extremaustralis* (strain BF11), *P. citronellolis* (A13). The genus *Pseudomonas* represents a large group of bacteria that are ecologically and functionally very diverse, including many pathogenic species for humans and plants [16], as well as mutualistic species, the most striking examples of which are biocontrol strains that protect plants from pathogens [17-18]. It has been established that *Pseudomonas* species can decompose various lipids and lipid-containing compounds [19-22]. The isolated strains phylogenetically belong to different groups of species of the genus *Pseudomonas*, in accordance with the modern classification. Strains *P. citronellolis* A13 and *P. nitroreducens* D13, D14, D151, P3-P5 belong to the *Pseudomonas aeruginosa* group. This group of species, in addition to numerous strains of *P. aeruginosa*, also includes subgroups of the species *P. nitroreducens/multiresinivorans*, *P. oleovorans/pseudoalcaligenes*, and some other species. Strains B21 and B24-B26 are classified within *P. synxantha/azotoformans/fluorescens/paralactis*, which belong to the group of fluorescent pseudomonads (*Pseudomonas fluorescens* group). 16S rRNA gene fragments analysis with a length of 600-800 bp does not allow a more accurate determination of the phylogenetic position. Strain B11 showed 100% similarity of the 16S rRNA gene sequence with *P. extremaustralis* isolated from a pond in the Antarctic, which has high resistance to heat and oxidative stress [23-24], as well as increased cold resistance [23, 25] and the ability to utilize hydrocarbons [23, 26]. The remaining representatives of *Pseudomonas*, closely related to the isolated strains, also possess various biotechnologically beneficial properties, such as psychrotolerance [27], the ability to utilize tributyrin [28], degradation of alkanes, terpenes [29], isoprenoids, hydrocarbons [30] and chloroaromatic compounds [31]. This indicates their significant potential for the biodegradation of organic compounds.

Analysis of the 16S rRNA gene fragment of strain 28D showed its 100% similarity to *Brevibacillus brevis* (*Firmicutes*). Species belonging to the genus *Brevibacillus* are a rich source of antimicrobial peptides such as gramicidin, gratisin, edein. Bioactive peptides produced by *Brevibacillus* spp. include antibacterial, antifungal, and anti-invertebrate agents. Representatives of this genus are widespread and can be found in various environments, including the intestinal tract of animals, sea water, and soil [32]. These microorganisms are widely used in agriculture and environmental biotechnologies [33].

Three new strains were related to Firmicutes of the genus *Bacillus*, namely *B. subtilis* (strains M22, B10) and *B. amyloliquefaciens* (strain B34). Representatives of *Bacillus* are among the most common producers of bacterial lipases [3]. The lipolytic enzymes of *B. subtilis* and *B. amyloliquefaciens* are of significant biotechnological interest and, therefore, many of them have been identified, cloned, and characterized to date [34-39]. In addition, *B. subtilis* and *B. amyloliquefaciens* are known for their activity as plant growth promoting rhizobacteria (PGPR) and are widely used to stimulate growth and counteract numerous plant pathogens [40-42]. Another potential use of such bacteria is related to their probiotic characteristics [43].

Strain B37 was assigned to the genus *Microvirgula*, a member of the *Betaproteobacteria* class. Representatives of *Microvirgula* grow well under aerobic and anaerobic conditions and have an atypical respiratory type of metabolism; they use oxygen and nitrogen oxides as final electron acceptors [44]. The genus *Microvirgula* (*M. aerodenitrificans*) was first described by Patureau et al. [44] and was characterized as a new denitrifying bacterium isolated from

activated sludge. Currently, the genus *Microvirgula* includes two species. The second species named as *Microvirgula curvata* was first isolated from hydrocarbon-contaminated soils [45] and today is represented by one strain. Sequenced 768 bp 16S rRNA gene fragment does not allow reliably attributing the strain B37 to a specific species.

For many representatives of *Pseudomonas* and *Bacillus*, including those close to the strains isolated by us, the lipolytic activity on various substrates has been studied quite well, and the genes of lipolytic enzymes have been deciphered and cloned. At the same time, for representatives of *Microvirgula* and *Brevibacillus* only lipolytic activity in diagnostic media was detected; a detailed study of lipolytic properties was not carried out. However, analysis of the *Microvirgula* and *Brevibacillus* genomes available in the NCBI database revealed genes for lipolytic enzymes. To date, the genomes of two strains of *Microvirgula aerodenitrificans* (JHVK01000000 and CP028519) isolated from different bioreactors and one strain of *Microvirgula* sp. AG722 (NZ\_QLTJ01000000) which is a bacterial rice endophyte with an unidentified phylogenetic position, have been published. The search of the lipolytic enzymes-coding genes in the listed genomes showed the presence of lipases and esterases. The available *Brevibacillus brevis* genomes (GCA\_002161835.1, GCA\_007725005.1, GCA\_001039275.2) found in the NCBI database also contain genes encoding lipolytic enzymes.

Thus, the detected phylogenetic and metabolic diversity of non-pathogenic bacterial strains with lipolytic activity indicates that the obtained isolates are of great interest for the application in various industrial biotechnologies, primarily for biodegradation of fat-containing waste.

## 4 Conclusion

Thirty bacterial isolates with lipolytic activity were obtained from wastewater samples from the containment pond of a milk plant and the grease trap of the treatment facilities of a meat processing plant. Phylogenetic analysis allowed us to identify and exclude potentially pathogenic microorganisms from the study. The remaining strains were assigned to the classes *Gammaproteobacteria* (*Pseudomonas nitroreducens*, *P. synxantha*, *P. extremaustralis* and *P. citronellolis*), *Betaproteobacteria* (*Microvirgula* sp.) and the phylum *Firmicutes* (*Bacillus subtilis*, *Bacillus amyloliquefaciens* and *Brevibacillus brevis*). For the first time, a representative of *Microvirgula* was isolated from fat-containing waste of the food industry. The strains demonstrate the great biotechnological potential for industrial bioconversion of lipids, including fats and oils. A number of producers can also be used as part of commercial biological products for plant growth stimulation in agriculture and for the disposal of industrial and agro-industrial waste.

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